The Genomics of Labour: Global Gene Expression Profiling and Oxytocin Receptor Gene Expression

by

Barbara Gould

Department of Experimental Medicine
McGill University, Montreal

Submitted November 2003

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements of the degree of Doctor of Philosophy.

Your file Votre référence
ISBN: 0-612-98262-9
Our file Notre référence
ISBN: 0-612-98262-9

Abstract

Premature labour and subsequent premature birth is a leading cause of neonatal morbidity and mortality. We studied murine labour at term and preterm with models of intrauterine infection and ovariectomy using Affymetrix microarray U74Av2 (containing 12,488 probe sets) and real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) to identify novel candidate genes involved in normal and preterm labour. Strict statistical analysis revealed 320, 188, and 74 genes to be significantly induced or suppressed during normal, infection-induced, and ovariectomy-induced labour, respectively. Novel genes identified include: associated with normal labour: alpha fetoprotein, apolipoprotein A1, fibrinogen polypeptides, cytochrome P45011a, Purkinje cell protein 4 (PCP4), and chloride channel calcium activated 3; associated with infection: numerous inflammatory mediators, alpha fetoprotein, apolipoprotein A1, and fibrinogen polypeptides. Small proline-rich protein 2 family genes were induced by ovariectomy and infection. PCP4 gene was induced after ovariectomy, but suppressed at normal labour. Only seven genes were significantly regulated (each induced) at labour in all models implying that unique gene networks are involved in normal and preterm labour induced by various stimuli. These included genes for plasminogen activator inhibitor 1 and for contraction associated proteins (CAPs) required for uterine activation and uterotonin stimulation of contractions.

The oxytocin receptor (OTR) gene encodes one such CAP. Northern blot and real-time RT-PCR demonstrated its up-regulation prior to labour in each model, preferentially in normal labour. Uterine contraction promotes increased central and peripheral oxytocin release and synaptic plasticity. To further examine the role of the OTR, we developed an OTR-lacZ reporter mouse. We mapped, by X-gal histochemistry, the distribution of OTR gene expression in the early postparturient mouse brain and identified novel regions of expression. These included the piriform cortex, entorhinal cortices, and parasubiculum, which support memory function. Dorsal tegmental, vestibular, and lateral reticular nuclei expression suggests the

transmission of locomotor inputs. Hypoglossal, facial, and spinal trigeminal nuclei support maternal behaviours. We also more accurately demarcated OTR gene expression in the solitary tract nucleus responsible for relaying contraction stimulation of oxytocin release.

These studies provide a more accurate knowledge base for the development of successful therapies to decrease the incidence of premature labour.

Résumé

Le début hâtif des contractions utérines menant aux prématurés est la principale cause de la morbidité et de la mortalité néonatale. Afin de mieux comprendre les mécanismes physiopatologiques menant aux contractions prématures, nous avons évalué l'expression génique à l'aide de la technologie des microchips d'Affymetrix U74Av2 (contenant 12 488 sondes) sur des souris ovarectomisées ou subissant une infection bactérienne intrauterine. L'identification de certains de ces nouveaux gènes liés au travail prématuré a été confirmée par RT-PCR en temps réel. Une analyse statistique robuste des résultats a révélé que 320 gènes sont modulés pendant le travail normal, 188 pendant le travail induit par infection et 74 pendant le travail induit par ovarectomie. Les nouveaux gènes identifiés incluent : liés au travail normal : alpha fétoproteine, apolipoproteine A1, les polypeptides du fibrinogène, cytochrome P45011a, Purkinje cell protein 4 et le chloride channel calcium activated 3; liés à l'infection : différents médiateurs de l'inflammation, de l'alpha fétoproteine, de l'apolipoprotein A1, et des polypeptides du fibrinogène. Les gènes appartenant à la famille des small proline rich protein 2 sont induits par l'ovariectomie et par l'infection. Le gène codant pour le Purkinje cell protein 4 est induit après ovariectomie mais est supprimé pendant le travail normal. Cependant, seulement sept gènes sont induits et par le travail normal et par le travail causé par l'infection et par l'ovariectomie. Ceci implique que de grandes différences existent entre les différents réseaux de gènes qui sont impliqués dans le travail normal et dans le travail prématuré. Ceux-ci inclus les gènes codant pour le plasminogen activator inhibitor 1 et ceux codant pour les proteines associées à la contraction (CAP).

Le récepteur de l'ocytocine (OTR) est un membre du groupe des CAPs. L'analyse des ARN messagers par Northern blot et par RT-PCR en temps réel a révélé que ce gène est stimulé avant le début de la parturition dans chacun des modèles investigués et surtout dans celui du travail normal. Il est bien connu que les contractions utérines causent une augmentation de la sécrétion centrale et périphérique d'ocytocine. Afin d'examiner en détail le rôle de l'OTR, nous avons développé un

modèle de souris exprimant le gène lac-Z dont l'expression est sous l'influence du promoteur du gène de l'OTR. La détection de l'activité de l'enzyme β-galactosidase par histochimie a permis de déterminer la distribution des sites d'expression du gène de l'OTR dans le cerveau de ces souris et nous avons identifié de nouvelles régions d'expression. Ceux-ci incluent le cortex piriforme, les cortex entorhinaux, et le parasubiculum qui sont des régions importantes liées à la fonction de la mémoire. L'expression dans les noyaux tegmentales dorsaux, vestibulaires et reticulaires lateraux suggère une implication de l'ocytocine dans la transmission afférente du système locomoteur. L'expression dans les noyaux hypoglossaux, faciaux et trigininaux spinaux implique un rôle de l'OTR dans le comportement maternelle. Nous avons aussi délimité l'expression de l'OTR dans le noyau du tractus solitaire qui est responsable de la stimulation de la sécrétion d'ocytocine en réponse à la contraction utérine.

En conclusion, ces études établissent les bases pouvant mener au développement de nouvelles thérapies destinées à diminuer l'incidence du travail prématuré.

Preface

The Guidelines for Submitting a Doctoral Thesis, Faculty of Graduate Studies and Research, McGill University, instructs the following in the preparation of this thesis:

Manuscript-Based Thesis

Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly-duplicated text of one or more published papers. These texts must be bound together as an integral part of the thesis with connecting texts that provide logical bridges between the different manuscripts, integrating all components of the thesis into a cohesive unit.

The thesis must conform to all other requirement of the "Guidelines for Thesis Preparation". The thesis must include a table of contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a comprehensive review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.

Additional material may be provided in appendices in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. Supervisors must attest to the accuracy of such statements at the doctoral defence. Under no circumstances can a co-author of any component of such a thesis serve as an examiner for that thesis.

Contributions to this Work

The work presented in Chapters 2 and 3 of this dissertation will be submitted for publication shortly. Chapter 4 provides additional and confirmatory evidence while serving as a bridge between studies. Chapter 5 represents work published in

Neuroscience. The work described in these chapters was performed, under the supervision of Dr. H. H. Zingg, by Barbara Gould except for the contributions made by the following people:

Chapters 2 and 3: Microarray experimental procedures were performed by the Montreal Genome Centre.

Chapter 3: Surgical assistance was provided by Maria Kontogiannea of Dr. R. Farookhi's laboratory (intrauterine infection) and by Deanna Collin of the McIntyre Animal Care Facility (ovariectomy).

Chapter 5: Technicians Caterina Russo, Levon Fendekian, and John Tam jointly prepared the 5'3'pGNA vector and supplied it to the late Naima Bachnou of Dr. A. Peterson's laboratory for electroporation into ES cells and subsequent growth of neomycin resistant recombinant ES cells. The work is recounted, for completeness, in the Appendix to Chapter 5. Selected recombinant ES cells were injected into developing mouse blastocysts and chimeric mice were generated with the help of the laboratory of Dr. M. Tremblay. Cryostat sectioning and β-galactosidase staining techniques were performed in demonstration to Barbara Gould by Priscila Valera of Dr. A. Peterson's laboratory.

Acknowledgements

I wish to express my deep gratitude and appreciation to my supervisor Dr. Hans. H. Zingg for his support, encouragement, insight, and patience, throughout all aspects of my PhD experience. My respect and appreciation to the members of my thesis committee, Dr. Hugh Bennett, Dr. Bernard Turcotte, and Dr. Geoff Hendy, for their critical assessment and guidance. I thank the members of the Laboratory of Molecular Endocrinology and Department of Experimental Medicine, past and present, who provided an environment of scientific discussion as well as friendship and include Esther Prince, Christine Chow, and Ghiabe Guibinga, with an extra thank you to Dominic Devost for his translation of my thesis abstract. Of special significance is Cindy Solomon whose friendship means the world to me. Thank you for the advice, support, laughter, and adventures throughout our PhD experiences, more of which are sure to come. Thank you to Ben Cadieux for being there and understanding. I wish to thank my family for their unending support, encouragement, and belief in my abilities, which strengthen my determination to achieve my goals. I most gratefully acknowledge the Canadian Institutes of Health Research and the McGill University Health Centre for providing financial support that made this research possible.

Table of Contents

List of Figures and Tables

Page

Chapter 3: Identification and Characterization of Global Gene Expression Changes During Infection - and Ovariectomy - Induced Premature Labour

Page

Chapter 5: Mapping Oxytocin Receptor Gene Expression in the Mouse Brain and Mammary Gland Using an OTR-LacZ Reporter Mouse

Chapter 6: General Discussion and Conclusions

List of Abbreviations

+	wild-type allele
AA	arachidonic acid
Abcb1b	ATP-binding cassette sub-family B (MDR/TAP) member 1B
Ach	acetylcholine
Afp	alpha fetoprotein
a.k.a.	also known as
Ap-1	activator protein 1
ApoA1	apolipoprotein A1
APRE	acute phase response element
Aqp	aquaporin
Arg1	liver arginase 1
ATP	adenosine triphosphate
bp	base pairs
C	Luria Broth control
Ca^{2+}	calcium
$[Ca^{2+}]_i$	intracellular calcium concentration
Ca-Cam	calcium-calmodulin complex
cAMP	cyclic adenosine monophosphate
CAP(s)	contraction associated protein(s)
Cdkn1c	cyclin-dependent kinase inhibitor 1C
cDNA	complementary DNA
cFos	FBJ osteosarcoma oncogene
cGMP	cyclic guanosine monophosphate
CGRP	calcitonin gene-related peptide
Chap10	chaperonin 10 (heat shock protein 10)
Cl^-	chloride
Clca	chloride channel calcium activated

COX	cyclooxygenase
Cpe	carboxypeptidase E
$cPLA_2$	cytosolic PLA_2
CRH	corticotrophin-releasing hormone
CRH-R	corticotrophin-releasing hormone receptor
cRNA	complementary RNA
Ctla2a	cytotoxic T lymphocyte-associated protein 2 alpha
Ctla2b	cytotoxic T lymphocyte-associated protein 2 beta
Cp	crossing point
Cx26	connexin 26
Cx43	connexin 43
Cyp11a	P45011a cholesterol side chain cleavage
D3	Ifi205 gene product
DAG	diacylglycerol
dChip	DNA-Chip Analyzer software
DEPC	diethylpyrocarbonate
DHEAS	dehydroepiandrosterone sulphate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
d.p.c.	days post coitus
Dscr	Down syndrome candidate/critical region
E	estrus
E.coli	*Escherichia coli*
eNOS	endogenous NOS
EP	prostaglandin E_2 receptor
EPF	early-pregnancy factor
ER	estrogen receptor
ERE	estrogen response element
ES cell	embryonic stem cell
EST	expressed sequence tag

ET	endothelin
FbgA	fibrinogen alpha polypeptide
FbgB	fibrinogen beta polypeptide
F2	prothrombin
F3	coagulation factor III
FP	prostaglandin $F_{2\alpha}$ receptor
$G_{\alpha i}$ (or G_i)	G protein α subunit type *i*
$G_{\alpha q}$ (or G_q)	G protein α subunit type *q*
$G_{\alpha s}$ (or G_s)	G protein α subunit type *s*
GAPDH	glutaraldehyde phosphate dehydrogenase
GARG	glucocorticoid attenuated response gene
GPCR	G protein coupled receptor
GTP	guanosine triphosphate
H	high-dose (10^{10}) of bacteria
HDL	high-density lipoprotein
HPA axis	hypothalamic-pituitary-adrenal axis
HPRT	hypoxanthine guanine phosphoribosyl transferase
Hsd3b1	3β-hydroxysteroid dehydrogenase 1
Hsd11b1	11β- hydroxysteroid dehydrogenase 1
Ifi202A –Ifi205	interferon activated "200 family genes" 202A to 205
Ifit	interferon-induced proteins with tetratricopeptide repeats
IFNγ	interferon γ
IGF	insulin-like growth factor
IGFBP	IGF binding protein
IL	interleukin
IL-1rn	IL-1 receptor antagonist
IP_3	inositol triphosphate
Irg1	immunoresponsive gene 1
IUGR	intrauterine growth restriction
kb	kilobase(s)

L (chapter 2)	labour
L (chapter 3)	low-dose (10^7) of bacteria
LB	Luria broth
LH	luteinizing hormone
LPS	lipopolysaccharide
LPSBP	LPS binding protein
MAPK	mitogen-activated protein kinase
M-Csf (or Csf-1)	macrophage-colony stimulating factor
MIP1α	macrophage inflammatory protein 1α
MLC-II	myosin light chain II
MLCK	myosin light chain kinase
MM	mismatch
MMP	matrix metalloproteinase
MP	myosin phosphatase
mRNA	messenger RNA
Na^+	sodium
NF-IL6 (or C/EBPβ)	nuclear factor IL-6
NFκB	nuclear factor κB
NO	nitric oxide
NOS	nitric oxide synthase
OT	oxytocin
OTR	oxytocin receptor
Ovx	ovariectomy
Ovx5	5 hours after Ovx
Ovx20-PTL	ovariectomy-induced PTL
P	postpartum
p202a	Ifi202A gene product
p203	Ifi203 gene product
p204	Ifi204 gene product
PAF	platelet activating factor

PAI-1	plasminogen activator inhibitor-1
pBS	pBluescript K/S
PBS	phosphate buffered saline
PCP4 (or Pep-19)	Purkinje cell protein 4
PCR	polymerase chain reaction
PG	prostaglandin
PGDH	PG dehydrogenase or 15-hydroxyprostaglandin dehydrogenase
PGE_2	prostaglandin E_2
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PIP_2	phosphatidyl-inositol 4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLA_2	phospholipase A_2
PM	perfect match
pPROM	premature prelabour rupture of membranes
PR	progesterone receptor
PTHrP	parathyroid hormone-related peptide
PTL	preterm labour
PVN	paraventricular nucleus
RGS	regulator of G protein signalling
RNA	ribonucleic acid
RNase	ribonuclease
RT-PCR	reverse transcription - polymerase chain reaction
SAA3	serum amyloid A3
Scy	small inducible cytokine
SE	standard error
Sham	sham operated
SON	supraoptic nucleus
Sox4	SRY-box containing gene 4
Sp-1	specificity protein 1

Sprr2	small proline-rich protein 2 family
SR	sarcoplamic reticulum
T	term
TGFβ	transforming growth factor β
T_h1	T helper cell type 1
T_h2	T helper cell type 2
TNFα	tumour necrosis factor α
Tnfip6	tumour necrosis factor induced protein 6
Tnfrsf11b	TNF receptor superfamily member 11b
TSH-R	thyroid stimulating hormone receptor
UTR	untranslated region
VCAM	vascular cell adhesion molecule
VIP	vasoactive intestinal peptide
VOC/AOC	voltage and agonist operated channels
vs.	versus
z	lacZ allele

Chapter 1

Introduction

1.1 Rationale and Objectives

It is a biological imperative for all creatures to reproduce. Successful survival of a species requires its mechanism of reproduction to be finely tuned and adaptable to ensure propagation under different environmental and physiological conditions. Reproduction in humans, and many other animals, is preceded by a variety of social interactions, a courtship, by which the best mate is selected and invited to copulate. Insemination around the time of ovulation often results in fertilization and in pregnancy. Development of the embryo proceeds under optimal conditions and when this has progressed sufficiently the offspring are delivered into the world. Survival still requires parental nurturing for some time. In mammals, this nurturing includes feeding, protection, the education of the progeny to provide for, and protect, themselves, as well as appropriate social conditioning. All of this effort culminates in the preparation of the individuals for self-sufficiency and future reproductive success.

Full and proper embryonic development is important for survival, however when environmental or genetic factors compromise successful reproduction, the process is often terminated. Termination early in embryonic development aborts offspring that would not likely have been able to survive. Cessation late in development often results from maternal environmental stresses; the progeny are prematurely delivered, escaping this environment with a chance for survival. The likelihood of survival increases with advanced development and therefore a premature conclusion of gestation is disadvantageous.

In humans, preterm delivery is the leading cause of perinatal morbidity and mortality. Preterm labour (between 22 and 37 weeks gestation) occurs in as much as 10% of all pregnancies with higher incidences in populations experiencing poverty where proper nutrition is difficult to maintain and access to medical and educational resources are limited [1]. Although therapies have been developed to halt the premature culmination of this process, they are only temporarily successful. In addition, the incidence of preterm labour is increasing. Our understanding of the

process of normal labour and the aberrations resulting in preterm labour is still limited despite considerable research.

To study the factors influencing normal and preterm labour, human tissue samples have been collected when possible, and animal models have been utilized when practical. Many studies investigate one or a few possible regulators at any given time, looking at changes in physiology, protein levels, or gene expression levels individually. The development of microarray technology, whereby the expression of thousands of genes can be monitored simultaneously, has enabled a more complete picture of the changes occurring under different, controlled conditions. We utilized this advancement to study the expression patterns of more than ten thousand identified genes and expressed sequences during normal and induced preterm labour in previously established mouse models [2, 3]. By extracting a thorough representation of the changes in gene expression under these conditions we intend to identify novel genes involved in these processes and develop a comprehensive investigation of the genetics of labour. Having a complete picture, we may more efficiently and effectively develop screening strategies for early identification of women likely to experience preterm labour and develop therapies to prevent the activation or progression of this process.

Several regulators of normal and preterm labour are known although the exact mechanisms of activation and action of these require further clarification. Many of these hormones, proteins, and genes are described in this introduction. One particular participant is the oxytocin receptor gene and its protein product. The oxytocin receptor (OTR) binds oxytocin (OT), the strongest uterotonic agent known. OT is often used to induce labour. For these reasons, the OT-OTR system has been studied to determine their roles in normal and preterm labour. Surprisingly, OT gene knock-out mice show no defect in their ability to undergo normal labour [4, 5] so the focus has turned towards its receptor. The OTR gene is not represented on the available Affymetrix mouse microarray chip. We investigated and confirmed its pattern of expression under normal labour conditions and that of induced preterm labour through traditional approaches: Northern blot and RT-PCR.

Further interest in the OTR inspired the development of an OTR knock-out mouse. During the process, an OTR-LacZ expressing reporter mouse was developed. We took advantage of this mouse to map the gene expression pattern within the brain of early post-parturient mothers. Our goal was to identify new regions that express the OTR gene in the mouse brain and to propose new functions of receptor activation and support suggestions presented by previous findings from other research groups. Some of these neuroendocrine/neurological roles include an involvement in the regulation of the production and release of OT and other hormones into the system, as well as influencing social, sexual, and maternal behaviours necessary for successful reproduction [6].

This thesis is a presentation of the evidence acquired by the study of the genes involved in the processes of normal and premature labour and an extended investigation of one gene, the oxytocin receptor gene, and its involvement in processes necessary for successful reproduction.

1.2 *Pregnancy, Labour, and Motherhood*

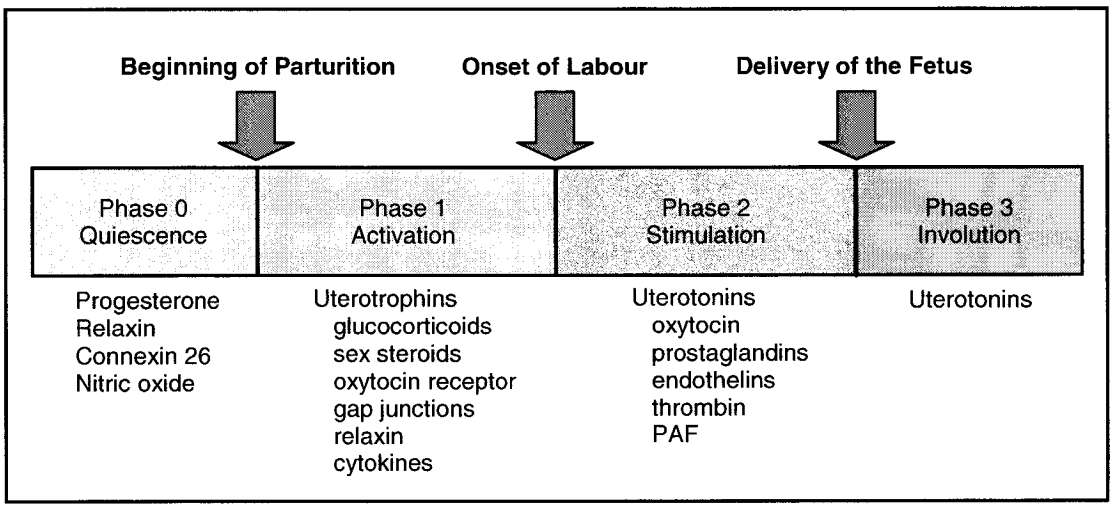


Figure 1.1 Distinct phases of pregnancy. During pregnancy, uterine function can be divided into four distinct phases. The longest period is gestation, phase 0 where the uterus grows to accommodate the developing fetus but is otherwise in a state of quiescence. During phase 1, the uterus enters into an activation state, priming for the process of labour. Phase 2 is the period when uterine contractions are stimulated by uterotonic factors resulting in the delivery of the fetus. Phase 3 involves the remodelling of the uterus. Uterotonins coordinate the involution of the uterus to a non-pregnant, receptive status and the process may begin again. Adapted from [7].

1.2.1 PREGNANCY

1.2.1.1 Ovulation, Fertilization, and Implantation

The establishment of pregnancy requires the successful sequential cascade of events initiated by ovulation followed by fertilization and subsequent migration of the embryo to the implantation site and depends on synchronicity between uterine receptivity and embryonic maturation for the attachment of the blastocyst.

The estrus (ovulatory) cycle proceeds from proestrus to estrus to metestrus (-1, followed by metestrus-2 in mice [8]) to diestrus and returns to proestrus. Proestrus is accompanied by increased growth of ovarian follicles and their production of high levels of estrogen. This peak in estrogen provokes a luteinizing hormone (LH) surge

from the anterior pituitary that induces the rupture and release of an oocyte [9]. Ovulation occurs at the estrus phase of the menstrual cycle while estrogen and progesterone levels are low. An artificially increased progesterone concentration by subcutaneous implants blocks ovulation and the estrus cycle [10]. The residual follicle transforms into the corpus luteum but in the absence of pregnancy, is degenerated.

With mating, sperm transport is facilitated by uterine and Fallopian tube contractions, increasing the chance of fertilization [8]. Successful fertilization of the ovum initiates embryogenesis.

The uterine endometrium, primed in response to increasing progesterone output from the corpus luteum of pregnancy, undergoes dramatic remodelling associated with cell proliferation and differentiation. Estrogen transforms the primed uterus to a receptive state during a period referred to as the "window of implantation" while its metabolite catecholestrogen activates an implantation-competent state in the blastocyst [11]. In women, this window of opportunity is approximately from day 20 to day 24 of the menstrual cycle (day 6-10 post ovulation) [12, 13] and in mice from day 3 to day 5 post coitum [14, 15]. The process begins with the attachment of the blastocyst to the luminal epithelium followed by invasion of the endometrial stroma. The endometrium becomes decidualized in response to the presence of the embryo, collaborating in the anchoring of the conceptus and the institution of a placental-maternal blood flow necessary for proper fetal development. The uterine environment, in return, receives cues to suppress an immune rejection against paternal genome-derived fetal antigens.

In an immunotolerant state, the uterus provides the nutrition and protection of the developing fetus. This tolerance is maintained through increased production of progesterone, which is capable of suppressing an immune response. Fetal antigens crossing the placenta can also induce a state of T cell anergy (non-responsiveness) [16]. The balance between pro-inflammatory T helper cell type 1 (T_h1) cytokines and anti-inflammatory T_h2 cytokines favours a T_h2 environment. The T_h2 cell is responsible for phagocytosis- and inflammation-independent host defense, and is characterized as central for tolerance of the developing fetus [17]. Interleukin (IL)-4,

IL-5, IL-10, and transforming growth factor β (TGFβ) are examples of the type 2 cytokines and are antagonistic to the production of T_h1 cytokines [16]. Significantly diminished production of macrophage-colony stimulating factor (M-Csf) by decidual T_h2 cells has been associated with recurrent abortion [17]. Throughout much of gestation, and to ensure its continuance, a T_h2 cytokine environment dominates and fetal acceptance is ensured.

1.2.1.2 Uterine Growth and Quiescence during Gestation (phase 0)

Myometrial Contractions

During gestation, a state of uterine quiescence is promoted through maternal-fetal tolerance and the maintenance of myometrial relaxation. The suppression of myometrial contractions during this period is paramount to complete fetal development. When development is complete, the fetus triggers a switch from a relaxatory state to that of myometrial contractility [18, 19]. Premature activation of uterine contractions leads to the early expulsion of the fetus resulting in either spontaneous abortion or premature delivery of a compromised offspring. Premature labour accounts for the majority of neonatal deaths.

Myometrial contractions, at both term and preterm, depend on conformational changes in muscle filament fibres actin and myosin. Phosphorylation of the actin-bound heads of myosin light chains causes the heads to bend, promoting a contraction by pulling actin (thin) filaments over myosin (thick) filaments, shortening the length of the muscle. This phosphorylation of myosin light chain II (MLC-II) is provided by myosin light chain kinase (MLCK). Myosin phosphorylation is essential to uterine force production. Alternate contractile pathways do not function in the myometrium [20]. MLCK is activated by binding of a calcium (Ca^{2+})/calmodulin-complex that induces a conformational change in the enzyme allowing it to phosphorylate myosin light chains. Calmodulin activity is dependent upon its binding of 4 Ca^{2+} ions. Factors that precipitate a net flux of Ca^{2+} into the cell promote uterine contraction. Uterotonin signalling through G protein coupled receptors (GPCRs) linked to G

protein $_{\alpha q}$ ($G_{\alpha q}$) subunits signal to promote inositol triphosphate (IP_3)-induced opening of Ca^{2+} channels. See Figure 1.2.

Uterine quiescence is mediated via factors that promote uterine cell signalling through second messengers cyclic adenosine monophosphate (cAMP) (including GPCR signalling via $G_{\alpha s}$ subunits) and cyclic guanosine monophosphate (cGMP) to prevent Ca^{2+} influx and subsequent phosphorylation of myosin light chain kinase necessary for smooth muscle contractions [19]. Up-regulation of protein kinase A (PKA) prevents the phosphorylation of MLC-II even in the presence of increasing intracellular Ca^{2+} levels. The events necessary for myometrial relaxation and contractility are outlined in reviews by Sanborn [21] and by Challis et al. [19].

In addition to these events necessary to prevent MLC-II phosphorylation, myometrial relaxation also relies on factors that promote the growth of the uterus in concert with that of the fetus. Increased uterine stretch in response to a growing conceptus in a no longer adapting environment leads to a stretch response which facilitates the induction of genes important for uterine activation and subsequent contraction, including the contraction associated protein (CAP) genes. These genes also rely on activation by estrogen. In the progesterone dominant environment maintained throughout much of pregnancy, these effects are not elicited and uterine quiescence is maintained.

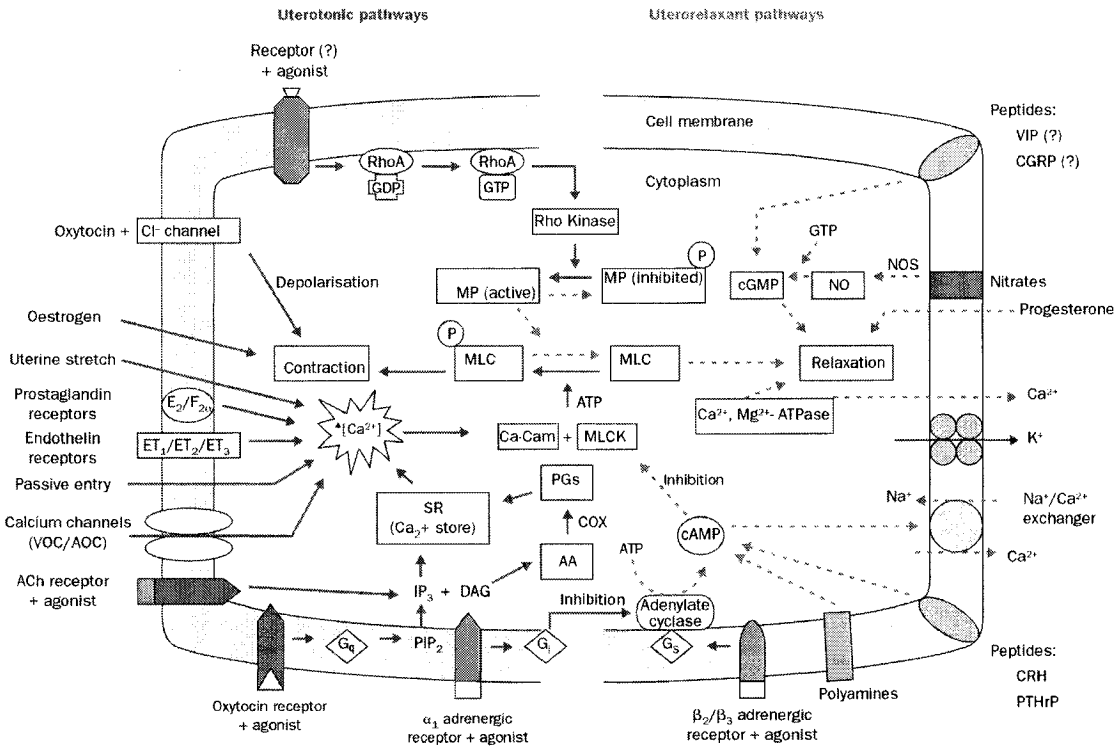


Figure 1.2 Major physiological pathways mediating myometrial contraction and relaxation. *Uterotonic pathways* (solid red arrows): Myometrial contraction and relaxation result from the phosphorylation or dephosphorylation of myosin light chains (MLC), respectively. Phosphorylation, by the enzyme myosin light chain kinase (MLCK), in the presence of adenosine triphosphate (ATP), is regulated by intracellular calcium concentrations ($[Ca^{2+}]_i$), in conjunction with the intermediate protein calmodulin (Cam), which together form the calcium-calmodulin (Ca-Cam) complex. Calcium channels (voltage and agonist operated channels (VOC/AOC), membrane endothelin (ET) receptors (ET_1, ET_2, ET_3), passive entry, membrane prostaglandin receptors (E_2, $F_{2\alpha}$) and stretch, all facilitate an increase in intracellular Ca^{2+} concentration ($\uparrow[Ca^{2+}]_i$) and result in smooth muscle contraction. Agonist-mediated activation of membrane acetylcholine (Ach) and oxytocin receptors stimulates the production of second messenger D-myoinositol 1,4,5-tiphosphate (IP_3), the latter through the action of the enzyme phosphoinositidase C (coupled to the oxytocin receptor by a stimulatory G-protein [G_q]), on the plasma membrane constituent phosphatidyl-inositol 4,5-bisphosphate (PIP_2). IP_3 releases Ca^{2+} from the sarcoplamic reticulum (SR) thus increasing $[Ca^{2+}]_i$ and resulting in cell contraction. A byproduct of IP_3 synthesis, the second messenger diacylglycerol (DAG) might promote cell contraction via intracellular prostaglandin synthesis from arachidonic acid

(AA) by cyclooxygenase (COX) enzymes. The steroid hormone oestrogen promotes cellular contractility by up-regulating COX enzymes, particularly the COX-2 isoform. The active isoform of myosin phosphatase (MP) dephosphorylates MLC, promoting cell relaxation. Receptor-agonist binding and the formation or upregulation of intracellular RhoA or Rho kinase could result in a shift in the equilibrium of intracellular MP in the direction of the inactive isoform, resulting in enhanced cell contraction -ie. calcium sensitization. Agonist binding of the $\alpha 1$ adrenergic receptor stimulates inhibitor G-proteins (G_i), which inactivate the adenylate cyclase mediated production of cAMP from ATP. cAMP results in cell relaxation in many ways, including inhibition of MLCK and the efflux of $[Ca^{2+}]_i$ through sodium/calcium (Na^+/ Ca^{2+}) exchanger channels. Chloride (Cl^-) channels, which might be activated by oxytocin, exert their uterotonic effect by depolarisation of the smooth muscle cell membrane.

Uterorelaxant pathways (broken blue arrows): Activation of beta-2 (β_2) and β_3 adrenergic receptors increases intracellular cAMP via G_s-mediated activation of adenylate cyclase, resulting in cell relaxation. Endogenous or exogenous nitrates are converted to nitric oxide (NO) within myometrial cells by nitric oxide synthase (NOS). The action of NO is mediated by activation of soluble guanylate cyclase, which in the presence of guanosine triphosphate (GTP) produces cyclic guanosine monophosphate (cGMP). cGMP activates protein kinases, ultimately leading to smooth muscle relaxation. Vasoactive intestinal peptide (VIP) and calcitonin gene-related peptide (CGRP) exert uterorelaxant effects by increasing concentrations of cGMP. Polyamines act via calcium antagonism, with the peptides corticotrophin-releasing hormone (CRH) and parathyroid hormone-related peptide (PTHrP) promote cell relaxation by increasing concentrations of cAMP. Cytosolic calcium-ATPase and magnesium-ATPase enzymes exert a tocolytic effect by decreasing $[Ca^{2+}]_i$. Reproduced with permission from Slatterly et al. with modifications [1]).

Progesterone

For many mammals, progesterone is produced in vast quantities by the corpus luteum during gestation. In humans, the placenta is the predominant source. Interaction with its nuclear progesterone receptor isoform B (PR-B) [22, 23], enables the complex to interact with specific gene promoter elements thereby allowing progesterone to modulate the expression of responsive genes, acting as a trophic factor to promote uterine growth. This genomic effect of this interaction on uterine smooth muscle relaxation is at numerous levels. It includes the down-regulation of estrogen receptor (ER) gene expression thereby reducing the responsiveness of the tissue to increasing ovarian synthesis of estrogen throughout gestation. Estrogen also stimulates DNA synthesis and cell proliferation. It acts specifically in the uterine luminal and glandular epithelia [24, 25] and not the endometrial stromal or myometrium [26] whereas progesterone supports this stromal and myometrial growth. Like progesterone, estrogen is capable of activating genes responsible for modulating myometrial contractility however, yet unlike progesterone, it prepares the uterus to respond to stimulation by uterotonins thereby facilitating contractions. Progesterone mediated down-regulation of ER expression during pregnancy maintains quiescence until labour. Progesterone also influences the expression and function of genes important for maintaining uterine relaxation.

Relaxin, Connexin 26, and Nitric Oxide

Relaxin is a peptide hormone secreted by the corpus luteum of pregnancy, decidua, and trophoblast [27, 28]. It functions to induce hypertrophy and DNA synthesis and cell division, promoting uterine growth during pregnancy and hence supporting myometrial relaxation. These actions are augmented by both progesterone and estrogen [29] and mediated by insulin-like growth factor (IGF) 1 and 2 and IGF binding protein (IGFBP)2 and IGFBP3 [28]. IGF1 acts as a mitogen induced during the early phase of pregnancy while IGF2 promotes uterine hyperplasia during the later stages of gestation. IGFBP2 and IGFBP3 are induced by relaxin and have been characterized as inhibitors of IGF-induced mitogenesis [30] and follow an expression

pattern similar to IGF2. The IGF-IGFBP system, supported by relaxin-induced synthesis and secretion of the peptides from decidual cells [28] promotes cell division during early gestation and hypertrophy with advancing pregnancy.

Relaxin, in concert with progesterone and estrogen, also influences uterine growth by increasing cell-cell communication in the myometrium and endometrium promoting the exchange of growth factors between cells [31]. This is facilitated through relaxin-induced up-regulation of connexin gene expression. Connexins are protein subunits of gap junctions. They assemble as hexamers arranged symmetrically around an aqueous pore in the plasma membrane forming a hemichannel. When hemichannels from adjacent cells connect, they form a gap junction, an intercellular conduit allowing direct communication between the cytoplasms of the cells. Gap junctions allow the passage of ions, such as Ca^{2+}, and small molecules, and propagate electrical impulses between cells allowing an improved coordination of smooth muscle cell contractions. Connexin 26 (Cx26) and connexin 43 (Cx43) are the predominant gap junction proteins expressed in the pregnant uterus. Their expression patterns within the uterus are spatially and temporally distinct. Cx26 is localized primarily in epithelial cells of the endometrium and the myometrium of the lower segment of the pregnant uterus [32, 33] and is implicated in facilitating uterine growth and maintaining uterine quiescence.

The Cx26 mRNA levels in rat uterus has been shown to increase around day 17 of pregnancy, reach its highest expression level during the last stages of pregnancy and then fall back to low expression prior to the initiation of active labour [33, 34]. The pattern of expression closely follows the level of progesterone in the uterus during gestation and labour. It was shown to be positively regulated by progesterone by Orsino et al. as progesterone administration to rats ovariectomized at day 17 of pregnancy removed the block to Cx26 gene expression and that progesterone administration prior to the onset of parturition prevented the typical decrease in Cx26 gene expression [33]. The expression of the Cx26 gene is also positively affected by increasing estrogen levels but is not further influenced by artificially increased progesterone levels beyond that found at term [35]. Cx26 gene expression is

described as highly sensitive to changes in estrogen levels as a relatively small increase in estrogen towards the end of gestation resulted in a quick increase in Cx26 transcript levels [35]. A further increase in estrogen vs. progesterone at the end of pregnancy does not induce further Cx26 gene expression and with the drop in progesterone preceding labour, so drops Cx26 gene transcription. This down-regulation at the time of labour may be an important switch in the activation of the quiescent uterus.

Relaxin also promotes a relaxed uterine state by inducing the expression of endogenous NO synthase (eNOS) and subsequently increasing endogenous nitric oxide (NO) levels in the endometrial luminal epithelium and longitudinal myometrium [27]. The relaxatory effect of NO is mediated by guanylate cyclase and guanosine triphosphate (GTP) to increase production of cGMP. This cGMP activates protein kinases that inhibit contractile signalling pathways and promote relaxatory mechanisms [21]. NO also suppresses the myometrial expression of Cx43 [21]. Cx43, a gap junction protein important for electrical coupling of uterine cells for the propagation of coordinated contractions during parturition, is classified as a contraction associated protein (CAP). In this manner, NO may effectively restrict relaxin induction of gap junction gene expression to Cx26 during gestation. Additionally, progesterone affects Cx43 gap junction formation by suppressing Cx43 gene transcription and protein trafficking through the Golgi apparatus [36]. The Cx43 gene is induced by increased amounts of estrogen and by stretch in activation of the uterus for parturition and will be discussed in greater detail subsequently.

Proportionate uterine growth, a blinding of the maternal immune system to the foreign tissue, and the prevention of coordinated uterine contractions throughout gestation are imperative to the maintenance of gestation and support of fetal development. The loss of even one of these can initiate the mechanism of labour, whether at term or, to the detriment of the offspring, prematurely.

1.2.2 LABOUR

1.2.2.1 Normal Labour: Activation (phase 1)

A withdrawal of progesterone is essential to the initiation of labour, whether by decreased production due to luteolysis as in most mammals or by rendering it ineffective by an induction of progesterone receptor (PR)-A (transcriptional repressor and competitor of PR-B) at the onset as found with humans [22, 23]. This withdrawal leads to a commensurate reduction of uterine growth and quiescence. At this point, numerous factors involved in activating the uterus to a responsive state and stimulation of the activated uterus are free to exert their functions.

Estrogen

An increasing level of circulating maternal estrogen is observed late in gestation. This is partially in response to placental CRH increasing fetal liver production of dehydroepiandrosterone sulphate (DHEAS) and its conversion to estrogen in the placenta, and partially due to increased maternal adrenal steroidogenesis [6, 19]. With the loss of progesterone, the repression of estrogen receptor gene expression is lifted and estrogen effects are felt. Estrogen promotes a decline in progesterone-supported plasma-bound PKA, which mediates cAMP signalling; its loss leads to Ca^{2+} influx and contraction [37]. Estrogen also supports a contractile uterus through induction of responsive genes.

cFos

The transcription factor cFos is a product of immediate-early gene expression. In the uterus, cFos expression is stimulated by a shift in balance from a progesterone dominant environment, maintained throughout pregnancy, to one in which estrogen is the prevailing force [25, 38]. The expression of cFos has been demonstrated in both myometrial and endometrial cell types in human and rat [24, 39, 40] but is specifically induced by estrogen in the endometrial and vascular endothelium of the mouse uterus only [26]. The effects of cFos expression in the myometrium of the rat and human may be provided to the mouse myometrium through the activation of other mediators.

The expression of cFos is also up-regulated by sheer force and intrauterine pressure. Mechanical stretch of the uterus caused by the ever-growing fetus has been demonstrated to activate mechanoreceptors of rat myometrial smooth muscle cells, with signalling events leading to cFos expression [41]. Heterodimerized with its partner cJun, cFos binds to the activator protein 1 (AP-1) promoter element, regulating the expression of responsive genes. With its up-regulation at the time of parturition, it induces the transcription of downstream affecters of uterine activation, particularly contraction associated proteins (CAPs).

Contraction Associated Proteins (CAPs)

Activation of the myometrium and stimulation of uterine contractions are necessary for the initiation and progression of labour. Activation of the parturient uterus arises as a result of significantly increased expression of contraction associated proteins (CAPs). CAPs include actin, myosin, Cx43, the OTR, and the receptor for prostaglandin (PG) $F_{2\alpha}$, FP. Stimulation of the contractions necessary for labour is dependent upon increases in agonists that interact with these CAPs, oxytocin and PGs. It is postulated that the expression of genes for these effectors are controlled by common transcriptional modulators. CAP mRNAs are at their highest levels at the time of normal labour but in the case of preterm labour, this maximal expression is found just prior to the event [42].

Cx43 and OTR gene expression are also induced by stretch under conditions of low progesterone [34, 43]. The gene for prostaglandin H_2 synthase-2 (a.k.a. cyclooxygenase -2 (COX-2)), which is responsible for increasing synthesis of PGs, is also up-regulated in the uterus by estrogen and by stretch [19]. Based on the expression profile of COX-2, Cook et al. suggest characterizing it as a CAP as well [42]. The exact mechanism of stretch-mediated increases in Cx43, OTR, and COX-2 gene expression requires further investigation.

Connexin 43

The promoter for Cx43 contains putative estrogen response element half palindromic sites [35, 44]. The shift in the progesterone:estrogen ratio at the end of

pregnancy alleviates the progesterone inhibition of estrogen-induced Cx43 gene expression and inhibition of Cx43 protein trafficking through the Golgi apparatus [36]. This results in increased levels of Cx43 mRNA and functional gap junctions.

Cx43 is expressed in both the endometrium and myometrium. In endometrial stroma, the Cx43 gene response to increased estrogen is slow, as Cx43 transcripts are not detected until 14 hours after estrogen treatment of ovariectomized rats [35]. In the myometrium at the onset of labour, clusters of Cx43 gap junctions increase in size and number. They are believed to participate in the initiation of labour by increasing the electrical conductivity of the myometrium, propagating synchronized contractions to pulse down through the uterus to expel the fetus [45]. In human, rat, and mouse the expression of Cx43 mRNA increases days prior to parturition reaching maximal levels at the time of labour and subsequently returning to low levels within a day or two postpartum [34, 42, 45].

Additional regulation of Cx43 is afforded by the activation of PKC. Stimulation of cultured human uterine smooth muscle cells with phorbol ester, a PKC activator, led to a transient increase in Cx43 gene expression by elevating transcription factor proteins [36, 46]. In human pregnant myometrium, levels of cJun, cFos, and Sp-1 were increased just prior to the onset of labour. These transcription factors interact with Ap-1 and specificity protein 1 (Sp-1) binding sites of the Cx-43 gene promoter [44, 47], further increasing its expression. As mentioned, the increase in myometrial Cx43 expression is facilitated by an increase in uterine stretch under conditions of increased estrogen and lowered effective progesterone.

Oxytocin Receptor

OTR gene expression is positively regulated by estrogen upon removal of the progesterone block to the expression of the ER. The human and rat OTR gene promoters contain non-classical estrogen response elements (ERE) in that only half palindromes are found [6, 48]. The mouse promoter includes four of these half-palindromes but also one complete ERE [49]. There are no progesterone responsive elements in the OTR gene promoter. Progesterone administration to non-pregnant and

pregnant rats decreases OTR mRNA levels and reversed the induction by estrogen treatment of ovariectomized, non-pregnant rats while progesterone receptor antagonist administration to late pregnant rats promoted a marked induction of OTR mRNA and precipitated premature parturition [50]. The mechanism of progesterone transcriptional repression is likely through modulation of uterine growth and the stretch responses. It is suggested that by an indirect, non-genomic action, progesterone interferes with OT binding and hence receptor signalling [51, 52].

The human OTR gene promoter contains a TATA-like motif, the rat and mouse promoters do not. While the TATA-box accurately indicates the transcription start site for a gene, this initiation may be provided by alternative sequences [49]. Additionally, the human and rat promoters contain Ap-1 sites while the mouse promoter does not. Once again, as cFos is induced by estrogen only in the endometrial and vascular endothelium of the mouse uterus [26] and not in the myometrium, as found for human and rat [24, 39, 40], this suggests cFos expression influences myometrial OTR gene expression via the Ap-1 site. It also suggests a possible alternative mechanism to cFos inducing endometrial OTR gene expression in humans and mice. If the rat promoter does not respond to this unique regulation in the endometrium, it would explain the lack of endometrial OTRs. This differential effect may indicate the presence of distinct, tissue specific OTRs. While variations of promoter elements suggest differential regulation of the OTR gene across different species and tissues, there are also numerous regulatory elements in common. For example, human, rat, and mouse OTR gene promoters each contain one or more acute phase response elements (APRE) and nuclear factor IL-6 (NF-IL6) binding sites indicating a regulation by inflammatory mediators.

In the non-gravid uterus, OTR gene expression and protein levels are high [53]. Larcher et al. described high levels of uterine OTR gene expression at proestrus, with this expression patterning that of estrogen. Increased gene expression at this time could support an OT-OTR interaction during mating which may play a role in fertilization by promoting sperm transport up the uterine horns. Levels of murine uterine OTR gene expression are significantly reduced by day 1 of pregnancy with

increasing progesterone and decreasing estrogen levels [49]. This repression is sustained throughout pregnancy, maintaining uterine quiescence. While uterine OTRs are suppressed during gestation, ovarian receptors are abundant [53]. Under low levels of circulating OT, these ovarian receptors contribute to maintaining the corpus luteum [53].

With advanced gestation and increasing OT concentrations, ovarian OTR levels decrease and uterine oxytocin receptor (OTR) gene expression and protein content increase thereby heightening uterine sensitivity to increasing levels of circulating oxytocin (OT). In this manner, a switch in OT target occurs. OTR gene expression achieves peak induction during labour. OTR levels in the myometrium increase dramatically in all mammals studied thus far, while humans and mice experience a moderate induction in the endometrial epithelium as well [54]. Interaction of OT with decidual OTRs promotes $PGF_{2\alpha}$ synthesis further promoting luteolysis and termination of pregnancy [6].

Even in the absence of OT, as demonstrated with OT knock-out mice, this dynamic regulation of OTR mRNA levels occurs [53]. This suggests an alternative ligand for this receptor and indicates that while OT may not be important for parturition [4, 5], receptor expression and function may be essential. An OTR knock-out mouse has not yet been generated leaving this question for future exploration.

Myometrial OTRs couple to the $G_{\alpha q}$ class of GTP binding proteins. Activation of the receptor leads to guanosine diphosphate (GDP) exchange for GTP by the $G_{\alpha q}$ subunit of the heterotrimeric G-protein and the dissociation of the α subunit from the $\beta\gamma$ dimer. This allows the dimer to activate membrane-bound phospholipase C_β and its production of IP_3 and DAG from PIP_2 (see Figure 1.2). IP_3 releases Ca^{2+} from intracellular stores in the SR. This increase in Ca^{2+} promotes an influx of Ca^{2+} from the extracellular space through channels in the plasma membrane. This increased Ca^{2+} induces the contractile mechanism for expulsion of the conceptus (see Figure 1.2). DAG stimulates PKC, an important substrate for mitogen-activated protein kinase (MAPK) signalling. This MAPK pathway promotes phospholipase A2 (PLA_2) and

COX-2 activity to increase PG synthesis [50], further promoting uterine contractions through prostaglandin receptor activation.

Prostaglandin Receptors

Prostaglandins (PGs) exert their action by binding to their GPCRs. Receptors for both $PGF_{2\alpha}$ (FP) and PGE_2 (EP1-4) are expressed in the myometrium. $PGF_{2\alpha}$ promotes myometrial contraction via the FP receptor, which is coupled to $G_{\alpha q}$ and IP_3 to increase intracellular signalling and uterine contraction. Of the 4 subtypes of PGE_2 receptors, EP1 signals via IP_3 to promote contraction, EP3 signalling inhibits the adenylate cyclase-cAMP uterorelaxant system, while EP2 and EP4 signal via cAMP to promote uterine relaxation [7]. These diverse receptor functions enable PGE_2 to exert various effects reflective of tissue specific or temporally regulated receptor expression [55]. In the rat uterus EP2 mRNA levels are high throughout pregnancy and experience a marked repression at the onset of labour. Uterine FP mRNA levels increase with gestation reaching maximal induction during parturition. The EP2 gene is induced by progesterone and unaffected by estrogen while the converse is true for FP gene expression, estrogen induces transcription and progesterone has no effect [56]. This temporal regulation of expressed receptors reflects the gestational state of the uterus.

In the ovary, EP2 binding is luteotrophic; EP2 knock-out mice have impaired ovulation and fertilization [57]. Receptors for maternal and fetal-derived prostaglandin $PGF_{2\alpha}$ are found in the corpus luteum facilitating luteolysis. In this way, the expression of each receptor gene promotes its further up-regulation. FP knock-out mice demonstrated successful ovulation, fertilization, and implantation however were unable to deliver their pups at term [58]. They did not respond to OT as induction of OTR gene expression was blocked. Ovariectomy, an artificially induced luteolysis, resulted in the loss of progesterone, an induction of OTR expression, and the restoration of parturition [58]. Initiation of labour clearly relies on luteolysis and PG activation of select receptors is important to this process. Once luteolysis is

accomplished, the oxytocinergic system is sufficient for activating and stimulating myometrial contractions and delivery of the offspring.

Cytokines

Throughout most of gestation, the uterus is receptive to the semi-allogeneic conceptus. An immunotolerant state exists under the influence of progesterone, which favours decidual T_h cell production of a T_h2 cytokine milieu and through this prevents the production of pro-inflammatory T_h1 cytokines. As normal parturition at term approaches there is a loss of T cell anergy, likely due to the drop in progesterone with luteolysis. T_h1 cytokines include IL-1α and β subtypes, TNFα, and IFNγ. IL-1 is also secreted by antigen presenting cells and endothelial cells. It supports a humoral response by promoting B cell maturation and clonal expansion. It also facilitates a cell-mediated response, acting as a chemoattractant for macrophages and neutrophils, increasing the expression of intercellular adhesion molecules on the vascular endothelium [59].

T_h1 cytokines, or suppressed T_h2 cytokine production, are permissive to placental production and passage of luteolytic and uterotonic factors PGs and CRH [19]. The loss of functional progesterone subsequently results in increased T_h1 cytokine production. Additionally, T_h1 cytokines lead to rejection and destruction of fetal tissues. The placenta is immunologically attacked and detached from the uterine wall. This leads to the loss of placenta-derived progesterone. Rupture of the chorion and amnion occurs through cytokine induction of matrix remodelling and apoptosis. The loss of these barriers allows fetal factors to participate in its own expulsion. Premature rupture of these fetal membranes is associated with preterm labour [60].

1.2.2.2 Uterine Stimulation (phase 2) and Involution (phase 3)

Once the uterus has been activated (phase 1), it becomes responsive to uterotonic factors. As a result of uterine stimulation by these factors (phase 2), a wave of uterine contractions ensues from the fundus through to the relaxed cervix, thrusting the fetus out of the uterus. Both fetal and maternal factors promote and sustain this

event. Subsequent to expulsion, maternal uterotonins persist. These continue to stimulate uterine contractions to encourage the remodelling of this organ to its compact, non-pregnant, receptive state and allow for subsequent pregnancy (phase 3).

Fetal Factors: Corticotrophin Releasing Hormone and Prostaglandins

Corticotrophin releasing hormone (CRH) is a product of both the maternal and fetal hypothalamic-pituitary-adrenal (HPA) axis. In human parturition, increasing placental levels of CRH acts as a "clock" to the onset of contractions. CRH levels reflect the development of the fetus and are prematurely increased by fetal stress [61]. Its synthesis is influenced by competing factors. Cortisone is converted to cortisol by 11β- hydroxysteroid dehydrogenase 1 (Hsd11b1). This cortisol is then able to promote CRH transcription. Cortisol also promotes COX-2 to increase PG synthesis that feeds forward to promote Hsd11b1 activity and further cortisol production. Cortisol directly, and indirectly through increased CRH, decreases prostaglandin dehydrogenase (PGDH) activity. This enzyme degrades PGs passing through the placenta, thereby inhibiting their influence on maternal tissues. CRH output from the placenta is increased by OT, T_h1 cytokines, and glucocorticoids to act as an uterotonin, stimulating myometrial contractions. Progesterone, produced from pregnenolone by 3β-hydroxysteroid dehydrogenase (Hsd3b1), as well as NO and T_h2 cytokines promote PGDH activity while inhibiting CRH production. Diminished functional progesterone with luteolysis and placental detachment alleviates this repression.

During much of gestation, low levels of maternal and fetal CRH interact with a myometrial receptor (CRH-R1) which signals through cAMP thereby participating in the maintenance of uterine quiescence. With uterine activation for parturition, a second receptor is expressed (CRH-R2) which signals through IP_3 and DAG to which high levels of CRH binds and thereby promote intracellular Ca^{2+} influx and promote uterine contractions. CRH-R1 expression is maintained in the lower uterine segment and in the cervix to allow the passage of the offspring. The regulation of CRH and its influences are outlined by Challis et al. [19] and Reis et al. [62].

It is important to note that while CRH in rodents is important for uterine vasculature relaxation to prevent high blood pressure and fetal stress due to hypoxia, it does not directly participate in initiation of labour [7, 63]. Placental prostaglandins have been shown to participate in the induction of luteolysis, thereby promoting uterine activation through CAP gene induction, and uterine stimulation by binding to myometrial receptors [64].

Maternal COX-2 Enzyme and its Prostaglandin Products

PG synthesis is accomplished first by the liberation of AA from cell membrane catalyzed by PLA_2 and conversion by COX. See Figure 1.3. Cytosolic PLA_2 is translocated from the cytoplasm to the cell membrane in response to increased intracellular Ca^{2+} where it mediates the release of AA. This AA is then metabolized to prostaglandins by the constitutively active COX-1 and the inducible COX-2 [65]. COX-2 gene expression is rapidly induced in response to mitogenic and pro-inflammatory stimuli in a tissue-/cell-dependent manner [7]. LPS, IL-1β, and TNFα induce COX-2 in the decidua; COX-1 expression is unaffected by pro-inflammatory cytokines. The promoter of the COX-2 gene contains regulatory elements [65] common to the OTR promoter indicating similar regulation and support for COX-2 classification as a CAP. As mentioned previously, COX-2 gene expression is also induced by shear stress [66].

COX-1 and COX-2 knock-out mice have been developed. COX-1 deficient mice experience normal fertility and while females experience a prolonged gestation, $PGF_{2\alpha}$ administration can induce parturition [57]. Maternal COX-1 emerges as indispensable and sufficient for murine parturition. COX-2 deficient mice, however, suffer complete infertility. Ovulation occurs but the egg cannot be fertilized. A lack of decidualization (even in the presence of transplanted wild-type embryos) prevents implantation [7]. Dual COX-1/COX-2 knock-out mice are unable to provide functional compensation and fare worse phenotypically than either single knock-out mouse. Surprisingly, dual COX-1 and OT knock-out mice restored timely parturition as luteolysis proceeded in the absence of OT [67]. This supports the necessity for the

constitutively expressed COX-1 to parturition and enforces a role for OT in the maintenance of the corpus luteum of pregnancy, binding to ovarian OTRs towards the end of gestation.

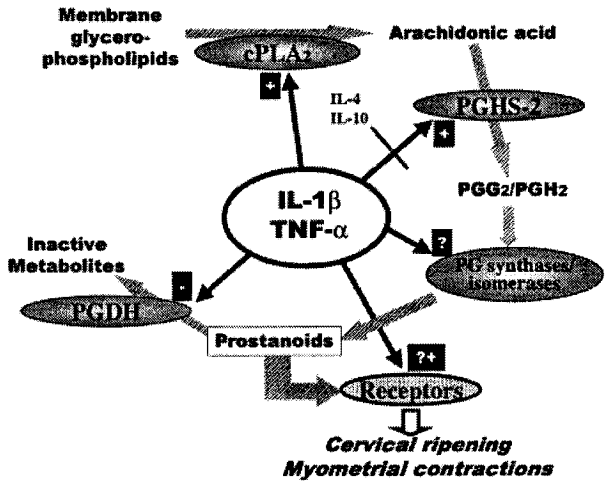


Figure 1.3 Coordinate regulation by cytokines of prostanoid biosynthesis and metabolism. $cPLA_2$, cytosolic phospholipase A2; PGHS-2, prostaglandin endoperoxide-H-synthase-2 (COX-2); PGDH, 15-hydroxyprostaglandin dehydrogenase. Reproduced with permission from Hansen et al. [65].

Investigation of $PGF_{2\alpha}$ receptor (FP) knock-out mice undergoing ovariectomy to induce a withdrawal of serum progesterone revealed a dramatic up-regulation of COX-2 gene expression in the myometrium and a decrease in endometrial COX-1 expression [68]. Progesterone administration prevented the changes in COX-2 gene expression while a COX-2 inhibitor was capable of delaying parturition in this model [68]. This study points to a critical function of COX-2 to the successful culmination of pregnancy.

The increased production of $PGF_{2\alpha}$ by the decidua promotes luteolysis at the end of pregnancy and myometrial production of both $PGF_{2\alpha}$ and PGE_2 stimulates their $G_{\alpha q}$- coupled receptors, promoting IP_3 turnover and increased intracellular Ca^{2+} levels necessary for MLCK phosphorylation and uterine smooth muscle contraction.

Oxytocin

Stimulation of uterine contraction is also accomplished by the binding of oxytocin to its myometrial receptor. Oxytocin is a cyclic nonapeptide hormone, produced in magnocellular neurons of the supraoptic nucleus (SON) and magnocellular and parvicellular neurons of the paraventricular nuclei of the hypothalamus (PVN). Parvicellular projections release OT into the median eminence of the pituitary where it can be transported to the anterior pituitary to affect hormone secretion. These projections also carry OT to different brain regions to elicit central effects. Via magnocellular neurons, OT is transported to the posterior pituitary (neurohypophysis) where it is released into the circulation [69]. It is also produced locally in numerous tissues where it can exert a paracrine effect. While OT is synthesized in the human and rat uterine epithelium with increasing levels prior to parturition [70], it is not expressed by murine uterine tissue [71]. Rat and human fetal tissues synthesize OT [19] and this may be true for mice as well.

Estrogen affects a rapid release of OT from the hypothalamus but with a less pronounced induction of transcription [70]. The expression of the OT gene in the uterus is positively modulated by estrogen as well as through competition between numerous enhancers and repressors still under investigation [6]. A synergistic stimulatory effect of estrogen and progesterone is proposed for regulation of uterine OT gene expression [72].

The phenomenal induction of myometrial OTR mRNA and protein during the activation of the uterus coincides with an increased synthesis of OT. Stimulation of the OTR and of uterine contraction leads to neuronal synaptic transmission to the solitary tract nucleus of the hindbrain. This is then relayed to the SON, provoking greater OT synthesis and release [73]. Sensory cues from the olfactory bulb feed into the SON as well, promoting this parturition-associated increase in pulsatile OT release [73]. In the endometrium/decidua, OT binding to its receptor induces $PGF_{2\alpha}$ synthesis while binding to myometrial OTRs provokes increased uterine contractility [6].

OT knock-out mice have been generated [4, 5]. Aside from an inability to eject milk for lactation and some social problems in males, there is no apparent

reproductive defect [74]. The OT/COX-1 double knock-out mice suggests a luteotrophic role in the ovary. Additionally, OT is suggested to promote uterine contraction through antagonism of CRH and inhibition of adenylate cyclase production [19].

1.2.3 MATERNAL and SOCIOSEXUAL BEHAVIOURS

In addition to provoking increased peripheral release of OT, vaginocervical stimulation during parturition elicits the expression and release of OT from parvicellular neurons of the PVN to induce remodelling of neuronal connections in the brain during parturition and lactation [75]. This rerouting of neural signals is central to the establishment of maternal behaviours and relies on the unique expression of the OTR. Species, sex, and hormonal status influence OTR gene expression in various, and often unique regions of the brain [76]. These differences give rise to varying maternal and sociosexual behaviours across individuals and species. Central OTR gene expression is induced by increasing estrogen levels at the end of pregnancy and in response to suckling early after parturition [77, 78]. The importance of OT appears to be the initial establishment of maternal behaviours and not for sustaining them.

Maternal behaviours towards offspring include scent recognition and attachment, licking and grooming, retrieval and protection, as well as nursing. These maternal behaviours provide additional neural patterning for the transmission of maternal success to subsequent generations [77].

OT is implicated in social behaviours such as affiliation, social memory, and inhibiting aggression and social avoidance [79]. Central OT in females increases sexual proceptive (soliciting) and receptive (lordosis) behaviours while in males, OT is a potent inducer of penile erection [6]. Elevated circulating levels are found during orgasm of both sexes increasing pair-bonding.

These maternal and sociosexual effects of OT encourage the propagation of the species through improved social dynamics and reproductive success.

The temporal and spatial coordination of the expression of genes central to the establishment, maintenance, and completion of pregnancy ensure the successful reproduction of the species. When one or more components become disregulated, the system may compensate or collapse. Loss of appropriate gene regulation during pregnancy can result in the early abortion of the conceptus or in the premature delivery of an underdeveloped, compromised offspring.

1.2.4 PREMATURE LABOUR

Approximately 10% of all deliveries are premature, occurring between 22 and 37 weeks gestation with an associated improved survival rate commensurate with gestational age [1]. The occurrence of preterm labour (PTL) may actually be increasing [80]. PTL accounts for 75% of all perinatal morbidity and 70% of infant deaths [81]. Factors that influence these rates include socio-economic status, ethnic origin (white women are experiencing a significantly increasing rate of PTL), increased multiple pregnancies due to assisted reproduction techniques, age of first pregnancy, numbers of pregnancies (parity), history of therapeutic or spontaneous abortion or previous PTL, and maternal infection [1].

1.2.4.1 Causes of Premature Delivery

Table 1.1 Causes of preterm delivery (from [1])

Cause	Frequency
Spontaneous preterm labour	31-50%
Multiple pregnancy and associated complications	12-28%
Preterm prelabour rupture of membranes (pPROM)	6-40%
Hypertensive disorders of pregnancy	12%
Intrauterine growth restriction	2-4%
Antepartum haemorrhage	6-9%
Miscellaneous – cervical incompetence, uterine malformation, etc.	8-9%

Preterm Premature Rupture of Fetal Membranes (pPROM)

Human fetal amnion and chorion membranes and the interstitial tissue provide a barrier between the maternal environment and that of the fetus. These membranes prevent undue stimulation of the uterus and maternal immune system by factors important for fetal maturation (i.e. CRH) and antigens derived from the paternal genome. These membranes also contain fluids, which buffer the fetus against its environment. The premature breakdown of these membranes leaves the fetus exposed and unduly stressed while allowing fetal factors to provoke uterine activation and contraction prematurely. Little is known about the factors initiating the breakdown of these membranes. It is suggested that they are present and exert their effects much earlier in gestation, making identification difficult by the time pPROM and PTL results. Relaxin gene expression in the decidua has been identified as inducing intramembrane angiogenesis and promoting the secretion of matrix metalloproteinases, which function in tissue breakdown and remodelling [60, 82-84]. Relaxin itself is capable of reducing membrane tensile strength by 30%. The further weakening of these membranes through matrix degradation leads to the rupture of fetal membranes under the stress of the growing fetus. Relaxin up-regulation in the decidua initiates pPROM in the absence of uterine infection. Intrauterine infection increases the incidence of pPROM, while membrane rupture predisposes to uterine and fetal infection [60].

Preeclampsia

Preeclampsia commonly presents as mild maternal hypertension and proteinuria and can quickly progress to a life-threatening state. Preeclampsia is the leading cause of maternal deterioration and death [85] and contributes to increased neonate morbidity and mortality as PTL is induced to prevent further deterioration of the mother and hence, the fetus [86]. The incidence of preeclampsia has increased quite dramatically over the last decade as a result of advanced maternal age and multiple uterine occupancy due to assisted reproductive techniques.

Vasoconstriction of fetal-maternal circulation and insufficient trophoblast invasion contribute to preeclampsia [85, 87]. Factors that are involved in vasoconstriction and dilation may provide markers for this disease. These include endothelins, and angiotensins as vasoconstrictors and vasoactive intestinal peptide (VIP), having vasodilatory effects [85]. It is interesting to note, referring to Figure 1.2, that endothelins promote uterotonic signalling pathways while VIP is involved in relaxation of the uterus. The drop in blood flow leads to toxicity of the fetus, increasing fetal stress and CRH production [19]. The culmination of increased stress due to vasoconstriction through increased endothelins and decreased VIP promotes the premature delivery of the fetus.

Preeclampsia is also associated with increased risk for intrauterine growth restriction [86], another cause of PTL.

Intrauterine growth restriction (IUGR)

A fetus experiencing intrauterine growth restriction (IUGR) is at risk for hypoxia and intrauterine death due to an unclear pathological change in the placenta [88]. There does appear to be an inflammatory response similar to that found for some women experiencing preeclampsia with most of the documented cases coinciding with malaria infection [89]. With IUGR maternal serum levels of insulin-like growth factor binding protein (IGFBP) 1 are increased and this may prevent insulin-like growth factor (IGF) 1 from passing through the placenta and promoting fetal growth. Fetal blood levels of IGF1 directly correlate with birth weight [88]. The contributions of vasoconstrictive substances to the hypoxic state of the fetus requires further investigation as does the pathological condition as a whole.

Intrauterine infection

Intrauterine infection (subclinical or otherwise) accounts for approx 50% of spontaneous preterm births. With low-grade infection, PTL is prevented as circulating factors mediate the immune response without provoking an inflammatory response. This insensitivity is provided by factors maintaining maternal acceptance of the fetal

graft and by the desensitization of the immune system to microbial constituents of the uterine normal flora. As a result, there is no tissue damage and hence no "sepsis-like" response leading to fetal rejection and expulsion. A bacterial invasion of significant numbers incites this inflammatory "sepsis" response through initial inflammation of the decidua followed by a local intrauterine inflammatory response [90, 91]. Detection is difficult as a mounting inflammation may be present but undetectable by normal bacterial culture methods and pro-inflammatory cytokine levels in amniotic fluid may be elevated (a predictor of inflammation-induced PTL) in women in the absence of an overt clinical infection [1]. This suggests fetal and/or chorioamnion infection may trigger PTL without a measurable evidence of infection.

Infection of the vagina and cervix (lower genital tract) usually results from bacterial vaginosis, an imbalance in the normal vaginal flora. Bacterial vaginosis is associated with ascending genital tract infection and a 2-fold increase in risk for PTL [80]. Upper vaginal tract infection involves bacterial overgrowth or heightened sensitivity in the decidua and placenta followed by bacterial invasion of fetal membranes, and the fetus itself. Colonization of fetal membranes accounts for a significant number of spontaneous preterm births and premature rupture of membranes [92]. Intrauterine infection may be present prior to conception and may result in a chronic low-grade inflammation that, over time mounts the necessary army to fight the infection and only at this later time triggers PTL [80].

The bacteria that cause the ascending intrauterine infection include *Escherichia coli*, *Pseudomonas*, and *Gonnococci*. Each of these is able to produce PLA_2 and PLC, liberating AA from the decidua and fetal membranes and increasing PG synthesis. This increase evokes luteolysis and uterine contractions (see Figure 1.4) [92]. A significant infection also induces a host inflammatory response.

Under the stimulus of sufficient invading bacterial infection, inflammatory cytokines are produced by activated macrophages and lymphocytes. The cytokine balance, so delicately regulated to favour a T_h2 cytokine milieu during implantation and throughout gestation is shifted, prematurely and dramatically, to a T_h1 environment activating a rejection response and prematurely expelling the fetus.

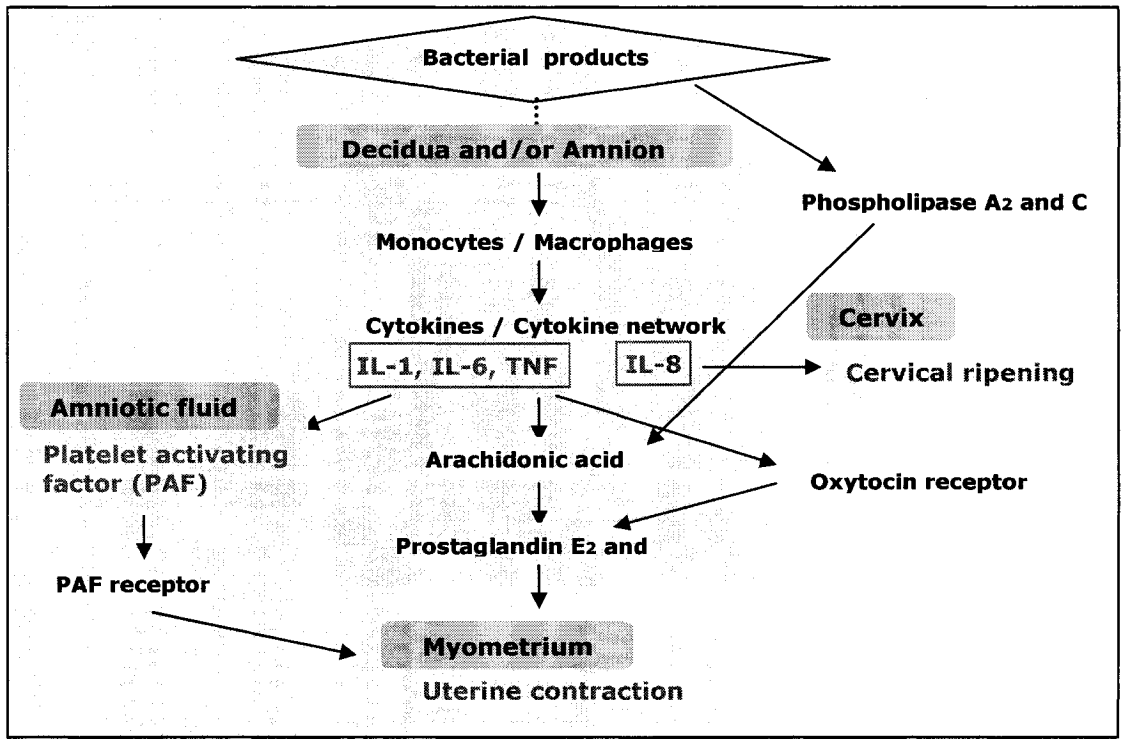


Figure 1.4 Hypothetical scheme of bacterial infection induced preterm labour. Reproduced with permission from Saji, et al. [92]).

Gram-negative bacteria have cell walls consisting of a plasma membrane covered by a thin peptidoglycan layer (unlike gram positive bacteria with its thick peptidoglycan layer), enclosed by a lipopolysaccharide (LPS) layer. The LPS molecule is extremely heat stable and is released in large quantities upon lysis of the bacterium. It imparts the toxicity attributed to pathogenic bacteria. It first binds to LPS-binding protein. It can be transported to a high-density lipoprotein (HDL), for neutralization of it toxic nature [93] during low-level infection, or with significant infection, LPS is transferred to its receptor CD14 on host macrophages thereby stimulating the production of pro-inflammatory cytokines [94]. During inflammation, maternal and fetal concentrations of IL-1, IL-6, TNFα, IL-8, macrophage inflammatory protein 1α (MIP1α), GRO, and platelet activating factor (PAF) are increased [92]. These repress the synthesis of progesterone while the stress of infection increases maternal and fetal cortisol production. Together these act to increase CRH and prostaglandin production in the placenta [19]. These uterotonic

factors interact with the ovary, supporting luteolysis, and with the uterus, activating it through induced CAP gene expression and stimulating myometrial contractions. PAF binds to myometrial receptors to further stimulate contractions while IL-8 is involved in cervical ripening [92]. The crescendo of this concert of gene products and interactions results in premature parturition, a deleterious event to mother and child.

1.2.4.2 Intervention

The identification of the factors involved in normal and premature labour has increased our understanding of the processes and genes involved. This has enabled the development of interventional therapies. Unfortunately, most diagnoses of conditions leading to PTL are made only after an advanced stage of pathology has manifested and intervention at this point is only minimally effective [95].

Therapeutic intervention of PTL predominantly sets out to inhibit or diminish the strength and frequency of uterine contractions to delay fetal expulsion, allowing further fetal development necessary to improve the prognosis upon delivery. The prophylactic use of the progesterone metabolite 17-α-hydroxyprogesterone caproate in women with a prior history for PTL showed, after weekly injections from mid gestation to delivery, a substantial reduction in recurrent PTL [96]. This is encouraging but addresses only those cases where a risk for PTL is established. In conditions of unexpected PTL, the use of tocolytic agents has been successful in temporarily delaying parturition. These agents are agonists of uterorelaxant pathways or antagonists of factors implicated in uterine contraction (see Figure 1.2). Agonists for β_2-adrenergic receptors, such as ritodrine and terbutaline, have been widely used to block PTL but are associated with severe adverse effects such as cardiac arrhythmia, pulmonary edema, and myocardial ischemia [95].

The development of new tocolytics has increased the options for management of PTL. The COX inhibitor, indomethacin, produces a suppression of uterine contraction lasting from 24 to 48 hours. Repeated administration is not indicated as extended treatment increases the risk to mother and child. Indomethacin crosses the placenta and is associated with severe fetal side effects such as the development of

oligohydramnios, leading to placental insufficiency and fetal demise [95]. The OTR antagonist, atosiban, has a similar duration of effectiveness with limited adverse maternal, fetal, or neonatal effects [1, 95]. Nifedipine, a Ca^{2+} channel blocker, again with short-term efficacy, is associated only with maternal hypotension [95]. It is recommended that atosiban and nifedipine should be administered before considering other established tocolytics [1].

The prophylactic use of antibiotics is controversial. Some reports caution against their use in the management of preterm labour as they do not prolong gestation and instead are associated with an increase in perinatal mortality [90]. Others, after meta-analysis of data from 14 trial studies of antibiotic treatment of women with PTL, suggest a small improvement in gestation length but data was insufficient to determine a positive effect on neonatal morbidity or mortality [97].

Unfortunately, current therapies are only moderate and short-lived successes. The available selection of candidate markers for screening and targets for intervention is somewhat exhausted and we must broaden our knowledge and understanding of the importance of additional contributors to the pathological state of premature labour. The use of microarray technology can expedite the discovery process.

1.3 MICROARRAY ANALYSIS

With the completion of whole genome sequencing for many organisms comes the identification of open reading frames and gene boundaries. This has enabled the sequences of transcribed genes and their mRNA to be documented and utilized. Microarray technology utilizes these known gene sequences and the trait of nucleic acid sequence complementarity to monitor changes in gene expression levels across physiological conditions. The only limits to the number of genes simultaneously monitored are the number of expressed sequences identified and the threshold of the application presenting these. What started out as an advancement to, and inversion of, Northern blot, with the probes for hundreds of known gene sequences linked to nylon membrane that was hybridized with the mRNA "target" of the sample of interest, has

escalated to the point where thousands of "probes" complementary to the entire transcriptome of a species can be printed on a glass matrix the size of a microscope slide [98]. Specialized microarray chips, having a particular collection of probes, can also be designed to meet the needs of the researcher. The terms microarray, array, and chip are used interchangeably when referring to the glass-based collection of probes. These chips monitor the changes in the transcriptome under various influences and in this way can provide insight into gene regulation, cellular function, and biochemical pathways [99].

There are two commonly used arrays: cDNA and oligonucleotide microarrays. The cDNA chips are usually made from polymerase chain reaction (PCR) products of the genes analysed covalently attached to the matrix (glass or nylon) in a defined location. These PCR-generated probes range in size from a few hundred base pairs to 2 kilobase pairs. Labelled targets are made by reverse transcription of the mRNAs to cDNAs and subsequent transcription to cRNAs using a fluorescently labelled ribonucleotide. This results in the theoretically proportionate amplification of the number of transcripts for a particular gene, which is tagged and ready for hybridization. The targets from two different biological samples are labelled with distinctly different fluorphors, Cy3-UTP (red) and Cy5-UTP (green) and hybridized simultaneously to one array [100]. Differences in transcript (target) hybridization are measured by the intensity and colour (red-orange-yellow-chartreuse-green) detected at the location of a known probe. The colour reflects the degree of similar gene expression in the two conditions analysed while the intensity reflects the number of transcripts hybridized to the probe. Interpretation of the differential gene expression between the samples is the next step. A disadvantage to cDNA is the relatively high background fluorescence (noise) and thus requires high levels of RNA (>10 μg) for adequate detection [98]. Also, the hybridization process must be repeated with the same samples labelled with the opposite tag to account for label discrepancies, adding another level of complexity and cost.

Oligonucleotide microarrays are synthesized based on nucleotide sequences available from a collection of databases and are much shorter than those for cDNA

arrays, usually in the range of 25-70 bases and they instead use several overlapping probes to represent a larger stretch of an expressed gene sequence [101]. Labelled targets are made in the same manner as for cDNA microarray however each biological sample is labelled with Cy5-UTP and hybridized to its own chip. In this way, each sample requires hybridization to one chip and assures a clear comparison between the transcriptomes analysed. The measure of fluorescence intensity reflects the level of expression of the gene represented at the particular location in response to the physiological condition analysed. This technology requires as little as 0.5 μg of starting RNA sample for accurate detection and only one microarray is required for each RNA sample to study.

The options available for analysis and interpretation of the data generated by microarray are diverse and numerous and unstandardized, leaving comparison between microarray output challenging, at best. Essentially, they follow a similar route. The fluorescence data measured off the chip is transformed and adjusted for background and control gene expression levels. Chip artefacts, array outliers, and probe outliers must be identified and removed from the remaining data to prevent a skew in the interpretation. Comparisons between samples, or between probe fluorescence values within a single set of chip data are made based on a predetermined significance criteria. Often a 2-fold ratio between the expression detected for a single gene under two different stimuli is deemed significant when matched with a probability (p value) of false reporting which is usually set at 5% ($p<0.05$), determined by a statistical test such as the Student's *t*-test [98].

Clustering data also provides insights into shared biological pathways and gene expression dynamics. The two components of clustering are (1) the "distance" or similarity between genes, and (2) an algorithm to identify groups of similar genes. This algorithm falls under two distinctions: hierarchical and partitioning methods. Hierarchical clustering refers to classification and organization of data identifying those genes closest to each other in expression and finding the next closest to the pair, and working outward from an arbitrarily determined starting gene, through successive iterations, until all genes have been assigned a relationship to all other genes [102].

The data are represented in the manner of a dendogram, similar to a phylogenetic tree. Partitioning, in contrast, divides the data into different clusters and then tries to minimize the "distance" within a cluster without trying to specify relationships between the data and maximize the "distance" between different clusters of data [98, 102]. The goal of clustering is to enable the identification of important gene expression patterns or subsets of genes involved in the biological phenomenon of interest.

Microarray technology and analysis greatly enhances the identification of the genetic participants involved in a biological process and the elucidation of the mechanisms of action of their products. Analysis of microarray data provides a wealth of novel candidates for further, individual, investigation using more traditional methodologies. These techniques include Northern blot and real-time relative quantitative RT-PCR for investigating gene expression levels and *in situ* hybridization to identify cell specificity of gene expression within a heterogeneous tissue. Promoter constructs and deletion mutants can provide detailed information as to the regulation of gene expression while immunohistochemistry can localize the protein, if indeed one is made. Immunoprecipitation and Western blot can elucidate protein-protein interactions positioning the gene product in a particular biological pathway. The development of transgenic and knock-out cells and organisms as well as the use of knock-down techniques such as the use of antisense RNA or small inhibitory RNA to interfere with the translation of a particular mRNA from a gene of interest can further elucidate the influence of a gene product. This is just a short list of techniques available to further characterize the structure, regulation, and function of a gene and its products identified as potentially important to a biological process through discovery by microarray analysis.

This thesis is a study of maternal factors influencing the outcome of normal labour and reproductive success utilizing both microarray and traditional analytical tools.

Chapter 2

Dynamic Changes in Gene Expression During Murine Parturition

Barbara R. Gould and Hans H. Zingg

Manuscript in Preparation

2.1 ABSTRACT

Our current understanding of the mechanism of labour is quite limited. As a result, the development of diagnostic, preventative, and therapeutic agents for the management of complicated and premature labour has been inadequate. To expand our knowledge of the genetic basis of the mechanism of normal labour we applied oligonucleotide microarray technology to identify novel genes, novel networks of known genes, and confirm those involved in the process of normal murine parturition. By comparing the levels of uterine gene expression at estrus, at term, at labour, and 12 hours postpartum and employing a strict selection criterion, we identified more than 300 genes and EST sequences involved in this process. The reproducible regulation patterns of known genes lent credence to the entire analysis. Further confidence was provided by performing relative quantitative real-time RT-PCR on selected transcripts. The greatest numbers of genes differentially regulated from estrus levels was observed at term suggesting the moment for successful intervention is in advance of apparent labour, increasing the importance of developing diagnostic methodologies.

Gene products involved in hormone biosynthesis and metabolism, immune regulation, as well as tissue growth and remodelling play a significant role in activating the quiescent uterus. Alpha fetoprotein and apolipoprotein A1 gene products are known to be involved in these functions and their significance at labour requires further investigation. Stimulation of the activated uterus is provided not only by oxytocin and prostaglandins but also by thrombin. The significance of the expression of many genes involved in blood clotting and fibrinolysis warrants further study. Fibrinogen alpha polypeptide gene is the most highly induced gene in the study reaching a 93-fold induction at term relative to its level of expression at estrus. Management of contractions may be provided by the novel uterine gene Purkinje cell protein 4 (PCP4). As a potential negative regulator of calcium/calmodulin-dependent uterine contractions, PCP4 gene induction, or agents promoting its protein stability or function may provide a therapy to maintain uterine quiescence.

We have identified novel targets for therapeutic intervention while providing numerous hypotheses for further investigation into the mechanism of normal murine labour, which may be extended to human parturition.

2.2 INTRODUCTION

Normal parturition is the culmination of pregnancy whereby the developmentally mature fetus is expelled from its uterine environment. The mechanism by which this phenomenon is achieved is contributed to by the interplay of fetal, placental, and maternal factors, which ultimately lead to cervical dilation, placental detachment, and coordinated uterine contractions. The exact nature of these contributions is of considerable interest to explain the occurrence of both premature labour, arising from the early activation of this cascade of events, and uterine dysfunction leading to difficult deliveries. Both of these aberrant conditions can lead to increased morbidity of the neonate, escalating the likelihood of early death or of physical or cognitive disabilities as a result. Although significant advances have been made over the last 40 years to better understand the process of parturition, the incidence of complications during pregnancy and premature delivery arising from them have not diminished. As found over the last decades, 8-10% of all pregnancies result in premature delivery, 75% of which result in neonatal morbidity and 70% of which result in death [81]. There has been limited success with current therapeutic management. These treatments, such as the use of the oxytocin receptor (OTR) antagonist atosiban, have arisen from the study of individual genes and their importance in the pathway to labour.

The process of normal labour requires the conversion of the uterus from a state of relative quiescence during gestation to one receptive to uterine stimulation by uterotonins. Factors important for maintaining the quiescent state of the uterus have been identified and characterized. These include the products of the relaxin and connexin 26 (Cx26) genes as well as progesterone, nitric oxide, and prostacyclin. Several gene products have been identified to be involved in the initial activation of the quiescent uterus allowing it to be responsive to uterotonic signals. They are referred to collectively as contraction-association proteins, CAPs. The OTR is a CAP, as is connexin 43 (Cx43), a gap junction protein responsible for communication between adjacent myometrial cells to coordinate the transmission of contraction signals. Prostaglandin F2α receptor (FP) is included in this group as it propagates the

uterotonic signal provided by its ligand. The effect of estrogen and uterine stretch on the regulation of these genes has also been investigated [34, 35, 43, 51, 56, 103]. Uterotonins stimulate the contractions necessary for delivery and for uterine involution postpartum. Again, many of these have been individually identified and characterized.

While significant advances have been made by studying these individual genes, their products and the network of products associated with them, little success has been achieved in the management of labour and its associated complications. We therefore undertook a broader investigation of the changes in gene expression during the progression of normal labour to identify novel participants in this process. Employing microarray technology allows the simultaneous determination of gene expression levels for thousands of genes. In this study, we utilized a murine model of parturition and the mouse genome oligonucleotide microarray U74Av2 from Affymetrix to reveal new participants in the process of labour, develop new hypotheses as to the nature of this process, and substantiate or refute previous beliefs. We hope to provide a foundation for further investigation of new candidates for both diagnosis and therapeutic management of preterm and otherwise complicated pregnancies.

2.3 MATERIALS AND METHODS

Animals

CD-1 female mice 8-12 weeks old were ordered from Charles River Laboratories, St. Constant, Quebec, to be mated overnight. Copulation was confirmed the next morning by the presence of a vaginal plug; 0.5 days post coitus (d.p.c.). Mice were caged separately. At 19 d.p.c. pregnant CD-1 mice deliver their pups. Animals were divided into two groups determined by the onset of parturition. Those animals in active labour (as determined by the presence of 1 or more pups in the cage) were identified as such (L). Their cohorts who had not yet entered into active labour were indicated as at term (T). When one mouse entered active labour, it was sacrificed and its uterus was removed; this was also done for one term pregnant mouse. An additional group of timed pregnant mice was allowed to proceed through parturition and then were sacrificed for their uteri approximately 12 hours postpartum (P). Mice in which fertilization was unsuccessful were used as non-pregnant controls and were evaluated for their stage in the estrus cycle. Staging was determined by microscopic inspection of collected vaginal smears. After 2 cycles had been completed, the uterine tissue was collected at the next estrus phase. This estrus (E) time-point is considered day 0 of pregnancy and provides a control for the time-course studied.

The mice were anesthetized using CO_2 and killed by exsanguination followed by cervical dislocation. All animals were maintained under a 12 hour light/dark cycle with access to food and water *ad libitum*. All animal experiments were carried out in accordance with the Bioethics Committee of the McGill University Health Centre. All efforts were made to minimize the number of animals used as well as their suffering.

RNA Extraction from Uteri

Animals were sacrificed at the described time-points. The uterus of each mouse was quickly removed and placed into a culture plate containing phosphate-buffered saline pH7.4 treated with diethylpyrocarbonate (Sigma-Aldrich, St. Louis, MO)(PBS-DEPC) kept on ice to minimize RNA degradation during preparation. Estrus sample tissue was rinsed quickly, cut in half and the halves frozen separately in

liquid nitrogen. Tissue from pregnant mice underwent additional manipulation. By carefully cutting around their borders, the placentas and decidual attachment sites were excised from the intact uterine tissue and discarded. Implantation sites where the placentas had detached (in the cases of delivered pups at labour and postpartum) were also dissected and discarded. Present embryos were removed along with their gestational sacs. The uterine tissue consisting of both myometrium and endometrium was rinsed in fresh cold PBS-DEPC, divided into two, and frozen in liquid nitrogen. Samples were stored at -70°C until RNA extraction was performed.

Total RNA was extracted from pools of uterine samples from 5 mice for each time-point using the TRIzol™ Reagent (Invitrogen, Burlington, ON) following manufacturer's instructions. RNA quantity and quality were determined by spectrophotometry at 260 nm and 280 nm absorbance and by formaldehyde-agarose gel electrophoresis. Sample concentrations were adjusted to 2 μg/μl. The RNA was stored at -70°C.

Affymetrix GeneChip Hybridization and Statistical Analysis

Total RNA samples (30 μg) were reverse-transcribed and the cDNA products were used as templates to synthesize biotin-labelled cRNA transcripts. The cRNA was fragmented to minimize target secondary structure and the quality of the cRNA was assessed by analysis with a test chip (Affymetrix, Santa Clara, CA). Hybridization of biotinylated cRNA fragments to the Affymetrix mouse genome array chip U74Av2 (version 2) followed. This array consists of 12,488 oligonucleotide targets, 66 of which are internal controls for the microarray experimental procedure. Additionally, some of the genes are represented by 2 or 3 different probe set pairs, consisting of perfect match sequences (PM) and mismatched sequences (MM) (for background correction), leaving ~12,000 unique gene probe sets to represent less than half of the predicted mouse genome.

Target cRNA that was specifically bound to the probe oligonuclotides of the mouse chip was detected using a streptavidin-phycoerythrin conjugate. The fluorescent signal was amplified using a biotinylated anti-streptavidin antibody and

was detected by confocal scanning [104]. All cRNA preparation, hybridization, and detection procedures were performed by the Montreal Genome Centre according to Affymetrix protocols. Fluorescence intensity data was provided to us after scaling and normalization with respect to the median intensity of each array for further analysis.

Each experimental time-point was repeated once (n=2) and a total of eight microarrays. Each replicate of four time-points was analysed as a separate experiment using the DNA-Chip Analyzer software (dChip) developed by Li and Wong [105, 106]. Affymetrix gene probe sets comprise 16 minimally overlapping 25 nucleotide long oligomers that are a perfect match (PM) to the sequence to be detected. A corresponding set of 16 oligomers, which include a mismatch (MM) at their 13^{th} nucleotide position, is used as a control for non-specific hybridization to these PM sequences. The PM and MM data for each probe set for an entire array and each array to be analyzed and compared was used by dChip to normalize all array data. Probe set outliers, array outliers, and array defects were viewed to assure satisfactory hybridization and detection of expressed genes. Outliers were excluded by the invariant set normalization procedure from further analysis. Model-based expression analysis was performed using the PM-only model [106]. Data generated included Affymetrix probe set ID, fluorescence intensity (normalized and modelled), a call describing the hybridization signal as present, marginal, or absent, and a standard error (SE) calculated by dChip based on the signal of those PM pixels which remain after outlier detection during normalization. Comparisons of gene expression, represented by signal intensity between arrays, were performed using the 2-tailed *t* test for significance using the Student's t test for independent samples and unequal variance according to the formula [107]:

$$t = \frac{\text{probe set mean gene}_i \text{ array}_1 - \text{probe set mean gene}_i \text{ array}_2}{\sqrt{(\text{SE gene}_i \text{ array}_1)^2 + (\text{SE gene}_i \text{ array}_2)^2}}$$

where the numerator indicates the difference between the mean fluorescence intensity of accepted PM probes for gene *i* on array 1 and on array 2, to which it is being compared. The denominator indicates the square root of the sum of the squared standard errors (SE) for gene *i* on the compared arrays and reflects the variance between the probe sets for gene *i*. Calculated *t* values allowed *p* values to be generated.

Gene lists were filtered to exclude those genes whose signal intensities were given absent calls in each array and to include only genes demonstrating a 2-fold or greater difference in expression between compared experiments with a statistically significant p value ≤ 0.05 in each replicate for a combined p value of ≤ 0.0025. Additionally, genes were eliminated from the list if the fold change in each replicate was not in the same direction. Gene expression comparisons were made between all four time-points. Expression values for genes passing the filtration criteria were averaged for each experimental point. The average values were used to organized gene expression patterns using GeneCluster software whereby the data were $\log_2$ transformed before normalization and median centering and a K-means clustering algorithm option was utilized [108]. The resultant list of cluster-organized genes was visualized using TreeView, companion software to GeneCluster [108]. Data lists were also organized by functional classification using Affymetrix annotation information.

Two Step Real-Time Relative Quantitative RT-PCR

Selected microarray quantitative results were corroborated by RT-PCR. Aliquots of extracted total RNA were treated with RNase-free DNase (Ambion, Inc, Austin TX) according to manufacturer's instructions. Samples were re-quantified and 10 µg of each sample were reverse-transcribed. First-strand cDNA was synthesized using random hexamers (Amersham Pharmacia Biotech, Baie d'Urfé, QC) and Moloney-Murine Leukemia Virus Reverse Transcriptase (Invitrogen). Reverse transcription (RT) was also performed without an RNA template as well as with RNA but lacking the reverse transcriptase and the preparations were used as negative controls.

RT products were diluted 1/50 for subsequent amplification and relative quantification by real-time PCR. PCR primer pairs are shown in Table 2.1. The expression of the housekeeping gene HPRT was used as an endogenous control as its expression showed minimal variation across the samples. Amplification of PCR products was achieved and detected by employing the FastStart SYBR Green reaction kit, the LightCycler thermal cycler and its software application (Roche Molecular Biochemicals, Laval, QC). The PCR reactions were optimized for high amplification profiles of specific products. Specificity was confirmed by LightCycler melting curve analysis and agarose gel electrophoresis.

To minimize error in estimation of PCR product concentration the "Calibrator Normalized Relative Quantification with PCR Efficiency Correction" method was performed using the Relative Quantification software (Roche). First, standard concentration curves were generated for each primer pair using an arbitrarily selected sample. Standard curve Crossing point (Cp) data reflecting PCR product concentration was generated by the second derivative maximum method of the LightCycler software application. The standard curve Cp values for each gene were exported to the Relative Quantification software and compared with the HPRT housekeeping standard curve. Coefficient (comparison) files were generated for each comparison using the Relative Quantification software.

Next, microarray samples were amplified by the parameters established during standard curve preparation for each of the genes of interest. A calibrator sample used to generate a point on the standard curve was included in each PCR run to reference the efficiency of amplification to that of the standard curve for that primer pair and to allow the Cp concentration values of each of the samples to be accurately determined for subsequent relative quantification. A PCR efficiency difference of > 0.2 between target (gene studied) and reference (housekeeping gene) can lead to a 10-fold or greater miscalculation of product concentration. An efficiency difference of > 0.05 is deemed unacceptable for concentration determination (Roche technical communication). Negative control samples were run with each experiment. Again, Cp data was generated and exported to the Relative Quantification software. Using

the Cp data from the genes studied and HPRT housekeeping gene amplification, and the correlated coefficient file previously generated containing the standard curves and efficiency correction, relative quantification of gene expression for each of the samples was performed by the software application. Arbitrary values of gene expression levels after housekeeping gene correction were generated. The data were plotted using Microsoft Excel software (Redmond, WA) and compared with plots of corresponding microarray data.

Animal and tissue preparation as well as Affymetrix microarray were performed in duplicate. Relative Quantification Real-Time PCR was performed in triplicate on each sample replicate subjected to microarray hybridization.

Table 2.1 Oligonucleotides for real-time RT-PCR.

Gene	Forward Primer	Reverse Primer	Product size (bp)
Afp	gcagaaacacatcgaggagag	tcatccctcagaaaactggtg	354
ApoA1	gtggctctggtcttcctgaca	tcctgtctcacccaatctgtttc	303
cFos	ctgtccgtctctagtgccaac	ctgctctactttgccccttct	237
Chap10	ggaagtttcttccgctctttg	gcctccatattctgggagaag	215
FbgA	cccttctgctctgatgatgact	ttatctcacggtttacagccct	421
HPRT	agtgttggatacaggccagac	atggccacaggactagaacac	232
PCP4	ggagataatgatgggcagaagaa	cagggtgtgtattgagtgaggatg	251
SAA3	catcttgatcctgggagttga	ttgagtcctctgctccatgtc	254

Afp, alpha fetoprotein; ApoA1, apolipoprotein A1; cFos, FBJ osteosarcoma oncogene; Chap10, chaperonin 10 (heat shock protein 10); FbgA, fibrinogen alpha polypeptide; HPRT, hypoxanthine guanine phosphoribosyl transferase; PCP4, Purkinje cell protein 4; SAA3, serum amyloid A3. Expected PCR amplicon length is given in base pairs (bp).

2.4 RESULTS

Microarray Analysis

Analysis of microarray data by our strict criterion resulted in the identification of 590 gene probe sets that reflected significantly changing gene expression levels between at least one of any two time-points in replicate 1 while in replicate 2 there were 719 probe sets which were indicated. The results of replicate 1 and replicate 2 were compared. First, probe sets that were unique to only one list were removed as non-reproducible. Next, replicate probe sets were removed if the fold change, *p* value, and number of absent calls assessments were not significant in both replicates for the same comparisons. A final filter was applied to remove probe sets if the significant fold changes for replicate 1 and replicate 2 were in opposite directions and this was the only significant time-point comparison. This left a final list of 342 probe sets that reflect a reproducibly significant gene expression change for one or more time-point comparison having a fold change value ≥ 2 or ≤ -2 and a combined *p* value of ≤ 0.0025. This represents 2.7% of the microarray probe sets. There are at least 22 genes that were represented by more than one probe set and 40 expressed sequences or named genes whose corresponding function is unknown at this time. The list of 342 probe sets represents a summary of genes most likely involved in the process of parturition but is by no means comprehensive.

The number of genes whose expression changed significantly was greatest at term, preceding the uterine contractions that are the characteristic indicator of labour. There were 192 probe sets representing genes significantly altered in their expression, 139 of which were up-regulated and 53 of which were down-regulated. The genes showing the greatest up-regulation included fibrinogen alpha and beta polypeptides (FbgA, FbgB); alpha fetoprotein (Afp); apolipoprotein A1 (ApoA1); coagulation factor II (a.k.a. prothrombin) (F2); insulin-like growth factor binding protein 2 (IGFBP2); 3β-hydroxysteroid dehydrogenase-1 (a.k.a. hydroxysteroid dehydrogenase-1, delta<5>-3-beta) (Hsd3b1); and cyclin-dependent kinase inhibitor 1C (Cdkn1c), all having expression levels more than 15-fold that found at estrus. Purkinje cell protein 4 (PCP4) and chloride channel calcium activated 3 (Clca3) genes both underwent a

repression of gene expression of more than 15-fold at term vs. estrus. At the time of labour, there were fewer genes regulated with 111 probe sets reflecting genes up-regulated and 30 sets representing genes experiencing a repression of transcription. The most significantly regulated expression levels were found for many of the genes most significantly regulated at term both for those up-regulated and for those down-regulated. Of the 80 probe sets that demonstrated an up-regulation in gene expression postpartum, IGFBP2 continued to be very significantly up-regulated (> 20-fold) in comparison with estrus levels. Matrix metalloproteinase 7 (MMP7) was more moderately up-regulated to levels 8.6-fold above that found at estrus. MMP7 is known as uterine metalloproteinase, playing a role in remodelling this tissue postpartum [109]. Additionally, many genes involved in the immune response were up-regulated at this time; cytotoxic T lymphocyte-associated protein 2 alpha (Ctla2a) and 2 beta (Ctla2b) with fold increases of 6.9 and 9, respectively. Again, as with term and labour, PCP4 and Clca3 showed the greatest repression in gene expression of the 43 probe sets displaying down-regulation at postpartum.

It is interesting to note when comparing term and labour that only two genes were significantly up-regulated as labour proceeded. The first of these genes was glucocortoid-regulated inflammatory prostaglandin G/H synthase, a.k.a. cyclooxygenase synthase -2 or COX-2. A fold change increase of 3.5 between labour and term, and a lack of up-regulation at term vs. estrus were demonstrated. This up-regulation was further increased postpartum. The second of these two up-regulated genes was the TNF receptor superfamily member 11b (Tnfrsf11b) (a.k.a. osteoprotegrin), which displayed a 2-fold increase from term to labour. It too was not significantly induced at term (vs. estrus). The gene that was down-regulated from term to labour was ATP-binding cassette sub-family B (MDR/TAP) member 1B (Abcb1b), which peaked at term and dropped 4.8-fold to levels at labour which were not statistically significant (vs. estrus).

Gene expression level changes between postpartum and its preceding time-points term and labour were expected. Versus term, there were 52 probe sets indicating genes showing a significant up-regulation and 97 probe sets identifying

genes exhibiting a down-regulation. Once again, COX-2 expression was up-regulated. Compared with gene expression levels at labour, there were less genes being significantly regulated as only 29 probe sets demonstrated an increase in gene expression and 49 showed a decrease. Insulin-like growth factor binding protein 5 (IGFBP5) was most significantly up-regulated compared to both term and labour levels and at postpartum Clca3 was being repressed to a lesser degree. Two probe sets for carboxypeptidase E (Cpe) showed significant gene up-regulation postpartum vs. both term and labour levels. Of the genes most greatly repressed, many were those initially up-regulated to very high levels at term, essentially switching off activation of genes important for parturition as it was completed. Table 2.2 summarizes the number of probe sets indicating changes in gene expression across the various time-point comparisons.

Table 2.2 Number of significant changes in gene expression between time-points.

	Term	**Labour**	**Postpartum**
Estrus	139, 53	111, 30	80, 43
Term		2, 1	52, 97
Labour			29, 49

The first number indicates the number of probe sets demonstrating an up-regulation from the time-point at the left of the row to the time-point at the head of the column. The second number indicates the number of probe sets demonstrating a down-regulation between the same time-points.

Cluster Analysis

Replicate array fluorescence values were averaged for each probe set and GeneCluster was used to organize the 342 probe sets based on their expression profiles. Log_2 transformed, median centred data were normalized and then clustered using the K-means algorithm [108]. The software utilized the data for all animal groups to assign the probe sets to one cluster or another. The genes were organized into nine clusters, which were depicted using TreeView software (Figure 2.1) [108]. Table 2.3 immediately follows Figure 2.1 and comprises the genes associated with

each cluster ordered by the magnitude of the fold change at the time point vs. estrus with the most numerous significant values for that cluster. The result of clustering was a clear portrait of the significant changes in gene expression and the various patterns those changes exhibit during the process leading into and out of parturition and perhaps most importantly, the moment of labour. By examining the probe set lists associated with each of the clusters, A to I, known patterns of expression can be seen and new ones are revealed. Clusters A to F contain genes whose expression increased from the time of estrus peaking at any of the other time-points. Genes in Cluster A showed an increase at term and at labour and a significant drop to near estrus level postpartum. An important representative is Cx43, which is represented by two probe sets. This gene exhibited its characteristic profile [34, 110], increasing in expression to a peak at labour and this is indicative of reliable microarray results. Cluster B contains genes that showed the highest up-regulation at term and labour from very low levels of expression at estrus. These levels were no longer significant postpartum. Genes for FbgA and FbgB polypeptides, Afp, ApoA1, Hsd3b1, and H19 mRNA were some of the most significantly up-regulated. Cluster C comprises genes that were more moderately expressed, peaking at term and then dropping quickly to previous levels. Apolipoproteins A4 and C1, as well as a second probe set for Hsd3b1 are among the most highly induced in this cluster. Cluster D includes genes that were moderately up-regulated at term and more slowly returned to estrus levels and includes integral membrane associated protein 1, 11β-hydroxysteroid dehydrogenase-1 (a.k.a. hydroxysteroid 11-beta dehydrogenase 1 (Hsd11b1), and serum amyloid A3 (SAA3). Cluster E genes demonstrated an increase throughout the end stages of pregnancy, sustaining these levels postpartum. IGFBP2 was most strongly regulated in this manner. Genes of Cluster F showed a peak expression postpartum and therefore are likely to be involved in the process of involution of the uterus and tissue remodelling. MMP7 had the greatest up-regulation in this group and as it is known to be involved in uterine tissue remodelling postpartum, provides support for this characterization of Cluster F genes. As well, COX-2 was significantly induced at labour to facilitate the PGE_2 and $PGF_{2\alpha}$ -induced uterine contractions. The level

continued to increase postpartum where contractions participate in returning the uterus to its non-pregnant form.

Clusters G to I contain genes whose expression decreased prior to and during the onset of parturition and while this encompasses fewer genes than those up-regulated at these time-points, it does not diminish their potential importance in the process. Cluster G shows genes that were down-regulated early and returned to estrus levels postpartum. IGFBP5 and chitinase 3-like 1 (a.k.a. glycoprotein 39) top this cluster in greatest gene expression changes between term and postpartum but more importantly is the down-regulation of liver arginase 1 (Arg1) and ceruloplasmin (a.k.a. ferroxidase) and others preceding the onset of labour which is then alleviated postpartum. Cluster H consists of genes that were for the most part, moderately down-regulated at all time-points vs. estrus levels with little variation from term to labour to postpartum. Only two genes stand out as strongly repressed across the late time-points. PCP4 and Clca3 genes exhibited the most significant decreases in gene expression vs. estrus levels. Also within this cluster is Cx26, which showed a maximal repression at the onset of labour, as expected [33, 34, 111], lending its support in validating the microarray data. Cluster I comprises genes that were significantly repressed particularly at the postpartum time. This gene cluster includes farnesyl diphosphate synthetase represented by two probe sets and squalene epoxidase, both of which are involved in cholesterol biosynthesis. Not surprisingly, actin alpha 1 skeletal muscle gene expression showed the greatest inhibition in this group. The postpartum period is a time for extracellular matrix and cytoskeletal breakdown as uterine remodelling proceeds.

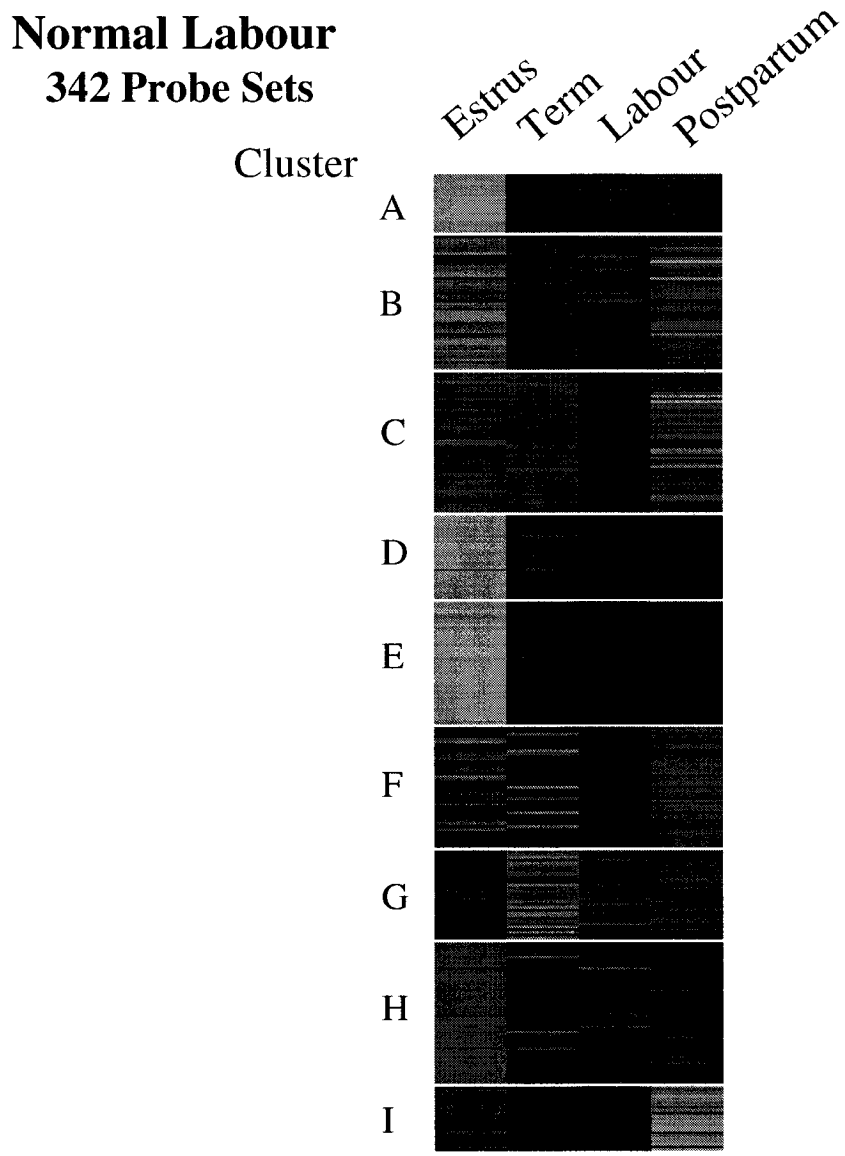


Figure 2.1 GeneCluster organization of probe sets representing significantly expressed genes.

K-means clustering of $\log_2$ transformed, normalized, and median centred average fluorescence data of 342 probe sets that were deemed to have statistically significant changes between one or more experimental time-points. The nine nodes are labelled Cluster A to I. Green colouring represents data that show a repression in gene expression with respect to the median value for the probe set. Red colouring represents data that show an induction of gene expression with respect to the median value for the probe set. The more intense the colour, the greater the deviation of the average expression value for that particular time-point from the median value for that particular probe set.

Cluster	GenBank	Title	T/E	L/E	P/E	L/T	P/L	P/T
A	M70642	connective tissue growth factor	7.22	14.28	2.63	---	-5.42	-2.74
A	AF028071	calbindin-D9K	9.30	11.16	---	---	---	---
A	U19118	activating transcription factor 3	---	7.54	---	---	---	---
A	AV037012	calbindin-D9K	6.69	6.80	---	---	---	---
A	U44027	prepronociceptin	---	6.52	---	---	---	---
A	X56304	tenascin C	---	5.98	3.01	---	---	---
A	AB007813	ficolin A	4.07	5.34	---	---	---	---
A	X61940	protein tyrosine phosphatase non-receptor type 16	3.15	4.77	---	---	-2.56	---
A	U94331	TNF receptor superfamily member 11b (osteoprotegerin)	---	4.38	2.45	2.08	---	---
A	AF117709	secreted frizzled-related sequence protein 4	3.63	4.33	---	---	---	---
A	M33960	serine proteinase inhibitor E1 (nexin plasminogen activator inhibitor 1)	---	4.09	---	---	---	---
A	AV230686	tenascin C	---	4.08	---	---	---	---
A	AW046181	serum/glucocorticoid regulated kinase	4.23	4.05	---	---	---	---
A	C85523	EST	2.88	2.87	---	---	---	---
A	M63801	gap junction membrane channel protein alpha 1 (connexin 43)	2.37	2.79	---	---	---	---
A	Y08361	reversion induced LIM gene	2.33	2.66	---	---	---	---
A	AW047643	EST	---	2.47	---	---	---	---
A	AA726364	lipoprotein lipase	---	2.40	---	---	---	---
A	M63801	gap junction membrane channel protein alpha 1 (connexin 43)	---	2.40	---	---	---	---
A	U04354	scinderin	2.26	2.35	---	---	---	---
A	M68898	mast cell protease 5	2.55	---	---	---	---	---
B	AI876446	fibrinogen alpha polypeptide	93.32	48.34	---	---	-31.35	-71.14
B	V00743	alpha fetoprotein	79.55	55.63	---	---	-50.34	-71.73
B	U79573	apolipoprotein A-I	43.21	29.21	---	---	-31.15	-46.62
B	X52308	coagulation factor II	40.12	23.99	---	---	-12.55	-28.78
B	AI196896	fibrinogen B beta polypeptide	31.16	18.85	---	---	-14.87	-29.49
B	M58567	hydroxysteroid dehydrogenase-1delta<5>-3-beta	24.92	11.99	---	---	-10.57	-18.38
B	X58196	H19 fetal liver mRNA	20.76	15.50	---	---	-9.80	-15.07
B	J04758	tryptophan hydroxylase	17.08	10.37	---	---	-9.73	-16.51
B	U22399	cyclin-dependent kinase inhibitor 1C (P57)	16.59	10.70	---	---	-5.38	-9.04
B	U63146	retinol binding protein 4 plasma	15.08	8.69	---	---	-5.41	-10.56
B	AI849587	voltage-dep calcium channel gamma subunit-like protein	10.05	5.95	---	---	-3.98	-7.20
B	V00722	hemoglobin beta adult minor chain	9.97	6.60	---	---	-5.42	-8.23
B	AI854771	EST: 92 % homologous to :SUI1_MOUSE Protein translation factor SUI1	9.65	6.73	---	---	-3.58	-5.32
B	AI786089	kininogen	9.52	5.86	---	---	-3.57	-5.47
B	J00413	hemoglobin beta adult major chain	7.29	5.29	---	---	-4.96	-6.95
B	U95182	guanylate cyclase activator 2b (retina)	7.14	---	---	---	---	-3.72
B	V00714	hemoglobin alpha adult chain 1	6.66	4.85	---	---	-3.92	-5.49
B	L39017	protein C receptor endothelial	6.59	---	---	---	---	-3.84
B	AI850953	RIKEN cDNA: Cl- ion pump-associated 55 kDa protein homolog (rat)	6.49	3.82	---	---	---	-4.09
B	AJ002390	annexin A8	5.83	3.70	---	---	---	-2.86

Cluster	GenBank	Title	T/E	L/E	P/E	L/T	P/L	P/T
B	X71922	insulin-like growth factor 2	5.63	---	---	---	---	-5.88
B	J05663	aldo-keto reductase family 1member B7	5.48	5.47	---	---	-3.62	-3.87
B	U15012	growth hormone receptor	5.24	3.81	---	---	-3.15	-4.45
B	AI836610	EST	5.13	4.02	---	---	-5.06	-6.67
B	X83569	neuronatin aka: Peg5	4.89	3.37	---	---	-2.70	-4.18
B	X66449	S100 calcium binding protein A6 (calcyclin)	4.02	3.35	---	---	-2.24	-2.69
B	AI842277	insulin-like growth factor binding protein 3	3.65	3.13	---	---	-3.15	-3.61
B	X95504	zinc finger protein regulator of apoptosis and cell cycle arrest	3.61	2.92	---	---	---	-2.97
B	AF036736	mRNA for immunoglobulin heavy chain mAb 667	3.28	---	---	---	---	-2.23
B	L33954	mRNA for immunoglobulin heavy chain mAb 667	3.17	---	---	---	---	-2.29
B	AA880988	extra cellular link domain-containing 1	3.14	---	---	---	---	---
B	AJ001633	annexin A3	2.59	2.22	---	---	---	---
B	AA939571	EST	2.43	---	---	---	---	---
B	X98471	epithelial membrane protein 1	2.37	---	---	---	-2.36	-2.70
B	AA871791	RIKEN cDNA: D20 protein homolog (human)	2.31	---	---	---	---	---
B	X06342	serine protease inhibitor Kazal type 3	---	11.64	---	---	-14.13	---
B	X81581	insulin-like growth factor binding protein 3	---	2.54	---	---	-2.52	-2.52
B	U83148	nuclear factor interleukin 3 regulated	---	2.47	---	---	---	---
B	AF031380	RIKEN cDNA: hypothetical Riboflavin kinase / FAD synthetase containing protein	---	---	---	---	---	-2.34
B	V00727	FBJ osteosarcoma oncogene (cFos)	---	3.92	---	---	-4.46	-2.68
B	AI503362	EST	---	2.91	---	---	-2.49	---
B	AW212475	RIKEN cDNA: Mitogen-inducible gene 6 protein homolog	---	---	---	---	-2.16	---
B	AF000236	chemokine orphan receptor 1	---	---	---	---	-3.28	-2.99
B	M32490	cysteine rich protein 61 aka: IFGBP10	---	2.93	---	---	-3.00	---
B	AI839150	RIKEN cDNA	---	---	---	---	-3.28	---
B	AF055638	growth arrest and DNA-damage-inducible 45 gamma	---	---	---	---	-3.17	-3.06
B	AB023957	ethanol induced gene product EIG180	---	---	---	---	-2.05	---
B	X55573	brain derived neurotrophic factor	---	2.16	---	---	-2.79	---
C	AI787317	EST	16.38	7.48	---	---	-6.25	-17.12
C	M64248	apolipoprotein A-IV	12.97	6.95	---	---	-7.20	-13.71
C	M60348	ATP-binding cassette sub-family B (MDR/TAP) member 1B	10.70	---	---	-4.82	---	---
C	AA895838	RIKEN cDNA	9.52	---	---	---	---	---
C	AV290268	hydroxysteroid dehydrogenase-1delta<5>-3-beta	8.95	---	---	---	---	-8.45
C	Z22661	apolipoprotein CI	7.97	---	---	---	---	-7.61
C	AA986050	EST: fibrinogen gamma polypeptide	7.02	3.46	---	---	-3.74	-7.94
C	AA790008	reduced expression 3	6.71	---	---	---	---	-6.45
C	AF026073	N-sulfotransferase	5.80	---	---	---	---	-5.15
C	AW061016	vitamin D receptor	5.52	---	---	---	---	-3.96
C	L24430	osteocalcin-related protein precursor (OC-X) (nephrocalcin)	5.34	2.76	---	---	-2.44	-4.93
C	AV378405	ectonucleoside triphosphate diphosphohydrolase 2	4.99	3.17	---	---	-2.96	-4.81
C	AF017994	mesoderm specific transcript formerly Peg1	4.66	---	---	---	---	-7.25

Cluster	GenBank	Title	T/E	L/E	P/E	L/T	P/L	P/T
C	X13060	albumin 1	4.60	---	---	---	---	-4.60
C	AV003378	hemoglobin beta adult major chain	4.59	3.03	---	---	-3.09	-4.66
C	AW121619	cytochrome P45011a cholesterol side chain cleavage	4.52	2.96	---	---	---	-4.27
C	D88792	cysteine-rich protein 2	4.38	---	---	---	-3.46	-5.37
C	AJ010338	mRNA for hypothetical protein	4.36	---	---	---	---	-4.14
C	M25529	serine protease inhibitor 1-2 aka:mouse alpha 1-antitrypsin	4.23	---	---	---	---	-4.63
C	X70393	inter-alpha trypsin inhibitor heavy chain 3	4.23	---	---	---	---	-3.69
C	AF032466	arginase type II	4.16	---	---	---	-2.48	-3.89
C	AI852838	maternally expressed 3	4.14	---	---	---	-3.88	-6.35
C	AF038939	paternally expressed 3	3.88	---	---	---	---	-3.95
C	M32484	placentae and embryos oncofetal gene	3.31	---	---	---	---	-3.63
C	AF009605	phosphoenolpyruvate carboxykinase 1 cytosolic	3.28	---	---	---	---	-3.51
C	AW049768	lipocalin 7	3.09	---	---	---	-2.33	-3.60
C	L29123	ferredoxin 1	2.91	---	---	---	---	-3.65
C	AW123955	DNA segment chr19 Wayne State University 57 expressed	2.88	---	---	---	---	-3.02
C	L11333	esterase 31	2.84	---	---	---	---	-3.04
C	AI843313	glypican 3	2.84	---	---	---	---	-2.56
C	L47600	troponin T2 cardiac	2.78	---	---	---	---	-3.27
C	AI846906	RIKEN cDNA	2.74	---	---	---	---	-2.46
C	AA655303	apolipoprotein M	2.72	---	---	---	---	-2.95
C	U25739	EST weakly similar to JC7182 Na+-dependent vitamin C (human)	2.59	---	---	---	---	-2.68
C	K02108	keratin complex 2 basic gene 6a	2.58	---	---	---	---	-2.59
C	AV303514	phosphatidyl inositol-4-phosphate 5-kinase type II gamma	2.55	---	---	---	---	-2.57
C	M55413	group specific component	2.36	---	---	---	---	-2.48
C	AB020741	Nik related kinase	2.29	---	---	---	---	-2.54
C	AB018421	cytochrome P4504a10	2.12	---	---	---	---	-2.63
C	U28419	eukaryotic translation initiation factor 1A	2.09	---	---	---	---	---
C	AA866768	EST	---	---	---	---	---	-3.54
C	AF009414	SRY-box containing gene 11	---	---	---	---	---	-2.87
C	U37799	scavenger receptor class B1	---	---	---	---	---	-2.15
C	AF084482	Wolfram syndrome 1 homolog (human)	---	---	---	---	---	-2.39
C	M20497	fatty acid binding protein 4 adipocyte	---	---	---	---	---	-2.95
C	AW123273	RIKEN cDNA	---	---	---	---	---	-2.10
C	U88327	cytokine inducible SH2-containing protein 2	---	---	---	---	---	-2.44
C	AW046694	RIKEN cDNA	---	---	---	---	---	-2.20
C	M15501	actin alpha cardiac	---	---	---	---	---	-2.67
C	X69620	inhibin beta-B	---	---	---	---	---	-2.53
D	AV059956	integral membrane-associated protein 1	36.18	18.27	---	---	---	-3.63
D	D88899	kidney-derived aspartic protease-like protein	14.00	12.83	6.90	---	---	---
D	X83202	hydroxysteroid 11-beta dehydrogenase 1	11.47	5.68	---	---	---	-4.32
D	X03505	serum amyloid A 3	10.32	5.94	3.77	---	---	-2.72

Cluster	GenBank	Title	T/E	L/E	P/E	L/T	P/L	P/T
D	U13705	glutathione peroxidase 3	7.04	6.55	5.07	---	---	---
D	AF051347	reduced expression 3	6.52	---	---	---	---	---
D	AW122893	RIKEN cDNA	6.36	4.13	3.03	---	---	-2.09
D	X94418	EST	5.95	4.19	---	---	---	---
D	X70922	lymphocyte antigen 6 complex locus F	4.68	3.94	---	---	---	-2.61
D	M80423	Mus castaneus IgK chain gene c-region 3' end	4.53	3.57	---	---	---	---
D	D86370	mesothelin	4.31	3.70	---	---	---	---
D	Y15163	Cbp/p300-interacting transactivator with Glu/Asp-rich C-term domain 2	4.26	3.94	2.20	---	---	---
D	U18869	disabled homolog 2 (Drosophila)	4.05	3.32	---	---	---	---
D	AI626942	EST	3.82	3.76	---	---	---	---
D	M73748	glycoprotein 38	3.15	2.95	---	---	---	---
D	U10410	recombinant antineuraminidase single chain Ig VH and VL domains	2.97	---	---	---	---	---
D	D31788	bone marrow stromal cell antigen 1	2.96	---	---	---	---	---
D	AI851255	cathepsin B	2.90	---	---	---	---	---
D	M63335	lipoprotein lipase	2.83	2.68	---	---	---	---
D	AJ001418	pyruvate dehydrogenase kinase 4	2.66	---	---	---	---	---
D	X06368	colony stimulating factor 1 receptor	2.64	2.30	---	---	---	---
D	V00835	metallothionein 1	2.63	2.27	---	---	---	---
D	X14961	fatty acid binding protein 3 muscle and heart	2.57	---	---	---	---	---
D	U03434	ATPase Cu++ transporting alpha polypeptide	2.27	---	---	---	---	---
D	AV376312	RIKEN cDNA: SPRY domain-containing SOCS box protein SSB-1 homolog (mouse)	2.27	---	---	---	---	---
D	AI596360	EST: SPRY domain-containing SOCS box protein SSB-1 homolog (mouse)	2.26	---	---	---	---	---
D	AI839138	thioredoxin interacting protein	2.26	---	---	---	---	---
D	AI746846	RIKEN cDNA	2.23	---	---	---	---	---
D	AF003348	Niemann Pick type C1	2.11	---	---	---	---	---
D	AW124633	NIMA (never in mitosis gene a)-related expressed kinase 7	2.09	---	---	---	---	---
E	X81580	insulin-like growth factor binding protein 2	27.22	30.36	20.88	---	---	---
E	X93037	extracellular proteinase inhibitor	6.94	12.62	17.46	---	---	---
E	X15592	cytotoxic T lymphocyte-associated protein 2 beta	12.24	14.15	8.99	---	---	---
E	AW124113	brain abundant membrane attached signal protein 1	7.29	8.65	7.76	---	---	---
E	D78265	olfactomedin 1	4.22	---	6.92	---	---	---
E	X15591	cytotoxic T lymphocyte-associated protein 2 alpha	7.56	7.21	6.91	---	---	---
E	M22810	kidney androgen regulated protein	8.86	8.84	6.52	---	---	---
E	AI852641	nuclear protein 1	4.49	5.97	5.49	---	---	---
E	X58861	complement component 1q subcomponent alpha polypeptide	---	4.43	5.14	---	---	---
E	Z80112	chemokine (C-X-C) receptor 4	---	2.85	4.98	---	---	---
E	M18237	immunoglobulin kappa chain variable 28 (V28)	4.33	3.20	4.74	---	---	---
E	M62470	thrombospondin 1	4.82	6.31	4.58	---	---	---
E	AV003873	DNA segment chr14 UCLA 3: 90% blast homology with Clusterin	---	3.11	4.39	---	---	---
E	M21050	lysozyme	2.87	2.91	4.39	---	---	---
E	D13664	osteoblast specific factor 2 (fasciclin I-like)	3.32	3.18	4.28	---	---	---

Cluster	GenBank	Title	T/E	L/E	P/E	L/T	P/L	P/T
E	D00466	apolipoprotein E	3.37	3.67	4.12	---	---	---
E	D14077	clusterin aka:apolipoprotein J	2.65	3.25	4.09	---	---	---
E	AI841076	EST: Highly similar to rat fasciculation and elongation protein zeta1	---	2.43	4.09	---	---	---
E	X51547	P lysozyme structural	3.14	3.12	4.05	---	---	---
E	AI840339	ribonuclease RNase A family 4	---	---	3.94	---	---	---
E	M22531	complement component 1q subcomponent beta polypeptide	---	2.86	3.76	---	---	---
E	AF035684	small inducible cytokine A21b (leucine)	---	2.37	3.73	---	---	---
E	X66295	complement component 1q subcomponent c polypeptide	---	2.93	3.63	---	---	---
E	AW227647	RIKEN cDNA	---	---	3.35	---	---	---
E	U28960	phospholipid transfer protein	---	2.33	3.04	---	---	---
E	AA612450	RIKEN cDNA	---	2.33	2.96	---	---	---
E	U49513	small inducible cytokine A9 aka:Macrophage inflammatory protein 1-gamma	2.73	3.13	2.87	---	---	---
E	M93275	adipose differentiation related protein	3.13	3.42	2.83	---	---	---
E	AI843884	EST	3.12	2.80	2.81	---	---	---
E	M58004	small inducible cytokine A6	---	2.44	2.77	---	---	---
E	X90778	histone gene complex 1	---	---	2.76	---	---	---
E	AW123904	GABA(A) receptor-associated protein-like 1	---	---	2.59	---	---	---
E	AI845237	chloride intracellular channel 4 (mitochondrial)	2.59	---	2.56	---	---	---
E	AV218205	cystatin C	---	---	2.23	---	---	---
E	U10098	cystatin C	---	---	2.21	---	---	---
E	AF041054	BCL2/adenovirus E1B 19 kDa-interacting protein 1NIP3	---	---	2.21	---	---	---
E	AI839417	moesin	---	---	2.12	---	---	---
E	X60367	retinol binding protein 1cellular	2.75	2.54	2.63	---	---	---
E	U92454	WW domain binding protein 5	---	---	2.10	---	---	---
E	AI849533	chloride intracellular channel 4 (mitochondrial)	2.22	---	2.08	---	---	---
E	X53928	biglycan	---	2.92	---	---	---	---
E	L19932	transforming growth factorbeta induced 68 kDa	---	2.51	---	---	---	---
E	M13805	keratin complex 1 acidic gene 17	---	2.33	---	---	---	---
E	AJ000990	legumain	2.18	---	---	---	---	---
F	L36244	matrix metalloproteinase 7	---	---	8.56	---	2.47	3.06
F	X13986	secreted phosphoprotein 1 aka: osteopontin precursor minopontin	---	---	7.04	---	2.36	---
F	M63695	CD1d1 antigen	---	---	5.62	---	4.36	6.03
F	X70298	SRY-box containing gene 4	---	2.16	4.94	---	2.28	2.77
F	M88242	prostaglandin-endoperoxide synthase 2 (COX-2)	---	3.21	4.60	3.53	---	4.73
F	AW124153	SRY-box containing gene 4	---	---	4.59	---	2.44	3.10
F	X61232	carboxypeptidase E	---	---	4.51	---	4.39	5.74
F	M65027	glycoprotein 49 A	---	---	4.30	---	---	3.03
F	U10551	GTP binding protein (gene overexpressed in skeletal muscle)	---	---	4.05	---	3.40	4.61
F	X70296	serine protease inhibitor 4	---	---	3.99	---	2.94	---
F	U43085	interferon-induced protein with tetratricopeptide repeats 2	---	2.24	3.78	---	---	3.23
F	X61232	carboxypeptidase E	---	---	3.67	---	4.14	5.63

Cluster	GenBank	Title	T/E	L/E	P/E	L/T	P/L	P/T
F	D00613	matrix gamma-carboxyglutamate (gla) protein	---	---	3.33	---	---	3.33
F	AI841689	RIKEN cDNA	---	---	3.16	---	---	2.53
F	AJ223208	cathepsin S	---	---	3.04	---	---	---
F	U05265	glycoprotein 49 B	---	---	2.97	---	---	2.45
F	AV370035	chemokine (C-C) receptor 5	---	---	2.96	---	---	2.59
F	L02914	aquaporin 1	---	---	2.81	---	2.29	---
F	X05862	H2B histone family member S	---	---	2.72	---	---	---
F	AW049730	glycogenin 1	---	---	2.62	---	2.21	2.62
F	AA793671	mRNA complete cds clone:c0103	---	---	2.55	---	---	3.00
F	AF006465	RAN binding protein 9	---	---	2.48	---	---	---
F	AW124544	endoplasmic reticulum membrane protein	---	---	2.42	---	---	2.46
F	AI844043	RIKEN cDNA	---	---	2.39	---	---	2.36
F	X97227	CD53 antigen	---	---	2.36	---	---	---
F	X99347	lipopolysaccharide binding protein	---	---	2.35	---	---	---
F	AV229143	EST: interferon activated gene 202A	---	---	2.31	---	---	---
F	U40930	sequestosome 1 aka: annexin A11	---	---	2.24	---	---	---
F	X75129	xanthine dehydrogenase	---	---	2.23	---	---	---
F	AI838836	RIKEN cDNA	---	---	2.21	---	---	---
F	M31419	interferon activated gene 204	---	---	2.19	---	---	3.13
F	M31418	interferon activated gene 202A	---	---	2.08	---	---	2.31
F	X89749	TG interacting factor	---	---	---	---	2.13	2.41
F	AA623874	RIKEN cDNA	---	---	---	---	---	2.10
F	AI840158	angiopoietin-like 2	---	---	---	---	---	2.30
F	AV357306	glycogenin 1	---	---	---	---	---	2.37
F	AI841295	RIKEN cDNA	---	---	---	---	2.13	2.48
F	AW122536	profilin 2	---	---	---	---	2.61	---
F	AF022889	latent transforming growth factor beta binding protein 1	---	---	---	---	---	2.36
F	M77196	CEA-related cell adhesion molecule 1	---	---	---	---	---	2.81
F	AW060971	RIKEN cDNA	---	---	---	---	---	2.12
F	M77196	CEA-related cell adhesion molecule 1	---	---	---	---	---	2.58
F	U73037	interferon regulatory factor 7	---	---	---	---	---	2.03
G	L12447	insulin-like growth factor binding protein 5	---	---	---	---	8.05	8.69
G	X93035	chitinase 3-like 1 aka: Glycoprotein 39	---	---	---	---	4.81	5.01
G	AF002283	PDZ and LIM domain 3	---	---	---	---	2.68	4.29
G	AV092014	peptidoglycan recognition protein	---	---	---	---	2.94	4.28
G	AF022371	interferon activated gene 203	---	---	---	---	---	3.64
G	AB001489	polymeric immunoglobulin receptor	---	---	---	---	---	3.54
G	AF076482	peptidoglycan recognition protein	---	---	---	---	2.29	3.11
G	AI504338	EST	---	---	---	---	---	2.84
G	U66166	SPARC-like 1 (mast 9 hevin) (Extracellular matrix protein 2)	---	---	---	---	2.20	---
G	L38444	T-cell specific GTPase	---	---	---	---	---	2.77

Cluster	GenBank	Title	T/E	L/E	P/E	L/T	P/L	P/T
G	AF062071	zinc finger protein 216	---	---	---	---	2.44	2.76
G	AJ005559	small proline-rich protein 2A	---	---	---	---	2.46	2.41
G	AI846197	RIKEN cDNA	---	---	---	---	2.45	2.40
G	AJ005561	small proline-rich protein 2C	---	---	---	---	---	2.37
G	AF039663	prominin	---	---	---	---	---	2.29
G	AA790307	onzin aka:Plac8: placenta-specific 8	---	---	---	---	---	2.26
G	AI747194	lysosomal membrane glycoprotein 2	---	---	---	---	---	2.22
G	AI987985	DNA segment chr16 Wayne State University 73 expressed	---	---	---	---	---	2.21
G	U30244	ephrin B2	---	---	---	---	---	2.20
G	AF004666	solute carrier family 8 (sodium/calcium exchanger) member 1	---	---	---	---	2.46	---
G	Y07688	nuclear factor I/X	-2.11	---	---	---	---	---
G	U22033	proteosome subunit beta type 8 (large multifunctional protease 7)	-2.24	---	---	---	---	2.47
G	AV349686	N-myc downstream regulated 2	-2.25	---	---	---	---	---
G	AJ005559	small proline-rich protein 2A	-2.26	-2.26	---	---	---	---
G	AW047343	D site albumin promoter binding protein	-2.27	-2.19	---	---	---	---
G	AV354117	RIKEN cDNA	-2.44	-2.67	---	---	5.48	4.99
G	U33629	myeloid ecotropic viral integration site 1	-2.56	---	---	---	---	---
G	U49430	ceruloplasmin aka:Ferroxidase	-2.61	---	---	---	---	2.80
G	AI197481	amiloride binding protein 1 (amine oxidase copper-containing)	-2.73	-2.42	---	---	---	---
G	M21038	myxovirus (influenza virus) resistance 1	-3.28	---	---	---	---	---
G	AI842065	EST	-4.16	-4.07	---	---	2.88	---
G	U51805	arginase 1 liver	-4.97	-3.70	---	---	2.72	3.54
H	AW123157	DNA segment chr11 Wayne State University 78 expressed	---	-2.32	---	---	---	---
H	AI843074	RIKEN cDNA	---	---	-2.27	---	---	---
H	AA763466	procollagen type I alpha 1	---	-2.21	-2.65	---	---	---
H	X71978	fused toes	---	---	-2.57	---	---	---
H	Z18272	procollagen type VI alpha 2	---	---	-2.71	---	---	---
H	M35523	cellular retinoic acid binding protein II	---	---	-2.30	---	---	---
H	AI841645	N-acetyltransferase ARD1 homolog (yeast)	---	---	-2.29	---	---	---
H	M22679	alcohol dehydrogenase 1 complex	---	-2.23	---	---	---	---
H	L42115	solute carrier family 1member 7	---	---	-2.34	---	---	---
H	AW045418	ribosomal protein L44	---	---	-2.41	---	---	---
H	AF011450	procollagen type XV	---	-2.66	---	---	---	---
H	L33779	desmocollin 2	---	---	-2.42	---	---	---
H	AA163960	EST	---	---	-2.28	---	---	---
H	AA684508	RNAU22 small nucleolar	-2.03	---	---	---	---	---
H	AF073993	heterogeneous nuclear ribonucleoprotein A2/B1	-2.07	---	---	---	---	---
H	AA656775	regulator for ribosome resistance homolog (yeast)	-2.12	---	---	---	---	---
H	M33934	inosine 5'-phosphate dehydrogenase 2	-2.14	---	-2.23	---	---	---
H	AF053232	nucleolar protein 5	-2.16	---	---	---	---	---
H	AA958560	RIKEN cDNA	-2.18	---	---	---	---	---

Cluster	GenBank	Title	T/E	L/E	P/E	L/T	P/L	P/T
H	AI843342	RIKEN cDNA	-2.20	---	---	---	---	---
H	AV252495	SWI/SNF related matrix associated actin dep regulator of chromatin d2	-2.24	---	---	---	---	---
H	AW122364	arginine-richmutated in early stage tumors	-2.26	---	---	---	---	---
H	U20344	Kruppel-like factor 4 (gut)	-2.28	---	-2.70	---	---	---
H	AI851250	EST	-2.30	---	---	---	---	---
H	AF009246	RAS dexamethasone-induced 1	-2.30	-2.79	-2.56	---	---	---
H	AA655369	translocase of inner mitochondrial membrane 8 homolog a (yeast)	-2.31	---	---	---	---	---
H	U09659	heat shock 10 kDa protein 1 (chaperonin 10)	-2.37	---	---	---	---	---
H	AI846708	ribophorin I	-2.39	---	---	---	---	---
H	M81445	gap junction membrane channel protein beta 2 (connexin 26)	-2.44	-2.94	---	---	---	---
H	AI122538	RIKEN cDNA	-2.44	-2.56	-3.20	---	---	---
H	AI849615	growth arrest specific 5	-2.44	---	---	---	---	---
H	X64837	ornithine aminotransferase	-2.51	-2.33	---	---	---	---
H	AI848479	ectodermal-neural cortex 1	-2.52	-2.66	---	---	---	---
H	D13458	prostaglandin E receptor 4 (subtype EP4)	-2.55	---	---	---	---	---
H	AF083464	REV3-like catalytic subunit of DNA pol zeta RAD54 like (yeast)	-2.55	-2.38	---	---	---	---
H	AI837110	heterogeneous ribonucleoproteins methyltransferase-like 2 (yeast)	-2.56	---	---	---	---	---
H	AI854510	thyroid hormone receptor-associated protein 100 kDa	-2.59	-2.43	-2.53	---	---	---
H	AW226939	carboxylesterase 3	-2.68	---	---	---	---	---
H	AI849271	RIKEN cDNA	-2.69	-2.79	---	---	---	---
H	AI845584	dual specificity phosphatase 6	-2.72	---	---	---	---	---
H	AW122030	Similar to phosphoserine aminotransferase	-2.86	-3.20	-3.73	---	---	---
H	X04591	creatine kinase brain	-2.99	-4.43	---	---	2.37	---
H	X67668	high mobility group box 2	-3.33	-2.88	-3.46	---	---	---
H	M12848	myeloblastosis oncogene	-3.36	-2.95	-3.65	---	---	---
H	M25944	carbonic anhydrase 2	-3.48	-3.27	---	---	---	---
H	AB016592	anterior gradient 2 (Xenopus)	-3.92	-3.66	-2.71	---	---	---
H	AI465965	EST	-4.09	-3.61	-2.60	---	---	---
H	AI314958	RIKEN cDNA	-4.13	-4.01	-4.58	---	---	---
H	U88623	aquaporin 4	-4.51	-3.84	---	---	---	---
H	X17320	Purkinje cell protein 4	-16.33	-11.26	-15.45	---	---	---
H	AV373378	chloride channel calcium activated 3	-23.24	-43.46	-7.45	---	5.76	---
I	AW122413	EST: Weakly similar to tyrosine-protein kinase JAK3 (mouse)	---	---	-2.06	---	---	---
I	X99273	aldehyde dehydrogenase family 1 subfamily A2	---	---	-2.18	---	---	---
I	AF002823	budding uninhibited by benzimidazoles 1 homolog (yeast)	---	---	-2.21	---	---	---
I	AI847054	RIKEN cDNA	---	---	-2.33	---	-2.88	-3.63
I	D55720	karyopherin (importin) alpha 2	---	---	-2.38	---	---	---
I	AI837621	RIKEN cDNA	---	---	-2.51	---	---	---
I	AA681998	RIKEN cDNA	---	---	-2.80	---	---	---
I	AI595322	EST: Similar to highly expressed in cancer rich in leucine heptad repeats	---	---	-2.81	---	---	---
I	AB017026	oxysterol binding protein-like 1A	---	---	-2.99	---	---	---

Cluster	GenBank	Title	T/E	L/E	P/E	L/T	P/L	P/T
I	AW124932	pre B-cell leukemia transcription factor 1	---	---	-3.02	---	---	---
I	AW125346	RIKEN cDNA	---	---	-3.07	---	---	---
I	AW122260	cytochrome P45051	---	---	-3.09	---	---	---
I	AA407599	EST	---	---	-3.16	---	-6.76	-4.20
I	D42048	squalene epoxidase	---	---	-3.17	---	---	-2.58
I	AI846851	farnesyl diphosphate synthetase	---	---	-3.22	---	---	---
I	AI849109	Ras-specific guanine releasing factor 1	---	---	-3.39	---	---	-3.01
I	AW045533	farnesyl diphosphate synthetase	---	---	-3.52	---	---	---
I	X82786	antigen identified by monoclonal antibody Ki 67	---	---	-4.03	---	---	-2.51
I	U01915	topoisomerase (DNA) II alpha	-2.27	-2.79	-4.12	---	---	---
I	AI850558	EST: Moderately similar to A2M2 murinoglobulin 2 precursor (mouse)	---	---	-4.58	---	---	-5.82
I	AW124988	apolipoprotein B editing complex 2	-2.85	---	-5.44	---	---	---
I	AA716963	EST: Similar to isopentenyl-diphosphate delta isomerase	-2.71	---	-8.28	---	-3.00	-3.04
I	M12347	actin alpha 1 skeletal muscle	---	---	-8.69	---	---	-3.44

Table 2.3 GeneCluster organized probe sets reflecting significant changes in gene expression levels.

Comparisons of average gene expression levels were made between term (T), labour (L), postpartum (P) and their estrus (E) baseline control. As well, fold changes between periparturient time-points L and T, P and L, and between P and T were calculated to further identify significant changes in gene expression levels. Criteria for significance were a fold change ≥ 2 or ≤ -2 and a p value ≤ 0.0025 for both replicates of a comparison. Significant fold change values are indicated. Comparisons that were not significant are represented by ---. Probe sets for each cluster are ordered by the magnitude of the fold change at the time point with the most numerous significant values for that cluster. EST, expressed sequence tag; RIKEN cDNA, Japanese consortium expressed sequence.

Functional Clustering

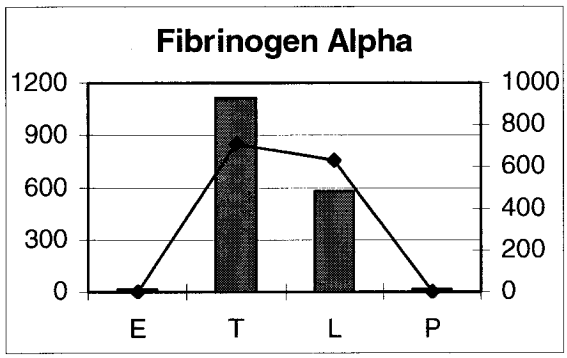
Genes were organized based on their known or inferred functions to identify networks of genes with similar or interrelated functions. Information was acquired from Affymetrix [112], Swiss-Prot [113], Unigene and Locus Link [114], and other available database websites. The list of 342 probe sets was functionally clustered in Table 2.4 found at the end of this chapter. As there was often more than one function associated with each gene product, alternative classifications were also included in the table. Cluster A genes fall mostly into the signalling group and the intercellular adhesion/communication, extracellular matrix, and blood clotting group. Cluster B genes are most prevalent in the cell growth/differentiation/apoptosis functional group, many of which are involved in the insulin-like growth factor system. As well, many genes fall under the intercellular adhesion/communication, extracellular matrix, and blood clotting classification, hormone biosynthesis/metabolism and transport functional groupings. Of note are the hemoglobin genes, whose protein products provide oxygen to the uterus during contraction and transporting away CO_2. Cluster C genes are also found to be involved in cell growth/differentiation/apoptosis but also participate in RNA/protein biosynthesis/metabolism/modification, cytoskeleton/muscle development, lipid binding/transport/metabolism, and in the electron transport chain preparing a source of energy to the cytoskeletal network for the contraction of the fibres during parturition. Genes of Cluster D fall mostly into the lipid binding/transport/metabolism (~13%) and cell defence/immune function (~20%) roles. Cluster E consists of genes that are involved in cell growth, differentiation, or apoptosis, RNA/protein biosynthesis/metabolism/modification and lipid binding/transport/metabolism. As well, a significant number of Cluster E genes fall into the intercellular adhesion/communication, extracellular matrix, and blood clotting functional group and even more (~29%) in the cell defence/immune functional classification. Once again, these genes were up-regulated and sustained throughout the periparturient period. Cluster F, which exhibited peak expression levels postpartum, includes genes that are involved in transcription regulation, RNA/protein biosynthesis/metabolism/ modification, intercellular adhesion/communication,

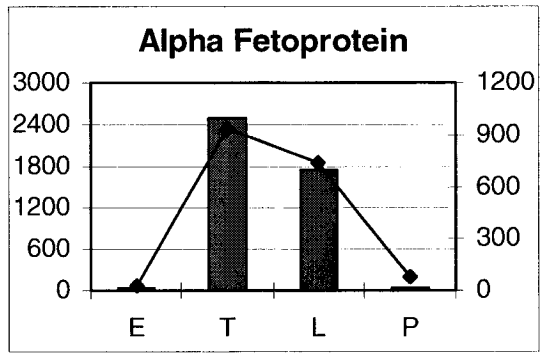
extracellular matrix, and blood clotting group, and in carbohydrate biosynthesis/metabolism and electron transport and once again, in greatest numbers in cell immune/immune function with ~23% of the cluster.

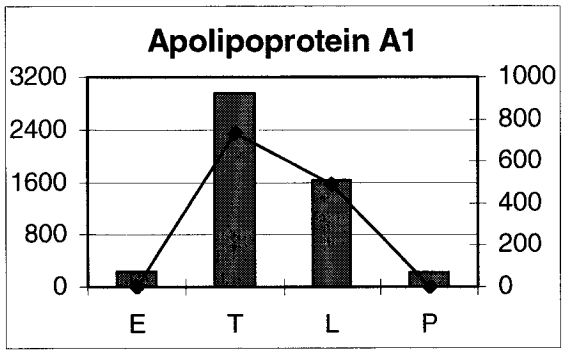
Clusters G-I comprise genes that were down-regulated during the periparturient period. Cluster G includes genes involved in cell growth/differentiation/ apoptosis as growth inhibitors, as activators of transcription, and in cell immune/immune function. Genes in Cluster H are involved in DNA/Nucleotide metabolism, some in transcriptional regulation, a significant number (~25%) in RNA/protein biosynthesis/metabolism/modification, and the intercellular adhesion/ communication, extracellular matrix, and blood clotting category where procollagen genes are represented. Cluster I, which demonstrated a repression most significant at postpartum includes genes which are important for hormone biosynthesis /metabolism, particularly in cholesterol biosynthesis.

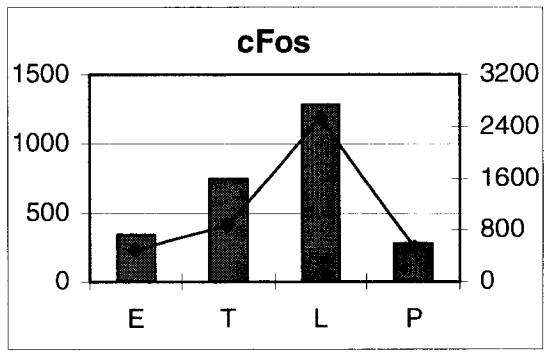
Validation of Microarray Results

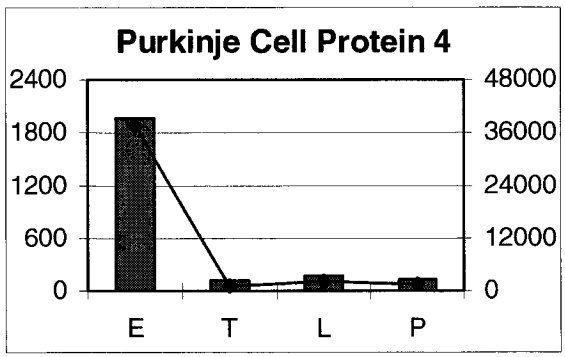
Additional to the support provided by the reiteration of well-described expression patterns for several genes, we chose to further corroborate the microarray results. To this end, relative quantitative real-time RT-PCR was performed for selected genes. Gene transcripts chosen for validation were FbgA, Afp, ApoA1, cFos transcription factor, PCP4, chaperonin 10 (Chap10)(a.k.a. heat shock protein 10), and SAA3. Primer pair sequences and amplification product size are listed in Table 2.1. Each experimental replicate was amplified three times for six RT-PCR replicates per time-point. RT-PCR product relative quantification data was plotted with microarray fluorescence values in Figure 2.2. In each case, the gene expression patterns observed by microarray analysis were reproduced by Relative Quantitative RT-PCR, validating the results.

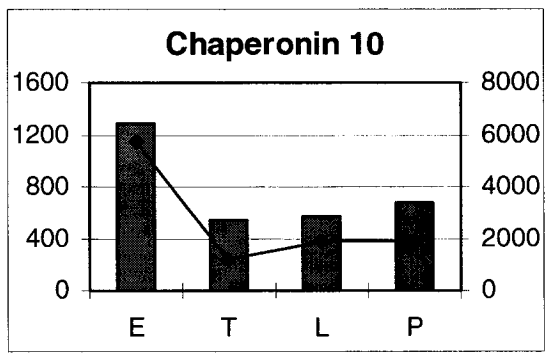

Fibrinogen Alpha
1200
900
600
300
0
1000
800
600
400
200
0
E
T
L
P


Alpha Fetoprotein
3000
2400
1800
1200
600
0
1200
900
600
300
0
E
T
L
P


Apolipoprotein A1
3200
2400
1600
800
0
1000
800
600
400
200
0
E
T
L
P


cFos
1500
1000
500
0
3200
2400
1600
800
0
E
T
L
P


Purkinje Cell Protein 4
2400
1800
1200
600
0
48000
36000
24000
12000
0
E
T
L
P


Chaperonin 10
1600
1200
800
400
0
8000
6000
4000
2000
0
E
T
L
P

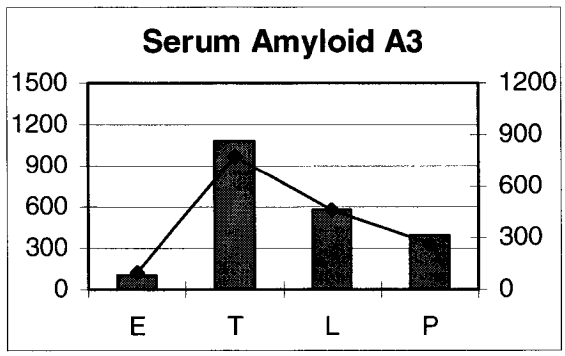

Serum Amyloid A3
1500
1200
900
600
300
0
1200
900
600
300
0
E
T
L
P

Figure 2.2 Validation of microarray data using relative quantitative real-time RT-PCR. Validation of microarray data was performed using seven differentially expressed genes. FbgA, fibrinogen alpha; Afp, alpha fetoprotein; ApoA1, apolipoprotein A1 and PCP4, Purkinje cell protein 4 represent genes which had significantly different expression levels across estrus (E), term (T), labour (L), and postpartum (P) time-points. SAA3, serum amyloid A3 expression changed moderately while cFos and Chap10, Chaperonin 10, had subtler changes in regulation. These genes also represent different expression patterns. The left axis indicates microarray fluorescence values after dChip modelling. The right axis specifies the relative quantitative real-time RT-PCR SYBR Green fluorescence values. Average values of two microarray replicates are plotted in bar graph form. Average values of triplicate RT-PCR amplification of two replicate RNA samples are plotted in line graph form.

2.5 DISCUSSION

Several studies utilizing Gene Chips and various multigene hybridization analysis platforms have been performed, particularly in humans, rats, and mice, to identify genes involved in the process of mammalian reproduction. Investigations range from estrus [115], to implantation and endometrial remodelling [12, 14, 15, 116, 117] needed to support the developing fetus, through the end stages of gestation to parturition [118-121]. In addition, models which include the application of known effectors of uterine gene expression such as estrogen [122] and vascular endothelial growth factor, a key regulator of angiogenesis and inducer of myometrial endothelial cell gene expression [123], have been studied using microarray technology. In the present study, we add to this body of knowledge. We have applied this technology to uncover the identities and expression patterns of important regulators/mediators of murine parturition. The inclusion of animals preceding parturition and early postpartum (~12 hours) have enabled us to identify the dynamic changes in gene expression associated with murine labour. Of the ~ 12,000 genes monitored by cRNA hybridization to the mouse Affymetrix microarray oligonucleotide chip U74Av2, we identified more than 300 genes that were differentially expressed during the end stages of pregnancy. Several of these are well described as having a role in the process of parturition, providing validity to the many more genes that are newly implicated in the process of parturition, their exact roles yet to be elucidated. The preponderance of genes was differentially regulated at term with respect to estrus levels suggesting the moment for successful intervention is in advance of apparent labour, increasing the importance of developing diagnostic methodologies. This discussion is not exhaustive, but instead focuses on presenting and supporting theories to the roles of gene products to the process of parturition.

Genes well described in the process of parturition- validation of results

Genes previously described as participating in the reproductive process and found to be differentially expressed by microarray analysis include the transcription factor cFos as here we found a 4-fold increase at the time of parturition (Cluster B).

The gene expression of cFos in the uterus has been demonstrated to be up-regulated by estrogen [25, 122] as well as mechanical stretch [41] and modulated by progesterone [38]. The hormonal regulation of cFos is further evidenced in the study of global gene expression changes during murine pregnancy put forth by Bethin et al. [118]. This similar, yet distinct study focused on transcription factors and cyclooxygenase-1 gene regulation through the last trimester of murine pregnancy. An increase in cFos is followed by transcription of target genes including Cx43 and the OTR, resulting in the increased production of these CAPs and activation of the uterus for labour.

The CAPs Cx43 and Cx26 are gap junction proteins and both demonstrated their prescribed pattern of gene expression throughout the end stages of pregnancy [34, 110]. Cx43 (Cluster A, 2 probe sets) was up-regulated in preparation for labour, reaching maximal levels at the moment of labour and declining to near-estrus levels postpartum. As term approaches, the progesterone levels increase, maintaining its dominance over increasing estrogen levels. Progesterone suppresses Cx43 gene expression, while estrogen stimulates gene expression within 14 hours after reaching effective concentrations. Stretch of the uterus, the switch to an estrogen dominant uterine environment, and up-regulation of the transcriptional activator cFos promote the expression of Cx43 at labour. The increase in Cx43 mRNA to maximal levels at labour is believed to maintain established gap junction communication between myometrial cells to coordinate pulsating contractions necessary to expel the fetus [45].

Cx26 had the opposite gene expression pattern at the end of pregnancy, falling into Cluster H. The pattern of gene expression in rats has been described as increasing during the last few days of pregnancy and then dropping in expression to low levels prior to the onset of labour [33, 34, 111]. A further drop in gene expression at the time of labour was observed here. Cx26 gene expression responds quickly after increases in estrogen levels with approaching parturition and is not repressed, but rather stimulated further by a balancing increase in progesterone levels [35]. The drop in Cx26 gene expression preceding the onset of uterine contractions follows that of progesterone levels and may also be accommodated by other factors including relaxin, which has a similar pattern of gene regulation during pregnancy. Cx26 is implicated

along with relaxin among others as mediators of uterine quiescence. Switching off these uterotrophins assists the activation of the uterus by stretch mechanisms prior to labour.

Growth Factors

Factors involved in uterine growth include insulin-like growth factors (IGFs) and their associated binding proteins (BPs) demonstrated significant changes in gene expression levels. IGF1 and IGF2 have known mitogenic effects on uterine endometrial epithelium. IGF1 is the dominant mitogen early in pregnancy contributing to increasing placental blood flow [124] and is appropriately, not detected in this study. IGF2 promotes uterine hyperplasia necessary during fetal growth. IGF2 expression levels are characterized as peaking mid-gestation [125]. We observed diminishing levels from moderately induced at term to insignificant at labour and postpartum (Cluster B). In addition to the decline in IGF2 gene expression, the H19 mRNA is known to down-regulate IGF2 transcription and translation by promoter/enhancer competition between the genes [126]. H19 mRNA levels followed IGF2 pattern of expression but at higher levels (Cluster B), showing significant diminishment at labour and a complete return to estrus levels postpartum. The high levels of H19 mRNA seen at term suggest this inhibition of IGF2 mitogenic activity, leading to decreased uterine growth and increased activation of the uterus for parturition through CAP gene induction. The down-regulation of both IGF2 and H19 mRNA as labour proceeds indicates that this growth inhibition is no longer necessary postpartum.

The mechanism of action of IGF binding proteins is dependent upon cell type and physiological condition. We correctly did not observe changes in IGFBP1 transcript levels in our model. This was because levels are known to increase early, from non-existent to prominent levels in luminal epithelium during decidualization [30]. IGFBP1 levels are not significantly expressed at the end of gestation. Cerro et al. demonstrated transcripts at day 18.5 in rat parturition (22-23 days to term). Our mouse model suggests a late stage down-regulation of the growth and IGF supportive

roles along with IGFBP1 mRNA levels. Microarray analysis demonstrated a marked up-regulation of IGFBP2 in mouse uterus at term, increasing further at labour, and subsequently returning to relatively more moderate levels postpartum (Cluster E). Unlike the common pattern of expression found in pig uterus [125], we find that IGF2 and IGFBP2 gene expression patterns do not mimic one another, proposing that the functions of their proteins are not collaborative in parturient mouse uterus. This also supports the classic observation that IGFBP2 inhibits DNA synthesis [127, 128]. The remarkable up-regulation of this mitogenic inhibitor at the time of labour furthers the notion that this IGF-IGFBP system mediates uterine stretch and activation of the uterus just prior to parturition. Consistent with this model is the up-regulation, albeit moderate, of IGFBP3 (Cluster B, 2 probe sets), characterized as an inhibitor of IGF-induced mitogenesis [30]. IGFBP5 is one of the most prominently expressed IGFBPs at estrus, specifically in the myometrium where it is believed to induce myometrial proliferation during the early stages of pregnancy [30]. More moderate levels of gene expression are seen later in gestation. This expression pattern is reiterated here (Cluster G) and reflects IGFBP5 down-regulation by estrogen [129]. A repression at term and at labour is observed but due to our strict criterion for determining significance these values are not reported. This is true for the induction of IGFBP5 gene expression postpartum. Statistical significance is however, reached upon direct comparison between the later time-points. A statistically significant 8-fold increase from term and labour gene expression levels to that at the postpartum time-point was observed. This down-regulation at term and labour suggests IGFBP5 to be an enhancer of IGF growth function. Its up-regulation postpartum suggests this function to participate in uterine remodelling. IGFBP10, in addition to its cell-proliferative properties, is annotated by Swiss-Prot [113] as promoting wound healing. The moderate induction of IGFBP10 (a.k.a. cysteine rich protein 61) gene expression specifically at the moment of labour (Cluster B) may participate in cutting off the blood supply to the placenta during detachment and repairing the remaining tissue.

The changes in gene expression of these members of the IGF-IFGBP system are reiterated in the microarray study of gene expression in parturient rat uterine tissue

[121] increasing the validity of these findings and hypotheses as to their role during the peri-parturient period.

Blood Clotting Factors

Blood clotting factors have long been described as influential in the process of parturition. Here we find the up-regulation of a large number of genes involved in the clotting mechanism, most notably for the polymers of fibrinogen. Fibrinogen alpha polypeptide (FbgA) (Cluster B) shows the most remarkable up-regulation of all the genes represented on the microarray chip with 93 times more transcripts detectable at term than at estrus. Fibrinogen beta (FbgB) (Cluster B) was induced 31-fold at term, and the EST for fibrinogen gamma (Genbank # AA986050) (Cluster C) was induced 7-fold at term. The expression levels of all three genes decreased significantly at labour and were not significantly expressed postpartum vs. estrus levels. Fibrinogen is composed of 2 strands of each polypeptide. The conversion of fibrinogen to the fibrin molecule deposited at the clot site is performed by thrombin, which is processed from the prothrombin precursor also known as coagulation factor II (F2) [130]. F2 gene transcription follows the same pattern as the fibrinogen polypeptide genes, a significantly high level (40-fold up-regulation) at term, approximately half that level at labour, and a level of expression postpartum which is no longer statistically significant (Cluster B). Plasminogen activator inhibitor-1 (PAI-1) is induced moderately only at the time of labour (Cluster A). This pattern was also identified by Bethin et al. [118]. PAI-1 is protective against fibrinolysis by preventing the conversion of plasminogen to plasmin, the factor responsible for lysing fibrin and fibrinogen. Fibrinogen degradation products are inhibitors of thrombin activity [130].

Thrombin itself is described as an uterotonic agent known to be up-regulated during preterm labour [131, 132] with effective protein levels much lower than that found in clotting blood. This surprising sensitivity of the uterus to thrombin leads to myometrial contractions during clot formation. Limited spotting during pregnancy can lead to spontaneous abortion.

There are also factors involved in inhibiting blood clotting which are differentially expressed at the mRNA level, including annexins 3 and 8, and protein C receptor (endothelial) (all in Cluster B). Each of these fibrinolytic factors is only moderately expressed at term, with decreasing levels found thereafter. There may be an up-regulation earlier in gestation to maintain uterine-placental blood flow and uterine quiescence during most of gestation.

The relevance of these clotting factors to the process of normal parturition is further delineated and substantiated through this microarray study.

Hormone Regulation

The dynamics of COX-2 gene expression was reproduced in this study as well. Falling into Cluster F, we found an up-regulation at the moment of labour, which is enhanced, postpartum. COX-2 is the inducible form of prostaglandin H synthase, the enzyme responsible for converting prostaglandin (PG) H_2 to both PGE_2 and $PGF_{2\alpha}$, effective uterotonins. COX-2 and PGs are provided by the placenta of the maturing fetus but maternal sources of COX-2 are dependent upon the increase in effective estrogen levels found at the end of parturition [19]. Glucocorticoids such as cortisol stimulate estrogen production and further augment COX-2 gene expression as well as directly affect CAP gene expression. Based on its expression profile, Cook et al. suggest characterizing COX-2 as a CAP [42].

Enzymes involved in cortisol metabolism include 11β-hydroxysteroid dehydrogenase 1 (Hsd11b1). This enzyme is capable of acting as an oxidizing agent converting biologically active cortisol to inactive cortisone and acting as a reductase, converting cortisone back to cortisol, depending on the tissue. In the decidua at term, $PGF_{2\alpha}$ promotes Hsd11b1 reductase activity by triggering phosphorylation of the enzyme as a result of prostaglandin receptor (FP) activation [133]. In the rat myometrium, Hsd11b1 is induced in late pregnancy under the stimulus of placental factors [134]. Local glucocorticoids also up-regulate Hsd11b1 gene expression in the myometrium and epithelium prior to parturition [135], providing a positive feedback loop to increase cortisol, estrogen and subsequently $PGF_{2\alpha}$, leading to activation and

stimulation of the uterus to deliver the fetus. In the microarray study on pregnant rat uterine samples performed by Girotti et al. Hsd11b1 was quite significantly increased by day 20 of gestation, three days before labour commences in the rat. This level was sustained at term but then diminished significantly at labour [121]. We found Hsd11b1, in Cluster D, showed an 11-fold up-regulation at term vs. estrus levels with lower, but significant levels at labour when the ascribed enzyme functions are relevant, and a slow diminishment postpartum as contractions for uterine remodelling lessen. The fold change, when directly comparing term and labour levels, did not pass our rigorous criteria for significance and is indicated as such. Our results regarding Hsd11b1 suggest that placental factors responsible for increased gene expression are cut off in preparation for placental detachment during parturition and that sufficient cortisol and estrogen production have precipitated a final "shutting off" of the positive feedback loop at which point downstream effectors of uterine contraction, namely $PGF_{2\alpha}$, Cx43, and OTR gene expression increase.

Also involved in cortisol production is 3β-hydroxysteroid dehydrogenase-1 (Hsd3b1). In fact, Hsd3b1 plays a crucial role in the biosynthesis of all classes of hormones and its up-regulation prior to the onset of labour is seen here. The two probe sets for this gene fell into Clusters B and C with Cluster B probe set (Genbank# M58567) showing a much greater level of induction than Cluster C probe set (Genbank# AV290268). The reason for the dual assignment is due to the level of gene expression detected by the unique oligonucleotide sequence of the individual probe sets. A potential explanation for this is that cross-hybridization of a transcript with sufficient homology to the sequence of one probe set (i.e. Genbank# M58567), would result in an artificially high fold change if this other transcript expression were induced in this same condition. Conversely, hybridization of an induced complementary cRNA sequence to the cRNA target of a probe set (i.e. Genbank# AV290268) would hinder the hybridization of the target cRNA sequence to the probe set and result in a lessened apparent fold increase. This phenomenon is found elsewhere among the probe sets (lipoprotein lipase -A and D, reduced expression 3 -C and D, hemoglobin beta adult major chain -B and -C,) but in all cases, both probe sets

showed similar up-regulation with approaching parturition. Cluster assignment is based on GeneCluster algorithm criteria and the median average fluorescence value for each probe set across all time-points.

We also find the enzyme for cytochrome P45011a cholesterol side chain cleavage (Cyp11a), which catalyzes the side-chain cleavage reaction of cholesterol to pregnenolone, to be induced at term and labour before diminishing to estrus levels postpartum (Cluster C). Circulating maternal estrogen levels are derived by both maternal adrenal and placental C19 steroid sources [19]. Cyp11a expression in term tissue may enable the uterus, otherwise incapable of converting cholesterol to C19 steroids [136], to catalyze this conversion and generate further, a local increase in estrogen concentration at the time preceding labour. The expression of Cyp11a was found in human endometrial endothelium [137] substantiating our findings.

Gene expression of Hsd11b1, Hsd3b1, and Cyp11a all follow the same pattern, increased expression at term with subsequent diminished expression as labour ensues. The profile for all three steroidogenic genes suggests that they provide for estrogen levels to topple the progesterone dominant environment at term, leading to activation of the uterus at labour.

Also involved in estrogen biosynthesis and metabolism and showing this same up-regulation at term with subsequent decreases at labour and further so postpartum are alpha fetoprotein (Afp) and apolipoprotein A1 (ApoA1) (both of Cluster B). Afp is characterized as having anti-estrogenic properties, reaching peak expression during the final trimester of pregnancy [138]. Afp is capable of binding steroids in addition to estrogen, of being taken up by cells to interfere with nuclear receptor function, and of exerting immunosuppressive effects on lymphocytes [139]. Gabant et al. developed an Afp knock-out mouse and found this gene and its product to be essential for female reproduction [139]. Uterine estrogen levels were unaffected but the uterus was continually stimulated by it. Additionally, increased risk of adverse pregnancy outcomes, such as premature birth, preeclampsia, and intrauterine growth restriction are associated with elevated maternal serum Afp levels [140]. These outcomes may

reflect a transcriptional repression of steroid-responsive genes, particularly those involved in cell proliferation.

The role Afp plays in the process of labour may include, (1) binding to increasing levels of estrogen, maintaining the functional progesterone balance over estrogen until the requisite time, (2) inhibiting the activation of estrogen-responsive genes, particularly for the CAPs, thereby (3) maintaining uterine quiescence, and also by (4) potentiating immune tolerance to the developing fetus. Uterine levels at term were up-regulated nearly 80-fold vs. estrus gene expression levels. The lessened gene expression at labour indicates the alleviation of these affects, rendering free estrogen to exert its role on activating the parturient uterus and enabling the rejection of fetal tissue. The significant drop in Afp gene expression postpartum signals the end of the requirement for these functions. Immunohistochemistry will be performed to determine the uterine cell types participating in this gene expression.

Apolipoprotein A1 (ApoA1) binds HDL-bound cholesterol transporting it to the liver for catabolism. This glycoprotein is found at higher concentrations in serum of pregnant women than non-pregnant women and a significant reduction is associated with the preeclampsia [141, 142]. High ApoA1 levels during normal pregnancy are associated with increased endothelial-dependent relaxation of myometrial arteries, likely through ApoA1 stimulation of endothelial prostacyclin secretion. Preeclampsia results in part from an impaired relaxatory mechanism leading to myometrial vessel contraction and vascular injury [142]. The down-regulation of this relaxation-promoting factor at normal labour, from high levels at term, may increase uterine tonicity and facilitate uterine stretch responses. ApoA1 also possesses anti-oxidative properties, transporting cell- and tissue-damaging reactive oxygen species to the liver for detoxification, reducing cell death and subsequent immune activation. An anti-inflammatory role is also assigned to ApoA1. It directly interferes with T cell-macrophage contact, preventing the stimulation of the macrophage to produce inflammatory T_h1 cytokines IL-1β and TNFα, and promoting the production of immunomodulatory T_h2 cytokines [143, 144]. Additionally, ApoA1 is involved in facilitating cholesterol efflux from macrophages through physical contact with the

cell's extracellular matrix and ATP-binding cassette transporter 1. The outpouring of cholesterol from circulating and infiltrating uterine macrophages at term provides a substrate for Cyp11a action in promoting estrogen biosynthesis in uterine tissue, facilitating the process of labour. ApoA1 also acts as a cofactor for lecithin cholesterol acyltransferase, which is capable of converting steroids to lipophilic steroid esters to prolong or potentiate the hormone's action. The steroid esters can be taken up directly by the cell via lipoprotein receptors and then be hydrolysed to free steroid capable of eliciting its physiological effect [145].

During blastocyst implantation, lipid accumulation in the uterine endometrium increases, reflecting low ApoA1-mediated transport. This accumulation is also found postpartum further defining the window of increased ApoA1 transcription [146]. Lipid accumulation associated with low ApoA1 levels leads to cell breakdown and tissue disruption, contributing to the uterine changes necessary at these times. Tissue remodelling during labour results in placental detachment. Postpartum, it returns the tissue to its non-pregnant state.

These many roles of ApoA1 may all participate in the process culminating in labour. Its up-regulation at term (43-fold) is one of the highest found in this study. The complete return to estrus levels postpartum defines this gene and its product as playing a key role in pregnancy and parturition. The similar term up-regulation (albeit to lesser extents) and subsequent down-regulation of many other mediators of lipid transport such as apolipoproteins A4, C1, E, M, J; serum amyloid A3 (SAA3); and fatty acid binding proteins 3 and 4, and of lipid metabolism, such as lipoprotein lipase, direct the spotlight on this system as a focus for further study with respect to understanding and regulating the process of labour.

Moderately down-regulated genes

Although not as numerous as those genes up-regulated with parturition, the importance of those genes suppressed at the pivotal moment should not be dismissed. Along with Cx26 repression, we found a large number of genes down-regulated in this

process. Term animals demonstrated the largest number of genes regulated in this manner and most of these genes continued to be repressed at the moment of labour.

Liver arginase 1 (Arg1) of Cluster G, which was significantly repressed at term and then began returning to estrus levels soon after, has been demonstrated to be up-regulated between days 16.5 and 19 in the Bethin murine labour study [118]. Microarray analysis of estrogen-regulated uterine genes has demonstrated Arg1 to be negatively regulated by estrogen [122]. As estrogen levels rise to a crescendo at labour one would expect the down-regulation of Arg1, as we have found. The role of Arg1 appears to be one of immunomodulation. T_h2 cytokines up-regulate the gene and lead to a block in T-cell proliferation [147]. This immunomodulation is lifted with increased estrogen, leading to a fetal tissue rejection and expulsion of the offspring. Arg1 has also been attributed to stimulating proliferation of smooth muscle cells [148]. A suppression of Agr1 with increased estrogen levels would contribute to limiting uterine growth and facilitating the stretch response.

Prostaglandin E_2 receptor subtype 4 (EP4) and Chaperonin 10 (Chap10) probe sets are representative of genes of Cluster H which demonstrated a repression specific to the prelabour time point. PGE_2 receptors have been characterized as participating in myometrial contraction (EP1 and EP3) via IP_3 production and calcium (Ca^{2+}) mobilization, or relaxation (EP2 and EP4) via the cAMP second messenger [149]. EP2 is well described in this relaxatory mechanism [150]. Here we found EP4 suppression at term (Cluster H) when uterine stretch affects are seen, with an abrogated response in gene regulation at the moment of labour when these affects become irrelevant. This suggests that EP4 in the mouse uterus plays a role in uterine quiescence during gestation. Additional to this relaxatory role is its immunomodulatory effect. At estrus EP4 mRNA was found in the endometrium and expression in the stroma increased dramatically during the window of implantation (days 3-5) [151]. This followed the increase in serum progesterone levels. Progesterone is known for its immunomodulatory effects, promoting a T_h2 over a T_h1 T cell response [152], and its ability to induce EP4 expression. Elevated cAMP levels have immunomodulatory properties in lymphocytes, leading to T cell anergy. EP4

receptors propagate the PGE_2 signal for this immunomodulation [153]. The importance of this immunomodulatory effect seen during the window of blastocyst implantation permits the invasion of trophoblast tissue, which is a product of paternal genome expression. The uterus, in the non-responsive state, does not reject the foreign peptides and the embryo with it. This suppression of immune response is maintained throughout pregnancy and wanes as parturition approaches. With EP4 gene regulation under the influence of progesterone a coincident drop occurs at term, allowing T_h1 cytokines to participate in the activation of the uterus. The use of an EP4-selective agonist may provide a means by which uterine activation may be suppressed, prolonging gestation in cases of women with a high risk for premature labour.

Chaperonin 10 (Chap10, a.k.a. heat shock protein 10) probe set indicates a suppression of Chap10 gene expression at term, which is subsequently lifted. A member of the Chap10 gene family with a nearly identical open reading frame to Chap10 encodes early-pregnancy factor (EPF), a secreted form of Chap10 that is differentially expressed during pregnancy [154]. Its pattern of expression throughout pregnancy begins with an increase within 24 hours of successful fertilization and this is sustained through most of gestation [155]. It is believed from the expression pattern found here that the probe set for Chap10 is detecting changes in gene expression of EPF in the uterus. EPF sources include activated platelets, stimulated ovaries, and dividing tumour, placental and regenerating cells [156]. It is known for its role in generating maternal immune tolerance to the implanting semi-allogeneic blastocyst and promoting growth [155]. It is described as a highly potent immunomodulator as minute quantities are able to elicit profound effects. The moderate gene repression measured between term and estrus (2.37-fold) may actually represent only part of the drop in gene expression if compared to levels found even a few days prior to term when gene expression is induced. This is likely the case for EP4 gene repression as well. This down-regulation at the end stages of pregnancy releases its block on tissue rejection, permitting the onslaught of factors triggering a rejection of the paternal genome derived fetal proteins, particularly of the placenta and a rejection of the fetus

itself, facilitating placental detachment and delivery. The levels of EPF gene expression at the moment of labour returned to pre-fertilization levels as the immunomodulation provided by EPF was no longer needed. As the uterine tissue consists of many different cells types, the source of this EPF gene expression may be provided by more than just stimulated circulating platelets. This remains to be determined.

The well-described roles in early pregnancy and the responses of these genes at the termination of gestation identify EP4 and Chap10/EPF gene products to be important regulators of the process of placental detachment and expulsion of the fetus. They present themselves as potential targets for intervention therapy in conditions of premature labour. It is of no surprise to find a substantial number of genes involved in immune response to be transcriptionally activated at this time.

Most significantly down-regulated gene products are calcium-dependent

We found, in the mouse uterus, a very significant down-regulation of Purkinje cell protein 4 (PCP4) mRNA across all end-stage time points relative to estrus levels. The low levels of PCP4 sustained across term, labour, and postpartum suggest two things. First, that a significant up-regulation at estrus is necessary for some unknown reason, and second, that the down-regulation of PCP4 is important for the termination of pregnancy and uterine involution postpartum. Pep-19, the rat homologue of murine PCP4 [157] is described as a neuron-specific transcript [158, 159]. Our study demonstrates, for the first time, evidence of extraneural PCP4 gene expression. PCP4/Pep-19 has been proposed to function in inhibiting calmodulin activity [160]. Calmodulin, in complex with Ca^{2+}, provides a relay of intracellular Ca^{2+} signalling with crossover effects to additional signalling cascades. This is achieved by activating enzymes such as kinases, phosphatases, adenylate cyclase, among others. Of interest in this case is the Ca^{2+}/calmodulin-dependent activation of myosin light chain kinase. Modulation of myosin light chain phosphorylation through the regulation of myosin light chain kinase activity alters myometrial responsiveness and contractility.

We propose that down-regulation of PCP4 at term leads to a decrease in protein at labour, enabling Ca^{2+} stimulation of uterine contraction at that time. This down-regulation is sustained through the early postpartum period to allow the Ca^{2+} stimulated contractions necessary for uterine involution. As myosin phosphorylation is essential to uterine force production and alternate contractile pathways do not function in the myometrium [20], the role for PCP4 in modulating myometrial contraction during labour is proposed.

Pep19 has also been characterized to have an anti-apoptotic effect on neuronal cells [161]. This function is executed by inhibiting Ca^{2+}-mediated cell death. In the uterus, this block may facilitate uterine growth during pregnancy. The inhibition of PCP4 gene expression at term may participate in restricting further growth and therefore mediating the expression of stretch induced genes leading to connexin 43 and OTR gene expression, activating the uterus for labour.

Calcium activated chloride channel (Clca) 3 was the most dramatically down-regulated gene with respect to estrus expression. Unlike PCP4, there was a significant fold-change in the expression levels between labour and postpartum. Postpartum, the repression was less, suggesting that the suppressed state, which reached a maximum at labour, was less important once delivery has concluded. The role for Clca3 (a.k.a. gob-5) is not well defined. Its expression in uterine goblet cells proposes a role in the preparation and/or secretion of mucins [162]. Secreted mucins in the uterus can trap bacteria and therefore be immunoprotective. Clca3 expression is up-regulated during inflammation to increase the production or secretion of mucins and facilitating the immune response to pathogens [163]. A possible role for Clca3 in apoptosis is suggested by the fact that Ca^{2+}-activated chloride channels 1 and 2 participate in this cellular event. Confounding is the fact that Clca1 is suggested to play an anti-apoptotic role while Clca2 is associated with a pro-apoptotic cell phenotype [164]. Considering the changes occurring in the mouse uterus during the final moments of pregnancy, we first propose that the dramatic down-regulation of Clca3, most notably at the moment of labour, leads to decreased mucus secretion, and hence a hyper-active state of immune response, amplifying the response to paternal genome-derived fetal

antigens, leading to placental detachment and fetal rejection and expulsion. Secondly, we propose that Clca3, in the uterus, is protective against apoptosis, similar to Clca1. Its down-regulation seen here is permissive to apoptotic induction or signalling, leading to decreased cellular proliferation, decreased uterine growth, and increased stretch of the uterus in response to the growing fetus. The role of Clca3 in parturition requires further study.

Genes known to be involved in parturition but not detected here

It is important to note that there is no probe set for the oxytocin receptor (OTR) gene present on the Affymetrix murine microarray chip U74Av2. Northern blot analysis using the uterine total RNA samples prepared for microarray analysis was performed. OTR gene expression increased at term and further so with labour, then demonstrated a marked suppression postpartum when compared with estrus levels (see Chapter 4). This recapitulation of the known pattern of OTR gene expression increases the reliability of our model and tissue and RNA preparations.

Several genes known to be regulated during normal parturition and represented on the Affymetrix microarray U74Av2 are not identified in this study. For example, interleukins -1β and -6 [165] are not found in our list. This is supported by an absence of gene transcripts for these cytokines in the Bethin study. The discrepancy may reflect species-specific gene expression in normal parturition. In rats, for example, IL-1α is differentially expressed during the periparturient period while IL-1β and IL-6 are not [121, 166]. The uterine expression of these interleukins are however, increased in association with infection-induced premature labour in mice [167].

Of no surprise is the absence of oxytocin from these murine studies. OT is not expressed by mouse uterine tissue [71] suggesting circulating sources play a more significant role than in other species. This absence further confirms the validity of our microarray analysis.

Comparison with the study by Bethin et al.

Our study varies from that of Bethin et al. [118] as we included a labour group as well a term group. Bethin did not study uteri from mice in active labour. Bethin also did not collect non-pregnant tissue samples for comparison. They focused on the changes taking place during the last trimester of pregnancy, from day 13.5 to day 16.5 to day 19. Additional to the different mouse groups used in ours and Bethin's analyses is the fact that different Affymetrix microarray chips, therefore different selections of genes were screened. The probe sets present on Mu11K microarray chips used by Bethin et al. are included on the U74Av2 Affymetrix chip, but only comprise about half the probe sets. U74Av2 represents the most up-to-date oligonucleotide array available from Affymetrix [112].

Bethin et al. conducted three experimental replicates, however one of their replicates represents a pool of two mouse uteri, and the remaining two replicates are of individual mouse samples [118]. Individual sample variation is a common problem in science. We believe our method of pooling five uterine samples for each time point reduces the effect of individual variability. Repeating this process for a second replicate increases the reliability of our analysis. These differences may account for the discrepancies between our studies and provide an explanation as to why Bethin et al. found so few similarities between their mouse and human results.

Validation of genes by relative quantitative real-time RT-PCR

The genes chosen to validate the microarray data cover a range of expression patterns and fold changes. Relative quantification by Real-Time RT-PCR confirmed our expression patterns and validated the genes examined. By extension, the reproducibility of data for this sample of genes can be extended to that of the whole microarray, providing support for our findings. The presence in our list, of genes known to be involved in parturition provides further credence to this study.

2.6 CONCLUSION

Gene transcription levels do not always reflect protein level as factors involved in mRNA stability, mRNA splicing, translation, and post-translational modification can effect the production and metabolism of the functional protein, or in some cases, functional RNA. Transcription, however, is the essential first step. By studying the dynamic regulation of gene expression for thousands of genes simultaneously, thanks to the advance of microarray technology, we have uncovered novel genes and gene networks whose regulation either pattern or oppose each other to the goal of a physiological outcome, in this case parturition. We have also provided confirmation and further clarification of the involvement of previously characterized genes and their expression patterns. Understanding the regulation of these genes provides insights into the proteins involved in such physiological processes, generating hypotheses for further investigation. This investigation has brought further evidence for the hypothesis that parturition is a reversal of the process by which blastocyst implantation, and pregnancy began. Opposite regulation of genes involved in uterine tissue growth, remodelling, and quiescence take place. As well, an alleviation of the suppression of immune reaction to paternal genome-derived fetal antigens initiated during decidualization contributes to the culmination of gestation, labour. We believe this body of evidence provides a springboard for future research and development of diagnostic or therapeutic interventions for premature or otherwise complicated labour.

2.7 ACKNOWLEDGEMENTS

We thank Dr. Robert Sladek and Dr. Yoshihiko Nagai from the Montreal Genome Centre for their assistance in Affymetrix GeneChip processing and analysis support. We most gratefully acknowledge the financial support of this project by Dr. Roberto Romero and the National Institute of Health as well as by grants from the Canadian Institutes of Health Research.

Functional Classification

Cluster	GenBank	Gene Symbol	Title
DNA/Nucleotide Metabolism			
C	AV378405	Entpd2	ectonucleoside triphosphate diphosphohydrolase 2
D	AI839138	Txnip	thioredoxin interacting protein
H	AW123157	D11Wsu78e	DNA segment chr11 Wayne State University 78 expressed
H	AI841645	Ard1	N-acetyltransferase ARD1 homolog (yeast)
H	M33934	Impdh2	inosine 5'-phosphate dehydrogenase 2
H	AF083464	Rev3l	REV3-like catalytic subunit of DNA pol zeta RAD54 like (yeast)
I	U01915	Top2a	topoisomerase (DNA) II alpha
Growth/Differentiation/Apoptosis			
A	M70642	Ctgf	connective tissue growth factor
A	U94331	Tnfrsf11b	TNF receptor superfamily member 11b (osteoprotegerin)
B	X58196	H19	H19 fetal liver mRNA
B	U22399	Cdkn1c	cyclin-dependent kinase inhibitor 1C (P57)
B	X71922	Igf2	insulin-like growth factor 2
B	U15012	Ghr	growth hormone receptor
B	X83569	Nnat	neuronatin aka: Peg5
B	X66449	S100a6	S100 calcium binding protein A6 (calcyclin)
B	AI842277	Igfbp3	insulin-like growth factor binding protein 3
B	X81581	Igfbp3	insulin-like growth factor binding protein 3
B	X95504	Zac1	zinc finger protein regulator of apoptosis and cell cycle arrest
B	X98471	Emp1	epithelial membrane protein 1
B	X55573	Bdnf	brain derived neurotrophic factor
B	AW212475	Mig-6 homolog	RIKEN cDNA: Mitogen-inducible gene 6 protein homolog
B	M32490	Cyr61	cysteine rich protein 61 aka: IGFBP10
B	AF055638	Gadd45g	growth arrest and DNA-damage-inducible 45 gamma
C	AA790008	Rex3	reduced expression 3
C	AF038939	Peg3	paternally expressed 3
C	AI843313	Gpc3	glypican 3
C	U88327	Cish2	cytokine inducible SH2-containing protein 2
C	X69620	Inhbb	inhibin beta-B
D	AF051347	Rex3	reduced expression 3
D	X06368	Csf1r	colony stimulating factor 1 receptor
E	X81580	Igfbp2	insulin-like growth factor binding protein 2
E	AI852641	Nupr1	nuclear protein 1
E	D78265	Olfm1	olfactomedin 1
E	AF041054	Bnip3	BCL2/adenovirus E1B 19 kDa-interacting protein 1NIP3
F	AI840158	Angptl2	angiopoietin-like 2

Cluster	GenBank	Gene Symbol	Title
G	L12447	Igfbp5	insulin-like growth factor binding protein 5
G	U30244	Efnb2	ephrin B2
G	AV349686	Ndr2	N-myc downstream regulated 2
G	AV354117	2410012A13Rik	RIKEN cDNA: candidate mediator of the p53-dependant G2 arrest homolog (human)
H	AI849615	Gas5	growth arrest specific 5
I	AF002823	Bub1	budding uninhibited by benzimidazoles 1 homolog (yeast)
I	AA681998	1110038L14Rik	RIKEN cDNA:cdk regulatory subunit 2 (XE-P9) homolog (Xenopus)
I	X82786	Mki67	antigen identified by monoclonal antibody Ki 67
Transcription regulation			
A	U19118	Atf3	activating transcription factor 3
A	AF117709	Sfrp4	secreted frizzled-related sequence protein 4
B	V00727	cFos	FBJ osteosarcoma oncogene
B	U83148	Nfil3	nuclear factor interleukin 3 regulated
C	AW061016	Vdr	vitamin D receptor
C	M32484	Pem	placentae and embryos oncofetal gene
C	AF009414	Sox11	SRY-box containing gene 11
D	Y15163	Cited2	Cbp/p300-interacting transactivator with Glu/Asp-rich C-term domain 2
E	AW124113	Basp1	brain abundant membrane attached signal protein 1
E	X90778	H2bfs	histone gene complex 1
F	X70298	Sox4	SRY-box containing gene 4
F	AW124153	Sox4	SRY-box containing gene 4
F	X05862	H2bfs	H2B histone family member S
F	U40930	Sqstm1	sequestosome 1 aka: annexin A11
F	X89749	Tgif	TG interacting factor
F	U73037	Irf7	interferon regulatory factor 7
G	AF062071	Zfp216	zinc finger protein 216
G	AI987985	Zfp288+C297	DNA segment chr16 Wayne State University 73 expressed
G	Y07688	Nfix	nuclear factor I/X
G	AW047343	Dbp	D site albumin promoter binding protein
G	U33629	Meis1	myeloid ecotropic viral integration site 1
H	AV252495	Smarcd2	SWI/SNF related matrix associated actin dep regulator of chromatin d2
H	U20344	Klf4	Kruppel-like factor 4 (gut)
H	AI854510	Trap100-pending	thyroid hormone receptor-associated protein 100 kDa
H	X67668	Hmgb2	high mobility group box 2
H	M12848	Myb	myeloblastosis oncogene
I	AW124932	Pbx1	pre B-cell leukemia transcription factor 1

Cluster	GenBank	Gene Symbol	Title
RNA/Protein Biosynthesis			
Protein Metabolism/Modification			
A	M68898	Mcpt5	mast cell protease 5
B	AI854771	C81439	EST: 92 % homologous to :SUI1_MOUSE Protein translation factor SUI1
B	AI850953	1200015P13Rik	RIKEN cDNA: Cl- ion pump-associated 55 kDa protein homolog (rat)
B	X06342	Spink3	serine protease inhibitor Kazal type 3
C	AF017994	Mest	mesoderm specific transcript formerly Peg1
C	M25529	Spi1-2	serine protease inhibitor 1-2 aka:mouse alpha 1-antitrypsin
C	X70393	Itih3	inter-alpha trypsin inhibitor heavy chain 3
C	AF032466	Arg2	arginase type II
C	L11333	Es31	esterase 31
C	U28419	Eif1a	eukaryotic translation initiation factor 1A
C	AF084482	Wfs1	Wolfram syndrome 1 homolog (human)
D	AV059956	Itmap1	integral membrane-associated protein 1
D	D88899	Kdap	kidney-derived aspartic protease-like protein
D	AI851255	Ctsb	cathepsin B
D	AI746846	2410004M09Rik	RIKEN cDNA: sorting nexin 10
E	X93037	Expi	extracellular proteinase inhibitor
E	AI841076		EST: Highly similar to rat fasciculation and elongation protein zeta1
E	AI840339	Rnase4	ribonuclease RNase A family 4
E	AJ000990	Lgmn	legumain
E	AV218205	Cst3	cystatin C
E	U10098	Cst3	cystatin C
F	AJ223208	Ctss	cathepsin S
F	AW060971	1300002A08Rik	RIKEN cDNA
G	AI846197	0610006H08Rik	RIKEN cDNA: histidyl-tRNA synthetase homolog (human)
G	U22033	Psmb8	proteosome subunit beta type 8 (large multifunctional protease 7)
G	U51805	Arg1	arginase 1liver
H	AA655369	Timm8a	translocase of inner mitochondrial membrane 8 homolog a (yeast)
H	X71978	Fts	fused toes
H	L42115	Slc1a7	solute carrier family 1 member 7
H	AW045418	Rpl44	ribosomal protein L 44
H	AF073993	Hnrpa2b1	heterogeneous nuclear ribonucleoprotein A2/B1
H	AA656775	Rrr-pending	regulator for ribosome resistance homolog (yeast)
H	AF053232	Nol5	nucleolar protein 5
H	AI843342	Sfrs1	RIKEN cDNA: splicing factor, arginine/serine-rich 1 (ASF/SF2)
H	U09659	Hspe1	heat shock 10 kDa protein 1 (chaperonin 10)
H	AI846708	Rpn1	ribophorin I
H	X64837	Oat	ornithine aminotransferase
H	AI837110	Hrmt1l2	heterogeneous ribonucleoproteins methyltransferase-like 2 (yeast)
H	AW122030	Psat-pending	Similar to phosphoserine aminotransferase

Cluster	GenBank	Gene Symbol	Title
I	D55720	Kpna2	karyopherin (importin) alpha 2
I	AW124988	Apobec2	apolipoprotein B editing complex 2
Signalling			
A	X61940	Ptpn16	protein tyrosine phosphatase non-receptor type 16
A	U44027	Pnoc	prepronociceptin
A	AW046181	Sgk	serum/glucocorticoid regulated kinase
A	Y08361	Ril-pending	reversion induced LIM gene
B	U95182	Guca2b	guanylate cyclase activator 2b (retina)
C	AV303514	Pip5k2c	phosphatidyl inositol-4-phosphate 5-kinase type II gamma
C	AB020741	Nrk	Nik related kinase
D	U18869	Dab2	disabled homolog 2 (Drosophila)
D	AV376312	4930422J18Rik	RIKEN cDNA: SPRY domain-containing SOCS box protein SSB-1 homolog (mouse)
D	AI596360	AI596360	EST: SPRY domain-containing SOCS box protein SSB-1 homolog (mouse)
D	AW124633	Nek7	NIMA (never in mitosis gene a)-related expressed kinase 7
F	U10551	Gem	GTP binding protein (gene over expressed in skeletal muscle)
G	AF039663	Prom	prominin
G	AI747194	Lamp2	lysosomal membrane glycoprotein 2
H	AA163960	AW112107	EST
H	AF009246	Rasd1	RAS dexamethasone-induced 1
H	D13458	Ptger4	prostaglandin E receptor 4 (subtype EP4)
H	AI845584	Dusp6	dual specificity phosphatase 6
I	AI849109	Rasgrf1	Ras-specific guanine releasing factor 1
Calcium Binding/Transport			
A	AF028071	Calb3	calbindin-D9K
A	AV037012	Calb3	calbindin-D9K
B	AI849587	Cacng4	voltage-dep calcium channel gamma subunit-like protein
C	L24430	Bglap-rs1	osteocalcin-related protein precursor (OC-X) (nephrocalcin)
D	AI626942	AI452351	EST
G	AF004666	Slc8a1	solute carrier family 8 (sodium/calcium exchanger) member 1
H	X17320	Pcp4	Purkinje cell protein 4
Intercellular adhesion/communication			
Extracellular Matrix		**Blood Clotting**	
A	M63801	Gja1	gap junction membrane channel protein alpha 1 (connexin 43)
A	M63801	Gja1	gap junction membrane channel protein alpha 1 (connexin 43)
A	X56304	Tnc	tenascin C
A	AV230686	Tnc	tenascin C
A	AB007813	Fcna	ficolin A

Cluster	GenBank	Gene Symbol	Title
A	M33960	Serpine1	serine proteinase inhibitor E1 (nexin plasminogen activator inhibitor 1)
B	AI876446	Fga	fibrinogen alpha polypeptide
B	X52308	F2	coagulation factor II
B	AI196896	Fgb	fibrinogen B beta polypeptide
B	AI786089	Kng	kininogen
B	L39017	Procr	protein C receptor endothelial
B	AJ002390	Anxa8	annexin A8
B	AA880988	Xlkd1	extra cellular link domain-containing 1
B	AJ001633	Anxa3	annexin A3
C	AA986050	AI256424	EST: fibrinogen gamma polypeptide
C	AF026073	Sultn	N-sulfotransferase
D	D86370	Msln	mesothelin
E	X53928	Bgn	biglycan
E	L19932	Tgfbi	transforming growth factor beta induced 68 kDa
E	M62470	Thbs1	thrombospondin 1
E	D13664	Osf2-pending	osteoblast specific factor 2 (fasciclin I-like)
E	AI845237	Clic4	chloride intracellular channel 4 (mitochondrial)
E	AI849533	Clic4	chloride intracellular channel 4 (mitochondrial)
F	L36244	Mmp7	matrix metalloproteinase 7
F	X70296	Serpine2	serine protease inhibitor 4
F	X13986	Spp1	secreted phosphoprotein 1 aka: osteopontin precursor minopontin
F	D00613	Mglap	matrix gamma-carboxyglutamate (gla) protein
F	AF022889	Ltbp1	latent transforming growth factor beta binding protein 1
F	M77196	Ceacam1	CEA-related cell adhesion molecule 1
F	M77196	Ceacam1	CEA-related cell adhesion molecule 1
G	U66166	Sparcl1	SPARC-like 1 (mast 9 hevin) (Extracellular matrix protein 2)
H	AA763466	Col1a1	procollagen type I alpha 1
H	Z18272	Col6a2	procollagen type VI alpha 2
H	AF011450	Col15a1	procollagen type XV
H	L33779	Dsc2	desmocollin 2
H	M81445	Gjb2	gap junction membrane channel protein beta 2 (connexin 26)
H	AI465965		EST
Cytoskeleton		**Muscle Development**	
A	U04354	Scin	scinderin
C	K02108	Krt2-6a	keratin complex 2 basic gene 6a
C	M55413	Gc	group specific component
C	M15501	Actc1	actin alpha cardiac
C	D88792	Csrp2	cysteine-rich protein 2
C	L47600	Tnnt2	troponin T2 cardiac
E	M13805	Krt1-17	keratin complex 1 acidic gene 17

Cluster	GenBank	Gene Symbol	Title
E	AW123904	Gabarapl1	GABA(A) receptor-associated protein-like 1
E	AI839417	Msn	moesin
F	AW122536	Pfn2	profilin 2
G	AF002283	Pdlim3	PDZ and LIM domain 3
G	AJ005559	Sprr2a	small proline-rich protein 2A
G	AJ005559	Sprr2a	small proline-rich protein 2A
H	AI848479	Enc1	ectodermal-neural cortex 1
I	M12347	Acta1	actin alpha 1 skeletal muscle
Lipid Binding/Transport/Metabolism			
A	AA726364	Lpl	lipoprotein lipase
B	U79573	Apoa1	apolipoprotein A-I
B	AA871791	1300003D03Rik	RIKEN cDNA: D20 protein homolog (human)
C	M64248	Apoa4	apolipoprotein A-IV
C	Z22661	Apoc1	apolipoprotein CI
C	X13060	Alb1	albumin 1
C	AW049768	Lcn7	lipocalin 7
C	AA655303	Apom	apolipoprotein M
C	U37799	Srb1	scavenger receptor class B1
C	M20497	Fabp4	fatty acid binding protein 4 adipocyte
D	X03505	Saa3	serum amyloid A 3
D	M63335	Lpl	lipoprotein lipase
D	X14961	Fabp3	fatty acid binding protein 3 muscle and heart
D	AF003348	Npc1	Niemann Pick type C1
E	D00466	Apoe	apolipoprotein E
E	M93275	Adfp	adipose differentiation related protein
E	D14077	Clu	clusterin aka:apolipoprotein J
E	AV003873	D14Ucla3	DNA segment chr14 UCLA 3: 90% blast homology with Clusterin
E	U28960	Pltp	phospholipid transfer protein
H	M35523	Crabp2	cellular retinoic acid binding protein II
H	AW226939	Ces3	carboxylesterase 3
H	AI849271	0610011L04Rik	RIKEN cDNA: (acetyl-CoA acyltransferase) (rat)
I	AB017026	Osbpl1a	oxysterol binding protein-like 1A
Hormone Biosynthesis/Metabolism			
B	V00743	Afp	alpha fetoprotein
B	M58567	Hsd3b1	hydroxysteroid dehydrogenase-1delta<5>-3-beta
B	J04758	Tph	tryptophan hydroxylase
B	J05663	Akr1b7	aldo-keto reductase family 1member B7
C	AV290268	Hsd3b1	hydroxysteroid dehydrogenase-1delta<5>-3-beta
C	AW121619	Cyp11a	cytochrome P45011a cholesterol side chain cleavage

Cluster	GenBank	Gene Symbol	Title
D	X83202	Hsd11b1	hydroxysteroid 11-beta dehydrogenase 1
F	M88242	Ptgs2	prostaglandin-endoperoxide synthase 2 (COX-2)
F	X61232	Cpe	carboxypeptidase E
F	X61232	Cpe	carboxypeptidase E
I	AW122260	Cyp51	cytochrome P45051
I	AA407599		EST
I	D42048	Sqle	squalene epoxidase
I	AI846851	Fdps	farnesyl diphosphate synthetase
I	AW045533	Fdps	farnesyl diphosphate synthetase
I	AA716963		EST: Similar to isopentenyl-diphosphate delta isomerase
Carbohydrate Biosynthesis/Metabolism			
Electron Transport -Energy			
B	AA939571	AI785303	EST
B	AF031380	KOI-4	RIKEN cDNA: hypothetical Riboflavin kinase / FAD synthetase containing protein
C	AF009605	Pck1	phosphoenolpyruvate carboxykinase 1 cytosolic
C	L29123	Fdx1	ferredoxin 1
C	AB018421	Cyp4a10	cytochrome P4504a10
C	AW123273	1200011C15Rik	RIKEN cDNA: similar to cytochrome P450 2S1 (human)
D	AJ001418	Pdk4	pyruvate dehydrogenase kinase 4
F	AW049730	Gyg1	glycogenin 1
F	AI844043	0610010I20Rik	RIKEN cDNA
F	X75129	Xdh	xanthine dehydrogenase
F	AA623874	3230402E02Rik	RIKEN cDNA
F	AV357306	Gyg1	glycogenin 1
G	X93035	Chi3l1	chitinase 3-like 1 aka: Glycoprotein 39
H	X04591	Ckb	creatine kinase brain
I	X99273	Aldh1a2	aldehyde dehydrogenase family 1 subfamily A2
Cell Defense/Immune Function			
B	AF036736		mRNA for immunoglobulin heavy chain mAb 667
B	L33954		mRNA for immunoglobulin heavy chain mAb 667
B	AF000236	Cmkor1	chemokine orphan receptor 1
D	U13705	Gpx3	glutathione peroxidase 3
D	X70922	Ly6f	lymphocyte antigen 6 complex locus F
D	M80423		Mus castaneus IgK chain gene c-region 3' end
D	M73748	Gp38	glycoprotein 38
D	U10410	LOC56304	recombinant anti-neuraminidase single chain Ig VH and VL domains
D	D31788	Bst1	bone marrow stromal cell antigen 1
E	X15592	Ctla2b	cytotoxic T lymphocyte-associated protein 2 beta
E	X15591	Ctla2a	cytotoxic T lymphocyte-associated protein 2 alpha

Cluster	GenBank	Gene Symbol	Title
E	X58861	C1qa	complement component 1q subcomponent alpha polypeptide
E	M18237	Igk-V28	immunoglobulin kappa chain variable 28 (V28)
E	U49513	Scya9	small inducible cytokine A9 aka:Macrophage inflammatory protein 1-gamma
E	X51547	Lzp-s	P lysozyme structural
E	X66295	C1qc	complement component 1q subcomponent c polypeptide
E	M21050	Lyzs	lysozyme
E	M22531	C1qb	complement component 1q subcomponent beta polypeptide
E	Z80112	Cmkar4	chemokine (C-X-C) receptor 4
E	M58004	Scya6	small inducible cytokine A6
E	AF035684	Scya21b	small inducible cytokine A21b (leucine)
F	M63695	Cd1d1	CD1d1 antigen
F	M65027	Gp49a	glycoprotein 49 A
F	U43085	Ifit2	interferon-induced protein with tetratricopeptide repeats 2
F	U05265	Gp49b	glycoprotein 49 B
F	AV370035	Cmkbr5	chemokine (C-C) receptor 5
F	X97227	Cd53	CD53 antigen
F	X99347	Lbp	lipopolysaccharide binding protein
F	AV229143		EST: interferon activated gene 202A
F	M31419	Ifi204	interferon activated gene 204
F	M31418	Ifi202a	interferon activated gene 202A
F	AI841295	0610025I19Rik	RIKEN cDNA
G	AV092014	Pglyrp	peptidoglycan recognition protein
G	AF022371	Ifi203	interferon activated gene 203
G	AB001489	Pigr	polymeric immunoglobulin receptor
G	AF076482	Pglyrp	peptidoglycan recognition protein
G	L38444	Tgtp	T-cell specific GTPase
G	AA790307	onzin	onzin aka:Plac8: placenta-specific 8
G	M21038	Mx1	myxovirus (influenza virus) resistance 1
H	M22679	Adh1	alcohol dehydrogenase 1 complex
H	M25944	Car2	carbonic anhydrase 2
Transport			
B	U63146	Rbp4	retinol binding protein 4 plasma
B	V00722	Hbb-b2	hemoglobin beta adult minor chain
B	J00413	Hbb-b1	hemoglobin beta adult major chain
B	V00714	Hba-a1	hemoglobin alpha adult chain 1
C	M60348	Abcb1b	ATP-binding cassette sub-family B (MDR/TAP) member 1B
C	AA895838	1300017J02Rik	RIKEN cDNA: transferrin (fragment) homolog (horse)
C	AV003378	Hbb-b1	hemoglobin beta adult major chain
C	AJ010338		mRNA for hypothetical protein
D	V00835	Mt1	metallothionein 1

Cluster	GenBank	Gene Symbol	Title
D	U03434	Atp7a	ATPase Cu++ transporting alpha polypeptide
E	X60367	Rbp1	retinol binding protein 1 cellular
F	AI841689	9430096L06Rik	RIKEN cDNA
F	L02914	Aqp1	aquaporin 1
F	AW124544	Ermelin-pending	endoplasmic reticulum membrane protein
G	AI197481	Abp1	amiloride binding protein 1 (amine oxidase copper-containing)
G	U49430	Cp	ceruloplasmin aka:Ferroxidase
H	U88623	Aqp4	aquaporin 4
H	AV373378	Clca3	chloride channel calcium activated 3
UNKNOWN			
A	C85523		EST
A	AW047643		EST
B	AI836610	AA407948	EST
B	AI839150	1110035L05Rik	RIKEN cDNA
B	AI503362		EST
B	AB023957	EIG180	ethanol induced gene product EIG180
C	AI852838	Meg3	maternally expressed 3
C	AI787317	AI315052	EST
C	AW123955	D19Wsu57e	DNA segment chr19 Wayne State University 57 expressed
C	AI846906	5033414D02Rik	RIKEN cDNA
C	U25739		EST weakly similar to JC7182 Na+-dependent vitamin C (human)
C	AA866768	AA407323	EST
C	AW046694	4631408O11Rik	RIKEN cDNA
D	AW122893	1810015C04Rik	RIKEN cDNA
D	X94418	AI893585	EST
E	M22810	Kap	kidney androgen regulated protein
E	AI843884	C75969	EST
E	AA612450	2310046B19Rik	RIKEN cDNA
E	AW227647	1110025J15Rik	RIKEN cDNA: N-methyl-D-Asp receptor Glu-binding chain homolog (rat)
E	U92454	Wbp5	WW domain binding protein 5
F	AA793671		mRNA complete cds clone:c0103
F	AF006465	Ranbp9	RAN binding protein 9
F	AI838836	6330403K07Rik	RIKEN cDNA
G	AI504338		EST
G	AJ005561	Sprr2c	small proline-rich protein 2C
G	AI842065	AU046135	EST
H	AI843074	2010012C24Rik	RIKEN cDNA
H	AA684508	Rnu22	RNA U22 small nucleolar
H	AA958560	1810035L17Rik	RIKEN cDNA
H	AW122364	Armet	arginine-rich mutated in early stage tumors

Cluster	GenBank	Gene Symbol	Title
H	AI851250	Spred2+C88	EST
H	AI122538	2810417H13Rik	RIKEN cDNA
H	AB016592	Agr2	anterior gradient 2 (Xenopus)
H	AI314958	2310047E01Rik	RIKEN cDNA
I	AW122413		EST: Weakly similar to tyrosine-protein kinase JAK3 (mouse)
I	AI847054	1110003O22Rik	RIKEN cDNA
I	AI837621	1100001I23Rik	RIKEN cDNA: tetraspan net-6 homolog (human)
I	AI595322		EST: Similar to highly expressed in cancer rich in leucine heptad repeats
I	AW125346	1110021D01Rik	RIKEN cDNA
I	AI850558		EST: Moderately similar to A2M2 murinoglobulin 2 precursor (mouse)

GenBank	Alternate Classifications	
AV378405	Protein Metabolism/Modification	
AI839138		
AW123157		
AI841645	RNA/Protein Biosynthesis	
M33934	Growth/Differentiation/Apoptosis	
AF083464	Growth/Differentiation/Apoptosis	
U01915		
M70642	DNA/Nucleotide Metabolism	Extracellular matrix
U94331	Extracellular matrix	Signalling
X58196		
U22399		
X71922		
U15012	Cell Defense/Immune Response	
X83569		
X66449	Calcium Binding/Transport	
AI842277		
X81581		
X95504	DNA/Nucleotide Metabolism	Transcription regulation
X98471	Intercellular adhesion/communication	
X55573		
AW212475	Signalling	
M32490		
AF055638	RNA/Protein Biosynthesis	
AA790008		
AF038939		
AI843313	Extracellular matrix	
U88327	Cell Defense/Immune Response	Signalling
X69620		
AF051347		
X06368	Cell Defense/Immune Response	Signalling
X81580	Intercellular adhesion/communication	Signalling
AI852641		
D78265		
AF041054	Calcium Binding/Transport	
AI840158	Blood Clotting	

GenBank	Alternate Classifications		
L12447			
U30244	Signalling		
AV349686			
AV354117			
AI849615			
AF002823	DNA/Nucleotide Metabolism		
AA681998			
X82786	DNA/Nucleotide Metabolism	RNA/Protein Biosynthesis	Transcription regulation
U19118			
AF117709	Signalling		
V00727	Growth/Differentiation/Apoptosis		
U83148			
AW061016	Calcium Binding/Transport		
M32484			
AF009414			
Y15163			
AW124113			
X90778	DNA/Nucleotide Metabolism		
X70298			
AW124153			
X05862	DNA/Nucleotide Metabolism		
U40930			
X89749			
U73037	Cell Defense/Immune Response		
AF062071			
AI987985			
Y07688	DNA/Nucleotide Metabolism		
AW047343			
U33629	Growth/Differentiation/Apoptosis		
AV252495	Cytoskeleton		
U20344			
AI854510	Growth/Differentiation/Apoptosis		
X67668	DNA/Nucleotide Metabolism		
M12848	Growth/Differentiation/Apoptosis		
AW124932	Growth/Differentiation/Apoptosis	Hormone Biosynthesis/Metabolism	

GenBank	Alternate Classifications	
M68898	Cell Defense/Immune Response	
AI854771		
AI850953		
X06342	Extracellular matrix	
AF017994		
M25529	Extracellular matrix	
X70393	Extracellular matrix	
AF032466		
L11333		
U28419		
AF084482		
AV059956	Extracellular matrix	
D88899		
AI851255		
AI746846	Signalling	
X93037		
AI841076		
AI840339		
AJ000990	Cell Defense/Immune Response	
AV218205		
U10098		
AJ223208		
AW060971		
AI846197		
U22033	Cell Defense/Immune Response	
U51805		
AA655369	Electron Transport -Energy	
X71978	Growth/Differentiation/Apoptosis	
L42115		
AW045418		
AF073993		
AA656775		
AF053232	Growth/Differentiation/Apoptosis	Transport
AI843342		
U09659	Transport	
AI846708		
X64837		
AI837110		
AW122030		

GenBank	Alternate Classifications
D55720	
AW124988	
X61940	Growth/Differentiation/Apoptosis
U44027	
AW046181	Transport
Y08361	
U95182	Transport
AV303514	
AB020741	
U18869	Cell Defense/Immune Response
AV376312	
AI596360	
AW124633	
U10551	
AF039663	
AI747194	Intercellular adhesion/communication
AA163960	
AF009246	
D13458	
AI845584	
AI849109	
AF028071	
AV037012	
AI849587	
L24430	
AI626942	
AF004666	
X17320	
M63801	Cell Defense/Immune Response
M63801	Cell Defense/Immune Response
X56304	
AV230686	
AB007813	

GenBank	Alternate Classifications			
M33960				
AI876446				
X52308	Calcium Binding/Transport	Cell Defense/Immune Response	Protein Metabolism/Modification	
AI196896				
AI786089	Protein Metabolism/Modification	Hormone Biosynthesis/Metabolism	Cell Defense/Immune Response	Signalling
L39017		Growth/Differentiation/Apoptosis		
AJ002390	Calcium Binding/Transport			
AA880988				
AJ001633	Calcium Binding/Transport	Hormone Biosynthesis/Metabolism	Signalling	
AA986050				
AF026073	RNA/Protein Biosynthesis			
D86370				
X53928				
L19932	Growth/Differentiation/Apoptosis			
M62470	Calcium Binding/Transport			
D13664				
AI845237				
AI849533				
L36244	Protein Metabolism/Modification			
X70296	Protein Metabolism/Modification			
X13986	Cell Defense/Immune Response			
D00613	Calcium Binding/Transport			
AF022889	Calcium Binding/Transport	Growth/Differentiation/Apoptosis		
M77196	Cell Defense/Immune Response			
M77196				
U66166	Calcium Binding/Transport			
AA763466				
Z18272				
AF011450				
L33779	Calcium Binding/Transport	Cytoskeleton		
M81445				
AI465965				
U04354	Calcium Binding/Transport			
K02108				
M55413	Cell Defense/Immune Response	Transport		
M15501				
D88792	Growth/Differentiation/Apoptosis			
L47600				
M13805				

GenBank	Alternate Classifications		
AW123904			
AI839417			
AW122536	Signalling		
AF002283			
AJ005559			
AJ005559			
AI848479	Growth/Differentiation/Apoptosis		
M12347			
AA726364	Extracellular matrix		
U79573	Hormone Biosynthesis/Metabolism		
AA871791			
M64248	Hormone Biosynthesis/Metabolism		
Z22661			
X13060	Protein Metabolism/Modification	Hormone Biosynthesis/Metabolism	Transport
AW049768	Protein Metabolism/Modification	Transport	
AA655303			
U37799			
M20497			
X03505	Cell Defense/Immune Response		
M63335	Extracellular matrix		
X14961	Growth/Differentiation/Apoptosis		
AF003348	Signalling		
D00466			
M93275	Growth/Differentiation/Apoptosis		
D14077	Growth/Differentiation/Apoptosis		
AV003873	Growth/Differentiation/Apoptosis		
U28960			
M35523	Growth/Differentiation/Apoptosis		
AW226939	Protein Metabolism/Modification		
AI849271	Electron Transport -Energy		
AB017026	Hormone Biosynthesis/Metabolism		
V00743	Cell Defense/Immune Response	Growth/Differentiation/Apoptosis	Transport
M58567			
J04758			
J05663	Carbohydrate Biosynthesis/Metabolism		
AV290268			
AW121619			

GenBank	Alternate Classifications	
X83202		
M88242	Cell Defense/Immune Response	
X61232	Protein Metabolism/Modification	
X61232	Protein Metabolism/Modification	
AW122260	Electron Transport -Energy	
AA407599	Cell Defense/Immune Response	
D42048		
AI846851	Growth/Differentiation/Apoptosis	Signalling
AW045533	Growth/Differentiation/Apoptosis	Signalling
AA716963		
AA939571		
AF031380		
AF009605		
L29123	Hormone Biosynthesis/Metabolism	
AB018421	Hormone Biosynthesis/Metabolism	
AW123273	Hormone Biosynthesis/Metabolism	
AJ001418		
AW049730		
AI844043		
X75129	DNA/Nucleotide Metabolism	
AA623874		
AV357306		
X93035	Extracellular matrix	
X04591		
X99273		
AF036736		
L33954		
AF000236	Signalling	
U13705	Transport	Lipid Binding/Transport/Metabolism
X70922	Intercellular adhesion/communication	
M80423		
M73748		
U10410		
D31788		
X15592		
X15591		

GenBank	Alternate Classifications	
X58861		
M18237		
U49513	Signalling	
X51547	Carbohydrate Biosynthesis/Metabolism	
X66295		
M21050	Carbohydrate Biosynthesis/Metabolism	
M22531		
Z80112	Signalling	Growth/Differentiation/Apoptosis
M58004	Blood Clotting	Signalling
AF035684	Growth/Differentiation/Apoptosis	
M63695		
M65027		
U43085		
U05265		
AV370035	Signalling	
X97227	Growth/Differentiation/Apoptosis	
X99347	Lipid Binding/Transport/Metabolism	
AV229143	Transcription regulation	
M31419	Transcription regulation	RNA/Protein Biosynthesis
M31418	Transcription regulation	
AI841295		
AV092014	Signalling	Growth/Differentiation/Apoptosis
AF022371		
AB001489		
AF076482	Signalling	Growth/Differentiation/Apoptosis
L38444	Signalling	
AA790307	Intercellular adhesion/communication	
M21038	Signalling	
M22679		
M25944		
U63146	Growth/Differentiation/Apoptosis	
V00722		
J00413		
V00714		
M60348	Cell Defense/Immune Response	
AA895838		
AV003378		
AJ010338		
V00835		

GenBank	Alternate Classifications	
U03434		
X60367		
AI841689		
L02914		
AW124544		
AI197481	Protein Metabolism/Modification	Extracellular matrix
U49430	Cell Defense/Immune Function	
U88623		
AV373378		
C85523		
AW047643		
AI836610		
AI839150		
AI503362		
AB023957		
AI852838		
AI787317		
AW123955		
AI846906		
U25739		
AA866768		
AW046694		
AW122893		
X94418		
M22810		
AI843884		
AA612450		
AW227647		
U92454		
AA793671		
AF006465		
AI838836		
AI504338		
AJ005561		
AI842065		
AI843074		
AA684508		
AA958560		
AW122364		

GenBank	Alternate Classifications
AI851250	
AI122538	
AB016592	
AI314958	
AW122413	
AI847054	
AI837621	
AI595322	
AW125346	
AI850558	

Table 2.4 Function-organized probe sets reflecting significant changes in gene expression levels.

Alternative functional classifications are indicated where applicable. EST, expressed sequence tag; RIKEN cDNA, Japanese consortium expressed sequence.

Chapter 3

Identification and Characterization of Global Gene Expression Changes during Infection- and Ovariectomy-Induced Premature Labour

Barbara R. Gould and Hans H. Zingg

Manuscript in Preparation

3.1 ABSTRACT

The incidence of premature labour has not decreased over the last 40 years. In spite of much interest and investigation into the participating gene products, our current view of the mechanisms of premature labour is quite narrow. As a result, the development of diagnostic, preventative, and therapeutic agents for the management of complicated and premature labour has met with limited success. To broaden our understanding of the participants in these processes we employed oligonucleotide microarray technology, studying global gene expression changes during the approach and culmination of preterm labour. A considerable number of genes were significantly expressed at the occurrence of preterm labour induced by infection and by ovariectomy. The reproducible expression patterns of genes known to be involved in these processes supports the validity of the entire analysis, as did performing relative quantitative real-time RT-PCR on selected transcripts.

In our infection-induced model, preterm parturition occurred in response to an injection of 10^{10} heat killed *E.coli*, 8 hours after the infection. Microarray analysis of gene expression demonstrated the induction ofgenes involved in an acute phase response and that this is, in part, responsible for preterm labour. A 10^{7} dose was not sufficient to induce this response and the genes regulated reflect this. IL-6 and cyclooxygenase-2 genes were among the most highly induced preceding and during premature parturition. Additionally, a substantial number of cytokine and interferon γ-induced genes were positively regulated. Alpha fetoprotein, apolipoprotein A1, and fibrinogen peptide genes were significantly suppressed with infection-induced preterm labour.

Ovariectomy of day 14.5 pregnant mice resulted in preterm labour after approximately 20 hours. Microarray analysis identified the up-regulation of SRY-box containing gene 4 (Sox4) and tumour necrosis factor induced protein 6 (Tnfip6) genes and the down-regulation of follistatin gene expression among others. Several genes were similarly expressed with normal labour and infection-induced preterm labour models suggesting that the genes involved in these processes to premature parturition

follow both common and divergent pathways, enabling the development of general and specific therapies in the management of preterm labour.

3.2 INTRODUCTION

Premature labour is as prevalent today as over the past several decades. The incidence in North America is approximately 10% of all births and it is the chief contributor to perinatal morbidity and mortality. Those infants who do survive often experience physical and/or mental impairments later in life [1]. Intrauterine infection (subclinical or otherwise) accounts for approximately 30% of preterm birth [91]. Through the arduous task of identifying and characterizing individual molecular participants in the process, several key components of normal and infection-induced preterm labour (PTL) have been identified. This body of research has enabled the development of tocolytics, which unfortunately have short-lived or adverse effects on the fetus, therefore limiting their use. Examples include agonists of uterorelaxants such as the β_2 adrenergic agonist Ritodrine and antagonists to uterotonic factors such as Atosiban to antagonize oxytocin receptor signalling. These new-generation tocolytics have less adverse effects than their predecessors do, however the inhibitory effect lasts only 24-48 hours after which the mother becomes desensitized and labour ensues [1]. An additional impediment to the management of PTL is the difficulty of diagnosis. Early detection and prevention of the causes inducing PTL would relieve the necessity of tocolytics. Infection-induced PTL is often associated with subclinical or asymptomatic bacterial infection, complicating this issue. The prophylactic use of antibiotics is not supported in the management of preterm labour as they do not prolong gestation and instead are associated with an increase in perinatal mortality [90].

Uterine infection sufficient to induce PTL is associated with a significant elevation of pro-inflammatory cytokines such as interleukin (IL) -1, which provokes an acute phase immune response. The most significantly induced gene target of IL-1 action is IL-6 such that the level of IL-6 in amniotic fluid is used as a diagnostic marker for infection-induced PTL [168, 169]. Much of the research on infection-induced PTL has focused on these effectors and one of their target genes, that encoding cyclooxygenase-2 (COX-2), in murine models of preterm labour (PTL). The models used included stimulation by live bacteria [167, 170], heat-killed bacteria [3,

171], the bacterial cell wall component LPS [93, 172, 173], and even ethanol injection [42]. Most of these studies have used traditional approaches to investigate the regulation of only a few genes at a time. Muhle et al. [2] used a microarray approach to identify changes in gene expression in an infection-induced PTL model utilizing heat-killed *E.coli.* The cDNA microarray used contained only 4963 spotted genes. They did not examine gene regulation at the time of infection-induced PTL as their focus was on early gene effects. Their analysis did include the examination of early gene regulation in response to ovariectomy (PTL without infection) to further clarify the genes specific to infection-induced PTL. We endeavoured a broader investigation, extending their analysis using the murine Affymetrix oligonucleotide array U74Av2, which consists of probe sets for approximately 12,000 different genes. Our analysis followed the changes in gene expression through to parturition induced by infection and by ovariectomy (Ovx). By examining the global gene expressionchanges associated with infection-induced PTL and PTL in the absence of infection (Ovx), we hope to reveal novel genes and their products participating in these processes, develop new hypotheses as to their mechanisms, and substantiate or refute previous beliefs. Comparing these different mechanisms to PTL, we can identify the commonalities and incongruities between them. Together, we hope to provide new candidates for both diagnosis and therapeutic management of preterm and otherwise complicated pregnancies.

3.3 MATERIALS AND METHODS

Animals

CD-1 female mice 8-12 weeks old were ordered from Charles River Laboratories, St.Constant, Quebec, to be mated overnight. Copulation was determined the next morning by the presence of a vaginal plug; 0.5 days post coitus (d.p.c.). At 14.5 d.p.c. (~ 75% through gestation), pregnant CD-1 mice were separated into Infection and Ovariectomy study groups and underwent the appropriate surgical procedure.

The **Infection** group received a 100 µl injection consisting of either 10^7 (low-dose (L)) or 10^{10} (high-dose (H)) heat-killed bacteria or 100 µl of sterile Luria broth (LB) as a control (C). The inoculation was administered into the right uterine horn between two adjacent fetuses. Animals were sacrificed at 3.5 hours or 8 hours post-injection as early and late time-points, respectively, and their uterine tissue was collected. These time-points are relative to the onset of infection-induced PTL, which occurred approximately 8 hours post-injection in response to the 10^{10} dose of bacteria. Those animals in active PTL (as determined by the presence of one or more pups in the cage) were identified as such (PTL). Their cohorts, who had not yet entered into active labour, were indicated as H8. When one mouse experienced PTL, it was sacrificed and its uterus was removed; this was also done for one H8 pregnant mouse not experiencing labour. Treatment of mice with the 10^7 dose of bacterium did not result in PTL. The low dose treatment groups provide insight into the changes in gene expression because of infection that are insufficient to lead to the process of labour.

The **Ovariectomy** group was divided into ovariectomized (Ovx) and sham surgery control groups. Bilateral ovariectomy was performed by clamping off the blood supply between each ovary and uterine horn and then removing the ovary. Sham operated mice were manipulated in a similar fashion but ovaries were not dissected. Uterine tissue was collected at 5 hours after surgery (early time-point) and after approximately 20 hours at the time of ovariectomy-induced PTL, (Ovx20-PTL) for both Ovx and Sham groups.

In preparation for surgery, pregnant mice were anaesthetized with 300 µl of a 2.5% Avertin solution. Isofluroane inhalant was used during surgery to maintain sedation. Incisions were closed using interrupted sutures through the peritoneal lining with sterile 4-0 silk and the skin was closed using wound clips. Animals quickly recovered from surgery and were caged individually. Prior to removing the uteri, the mice were anesthetized using CO_2 and killed by exsanguination and cervical dislocation. All animals were maintained under a 12 hour light/dark cycle with access to food and water *ad libitum*. All animal experiments were carried out in accordance with the Bioethics Committee of the McGill University Health Centre. All efforts were made to minimize the number of animals used as well as their suffering.

Heat-killed Bacteria

A blood-agar plate of pathogenic *E.coli* was procured from the clinical microbiology laboratory at the hospital. A colony was selected and used to inoculate 5 ml of sterile LB (no antibiotics) and grown overnight at 37°C. The following day the 5 ml liquid culture was added to 2 l of sterile LB and again grown overnight. An aliquot of culture was removed the following day for cell counting by spectroscopy. The absorbance at 600 nm was determined based upon 1 $OD_{600} \cong 8x10^8$ cells/ml [174] using serial dilutions. Averaged concentration values provided for accurate measurement. An LB sample was used to adjust background absorbance. After the number of bacteria was calculated, the 2 l culture was concentrated to 10^{11}/ml by centrifugation at 4°C. The bacteria were then killed according to Mussali et al. [3] by boiling the tube of cells in a water bath for 5 minutes and then placing it on ice before storing it at -20°C until needed. An aliquot of the cooling solution was cultured overnight at 37°C to confirm that the bacteria had been killed. A 1/100 dilution of the 10^{11}/ml stock in LB was prepared as needed for the 10^7-infection group at the time of surgery.

RNA Extraction from Uteri

Animals were sacrificed at the described time-points. The uterus of each mouse was quickly removed and placed into a culture plate containing phosphate buffered saline pH7.4 treated with diethylpyrocarbonate (PBS-DEPC) kept on ice to minimize RNA degradation during preparation. By carefully cutting around their borders, the placentas and decidual attachment sites were excised from the uterine tissue and discarded. The embryos present were removed along with their gestational sacs. Implantation sites where the placentas had detached (in the case of PTL) were also dissected and discarded. The uterine tissue consisting of both myometrium and endometrium was rinsed in fresh cold PBS-DEPC, divided into two, and frozen in liquid nitrogen. Samples were stored at -70°C until RNA extraction was performed.

Total RNA was extracted from pools of uterine samples from 5 mice for each time-point using the TRIzol™ Reagent (Invitrogen, Burlington, ON) as specified by manufacturer's instructions. RNA quantity and quality were determined by spectrophotometry at 260 nm and 280 nm absorbance and by formaldehyde agarose gel electrophoresis. Sample concentrations were adjusted to 2 μg/μl. The RNA was stored at -70°C.

Affymetrix GeneChip Hybridization and Statistical Analysis

Total RNA samples (30 μg) were reverse transcribed and the cDNA products were used as templates to synthesize biotin-labelled cRNA transcripts. The cRNA was fragmented to minimize target secondary structure and the quality of the cRNA was assessed by analysis with a test chip (Affymetrix, Santa Clara, CA). Hybridization of biotinylated cRNA fragments to the Affymetrix mouse genome array chip U74Av2 (version 2) followed. This array consists of 12,488 oligonucleotide targets, 66 of which are internal controls for the microarray experimental procedure. Additionally, some of the genes are represented by 2 or 3 different probe set pairs, consisting of perfect match sequences (PM) and mismatched sequences (MM) (for background correction), leaving ~12,000 unique gene probe sets to represent less than half of the predicted mouse genome.

Target cRNA that was specifically bound to the probe oligonuclotides of the mouse chip was detected using a streptavidin-phycoerythrin conjugate. The fluorescent signal was amplified using a biotinylated anti-streptavidin antibody and was detected by confocal scanning [104]. All steps in cRNA preparation, hybridization, and detection were performed by the Montreal Genome Centre according to Affymetrix protocols. Fluorescence intensity data were provided, after scaling and normalization with respect to the median intensity of each array, for further analysis by us.

Each experimental time-point was repeated once (n=2) for a total of 14 infection microarrays and 8 ovariectomy microarrays. Each replicate set of infection and ovariectomy time-points was analysed as separate experiments using the DNA-Chip Analyzer software (dChip) developed by Li and Wong [105, 106]. Affymetrix gene probe sets comprise 16 minimally overlapping 25 nucleotides long oligomers that are a perfect match (PM) to the sequence to be detected. A corresponding set of 16 oligomers, which include a mismatch (MM) at their 13^{th} nucleotide position, is used as a control for non-specific hybridization to these PM sequences. The PM and MM data for each probe set for an entire array and each array to be analyzed and compared were used by dChip to normalize all array data. Probe set outliers, array outliers, and array defects were viewed to assure satisfactory hybridization and detection of expressed genes. Outliers were excluded by the invariant set normalization procedure from further analysis. Model-based expression analysis was performed using the PM-only model [106]. Data generated included Affymetrix probe set ID, fluorescence intensity (normalized and modelled), a call describing the hybridization signal as present, marginal, or absent, and a standard error (SE) calculated by dChip based on the signal of those PM pixels which remain after outlier detection during normalization. Comparisons of gene expression, represented by signal intensity between arrays, were performed using the 2-tailed t test for significance using the Student's t test for independent samples and unequal variance according to the formula [107]:

$$t = \frac{\text{probe set mean gene}_i \text{ array}_1 - \text{probe set mean gene}_i \text{ array}_2}{\sqrt{(\text{SE gene}_i \text{ array}_1)^2 + (\text{SE gene}_i \text{ array}_2)^2}}$$

where the numerator indicates the difference between the mean fluorescence intensity of accepted PM probes for gene *i* on array 1 and on array 2, to which it is being compared. The denominator indicates the square root of the sum of the squared standard errors (SE) for gene *i* on the compared arrays and reflect the variance between the probe sets for gene *i*. Calculated *t* values allowed *p* values to be generated.

Gene lists were filtered to exclude those genes whose signal intensities were given absent calls in each array and to include only genes demonstrating a 2-fold or greater difference in expression between compared experiments with a statistically significant p value ≤ 0.05 in each replicate for a combined p value of ≤ 0.0025. Additionally, genes were eliminated from the list if the change in each replicate was not in the same direction. Gene expression comparisons were made between each group receiving a bacterial inoculation and its appropriate time-point control. As well, comparisons were made between high and low doses at 3.5 hours, 8 hours, and PTL, and across the time points for a particular infection dose. These last two sets of comparisons would allow the identification of genes undergoing changes in expression specific to the high, PTL-inducing dose vs. a milder infection response and to observe which changes occur early and late with respect to the time of infection, leading to speculation about the order of events necessary to culminate in premature parturition. Ovariectomy data were examined by comparing ovariectomized animal groups with their time-point equivalent sham-operated control group. Expression values for genes passing the filtration criteria were averaged for each experimental point. The average values were used to organize gene expression patterns using the GeneCluster software whereby the data were $\log_2$ transformed before normalization and median centering and a K-means clustering algorithm option was utilized [108]. The resultant list of cluster-organized genes was visualized using TreeView, companion software to GeneCluster. Data lists were also organized by functional classification using Affymetrix annotation information.

Two Step Real-Time Relative Quantitative RT-PCR

Selected microarray results were corroborated by RT-PCR. Aliquots of extracted total RNA were treated with RNase-free DNase (Ambion, Inc, Austin TX) according to manufacturer's instructions. Samples were re-quantified and 10 μg of each sample were reverse-transcribed. First strand cDNA was synthesized using random hexamers (Amersham Pharmacia Biotech, Baie d'Urfé, QC) and Moloney-Murine Leukemia Virus Reverse Transcriptase (Invitrogen). Reverse transcription (RT) was also performed without using an RNA template as well as with RNA but lacking the reverse transcriptase and the preparations were used as negative controls.

RT products were diluted 1/50 for subsequent amplification and relative quantification by real-time PCR. PCR primer pairs are shown in Table 3.1. The expression of the housekeeping gene HPRT was used as an endogenous control as its expression showed minimal variation across the samples. Amplification of PCR products was achieved and detected by employing the FastStart SYBR Green reaction kit, the LightCycler thermal cycler and its software application (Roche Molecular Biochemicals, Laval, QC). The PCRs were optimized for high amplification profiles of specific products. Specificity was confirmed by LightCycler melting curve analysis and agarose gel electrophoresis.

To minimize error in estimation of PCR product concentration the "Calibrator Normalized Relative Quantification with PCR Efficiency Correction" method was performed using the Relative Quantification software (Roche). First, standard concentration curves were generated for each primer pair using an arbitrarily selected sample. Standard curve Crossing point (Cp) data reflecting PCR product concentration was generated by the second derivative maximum method of the LightCycler software application. The standard curve Cp values for each gene were exported to the Relative Quantification software and compared with the HPRT housekeeping standard curve. Coefficient (comparison) files were generated for each comparison using the Relative Quantification software.

Next, microarray samples were amplified by the parameters established during standard curve preparation for each of the genes of interest. A calibrator sample used

to generate a point on the standard curve was included in each PCR run to reference the efficiency of amplification to that of the standard curve for that primer pair and to allow the Cp concentration values of each of the samples to be accurately determined for subsequent relative quantification. A PCR efficiency difference of > 0.2 between target (gene studied) and reference (housekeeping gene) can lead to a 10-fold or greater miscalculation of product concentration. An efficiency difference of > 0.05 is deemed unacceptable for concentration determination (Roche technical communication). Negative control samples were run with each experiment. Again, Cp data was generated and exported to the Relative Quantification software. Using the Cp data from the genes studied and HPRT housekeeping gene amplification, and the correlated coefficient file previously generated containing the standard curves and efficiency correction, relative quantification of gene expression for each of the samples was performed by the software application. Arbitrary values of gene expression levels after housekeeping gene correction were generated. The data were plotted using Microsoft Excel software (Redmond, WA) and compared with plots of corresponding microarray data.

Animal and tissue preparation as well as Affymetrix microarray analysis were performed in duplicate. Relative quantification real-time PCR was performed in triplicate on each sample replicate subjected to microarray hybridization.

Table 3.1 Oligonucleotides for real-time RT-PCR

Model	Gene	Forward Primer	Reverse Primer	Product size (bp)
*	Afp	gcagaaacacatcgaggagag	tcatccctcagaaaactggtg	354
*	ApoA1	gtggctctggtcttcctgaca	tcctgtctcacccaatctgtttc	303
* °	cFos	ctgtccgtctctagtgccaac	ctgctctactttgccccttct	237
*	FbgA	cccttctgctctgatgatgact	ttatctcacggtttacagccct	421
* °	HPRT	agtgttggatacaggccagac	atggccacaggactagaacac	232
* °	M-Csf	cagaccctcgagtcaacagag	tctgtcagtctctgcctggat	240
°	PCP4	ggagataatgatgggcagaagaa	cagggtgtgtattgagtgaggatg	251
* °	RGS-2	ggaagacccgtttgagctact	agtcttcacaagccaaccaga	234
*	SAA3	catcttgatcctgggagttga	ttgagtcctctgctccatgtc	254
*	Sprr2D	aagtgtcctgagccttgtcct	acttgctcttaggtgggcact	166

* Infection; ° Ovariectomy; Afp, alpha fetoprotein; ApoA1, apolipoprotein A1; cFos, FBJ osteosarcoma oncogene; FbgA, fibrinogen alpha polypeptide; HPRT, hypoxanthine guanine phosphoribosyl transferase; M-Csf, macrophage colony stimulating factor; PCP4, Purkinje cell protein 4; RGS-2, regulator of G protein signalling 2; SAA3, serum amyloid A3; Sprr2D, small proline-rich protein 2D.

3.4 RESULTS

Microarray Analysis

Analysis of microarray data by our strict criterion generated lists of probe sets that reflected significantly changing gene expression levels in our models of PTL. In the case of the intrauterine infection experiment, the analysis left 371 probe sets indicating significantly different gene expression between any two relevant time-points in replicate 1 and 352 probe sets indicating significantly different gene expression between any two relevant time-points in replicate 2. In the case of ovariectomy induced PTL, replicate 1 dChip and subsequent analysis resulted in a list of 235 probe sets demonstrating significant changes between ovariectomy and sham groups at either 5 or 20 hours post surgery while replicate 2 analysis produced a list of 141 probe sets.

The results of replicate 1 and replicate 2 were compared. First, probe sets that were unique to only one list were removed as non-reproducible. Next, replicate probe sets were removed if the fold change, p value, and number of absent calls assessments were not significant in both replicates for the same comparisons. A final filter was applied to remove probe sets if the significant fold changes for replicate 1 and replicate 2 were in opposite directions and this was the only significant time-point comparison. This left a final list of 204 probe sets in our infection model and a list of 79 probe sets in our ovariectomy model that were reproducibly demonstrating a significant change in gene expression for one or more relevant time-point comparison having a fold change value ≥ 2 or ≤ -2 and a combined p value of ≤ 0.0025. For the infection study, the list of 204 probe sets represents 1.63% of the microarray probe sets. There are at least 16 genes that were represented by more than one probe set and 23 (expressed sequences or named genes) whose corresponding function is unknown at this time. The list of 204 probe sets represents a summary of genes most likely involved in the process of infection-induced PTL but is by no means comprehensive. For the ovariectomy study, the list of 79 probe sets represents 0.63% of the microarray probe sets. At least five genes that were represented by more than one probe set and 15 probe sets for which no function has as of yet been assigned. This probe set list

represents a summary of genes most likely involved in the process of premature labour induced by ovariectomy.

Infection-Induced Changes in Gene Expression: As PTL approached, an increasing number of genes were differentially regulated. At the time of infection-induced PTL, 125 probe sets were reflecting genes that were significantly up-regulated in their expression and 32 that were representing those significantly repressed. The genes that experienced the greatest up-regulation at PTL were chitinase 3-like 1 (glycoprotein 39) and IL-6 with 45- and 36-fold increases, respectively, compared with control levels at 8 hours. This pattern for IL-6 has been well described during infection-induced PTL [91, 92]. Small inducible cytokine (Scy) A2, A5, B2, B5, and B10; GRO1 oncogene, with two probe set representatives on the DNA chip; the small proline-rich protein 2 family member (Sprr2) D; immunoresponsive gene 1 (Irg1) (also twice represented); and most notably glucocorticoid-regulated inflammatory prostaglandin G/H synthase, also known as COX-2, were substantially up-regulated at the time of PTL. Thrombospondin 1 showed a moderate induction at PTL. The genes most significantly down-regulated at the onset of PTL were for alpha fetoprotein (Afp) and apolipoprotein A1 (ApoA1). Fibrinogen alpha and beta polypeptide genes (FbgA and FbgB, respectively) and H19 mRNA were more moderately repressed.

Just preceding the onset of uterine contractions due to high dose infection (high dose, 8 hour time-point (H8)), the number of probe sets detecting significant changes in gene expression, compared to the 8 hour control (C8) levels, was 100 as induced and 7 as repressed. Those up-regulated to the greatest extent were those same genes indicated at PTL, albeit to varying levels. The 7 probe sets that were down-regulated prior to the onset of uterine contractions remained so through PTL. These genes are: for regulator of G-protein signalling 2 (RGS-2); transforming growth factor beta 2 (TGFβ2); small inducible cytokine A6 (ScyA6); RIKEN cDNA 311038L01; thyroid stimulating hormone receptor (TSH-R); EST AW061234; and macrophage colony stimulating factor (M-Csf).

It is remarkable that in comparison between PTL and H8 (time and dose cohort to PTL yet not actively in labour) only one gene was found to be changing statistically significantly in its expression level. This was the gene for alpha fetoprotein and a greater than 13-fold suppression was observed.

Early changes in gene expression with the PTL-producing high dose of bacteria were detected by increased fluorescence of 95 DNA Chip probe sets. Immunoresponsive gene 1 probe sets reflected an up-regulated expression level most significantly early after the 10^{10} dose of bacteria had been administered. The levels diminished gradually as time (and PTL) proceeded. COX-2; small inducible cytokines B2, B5, B10, and A5; IL-6; and GRO-1 were highly up-regulated as well. The expression of small inducible cytokine A2 (ScyA2) gene was more moderately induced, as was the expression of Sprr2D. Of the seven genes down-regulated at 3.5 hours, most of these continued to be repressed at these moderate levels through 8 hours and to PTL. Chitinase 3-like 1 mRNA was not significantly changed in its level of expression at this time, identifying it as a late-stage specific gene with respect to PTL.

When examining the changes between H3.5 and H8 or PTL, we find the granzymes G, E, and D most significantly up-regulated as PTL approached and an even greater increase in gene expression at the time of PTL. Granzyme C, while not statistically significantly up-regulated in its expression at 8 hours does reach statistically significant levels at PTL. The expression of Granzyme F does not seem to be changing in its expression under the conditions of this study. Granzymes are serine proteases and it has been demonstrated that they are produced by the granulated metrial gland cells of the decidualized endometrium [175] and by cytotoxic T cells [176] where they function to induce apoptosis [177]. The impressive magnitude of the increase in the gene expression of these granzymes with the progression towards PTL is tempered by the natural increase in granzyme expression during this time in control pregnant mice. M. P. Allen found an increase in the expression of granzymes, particularly D-G, which rises around day 11 of gestation, levelling to a peak at 15 d.p.c., and dropping sharply to nearly undetectable levels by day 17 and remaining so

through to normal labour at day 19 [175]. Our 8 hour time-point was consecutive to the 14.5 d.p.c. time when the surgery was performed. Our control mice were at their natural peak. When comparing with the levels found after a low dose infection there is a significant increase in gene expression at 8 hours and PTL. This pattern is seen for the additional genes significantly changing in their expression from early to later time-points with the high dose of bacterium. These genes are for the serine proteinase plasminogen activator inhibitor (PAI-1 or Serpine1), matrix metalloproteinase (MMP)3, and tenascin C (represented twice on the microarray). It is important to note that at PTL several genes are significantly down-regulated with respect to their 3.5 hour time-point levels. Afp and ApoA1, FbgB, and H19 mRNA, genes most significantly down-regulated at the onset of PTL (vs. C8), were demonstrated to be specifically repressed just at the time of PTL.

Again, looking at differences between high dose effects and low dose effects there are many genes differentially regulated. Between H8 and L8 (low dose, 8 hours) there are 48 probe sets depicting genes up-regulated specifically with the dose leading to PTL and 4 genes that are down-regulated. These numbers increase when comparing PTL with its low dose time cohort (57 increasing, 19 decreasing at PTL). These genes are likely to be the key to triggering PTL, as their level of gene expression with a low-grade infection does not result in the termination of pregnancy. The most significantly up-regulated genes are IL-6, granzyme G, and COX-2, followed by many small inducible cytokines, the granzymes C, D, and E, as well as PAI-1, MMP13 and MMP3, GRO-1, Sprr2D, cFos, and IL-1α and IL-1β, and thrombospondin 1, just to mention a few. The gene for Chitinase 3-like 1 was significantly induced just prior to PTL, at H8. The most significantly down-regulated genes at PTL vs. L8 levels are Afp, ApoA1, H19 mRNA, FbgA, TSH-R, and RGS-2. Many of these genes were found to be differentially regulated at the early, 3.5 hour, time-point comparison as well (H3.5 vs. L3.5). Irg1 is specific to the high dose soon after infection, identifying it as a potential trigger of events culminating in PTL.

Changes with the low dose infection were few with only 6 genes up-regulated with respect to control levels at 3.5 hours and only 15 genes up-regulated at 8 hours.

One of the probe sets for Irg1 is up-regulated early but the levels seen with the high dose well surpass those with the low dose of bacteria. This suggests a threshold that must be reached for PTL to ensue. The small inducible cytokine B2 appears to follow this pattern as well. Comparing levels between L8 and L3.5 demonstrates that MMP7, the uterine metalloproteinase, which plays a role in tissue remodelling postpartum, is induced over time with low infection. As this gene is induced at H3.5 and at L8 to similar levels it suggests that while it may play a part in PTL it is unlikely to be a determining factor. The low dose infection never resulted in PTL, proceeding along to labour at full term. The expression of RIKEN cDNA 1810037I17 (Genbank# AW047207) is significantly down-regulated between L8 and L3.5 only and is not implicated in the process of PTL.

Table 3.2 summarizes the number of probe sets demonstrating changes in gene expression for the relevant comparisons of the infection induced PTL model.

Table 3.2 Number of significant changes in gene expression between relevant infection experiment time-points (up-regulated, down-regulated).

	L3.5	**H3.5**	**L8**	**H8**	**PTL**
C3.5	6[a], 0	**95,7[b]**			
L3.5		32,1[c]	1[d], 4		
H3.5				**8[e], 1[f]**	**18, 14**
C8			15, 0	**100, 7[b]**	**125, 32**
L8				48, 4[g]	57, 19
H8					**0, 1[h]**

[a] these genes are significantly changing in other comparisons as well; [b] 5 of these are common to both comparisons; [c] RIKEN cDNA 31100386O1 (Genbank # AI844396); [d] MMP7, also up-regulated at H3.5 vs. C3.5; [e] genes of Cluster F; [f] Hemoglobin Y beta-like embryonic chain repressed with approaching PTL; [g] genes repressed at 8 hours with a high bacterial dose: TSH-R, RGS-2, ScyA6, and EST AW047237; [h] Alpha fetoprotein is down-regulated 13.6 fold at the onset of PTL vs. H8.

Ovariectomy-Induced Changes in Gene Expression: As with infection-induced PTL, an increasing number of genes were differentially regulated with time. Early after ovariectomy (Ovx5) seven genes were significantly but moderately down-regulated. EST AW047643 exhibited the greatest repression in gene expression with a 3 fold decrease compared with Sham5 levels. To lesser degrees RGS-2, small inducible cytokines A2 and B2, follistatin, EST AI467657 (Genbank# AI553024) and RIKEN cDNA 1300002F13 (mitogen-inducible gene 6 protein homologue) were repressed. Only follistatin, RGS-2, and EST AI467657 continued to be repressed at the time of ovariectomy-induced PTL (Ovx20-PTL) and each to a greater degree.

The RIKEN cDNA 1300007C21 (GAG homologue), cFos, and COX-2 showed the greatest up-regulation at Ovx20-PTL. These were followed closely by thrombospondin 1, PAI-1, small proline rich proteins 2E (with 2 probe sets), 2I, 2G, 2B, osteoprotegerin (TNF-R superfamily member 11b), tenascin C, lipoprotein lipase (represented twice), Purkinje cell protein 4 (PCP4), and connexin 43 (Cx43), just to name a few of the 42 genes significantly up-regulated with respect to the Sham20 control levels. The genes for cFos, COX-2, and Connexin 43 are well known in the process of labour and continue this pattern under the stimulus of ovariectomy.

There were nearly as many genes down-regulated at the time of PTL due to ovariectomy with 33 probe sets passing the strict filtration criteria. Connexin 26 (Cx26) shows its typical pattern of repression at the time of labour [33, 34]. M-Csf, coagulation factor III (F3), and genes for cardiac actin alpha and skeletal muscle actin alpha 1 were moderately repressed along with RGS-2 and follistatin. Genes most significantly down-regulated include EST AI467657, TGFβ2, aquaporin 8, and hydroxyacid oxidase (glycolate oxidase) 3.

Cluster Analysis

Fluorescence values from replicate assays were averaged for each probe set and Gene Cluster was used to organize the probe sets (204- infection; 79-ovariectomy) based on their expression profiles. Log_2 transformed, median centred data were normalized and then clustered using the K-means algorithm [108]. The

software utilizes the data for all animal groups to assign the probe sets to one cluster or another. The genes were organized into clusters (six clusters for infection; eight clusters for ovariectomy), which were depicted using TreeView software (Figure 3.1-infection; Figure 3.2-ovariectomy). Table 3.3 lists the genes associated with each infection cluster and Table 3.4, those for ovariectomy; ordered by the magnitude of the fold change at the time-point with the most numerous significant values for that cluster. The results of clustering were clear depictions of the significant changes in gene expression and the various patterns those changes exhibit during the process leading into and during the moment of PTL. By examining the probe set lists associated with each of the clusters, known patterns of expression can be seen and new ones are revealed.

Infection Study (Figure 3.1; Table 3.3): Cluster A contains genes whose expression decreased with high bacterial infection with a maximal repression reached at the PTL time-point. This group includes Afp, ApoA1, H19 mRNA, the fibrinogen alpha and beta polypeptides, M-Csf, and RGS-2. Low dose infection showed no appreciable deviation from control gene expression levels at either 3.5 or 8 hours.

Clusters B to F encompass the remaining probe sets, portraying an up-regulated gene expression with high doses of bacterial infection. Cluster B exhibits those probe sets that display a pattern of increased gene expression at 3.5 hours compared with control and low dose levels. The levels at 8 hours and PTL remained high but appear to be less strongly induced based on the clustering algorithm. Immunosuppressive gene 1; ScyB2, B5 and B10; and GRO1 exemplify this group as do several interferon activated genes. Low infection caused an early increase in expression which then returned to near control levels by 8 hours. Cluster C includes probe sets whose genes are up-regulated to highest levels at PTL. The small proline-rich proteins fall into this cluster. There is an increase in expression of these genes with a low infection by 8 hours. These, with their representative MMP7, may be important but not critical for the onset of parturition.

Clusters D and E contain probe sets for genes that demonstrate some of the greatest fold changes. Cluster D consists of probe sets that reflect high levels of gene expression that are sustained from the 3.5 hour time-point to PTL while the control and low dose groups show a decrease with time. Here reside probe sets for IL-6, COX-2, small inducible cytokines, cFos, IL-1 α and β, among others. Cluster E shows those probe sets that depict genes with a small up-regulation with low dose infection, increasing further with time and this pattern is even more substantial with the high dose of bacteria. Chitinase 3-like 1 and Sprr2D gene probe sets characterize this cluster.

Cluster F probe sets exhibit a slight repression in gene expression levels with low dose infection which somewhat alleviates itself with time. With the 10^{10} PTL-inducing dose of bacteria, there is an initial (albeit statistically insignificant) suppression of gene expression at 3.5 hours. This suppression is reversed at 8 hours and at PTL, where peak expression is observed. This group is represented by probe sets for the granzyme genes C, D, E, and G, thrombospondin 1, tenascin C, and Cx43 genes. Cx43 is known to peak at labour as a member of the contraction associated proteins (CAPs) and this pattern is indicative of reliable microarray results. The importance of the early suppression or time-regulated up-regulation in gene expression may indicate an attempt to resist the changes that ultimately lead to PTL or may instead indicate genes which are induced downstream in a cascade of events which lead to PTL.

Ovariectomy Study (Figure 3.2; Table 3.4): The GeneCluster and TreeView software necessitated the need for 8 clusters for the ovariectomy data. This gave the clearest organization of the 79 significant genes. Perhaps this was required since relatively few genes were available to which patterning could be assessed and assigned. Clusters A-D include probe sets for genes that exhibit their greatest up-regulation at ovariectomy-induced PTL (Ovx20-PTL). Probe sets in Clusters A and C show a slight up-regulation early after ovariectomy (Ovx5) compared with its Sham5 control while probe sets in Clusters B and D show a slight suppression at Ovx5 vs.

Sham5 control. Cluster A probe sets showed the greatest up-regulation in gene expression and include most of the small proline-rich proteins, lipoprotein lipase (2 separate probe sets), and PCP4. Cluster B includes tenascin C, small proline-rich protein 2B, and liver arginase 1 probe sets. Cluster C contains probe sets for the mouse GAG homologue RIKEN cDNA, thrombospondin 1, osteoprotegerin, nuclear protein 1, and Cx43. Cluster D is represented by probe sets for cFos, COX-2, and PAI-1 genes. Cluster E is a transition grouping with only TNF-induced protein 6 (Tnfip6) being depicted as up-regulated at Ovx20-PTL and ScyA2 depicted as being down-regulated at Ovx5 compared with their controls.

Clusters F to H consist of probe sets that depict a repression in gene expression, most significantly at Ovx20-PTL, with those having the greatest magnitude found in Cluster H. Cluster F probe sets appear to portray little changes in gene expression at 5 hours but more substantial increases at 20 hours. Profilin 2, prolactin-like protein C, cytochrome P450 retinoic acid A1, and CEA-related cell adhesion molecule 11 comprise this group. Cluster G contains probe sets for genes that are repressed early after ovariectomy and remain so (if not statistically significantly) at Ovx20-PTL. Probe sets for follistatin and RGS-2 are grouped here. Cluster H includes probe sets whose genes have the greatest repression at Ovx20-PTL. M-Csf, Cx26 (following its well described pattern), actin alpha genes from cardiac and skeletal muscle, and TGFβ genes are representatives of this largest cluster.

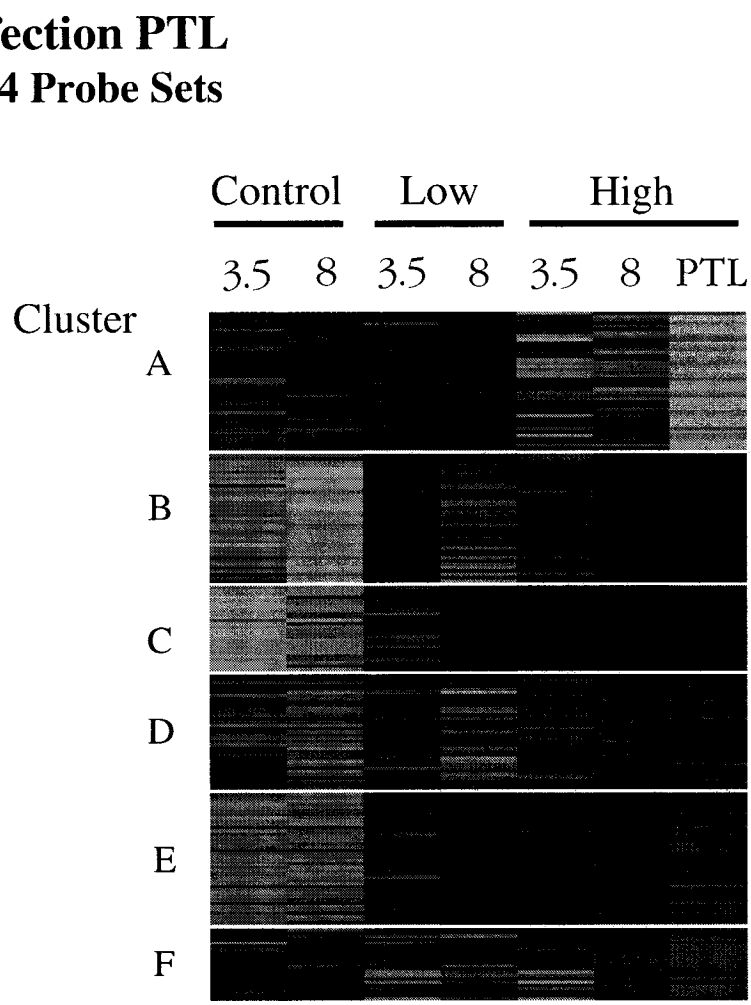


Figure 3.1 GeneCluster organization of Affymetrix gene probe sets displaying significant changes in gene expression due to intrauterine infection.
K-means clustering of $\log_2$ transformed, normalized, and median centred average fluorescence data of 204 probe sets that were deemed to have statistically significant changes between one or more relevant experimental groups. The 6 nodes are labelled Cluster A to F. Green colouring represents data that show a repression in gene expression with respect to the median value for the probe set. Red colouring represents data that show an induction of gene expression with respect to the median value for the probe set. The more intense the colour, the greater the deviation from the median value for that particular experimental group.

Cluster	GenBank ID	Title	H3.5/C3.5	H8/C8	PTL/C8
A	AF009246	RAS dexamethasone-induced 1	---	---	---
A	AI853714	cathepsin B	---	---	---
A	AV373027	small inducible cytokine A6	---	---	---
A	AW047237	EST	---	---	---
A	D88792	cysteine-rich protein 2	---	---	---
A	AA986050	EST: fibrinogen gamma polypeptide	---	---	---
A	X14061	hemoglobin Z beta-like embryonic chain	---	---	---
A	V00726	hemoglobin Y beta-like embryonic chain	---	---	---
A	X13060	albumin 1	---	---	---
A	AI196896	fibrinogen B beta polypeptide	---	---	---
A	AI852838	maternally expressed gene 3	---	---	-2.11
A	AI005782	transmembrane protease serine 2	---	---	-2.13
A	D50086	neuropilin	---	---	-2.17
A	AF110520	ribosomal protein S28	---	---	-2.24
A	AI840130	Src activating and signaling molecule	---	---	-2.30
A	AI845735	polymerase (RNA) II (DNA directed) polypeptide E	---	---	-2.35
A	X66295	complement component 1q subcomponent c polypeptide	---	---	-2.36
A	X58861	complement component 1q subcomponent alpha polypeptide	---	---	-2.39
A	AI843106	p53 regulated PA26 nuclear protein	---	---	-2.40
A	AF026073	N-sulfotransferase	---	---	-2.42
A	AW125884	acetyl-Coenzyme A synthetase 2	---	---	-2.47
A	AW124483	RIKEN cDNA	---	---	-2.52
A	AI843448	microsomal glutathione S-transferase 3	---	---	-2.54
A	AF018952	aquaporin 8	---	---	-2.63
A	AW047919	EST	---	---	-2.65
A	AI854285	Nd1	---	---	-2.77
A	M60348	ATP-binding cassette sub-family B (MDR/TAP) member 1B	---	---	-2.87
A	M21952	colony stimulating factor 1 (macrophage)	-2.68	-2.10	-2.89
A	M58004	small inducible cytokine A6	---	-2.48	-3.02
A	AF009605	phosphoenolpyruvate carboxykinase 1 cytosolic	-3.15	---	-3.14
A	AW061234	EST	-2.99	-2.21	-3.30
A	AF084482	Wolfram syndrome 1 homolog (human)	-2.42	---	-3.31
A	U67187	regulator of G-protein signaling 2	---	-2.57	-3.60
A	AI844396	RIKEN cDNA	---	-2.47	-3.71
A	U22399	cyclin-dependent kinase inhibitor 1C (P57)	---	---	-3.96
A	X57413	transforming growth factor beta 2	-2.26	-2.49	-4.20
A	U02602	thyroid stimulating hormone receptor	-2.38	-2.23	-4.20

Cluster	GenBank ID	Title	H3.5/C3.5	H8/C8	PTL/C8
A	M13125	hemoglobin X alpha-like embryonic chain in Hba complex	---	---	-4.63
A	AI876446	fibrinogen alpha polypeptide	---	---	-5.30
A	X58196	H19 fetal liver mRNA	---	---	-5.83
A	U79573	apolipoprotein A-I	---	---	-9.47
A	V00743	alpha fetoprotein	---	---	-17.09
B	L38281	immunoresponsive gene 1	45.51	13.68	---
B	AI323667	immunoresponsive gene 1	21.77	11.41	10.63
B	X53798	small inducible cytokine subfamily member 2	17.85	27.66	27.97
B	M33266	small inducible cytokine B subfamily (Cys-X-Cys) member 10	12.47	20.43	16.18
B	AA204579	EST: Viral hemorrhagic septicemia virus (VHSV) induced gene 1	9.68	8.45	9.70
B	U27267	small inducible cytokine B subfamily member 5	8.33	29.10	33.25
B	U43084	interferon-induced protein with tetratricopeptide repeats 1	8.30	7.99	8.23
B	J04596	GRO1 oncogene	7.47	22.47	20.52
B	J04596	GRO1 oncogene	7.21	10.05	9.38
B	D78354	phospholipid scramblase 1	6.18	4.58	4.03
B	M21038	myxovirus (influenza virus) resistance 1	6.07	5.61	6.05
B	L32973	thymidylate kinase family LPS-inducible member	5.93	---	5.59
B	AF019385	heparan sulfate (glucosamine) 3-O-sulfotransferase 1	5.58	---	---
B	M84487	vascular cell adhesion molecule 1	4.98	7.25	6.70
B	L24118	tumor necrosis factor alpha-induced protein 2	4.38	3.94	3.48
B	M90551	intercellular adhesion molecule 1	4.04	5.42	5.47
B	U57524	nuclear factor of kappa light chain gene enhancer in B-cells inhibitor alpha	3.32	3.54	3.58
B	AF099973	schlafen 2	3.07	3.53	3.79
B	U23781	B-cell leukemia/lymphoma 2 related protein A1d	2.97	---	---
B	AI642048	nuclear factor of kappa light chain gene enhancer in B-cells inhibitor alpha	2.95	3.47	3.38
B	U88908	baculoviral IAP repeat-containing 2	2.94	---	---
B	M12731	neuroblastoma myc-related oncogene 1	2.93	---	---
B	M31419	interferon activated gene 204	2.82	4.17	4.78
B	AJ007972	interferon-g induced GTPase	2.79	3.47	3.01
B	AW122207	down-regulator of transcription 1	2.66	---	---
B	Y15907	human immunodeficiency virus type I enhancer binding protein 2	2.65	---	---
B	M57999	nuclear factor of kappa light chain gene enhancer in B-cells 1p105	2.65	2.49	2.31
B	AW047899	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2p49/p100	2.58	2.24	2.20
B	AF099973	schlafen 2	2.53	2.90	2.72
B	M74123	interferon activated gene 205	2.36	3.87	3.81
B	AI849533	chloride intracellular channel 4 (mitochondrial)	2.19	---	---
B	AI845237	chloride intracellular channel 4 (mitochondrial)	2.08	---	---

Cluster	GenBank ID	Title	H3.5/C3.5	H8/C8	PTL/C8
B	AI853875	zinc ring finger protein 1	2.05	---	---
B	X83601	pentaxin related gene	---	6.16	5.97
B	AV229143	EST: interferon activated gene 202A	---	---	3.64
B	AV374868	cytokine inducible SH2-containing protein 3	---	3.67	3.52
B	X61800	CCAAT/enhancer binding protein (C/EBP) delta	---	3.42	3.51
B	Y13089	caspase 11	---	2.83	2.82
B	AA960657	EST: interferon activated gene 203	---	---	2.65
C	X56602	interferon-stimulated protein (15 kDa)	5.13	5.61	6.06
C	AJ007970	guanylate nucleotide binding protein 2	3.50	4.44	4.50
C	M31419	interferon activated gene 204	---	---	4.32
C	AA959954	RIKEN cDNA: helicard	4.48	4.34	4.32
C	AJ005560	small proline-rich protein 2B	4.29	2.65	4.19
C	AF099977	schlafen 4	2.33	3.21	4.17
C	AF076482	peptidoglycan recognition protein	3.38	3.28	4.10
C	AW047476	guanylate nucleotide binding protein 3	3.06	3.78	3.99
C	AW047653	ubiquitin specific protease 18	3.60	3.77	3.93
C	X13333	CD14 antigen	3.84	3.37	3.84
C	X03505	serum amyloid A3	4.15	3.05	3.49
C	AA591002	solute carrier family 5 member 1	3.87	3.24	3.26
C	AJ005561	small proline-rich protein 2C	---	---	3.16
C	U73037	interferon regulatory factor 7	2.25	2.74	3.02
C	U22033	proteosome subunit beta type 8 (large multifunctional protease 7)	---	2.48	3.01
C	AJ005567	small proline-rich protein 2I	3.58	---	2.85
C	AJ005563	small proline-rich protein 2E	3.41	2.19	2.84
C	X81627	lipocalin 2	3.75	2.55	2.72
C	U49430	ceruloplasmin aka:Ferroxidase	---	---	2.69
C	AV210775	lipocalin 2	3.01	---	2.61
C	AJ005565	small proline-rich protein 2G	2.73	---	2.41
C	L36244	matrix metalloproteinase 7	3.03	---	---
C	U12884	vascular cell adhesion molecule 1	---	2.51	---
C	M25944	carbonic anhydrase 2	2.68	---	---
C	AJ005564	small proline-rich protein 2F	2.91	---	---
C	K02108	keratin complex 2 basic gene 6a	2.18	---	---
D	X54542	interleukin 6	6.83	36.88	36.65
D	M88242	prostaglandin-endoperoxide synthase 2 (COX-2)	14.24	18.40	28.17
D	M19681	small inducible cytokine A2	2.81	15.58	15.84
D	M34815	small inducible cytokine B subfamily (Cys-X-Cys) member 9	3.22	6.11	8.06

Cluster	GenBank ID	Title	H3.5/C3.5	H8/C8	PTL/C8
D	X66473	matrix metalloproteinase 13	5.20	5.59	6.90
D	X70058	small inducible cytokine A7	2.28	5.53	5.69
D	M13926	colony stimulating factor 3 (granulocyte)	5.68	4.45	4.64
D	L32838	interleukin 1 receptor antagonist	---	3.28	3.91
D	U44088	T-cell death associated gene	4.24	3.27	3.80
D	AW047630	carbon catabolite repression 4 homolog (yeast)	---	3.57	3.63
D	V00727	FBJ osteosarcoma oncogene (cFos)	---	2.69	3.60
D	AB020886	A kinase (PRKA) anchor protein (gravin) 12	---	2.83	3.52
D	AF071180	formyl peptide receptorrelated sequence 2	3.00	3.45	3.52
D	M14639	interleukin 1 alpha	4.88	3.46	3.42
D	U53219	interferon gamma induced GTPase	2.53	3.30	3.27
D	AA614971	molecule possessing ankyrin-repeats induced by lipopolysaccharide	3.17	3.52	3.26
D	M15131	interleukin 1 beta	3.31	---	3.25
D	AW060549	RIKEN cDNA: GAG homolog (mouse)	---	---	3.05
D	M61007	CCAAT/enhancer binding protein (C/EBP) beta	---	2.88	3.01
D	AI846152	Down syndrome critical region homolog 1 (human)	---	2.49	2.92
D	AI642662	RIKEN cDNA: MAP kinase phosphatase-7	2.82	2.35	2.59
D	Y07836	basic helix-loop-helix domain containing class B2	---	2.20	2.44
D	AW124544	endoplasmic reticulum membrane protein	2.67	---	2.41
D	AV138783	growth arrest and DNA-damage-inducible 45 beta	---	---	2.40
D	X61940	protein tyrosine phosphatase non-receptor type 16	---	---	2.33
D	M21065	interferon regulatory factor 1	---	2.36	2.17
D	AF059213	cholesterol 25-hydroxylase	---	3.89	---
D	AA914345	interferon-inducible GTPase	---	2.80	---
D	AI844128	EH-domain containing 1	2.19	---	---
D	J04491	small inducible cytokine A3	2.28	---	---
D	U20344	Kruppel-like factor 4 (gut)	---	---	---
D	U19118	activating transcription factor 3	---	---	---
D	M28845	early growth response 1	---	---	---
D	X67863	simple repeat sequence-containing transcript	2.12	---	---
D	AW047207	RIKEN cDNA 1810037I17 gene	---	---	---
E	X93035	chitinase 3-like 1	---	18.88	45.35
E	AF065947	small inducible cytokine A5	13.50	12.80	15.62
E	L38444	T-cell specific GTPase	8.40	13.19	12.83
E	AJ005562	small proline-rich protein 2D	3.74	7.21	11.77
E	AJ007971	interferon-inducible GTPase	4.15	10.33	9.31
E	M83218	S100 calcium binding protein A8 (calgranulin A)	2.16	6.81	9.09

Cluster	GenBank ID	Title	H3.5/C3.5	H8/C8	PTL/C8
E	M27695	urate oxidase	3.41	3.62	8.96
E	AV152244	RIKEN cDNA: interferon-stimulated protein (15 kDa)	7.27	7.30	8.68
E	U43086	interferon-induced protein with tetratricopeptide repeats 3	6.67	7.69	6.95
E	U43085	interferon-induced protein with tetratricopeptide repeats 2	6.97	6.31	6.77
E	J03298	lactotransferrin	7.35	5.48	6.57
E	AI121305	RIKEN cDNA	6.36	4.68	5.59
E	AV092014	peptidoglycan recognition protein	---	3.73	5.48
E	L35528	superoxide dismutase 2 mitochondrial	3.04	4.56	5.42
E	M83219	S100 calcium binding protein A9 (calgranulin B)	---	3.76	5.08
E	U12884	vascular cell adhesion molecule 1	3.20	4.14	4.37
E	L09737	GTP cyclohydrolase 1	3.55	3.46	4.30
E	AF002719	secretory leukocyte protease inhibitor	4.69	4.34	4.18
E	M31418	interferon activated gene 202A	---	4.84	3.97
E	AV206059	motilin-related peptide	2.39	3.06	3.96
E	AF099974	schlafen 3	---	---	3.84
E	AF022371	interferon activated gene 203	---	3.37	3.77
E	J03776	tripartite motif protein 30	2.47	3.06	3.38
E	U19119	interferon inducible protein 1	2.79	---	3.33
E	U60020	ATP-binding cassettesub-family B (MDR/TAP) member 2	2.32	3.19	3.22
E	M35247	histocompatibility 2T region locus 17	---	2.59	3.09
E	AA816121	EST	3.13	3.66	2.99
E	M69260	annexin A1	---	---	2.89
E	M35244	histocompatibility 2T region locus 10	---	2.29	2.72
E	D44456	proteosome subunit beta type 9 (large multifunctional protease 2)	---	2.49	2.63
E	AI852641	nuclear protein 1	---	---	2.54
E	Y00629	histocompatibility 2T region locus 23	---	---	2.52
E	U15635	SAM domain and HD domain 1	---	---	2.47
E	U55060	lectin galactose binding soluble 9	---	2.25	2.17
E	AV049898	lectin galactose binding soluble 9	---	---	2.08
E	AA958903	EST	4.22	3.85	---
E	M64085	serine protease inhibitor 2-1	---	2.62	---
E	X87128	TNF receptor superfamily member 1b	2.13	---	---
E	AI746846	RIKEN cDNA: sorting nexin 10	---	2.16	---
E	AF087825	claudin 7	2.42	---	---
F	M33960	serine proteinase inhibitor clade E1 (nexin plasminogen activator inhibitor type 1)	---	6.29	8.03
F	J02872	granzyme G	---	---	7.40
F	X66402	matrix metalloproteinase 3	---	3.02	4.00

Cluster	GenBank ID	Title	H3.5/C3.5	H8/C8	PTL/C8
F	U94828	regulator of G-protein signaling 16	---	3.15	3.58
F	M36901	granzyme E	---	---	3.02
F	M62470	thrombospondin 1	---	---	2.93
F	AB004315	regulator of G-protein signaling 4	---	---	2.62
F	M22527	granzyme C	---	---	2.57
F	M63801	gap junction membrane channel protein alpha 1 (connexin 43)	---	---	2.56
F	X56990	granzyme D	---	---	2.45
F	M95200	vascular endothelial growth factor A	---	---	2.43
F	M32490	cysteine rich protein 61 aka: IGFBP10	---	---	2.42
F	U96700	serine protease inhibitor 6	---	---	2.40
F	M22998	solute carrier family 2 (facilitated glucose transporter) member 1	---	---	2.20
F	AV230686	tenascin C	---	---	---
F	X56304	tenascin C	---	---	---
F	AF030636	small inducible cytokine subfamily B (Cys-X-Cys) member 13	---	---	---
F	AV223216	interleukin 1 receptor type II	---	---	---
F	AW123751	RIKEN cDNA: growth and transformation-dependent (fragment) homolog (rat)	---	---	---
F	AF041054	BCL2/adenovirus E1B 19 kDa-interacting protein 1NIP3	---	---	---
F	M70642	connective tissue growth factor	---	---	---
F	M32486	Mouse 19.5 mRNA	---	---	---

Cluster	GenBank ID	H8/H3.5	PTL/H3.5	PTL/H8	H3.5/L3.5	H8/L8	PTL/L8	L3.5/C3.5	L8/C8	L8/L3.5
A	AF009246	---	---	---	---	---	-2.45	---	---	---
A	AI853714	---	---	---	---	---	-2.47	---	---	---
A	AV373027	---	---	---	---	---	-2.07	---	---	---
A	AW047237	---	---	---	---	-2.28	-3.15	---	---	---
A	D88792	---	-2.53	---	---	---	---	---	---	---
A	AA986050	---	-3.48	---	---	---	---	---	---	---
A	X14061	---	-5.46	---	---	---	---	---	---	---
A	V00726	-2.49	-4.90	---	---	---	---	---	---	---
A	X13060	---	-3.10	---	---	---	---	---	---	---
A	AI196896	---	-8.24	---	---	---	---	---	---	---
A	AI852838	---	---	---	---	---	---	---	---	---
A	AI005782	---	---	---	---	---	---	---	---	---
A	D50086	---	---	---	---	---	---	---	---	---
A	AF110520	---	---	---	---	---	---	---	---	---
A	AI840130	---	---	---	---	---	---	---	---	---
A	AI845735	---	---	---	---	---	---	---	---	---
A	X66295	---	---	---	---	---	-2.36	---	---	---
A	X58861	---	---	---	---	---	-2.31	---	---	---
A	AI843106	---	---	---	---	---	---	---	---	---
A	AF026073	---	---	---	---	---	---	---	---	---
A	AW125884	---	---	---	---	---	---	---	---	---
A	AW124483	---	---	---	---	---	---	---	---	---
A	AI843448	---	---	---	---	---	---	---	---	---
A	AF018952	---	---	---	---	---	---	---	---	---
A	AW047919	---	-2.32	---	---	---	---	---	---	---
A	AI854285	---	---	---	---	---	---	---	---	---
A	M60348	---	---	---	---	---	---	---	---	---
A	M21952	---	---	---	---	---	---	---	---	---
A	M58004	---	---	---	---	-2.44	-3.05	---	---	---
A	AF009605	---	---	---	---	---	---	---	---	---
A	AW061234	---	---	---	---	---	-2.91	---	---	---
A	AF084482	---	---	---	---	---	-2.81	---	---	---
A	U67187	---	---	---	---	-2.89	-3.84	---	---	---
A	AI844396	---	---	---	-2.72	---	-3.21	---	---	---
A	U22399	---	-3.01	---	---	---	-2.68	---	---	---
A	X57413	---	---	---	---	---	-2.91	---	---	---
A	U02602	---	---	---	---	-2.20	-4.11	---	---	---

Cluster	GenBank ID	H8/H3.5	PTL/H3.5	PTL/H8	H3.5/L3.5	H8/L8	PTL/L8	L3.5/C3.5	L8/C8	L8/L3.5
A	M13125	---	-6.59	---	---	---	-3.73	---	---	---
A	AI876446	---	---	---	---	---	-4.70	---	---	---
A	X58196	---	-5.41	---	---	---	-4.19	---	---	---
A	U79573	---	-10.05	---	---	---	-6.98	---	---	---
A	V00743	---	-15.66	-13.59	---	---	-14.73	---	---	---
B	L38281	---	---	---	2.87	---	---	---	---	---
B	AI323667	---	---	---	2.22	---	---	9.84	6.23	---
B	X53798	---	---	---	2.63	7.68	8.08	6.66	---	-3.75
B	M33266	---	---	---	4.39	7.93	6.64	---	---	---
B	AA204579	---	---	---	---	---	3.66	---	---	---
B	U27267	---	---	---	---	---	---	---	---	---
B	U43084	---	---	---	2.74	---	2.67	3.07	3.16	---
B	J04596	---	---	---	---	4.80	4.52	---	---	---
B	J04596	---	---	---	2.23	4.43	4.18	---	---	---
B	D78354	---	---	---	---	---	---	---	2.42	---
B	M21038	---	---	---	---	---	3.31	---	---	---
B	L32973	---	---	---	2.55	---	---	---	---	---
B	AF019385	---	-2.31	---	---	---	---	---	---	---
B	M84487	---	---	---	2.39	---	---	---	4.90	---
B	L24118	---	---	---	2.30	---	---	---	---	---
B	M90551	---	---	---	---	2.36	2.43	---	---	---
B	U57524	---	---	---	---	---	---	---	---	---
B	AF099973	---	---	---	---	---	---	---	---	---
B	U23781	---	---	---	---	---	---	---	---	---
B	AI642048	---	---	---	---	2.13	2.08	---	---	---
B	U88908	---	---	---	---	---	---	---	---	---
B	M12731	---	-2.51	---	---	---	---	---	---	---
B	M31419	---	---	---	---	---	---	---	---	---
B	AJ007972	---	---	---	---	2.46	---	---	---	---
B	AW122207	---	---	---	---	---	---	---	---	---
B	Y15907	---	---	---	---	---	---	---	---	-2.21
B	M57999	---	---	---	---	2.24	---	---	---	---
B	AW047899	---	---	---	---	---	---	---	---	---
B	AF099973	---	---	---	---	---	---	---	---	---
B	M74123	---	---	---	---	---	---	---	2.46	---
B	AI849533	---	---	---	---	---	---	---	---	---
B	AI845237	---	---	---	---	---	---	---	---	---

Cluster	GenBank ID	H8/H3.5	PTL/H3.5	PTL/H8	H3.5/L3.5	H8/L8	PTL/L8	L3.5/C3.5	L8/C8	L8/L3.5
B	AI853875	---	---	---	---	---	---	---	---	---
B	X83601	---	---	---	---	3.85	3.73	---	---	---
B	AV229143	---	---	---	---	---	---	---	---	---
B	AV374868	---	---	---	---	---	---	---	---	-2.39
B	X61800	---	---	---	---	---	---	---	---	---
B	Y13089	---	---	---	---	---	---	---	---	---
B	AA960657	---	---	---	---	---	---	---	---	---
C	X56602	---	---	---	2.54	---	---	---	2.93	---
C	AJ007970	---	---	---	---	---	---	---	2.59	---
C	M31419	---	---	---	---	---	---	---	---	---
C	AA959954	---	---	---	2.50	---	---	---	2.64	---
C	AJ005560	---	---	---	---	---	---	---	---	---
C	AF099977	---	2.22	---	---	---	2.16	---	---	---
C	AF076482	---	---	---	---	---	---	---	---	---
C	AW047476	---	---	---	---	---	---	---	2.83	---
C	AW047653	---	---	---	---	---	---	---	---	---
C	X13333	---	---	---	---	---	---	---	2.95	---
C	X03505	---	---	---	---	---	---	---	---	---
C	AA591002	---	---	---	2.29	---	---	---	---	---
C	AJ005561	---	---	---	---	---	---	---	---	---
C	U73037	---	---	---	---	---	---	---	---	---
C	U22033	---	---	---	---	---	---	---	---	---
C	AJ005567	---	---	---	---	---	---	---	---	---
C	AJ005563	---	---	---	---	---	---	---	---	---
C	X81627	---	---	---	---	---	---	---	---	---
C	U49430	---	---	---	---	---	---	---	---	---
C	AV210775	---	---	---	---	---	---	---	---	---
C	AJ005565	---	---	---	---	---	---	---	---	---
C	L36244	---	---	---	---	---	---	---	3.48	2.39
C	U12884	---	---	---	---	---	---	---	---	---
C	M25944	---	---	---	---	---	---	---	---	---
C	AJ005564	---	---	---	---	---	---	2.22	---	---
C	K02108	---	---	---	---	---	---	---	---	---
D	X54542	---	---	---	6.75	18.29	19.03	---	---	---
D	M88242	---	---	---	4.72	10.01	14.61	---	---	---
D	M19681	---	---	---	2.76	6.67	6.92	---	---	---
D	M34815	---	2.44	---	2.58	4.09	5.40	---	---	---

Cluster	GenBank ID	H8/H3.5	PTL/H3.5	PTL/H8	H3.5/L3.5	H8/L8	PTL/L8	L3.5/C3.5	L8/C8	L8/L3.5
D	X66473	---	---	---	4.03	5.50	6.86	---	---	---
D	X70058	---	---	---	2.10	3.80	3.88	---	---	---
D	M13926	---	---	---	3.12	3.57	3.75	---	---	---
D	L32838	---	---	---	2.36	2.61	3.12	---	---	---
D	U44088	---	---	---	2.58	3.71	4.30	---	---	---
D	AW047630	---	---	---	---	3.03	3.14	---	---	---
D	V00727	---	---	---	---	4.21	5.68	---	---	---
D	AB020886	---	---	---	---	2.79	3.48	---	---	---
D	AF071180	---	---	---	---	2.22	2.27	---	---	---
D	M14639	---	---	---	3.16	2.91	2.89	---	---	---
D	U53219	---	---	---	---	2.33	2.31	---	---	---
D	AA614971	---	---	---	2.42	2.82	2.67	---	---	---
D	M15131	---	---	---	2.75	---	2.30	---	---	---
D	AW060549	---	---	---	2.70	---	4.18	---	---	---
D	M61007	---	---	---	---	2.32	2.42	---	---	---
D	AI846152	---	---	---	---	2.31	2.71	---	---	---
D	AI642662	---	---	---	---	---	---	---	---	---
D	Y07836	---	---	---	---	2.15	2.38	---	---	---
D	AW124544	---	---	---	---	---	---	---	---	---
D	AV138783	---	---	---	---	---	2.36	---	---	---
D	X61940	---	---	---	---	---	2.35	---	---	---
D	M21065	---	---	---	---	---	---	---	---	---
D	AF059213	---	---	---	---	2.92	---	---	---	---
D	AA914345	---	---	---	---	---	---	---	---	---
D	AI844128	---	---	---	---	---	---	---	---	---
D	J04491	---	---	---	---	---	---	---	---	---
D	U20344	---	---	---	---	---	2.14	---	---	---
D	U19118	---	---	---	---	2.35	2.69	---	---	---
D	M28845	---	---	---	---	2.20	---	---	---	---
D	X67863	---	---	---	---	---	---	---	---	---
D	AW047207	---	---	---	---	---	---	---	---	-2.20
E	X93035	---	---	---	---	2.57	---	---	---	---
E	AF065947	---	---	---	6.43	4.52	5.48	---	---	---
E	L38444	---	---	---	---	4.78	4.65	---	---	---
E	AJ005562	---	---	---	---	3.81	6.49	---	---	---
E	AJ007971	---	---	---	---	3.72	3.33	---	2.79	---
E	M83218	---	3.44	---	---	---	3.36	---	---	---

Cluster	GenBank ID	H8/H3.5	PTL/H3.5	PTL/H8	H3.5/L3.5	H8/L8	PTL/L8	L3.5/C3.5	L8/C8	L8/L3.5
E	M27695	---	---	---	2.36	---	---	---	---	---
E	AV152244	---	---	---	3.33	2.27	2.70	---	3.24	---
E	U43086	---	---	---	2.82	---	---	---	---	---
E	U43085	---	---	---	---	---	---	---	---	---
E	J03298	---	---	---	2.68	---	---	2.82	---	---
E	AI121305	---	---	---	2.41	---	---	2.68	2.67	---
E	AV092014	---	---	---	---	---	---	---	---	---
E	L35528	---	---	---	---	---	---	---	2.58	---
E	M83219	---	---	---	---	---	---	---	---	---
E	U12884	---	---	---	---	---	---	---	---	---
E	L09737	---	---	---	---	---	2.09	---	---	---
E	AF002719	---	---	---	2.37	---	---	---	---	---
E	M31418	---	---	---	---	---	---	---	---	---
E	AV206059	---	---	---	---	---	---	---	---	---
E	AF099974	---	---	---	---	---	---	---	---	---
E	AF022371	---	---	---	---	---	---	---	---	---
E	J03776	---	---	---	---	---	---	---	---	---
E	U19119	---	---	---	---	---	---	---	---	---
E	U60020	---	---	---	---	---	---	---	---	---
E	M35247	---	---	---	---	---	---	---	---	---
E	AA816121	---	---	---	---	---	---	---	---	---
E	M69260	---	---	---	---	---	---	---	---	---
E	M35244	---	---	---	---	---	---	---	---	---
E	D44456	---	---	---	---	---	---	---	---	---
E	AI852641	---	---	---	---	---	---	---	---	---
E	Y00629	---	---	---	---	---	---	---	---	---
E	U15635	---	---	---	---	---	---	---	---	---
E	U55060	---	---	---	---	---	---	---	---	---
E	AV049898	---	---	---	---	---	---	---	---	---
E	AA958903	---	---	---	---	---	---	---	---	---
E	M64085	---	---	---	---	---	---	---	---	---
E	X87128	---	---	---	---	---	---	---	---	---
E	AI746846	---	---	---	---	---	---	---	---	---
E	AF087825	---	---	---	---	---	---	---	---	---
F	M33960	3.29	4.02	---	---	6.73	8.50	---	---	---
F	J02872	27.68	44.19	---	---	13.95	10.76	---	---	---
F	X66402	2.91	3.80	---	---	3.61	4.68	---	---	---

Cluster	GenBank ID	H8/H3.5	PTL/H3.5	PTL/H8	H3.5/L3.5	H8/L8	PTL/L8	L3.5/C3.5	L8/C8	L8/L3.5
F	U94828	---	---	---	---	3.32	3.77	---	---	---
F	M36901	6.44	8.85	---	---	4.69	5.08	---	---	---
F	M62470	---	---	---	---	2.90	3.45	---	---	---
F	AB004315	---	---	---	---	---	2.69	---	---	---
F	M22527	---	5.45	---	---	4.22	5.19	---	---	---
F	M63801	---	---	---	---	---	---	---	---	---
F	X56990	4.88	6.52	---	---	3.67	4.03	---	---	---
F	M95200	---	2.69	---	---	---	2.58	---	---	---
F	M32490	---	---	---	---	---	2.97	---	---	---
F	U96700	---	---	---	---	---	---	---	---	---
F	M22998	---	---	---	---	2.14	2.63	---	---	---
F	AV230686	2.43	3.58	---	---	2.25	3.35	---	---	---
F	X56304	2.51	3.30	---	---	2.18	2.92	---	---	---
F	AF030636	2.50	3.02	---	---	---	---	---	---	---
F	AV223216	---	2.86	---	---	---	---	---	---	---
F	AW123751	---	2.59	---	---	---	---	---	---	---
F	AF041054	---	2.47	---	---	---	---	---	---	---
F	M70642	---	2.37	---	---	---	---	---	---	---
F	M32486	---	2.34	---	---	---	---	---	---	---

Table 3.3 GeneCluster organized probe sets reflecting significant intrauterine infection-induced changes in gene expression levels. Comparisons of average gene expression levels were made between each group receiving a bacterial inoculation and its appropriate time-point control (C). As well, comparisons were made between gene expression levels at high (H) and low (L) doses at 3.5 hours, 8 hours, and PTL, and across the time-points for a particular infection dose. Criteria for significance were a fold change ≥ 2 or ≤ -2 and a p value ≤ 0.0025 for both replicates of a comparison. Significant fold change values are indicated. Comparisons that were not significant are represented by ---. Probe sets for each cluster are ordered by the magnitude of the fold change at the time-point with the most numerous significant values for that cluster. EST, expressed sequence tag; RIKEN cDNA, Japanese consortium expressed sequence.

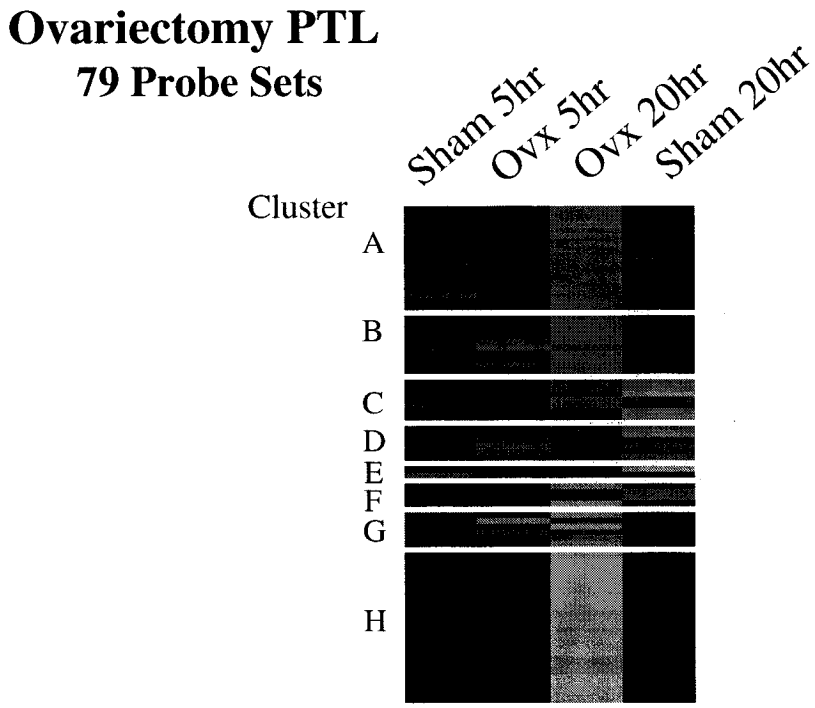


Figure 3.2 GeneCluster organization of gene probe sets displaying significant changes in gene expression during ovariectomy-induced preterm labour.
K-means clustering of $\log_2$ transformed, normalized, and median centred average fluorescence data of 79 probe sets that were deemed to have statistically significant changes between ovariectomy (Ovx) and Sham-operated animal groups for early (5 hours) at late (20 hours- PTL) one or more relevant experimental groups. The 8 nodes are labelled Cluster A to H. Green colouring represents data that show a repression in gene expression with respect to the median value for the probe set. Red colouring represents data that show an induction of gene expression with respect to the median value for the probe set. The more intense the colour, the greater the deviation from the median value for that particular experimental group.

Cluster	GenBank ID	Title	Ovx/Sham 5hr	Ovx/Sham 20hr
A	AA606367	RIKEN cDNA: keratin 21, type I, cytoskeletal homolog (rat)	---	4.28
A	AJ005561	small proline-rich protein 2C	---	3.78
A	D10049	melanoma antigen 80 kDa	---	3.58
A	AJ005567	small proline-rich protein 2I	---	3.54
A	M26005	RIKEN cDNA: GAG homolog (mouse)	---	3.43
A	AJ005563	small proline-rich protein 2E	---	3.37
A	AJ005565	small proline-rich protein 2G	---	3.33
A	X70298	SRY-box containing gene 4	---	3.17
A	AB013848	peptidyl arginine deiminase type I	---	3.09
A	AW124153	SRY-box containing gene 4	---	3.04
A	M60493	cystic fibrosis transmembrane conductance regulator homolog	---	2.80
A	L19932	transforming growth factor beta induced 68 kDa	---	2.70
A	M63335	lipoprotein lipase	---	2.60
A	X17320	Purkinje cell protein 4	---	2.56
A	AA726364	lipoprotein lipase	---	2.50
A	AF016294	E74-like factor 3	---	2.48
A	M77196	CEA-related cell adhesion molecule 1	---	2.46
A	AF007769	baculoviral IAP repeat-containing 1a	---	2.09
B	M17327	clone IMAGE:5053066 mRNA partial cds	---	3.23
B	AJ005560	small proline-rich protein 2B	---	3.16
B	X56304	tenascin C	---	3.05
B	M17327	clone IMAGE:5053066 mRNA partial cds	---	2.92
B	AF041847	cardiac responsive adriamycin protein	---	2.68
B	AI850090	RIKEN cDNA	---	2.60
B	U67840	distal-less homeobox 5	---	2.51
B	L12029	stromal cell derived factor 1	---	2.22
B	U51805	arginase 1liver	---	2.21
B	AV260677	enabled homolog (Drosophila)	---	2.04
C	AW060549	RIKEN cDNA: GAG homolog (mouse)	---	7.73
C	M62470	thrombospondin 1	---	4.28
C	AI846152	Down syndrome critical region homolog 1 (human)	---	4.17
C	AF096875	deiodinaseiodothyronine type II	---	3.36
C	U94331	TNF receptor superfamily member 11b (osteoprotegerin)	---	3.33
C	AI852641	nuclear protein 1	---	2.69
C	M63801	gap junction membrane channel protein alpha 1 (connexin 43)	---	2.16
D	V00727	FBJ osteosarcoma oncogene (cFos)	---	6.21
D	M88242	prostaglandin-endoperoxide synthase 2 (COX-2)	---	6.15
D	M33960	serine proteinase inhibitor E1 (nexin plasminogen activator inhibitor type 1)	---	4.21
D	U19118	activating transcription factor 3	---	3.06
D	U94828	regulator of G-protein signaling 16	---	2.50

Cluster	GenBank ID	Title	Ovx/Sham 5hr	Ovx/Sham 20hr
D	X61940	protein tyrosine phosphatasenon-receptor type 16	---	2.47
E	U83903	tumor necrosis factor induced protein 6	---	2.53
E	M19681	small inducible cytokine A2	-2.36	---
F	AW122536	profilin 2	---	-2.40
F	AF090140	prolactin-like protein C	---	-2.57
F	Y12657	cytochrome P45026 retinoic acid A1	---	-3.15
F	AI156095	CEA-related cell adhesion molecule 11	---	-3.67
G	AF077861	inhibitor of DNA binding 2	---	-2.79
G	Z29532	follistatin	-2.37	-2.85
G	AI853531	RIKEN cDNA: Mitogen-inducible gene 6 protein homolog	-2.57	---
G	U67187	regulator of G-protein signaling 2	-2.60	-3.40
G	X53798	small inducible cytokine subfamily member 2	-2.77	---
G	AW047643	EST	-3.13	---
H	AI845735	EST:Similar to polymerase (RNA) II (DNA directed) polypeptide E (25kD)	---	-2.04
H	AF026073	N-sulfotransferase	---	-2.05
H	AI846308	sideroflexin 1	---	-2.17
H	D17444	leukemia inhibitory factor receptor	---	-2.25
H	M21952	colony stimulating factor 1 (macrophage)	---	-2.32
H	M81445	gap junction membrane channel protein beta 2 (connexin 26)	---	-2.35
H	AI846600	monoglyceride lipase	---	-2.40
H	U35374	purine-nucleoside phosphorylase	---	-2.40
H	AA986114	T-cell immunoglobulin and mucin domain containing 2	---	-2.42
H	AI844396	RIKEN cDNA	---	-2.47
H	AI853714	cathepsin B	---	-2.50
H	AW125884	acetyl-Coenzyme A synthetase 2	---	-2.64
H	M26071	coagulation factor III	---	-2.66
H	M15501	actin alpha cardiac	---	-2.68
H	AW061234	EST	---	-2.75
H	AW047919	EST	---	-2.75
H	AW122851	RIKEN cDNA: FK506 binding protein precursor homolog (human)	---	-2.78
H	U62021	neuronal pentraxin 1	---	-2.85
H	AI851255	cathepsin B	---	-2.88
H	M14223	ribonucleotide reductase M2	---	-3.16
H	M12347	actin alpha 1 skeletal muscle	---	-3.33
H	AI317360	elongation of very long chain fatty acids (FEN1/Elo2SUR4/Elo3yeast)-like 2	---	-5.38
H	AI553024	EST: Highly similar to promyelocyte leukemia Zn finger protein (mouse)	-2.58	-5.60
H	X57413	transforming growth factor beta 2	---	-6.11
H	AF018952	aquaporin 8	---	-6.30
H	AI648067	hydroxyacid oxidase (glycolate oxidase) 3	---	-7.54

Table 3.4 GeneCluster organized probe sets reflecting significant ovariectomy-induced changes in gene expression levels. Examinations of average gene expression levels were made between ovariectomized (Ovx) animal groups and their time-point equivalent sham-operated (Sham) control group. Significance was determined if a fold change ≥ 2 or ≤ -2 and a p value ≤ 0.0025 were achieved for a comparison in both experimental replicates. Significant fold change values are indicated. Comparisons that were not significant are represented by ---. Probe sets for each cluster are ordered by the magnitude of the fold change at the time-point with the most numerous significant values for that cluster. EST, expressed sequence tag; RIKEN cDNA, Japanese consortium expressed sequence.

Functional Clustering

Genes were organized based on their known or inferred functions to identify networks of genes with similar or interrelated functions. Information was acquired from Affymetrix [112], Swiss-Prot [113], Unigene and Locus Link [114], and other available database websites. The infection study list of 204 probe sets was functionally clustered in Table 3.5 and the ovariectomy study list of 79 probe sets was functionally clustered in Table 3.6. As there was often more than one function associated with each gene product, alternative classifications were also included in the tables. These tables are found at the end of this chapter.

From the **infection study**, Cluster A with its down-regulation of genes at PTL were found to fall under the functional categories of RNA/protein biosynthesis-metabolism/modification; signalling; intercellular adhesion/communication, extracellular matrix, and blood clotting group; cell immune/immune function with complement 1q subcomponents and ScyA6; and the transport classification where there are several hemoglobin genes listed. Of the genes up-regulated with PTL in Clusters B-F, Cluster B genes are classified predominantly under growth/differentiation/apoptosis with schlafen 2 and chloride intracellular channel 4 (mitochondrial) each represented twice; transcription factor genes, many involved in NFκB expression; and most significantly cell immune/immune function (not surprisingly considering the model). Cluster C probe sets also fall into the cell immune/immune function group. Cluster D genes are mainly classified under transcription regulation, including cFos; signalling; and once again under cell immune/immune function where IL-6 and IL-1 α and β are found. Probe sets found in Cluster E are classified mostly under the cell immune/immune function heading. Cluster F, with its late up-regulation of genes with the PTL-producing dose of bacteria include the granzymes C, D, E, and G and others under the heading of growth/differentiation/apoptosis; also are found genes which play a role in intercellular adhesion/communication, extracellular matrix, and blood clotting.

From the **ovariectomy study**, Clusters A-D consist of probe sets for genes up-regulated at the time of ovariectomy induced PTL. Much of Clusters A and B are

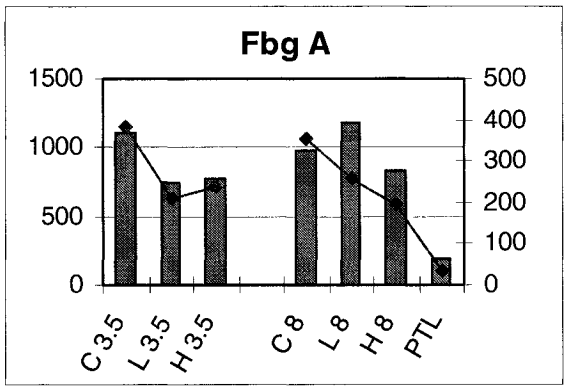
comprised of probe sets for genes with unknown functions, particularly the small proline-rich proteins. Cluster C genes fall mostly within the cell growth/differentiation/apoptosis functional group and the intercellular adhesion/communication, extracellular matrix, and blood clotting classifications. Genes of Cluster D fall under transcriptional regulation (cFos and activating transcription factor 3), and signalling headings. The transitional Cluster E contains Tnfip6 and ScyA2 gene probe sets. Tnfip6 is important for cell-cell and cell-matrix interactions during inflammation and is a target of prostaglandin action, facilitating ovulation [178]. ScyA2 is important for signalling during an immune response and promotes chemotaxis.

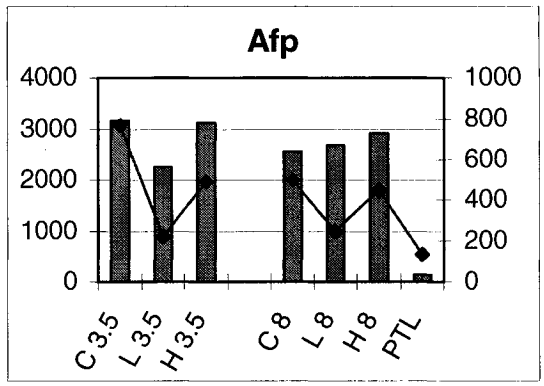
The genes whose expression is down-regulated with ovariectomy are found in Clusters F-H. The 4 Cluster F genes each fall under a different classification. Cluster G contains some genes important for cell signalling. Cluster H contains the genes involved in DNA/nucleotide metabolism; RNA/protein biosynthesis, metabolism/modification; intercellular adhesion/communication, extracellular matrix, and blood clotting classification including Cx26 and F3, respectively; and cell immune/immune function.

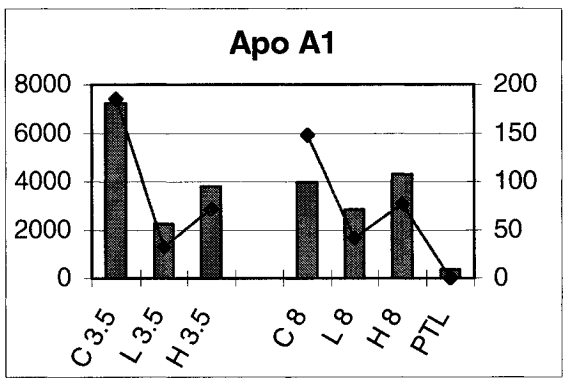
Validation of Microarray Results

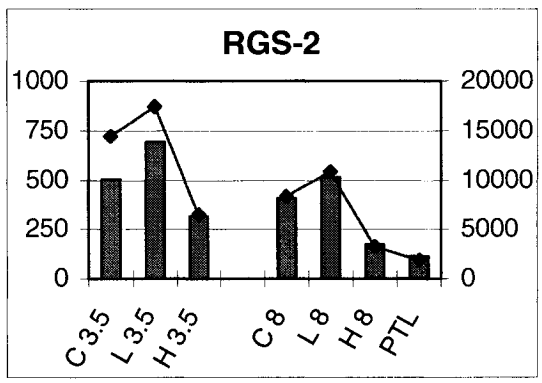
To corroborate the microarray results, relative quantitative real-time RT-PCR was performed for selected genes. Gene transcripts chosen for validation of infection microarray data were alpha fetoprotein (Afp), apolipoprotein A1 (ApoA1), cFos transcription factor, fibrinogen alpha polypeptide (FbgA), macrophage-colony stimulating factor (M-Csf), regulator of G protein signalling 2 (RGS-2), serum amyloid A3 (SAA3), and small proline-rich protein 2D (Sprr2D). Validation of ovariectomy microarray data was performed using PCR primers to cFos, M-Csf, Purkinje cell protein 4 (PCP4), and RGS-2. Primer pair sequences and amplification product size are listed in Table 3.1. Each experimental replicate was amplified in triplicate for 6 RT-PCR replicates per time-point. RT-PCR product relative quantification data were plotted with microarray fluorescence values in Figure 3.3

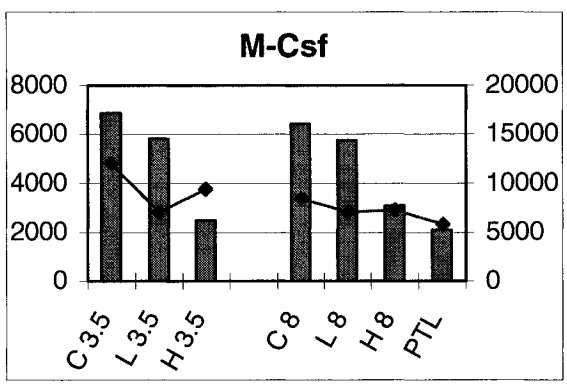
(Infection) and Figure 3.4 (Ovariectomy). In each case, the changes in gene expression observed by microarray analysis were reproduced by relative quantitative real-time RT-PCR, validating the results.

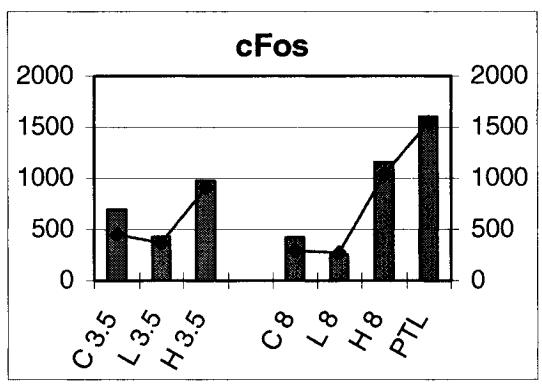

Fbg A
1500
1000
500
0
500
400
300
200
100
0
C 3.5
L 3.5
H 3.5
C 8
L 8
H 8
PTL

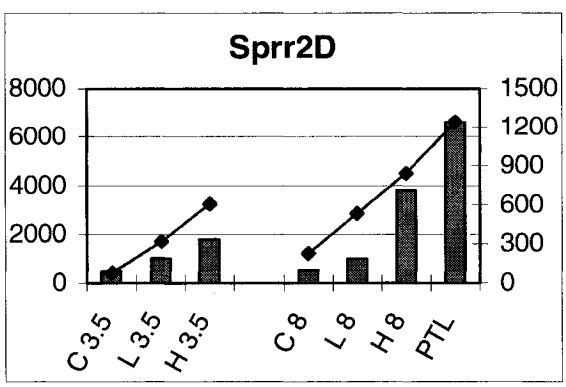

Afp
4000
3000
2000
1000
0
1000
800
600
400
200
0
C 3.5
L 3.5
H 3.5
C 8
L 8
H 8
PTL


Apo A1
8000
6000
4000
2000
0
200
150
100
50
0
C 3.5
L 3.5
H 3.5
C 8
L 8
H 8
PTL


RGS-2
1000
750
500
250
0
20000
15000
10000
5000
0
C 3.5
L 3.5
H 3.5
C 8
L 8
H 8
PTL


M-Csf
8000
6000
4000
2000
0
20000
15000
10000
5000
0
C 3.5
L 3.5
H 3.5
C 8
L 8
H 8
PTL


cFos
2000
1500
1000
500
0
2000
1500
1000
500
0
C 3.5
L 3.5
H 3.5
C 8
L 8
H 8
PTL


Sprr2D
8000
6000
4000
2000
0
1500
1200
900
600
300
0
C 3.5
L 3.5
H 3.5
C 8
L 8
H 8
PTL

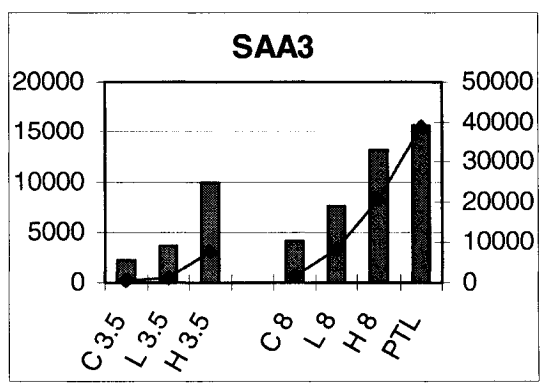

SAA3
20000
15000
10000
5000
0
50000
40000
30000
20000
10000
0
C 3.5
L 3.5
H 3.5
C 8
L 8
H 8
PTL

Figure 3.3 Validation of microarray data from the intrauterine-infection model for preterm labour using relative quantitative real-time RT-PCR. Validation of microarray data was performed using eight genes with distinct differential expression patterns: FbgA, fibrinogen alpha polypeptide; Afp, alpha fetoprotein; ApoA1, apolipoprotein A1; RGS-2, regulator of G protein signalling 2; M-Csf, macrophage colony stimulating factor; cFos, FBJ osteosarcoma oncogene; Sprr2D, small proline-rich protein 2D; and SAA3, serum amyloid A3. The genes down-regulated with PTL are all representatives of Cluster A. SAA3 represents Cluster C, cFos represents Cluster D, and Sprr2D represents Cluster E, each having different expression patterns. The axis on the left indicates microarray fluorescence values after dChip modelling. The axis on the right specifies the relative quantitative real-time RT-PCR SYBR Green fluorescence values. Average values of two microarray replicates are plotted in bar graph form. Average values of triplicate RT-PCR amplification of two replicate RNA samples are plotted in line graph form.

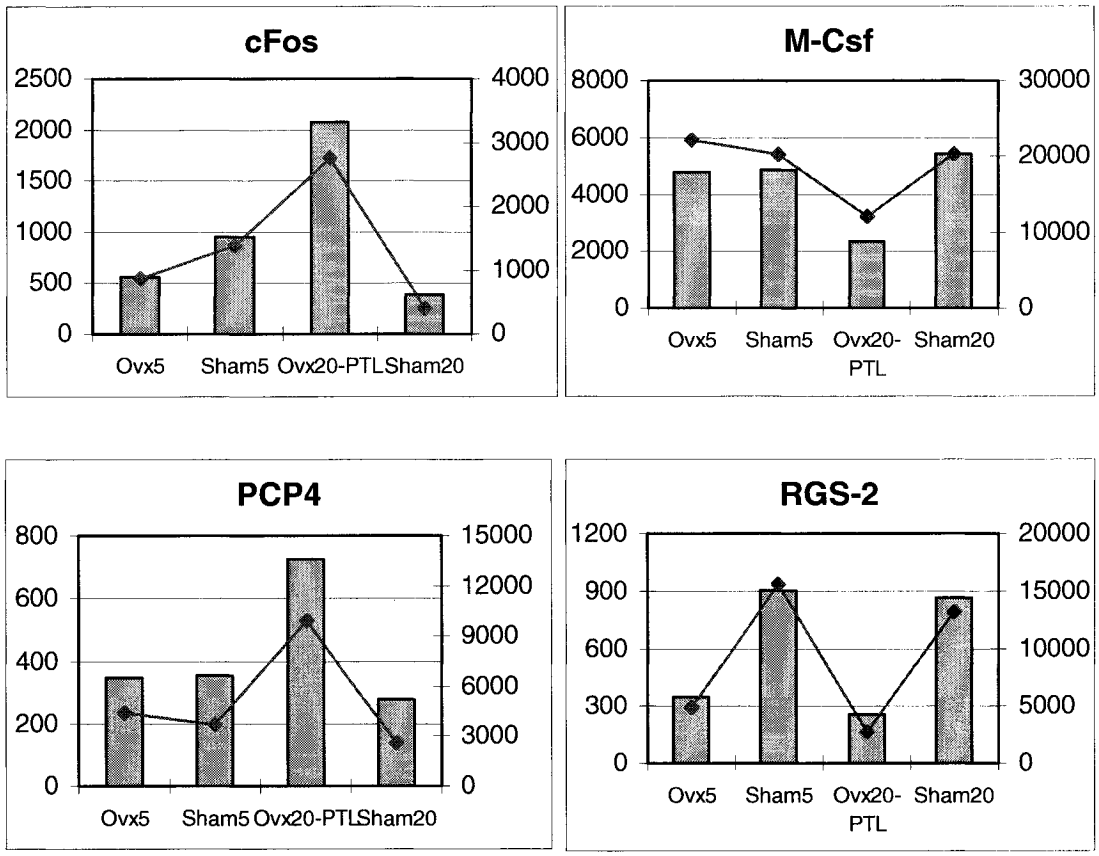


Figure 3.4 Validation of microarray data from the ovariectomy model for preterm labour using relative quantitative real-time RT-PCR.

Validation of microarray data was performed using four genes with distinct differential expression patterns. The genes cFos, FBJ osteosarcoma oncogene; and PCP4, Purkinje cell protein 4 both represent genes which are up-regulated with ovariectomy-induced PTL. M-Csf, macrophage colony stimulating factor represent genes that are down-regulated with ovariectomy-induced PTL. RGS-2, regulator of G protein signalling 2 represents genes down-regulated as early as 5 hours after ovariectomy. These genes also represent different expression patterns as cFos represents Cluster D while PCP4 represents Cluster A. M-Csf represents Cluster H while RGS-2 represents Cluster G. The axis on the left indicates microarray fluorescence values after dChip modelling. The axis on the right specifies the relative quantitative real-time RT-PCR SYBR Green fluorescence values. Average values of 2 microarray replicates are plotted in bar graph form. Average values of triplicate RT-PCR amplification of two replicate RNA samples are plotted in line graph form.

3.5 DISCUSSION

Changes in Gene Expression in Infection-Induced Preterm Labour

We identified 204 probe sets depicting genes that were differentially regulated in our infection study. Several of the genes represented by the probe sets are well described as having a role in the process of infection-induced premature parturition, providing validity to those that are newly implicated in the process, their exact roles and relative importance to the overall mechanism yet to be elucidated. A significant number of gene probe sets (~80%) represented genes induced with a high dose of killed *E.coli* relative to levels found in mice receiving a low dose or vehicle control. The high dose infection was the only stimulus to successfully induce PTL. By examining the genes and their products indicated by this model of PTL, we may identify novel pathways important to the culmination of infection-induced PTL and allow diagnostic and/or preventative therapies to be developed. Discussion of all genes identified is not realistic in this forum. We instead focus on presenting and supporting theories to the roles of gene products to the process of infection-induced PTL.

Up-regulation of Immune Response Genes and Growth Factors

Pro-inflammatory Mediators

Of no surprise is the large number of genes involved in cell defence/immune functions that are differentially regulated throughout this process. Initially, LPS, the toxic cell wall component of pathogenic bacteria is bound to host-produced LPS-binding protein (LPSBP) and in this complex is presented to the CD14 receptor molecule on the surface of macrophages. We found that although LPSBP was unchanged in this model and therefore is not found in our list, the CD14 probe set indicated a significant up-regulation (nearly 4-fold) with the high dose infection as early as 3.5 hours post-infection and this was sustained through PTL. An induction was found with low infection but peaked at 8 hours relative to control levels. This suggests that CD14 activation is only one factor in initiating an immune response significant to induce PTL. Additionally, bacterial antigens may bind membrane-

bound antibodies of circulating B cells. Macrophages and B cells present the processed antigen to T helper (T_h) cells. Macrophages secrete interleukin (IL)-1, acting as a growth factor for B cells and activating T_h cells after interaction with presented antigen. The cytokines released by macrophages and activated T_h cells activate an entire network of cells mediating an immune response. The properties of the cytokines, pro-inflammatory or anti-inflammatory, produced by T_h cells classify them as T_h1 cells or T_h2 cells, respectively.

Throughout much of gestation a T_h2 cytokine environment dominates. IL-4, IL-5, IL-10, and transforming growth factor β (TGFβ) are examples of the type 2 cytokines produced by T_h cells. The T_h2 cell is responsible for phagocytosis- and inflammation-independent host defence, promoting tolerance to the developing fetus [17]. They are characterized as central to allograft tolerance and maintenance. As normal parturition at term approaches a shift in cytokine production tips the balance to a T_h1 cytokine environment, activating an inflammatory response to the allogeneic fetus and promoting a rejection resulting in birth. Under the stimulus of an invading bacterial infection, the cytokine balance is shifted, prematurely and dramatically, to a T_h1 environment and PTL results. The production of T_h1 cytokines during experimentally induced PTL is well known and was found in our analysis, providing legitimacy to our results as a whole.

T_h1 cytokines include IL-1α and β subtypes, TNFα, and IFNγ. IL-1 is secreted by antigen presenting cells and endothelial cells. It supports a humoral response by promoting B cell maturation and clonal expansion. It also facilitates a cell-mediated response, acting as a chemoattractant for macrophages and neutrophils, increasing the expression of intercellular adhesion molecules on vascular endothelial cells, facilitating immune cell influx and inducing the synthesis of proteins necessary for an acute phase response, as well as participating in the activation of T_h cells [59]. We found IL-1α and β (Cluster D) to be moderately induced at the transcription level early after high bacterial injection. These elevated levels were significantly induced

relative to the low dose treatment. The induction was maintained through PTL. The receptor for IL-1α and β (Cluster F) is induced at PTL relative to levels early after high dose infection. This specific up-regulation likely facilitates the increase in IL-1, promoting an inflammatory response. Conversely, it can also bind the IL-1 receptor antagonist (IL-1rn) (Cluster D), which is expressed by decidual macrophages [16] in a similar manner to IL-1α and IL-1β. As was suggested by Romero et al. [179], the IL-1rn may be capable of preventing PTL induced by IL-1 administration. In a live *E.coli* induction of PTL [170], an early increase in transcripts for both IL-1α and IL-1β were recorded along with a more moderate induction of the receptor antagonist. These results together suggest an attempt by the uterine environment to prevent an inflammatory response during gestation. The level of IL-1rn within the uteri of infected mice was suggested to be insufficient to inhibit this response exclusively [167], indicating fetal IL-1rn and other factors may participate.

IL-1 and LPS directly induce IL-6 gene expression. IL-6 is well described to be highly induced during infection-induced premature labour and is characterized as a diagnostic marker for this event [3, 92, 167]. Not surprisingly, IL-6 was the most significantly up-regulated cytokine gene in our study. An increase in gene expression was found after 3.5 hours of high (10^{10}) bacteria inoculation (~6.8-fold). This rose quickly to a 37-fold increase after 8 hours compared with control and this level was sustained at PTL. A significant induction relative to low (10^{7}) bacterial injection was observed. A minimal increase was found after 8 hours of treatment with 10^{7} bacterium but did not significantly vary from levels of control uteri. This suggests that this high dose-related increase is important to the precipitation to PTL. Increased IL-6 production also results in response to mechanical stretch [180], indicative of a restriction on uterine growth similar to that found to trigger CAP gene important for uterine contractions.

IL-1 and IL-6 are able to regulate the transcription of acute phase response genes. Binding of interleukin to its plasma membrane receptor leads to internalization, translocation to the nucleus, and activation of the transcription factors NFκB and NF-IL6 (a.k.a. C/EBPβ). NFκB then feeds back to enhance IL-1 and IL-6

expression and numerous acute phase proteins such as IL-8, GRO1, VCAM-1, ICAM-1, and serum amyloid A3 (SAA3) [181]. NF-IL6 functions similarly. Both IL-1 and IL-6 promote the synthesis of prostaglandins through the up-regulation of cyclooxygenase-2 (COX-2), which possesses at least one NFκB and one NF-IL6 promoter element [182]. Induction of these transcription factors also leads to the regulated expression of the oxytocin receptor (OTR) gene as its promoter includes binding sites for both NFκB and NF-IL6 [49, 183]]. In our model, except for IL-8 and the OTR, which are not represented on the murine array, all were transcriptionally activated after high dose treatment leading to PTL. Unlike the IL-1rn, no antagonist of IL-6 function in PTL has been studied. It is likely that such an antagonist would have a positive effect on preventing infection-induced PTL by temporarily limiting the inflammatory response and suppressing the activation of genes known to be induced during normal parturition.

Consistent with these results was the induction of COX-2 in our study. COX-2 is the inducible form of prostaglandin H synthase, the enzyme responsible for converting arachidonic acid (AA) to prostaglandin (PG) $H_{2,}$, the precursor to both PGE_2 and $PGF_{2\alpha}$, effective uterotonins. COX-2 and PGs are provided by the placenta of the maturing fetus but maternal sources of COX-2 are dependent upon the increase in effective estrogen levels found at the end of normal parturition [19]. Following bacterial exposure, it is found at high levels in macrophages and other inflammation-associated cell types [173]. In our model, we found the uterus produced a significant amount of COX-2 transcript. An early gene induction of 14-fold vs. control levels was measured. This level increased to 18-fold by 8 hours and peaked at an impressive 28-fold difference compared with control. This induction was specific, once again, to the high infection dose as a significant up-regulation compared with low dose levels was observed and low dose levels did not vary significantly with respect to time-matched controls. Inhibiting prostaglandin production by the administration of nonsteroidal anti-inflammatory drugs, such as indomethacin, which targets both COX-1 and COX-2 results in the attenuation of PTL. Unfortunately, its use also leads to significant fetal side effects [1]. The use of a selective COX-2 inhibitor, SC-236, was

demonstrated to be efficient in abrogating PTL induced by inflammation, while a COX-1 inhibitor was less successful [173]. The effect of SC-236 on fetal morbidity and mortality still needs to be tested. As IL-6 provokes a significant production of prostaglandins, IL-6 is indicated a potential target for both diagnostic and intervention therapies.

Pro-inflammatory cytokine tumour necrosis factor (TNF) α gene expression did not reach statistical significance at any comparison made but was increased early after high dose infection relative to control. This expression subsequently dropped at PTL, again without statistical significance. The importance of this factor in inflammation-mediated PTL is questionable.

Interferon (IFN)γ was given an "A" absent call in each experimental time-point for both microarray replicates. This may reflect an expression below the threshold of the Affymetrix chip scanner or may indicate a defect with the probe set for this gene. There were however, numerous interferon-induced genes differentially regulated by infection leading to PTL, facilitating the pro-inflammatory effects of IFNγ. This long list includes the interferon activated "200 family genes" (Ifi) 202A, 203, 204, and 205 referred to as such because they each contain partially conserved 600 nucleotide segments encoding 200-amino acid domains [184]. Ifi202A encodes a protein (p202a), whose over-expression inhibits cell proliferation by preventing the binding of transcription factors cFos, cJun, NFκB p50 and p65, among others to their specific DNA sequences [185]. p202a is induced during the differentiation of muscle, modulating myogenic transcription factors and inhibiting apoptosis [186]. Ifi204 gene product inhibits cell proliferation by inhibiting the transcription of ribosomal RNA. It is also suggested to participate in the differentiation of the monocyte [185] and is involved in the differentiation of muscle fibres [186]. Ifi203 is constitutively expressed in the thymus and bone marrow where its protein product may regulate T cell and B cell development and maturation. Expression of the Ifi203 gene product p203 in the spleen, where B cell proliferation proceeds in response to the presence of blood-borne antigen, suggests a regulatory effect on the modulating or facilitating an immune response to systemic infection. The p203 possesses only one "200 amino

acid motif" unlike p202 and p204, which have two each, and as a result fails to exert an anti-proliferative activity [187]. The expression of Ifi203 in the uterus is a novel finding and likely plays a role in the amplification of immune response leading to PTL. Ifi205 is also known as D3 and is induced in macrophages by LPS [185]. D3 protein is more than 90% homologous to p204 [184] suggesting it too may participate in the specific production of macrophages, further amplifying the immune response. The Ifi200 family is apparently involved in both inhibiting and promoting cell differentiation and proliferation depending on the cell context. In the condition of uterine infection during gestation, Ifi200 gene products may promote an immune response by stimulating the differentiation and proliferation of antigen presenting cells and may facilitate the organization of muscle fibres for uterine contraction during PTL. Preventing the expression of the Ifi202A-205 genes or the functions of their products may limit the immune response and the ability for contractions to occur. These genes and their products require further analysis in the context of intrauterine infection leading to PTL.

In our model of infection-induced PTL, we found these genes to be moderately activated exclusively with the high dose of bacteria with a consistently significant mRNA level at the moment of PTL. The two probe sets for each Ifi202A, Ifi203, and Ifi204, were assigned into different clusters (Ifi202A -B and E, Ifi203 -B and E, Ifi204 -B and C). GeneCluster assigned the probe sets based on the average expression level of each experimental group relative to the median value. The reason for the dual assignment is due to the significant level of gene expression detected by the unique oligonucleotide sequence of the individual probe sets. A potential explanation for this is that cross-hybridization of a transcript with sufficient homology to the sequence of one probe set would result in an artificially high fold change if this other transcript expression were induced in this same condition. Conversely, hybridization of an induced complementary cRNA sequence to the cRNA target of a probe set would hinder the hybridization of the target cRNA sequence to the probe set and result in a lessened apparent fold increase. In this case, we are examining a family of genes that may have sequence similarities affecting hybridization of the true target to the probe.

This phenomenon of multiple cluster assignment is found elsewhere among the probe sets (interferon-inducible GTPase Iigp-pending and vascular cell adhesion molecule (VCAM)-1). In each case, the probe sets showed an up-regulation with approaching PTL.

The genes for interferon-induced proteins with tetratricopeptide repeats (Ifit) 1, 2, and 3 are induced in our model. These genes are also referred to as glucocorticoid attenuated response gene (GARG)-16, -39, and -49, respectively. Glucocorticoids have potent anti-inflammatory effects. LPS and interferons have been demonstrated to induce the expression of these GARGs and dexamethasone, a synthetic glucocorticoid agonist, attenuated this response [188]. While macrophages express these genes, other cell types may as well. In our hands, an up-regulation of Ifit1/GARG-16 was found with both low and high bacterial doses as early as 3.5 hours post-injection. This induction of gene expression was sustained through 8 hours for the low dose and to PTL with the high dose. There was a significant difference in the stimulation of Ifit1 gene expression, with nearly 3 times as much mRNA detected with the high infection. Similar changes in gene expression were observed for Ifit2/GARG-39 and for Ifit3/GARG-49. The functions of these genes still await determination but it appears that they may participate in mounting an immune response.

Additional LPS-induced GARGs (without tetratricopeptide repeats) identified include small inducible cytokine (Scy) genes ScyA2/MCP-1 and ScyA7/MCP-3, which are chemoattractants specific for monocytes. ScyA2 gene experienced an early moderate increase in transcription in response to the high dose. It was greatly up-regulated to more than 15 times control levels by the 8 hour time-point. This increased further by the time PTL ensued. The level of expression induced by low level bacterial infection followed the same pattern but was more moderate. The high level of bacteria induced a change which was significantly greater than that found with the low inoculation. ScyA7 mRNA levels followed a similar pattern but with increased expression peaking at nearly 6-fold more than time-control levels during PTL. A fibrotic activity has recently been annotated for this gene product [189]. Thrombospondin 1 is included in this list of GARGs and possesses the ability to

mediate cell-to-cell and cell-to-matrix interactions. Also included is the gene for cysteine-rich protein 61/insulin-like growth factor binding protein (IGFBP) 10, which can promote cell proliferation, chemotaxis, angiogenesis and cell adhesion [188]. Both fall into Cluster F with an induction with the onset of PTL to levels significantly greater with the high than the low bacterial infection. Thrombospondin 1 is capable of binding fibrinogen while IGFBP10 facilitates wound healing by up-regulating MMP3 and PAI-1 genes [113]. The emerging roles assigned to these factors suggest an importance for fibrin deposition during PTL.

Additional members of family A include ScyA3; this gene is up-regulated moderately in the H3.5 group. This cytokine promotes neutrophil activation and is chemotactic for eosinophils. ScyA5, also known as RANTES, was up-regulated to more than 13-fold control levels by 3.5 hours following injection of the high dose. This level increased slightly by PTL. The increase was significantly greater than that found with the low dose infection. Members of the small inducible cytokine B family were also differentially expressed. These include Scyb2/macrophage inflammatory protein 2, Scyb5, Scyb9/MIG, and Scyb10/IP-10. Each of these genes were dramatically induced by high levels of killed bacteria as early as 3.5 hours after infection and further so by 8 hours and PTL, these levels being substantial relative to those found after low dose stimulation. Both Scyb9 and Scyb10 promote T_h lymphocyte recruitment and are angiostatic, thus contributing to tissue injury, supporting the generation of a T_h1 cytokine response to bacterial invasion and promoting graft rejection [190, 191]. They accomplish this by binding to a common receptor.

The list of cytokines significantly induced at the transcriptional level is, not unexpectedly, extensive. They highlight the immune response in this process for premature parturition in response to a bacterial stimulus. This discussion should not overlook immunoresponsive gene 1 (Irg1) which showed a most dramatic induction at 3.5 hours after high dose stimulus. A fold change relative to control levels were calculated at approximately 45 and 22 for the two probe sets representing this gene (Cluster B). The levels diminished to still significant levels at 8 hours and diminished

further at PTL. While this appears quite remarkable, it should be pointed out that it is less than a 3-fold difference at 3.5 hours compared with the low dose response and the difference is not statistically significant by 8 hours. In the mouse uterus, this gene is induced by progesterone in luminal epithelial cells participating in preparation of the uterus for blastocyst implantation [192].

Chitinase 3-like 1 gene encoding glycoprotein 39 (Cluster E) was most impressively induced at the moment of infection-induced PTL. A fold change reflecting approximately 45 times more transcripts with high dose infection than that found in the time-matched control. As with Irg1, this impressive display was not significant relative to levels found in mice treated with the low dose. Glycoprotein 39 is secreted from smooth muscle cells, macrophages, and other cell types and is suggested to function in the late-differentiation of monocytes and may also affect the extracellular matrix, promoting increased turnover and connective-tissue cell growth and remodelling [193]. It was first identified in the mouse uterus under the condition of normal labour (Chapter 2) where a significant induction postpartum was found, substantiating its role in tissue remodelling.

The importance of these two genes, Irg1 and chitinase-3 like 1, exhibiting the greatest apparent induction after treatment with high levels of killed bacteria remains to be seen. What is brought to light is the importance of a thorough investigation of genes monitored and analysed by microarray chip technology and associated analysis software. In this situation, the inclusion of the low dose group allowed the fine discernment of the validity of otherwise unquestioned results.

Anti-inflammatory Mediators

Calgranulins A (S100A8/MRP8) and B (S100A9/MRP14), produced by neutrophils and monocyte/macrophages upon stimulation by IFNγ and TNFα, heterodimerize to form calprotectin. In complex form, they possess an antimicrobial activity, preventing bacterial colonization by inhibiting the binding of microorganisms to epithelial cells and competitive binding of the nutrient zinc [194, 195]. Calprotectin also has pro-inflammatory effects, acting as a chemokine to inducing neutrophil and

macrophage migration to the site of inflammation. At marginal concentrations, the complex promotes cell growth, but upon cytokine stimulation, an increased amount of calgranulin A and B proteins commensurate with increased transcription, and hence, increased calprotectin, converts to growth inhibitory and apoptotic affects [195, 196]. In the condition of inflammatory muscle diseases, increased levels of calprotectin are synthesized. The effect was inhibited proliferation and tissue destruction. In murine myoblast cells treated with the calprotectin complex, a significant dose-dependent inhibition of cell proliferation was observed accompanied by an increase in apoptosis [196]. Differentiation from myoblast to myocyte (and myotuble formation) was also blocked in a dose-dependent manner. The cumulative result, under the influence of infection, was the degeneration of muscle tissue [196]. In the context of gestational myometrium, inflammation would lead to an increased influx of neutrophils and macrophages, stimulating increased calprotectin formation by their increased gene expression of calgranulins A and B and lead to growth inhibition of myometrial cells. The result would be increased uterine stretch by the growing and moving fetus. Uterine stretch has been demonstrated to be necessary for full induction of genes involved in uterine activation, the contraction associated proteins (CAPs), during normal labour. In this manner, calgranulin A and B genes are implicated in the process of uterine contractions during infection-induced PTL. The calprotectin complex accounts for approximately 40-50% of neutrophil cytosolic proteins where, it exclusively, under the influence of calcium, binds arachidonic acid, implicating it in the mobilization, metabolism, or release of arachidonic acid [197]. Calprotectin may partake in the increased synthesis of prostaglandins from arachidonic acid by providing it as a substrate for COX-2 activity.

In our investigation, we observed a significant induction of both calgranulin A and B genes (both Cluster E) solely under the stimulus of high dose infection with a significant increase in expression with time, peaking at PTL. Aguan et al. [120] reported a down-regulation in human myometrium during normal labour compared with non-labouring myometrium. In our murine model of normal labour (Chapter 2), we did not observe a significant change in the expression of either of these genes. As

expression is dependent upon activation by pro-inflammatory cytokines, it is of no surprise that they are not significantly expressed during normal labour. The proposed participation in PTL is not required during normal labour as other factors promote a uterine stretch response. In another microarray analysis of genes induced by uterine infection during pregnancy calgranulin A was reported to be up-regulated with 10^{10} and 10^{7} bacterial infection as well as with ovariectomy [2]. Calgranulin B gene induction was not observed, raising questions to their analysis methods. In the condition of infection-induced PTL, the use of an inhibitor of calprotectin may prolong gestation by limiting macrophage and neutrophil invasion, preventing myometrial tissue destruction and blocking the synthesis of prostaglandins and hence contribute to uterine quiescence. Yui et al. identified lycoricidinol (narciclasine), as having a very potent inhibitory effect on calprotectin's cytotoxicity [195]. Use of this compound may provide a useful therapy to infection-mediated PTL.

Lactotransferrin (a.k.a. lactoferrin) was first described in milk and has since been indicated in epithelial cell secretions where its product in barrier fluids is protective against pathogenic invasion [198] and as a neutrophil product participating in the cytotoxic function of calprotectin [199]. In addition, lactotransferrin is characterized as immunomodulatory, down-regulating the expression of pro-inflammatory cytokines induced by bacteria/LPS. These include TNFα, granulocyte-macrophage colony stimulating factor, and perhaps, more importantly, IL-6 [200]. In a murine model of LPS-induced PTL, pre-treatment with lactoferrin significantly suppressed LPS enhanced plasma IL-6 levels and allowed the continuance of gestation [201]. In our model, we found a considerable increase in lactotransferrin gene expression (Cluster E) 3.5 hours after high dose infection that continued through to PTL. This increase was significant over levels induced by the low-grade infection. This dynamic expression was not detected by Muhle et al. [2]; perhaps the gene was not represented on the microarray they chose.

The lactotransferrin gene, under the influence of estrogen, is expressed in the uterine endometrium where it contributes to the mucosal barrier of the uterus binding iron necessary for bacterial cell growth therefore providing a defence against infection

[200]. An increased level of gene expression and consequently increased lactotransferrin was observed with advancing gestational age and a further induction was found in women experiencing intra-amniotic infection [202]. In the context of normal murine labour, this gene was not detected by our analysis (Chapter 2). This might reflect the relatively high levels of lactotransferrin transcription in the mouse uterus at estrus [115]. Comparison of the term, labour, and postpartum levels to that at estrus did not amount to a statistically significant increase. The increase and protective nature of lactotransferrin during infection-induced PTL suggests it as a means to halting the progression of PTL. Generation of a lactotransferrin pharmacotherapy with an increased efficacy may prove a successful tool for the management of premature parturition resulting from pathogenic invasion.

The serum amyloid A3 gene (SAA3), similarly to the lactotransferrin gene, is expressed in the breast and its product is found in colostrom and milk. It has since been found expressed in other tissues where it stimulates the production of mucins, protecting the tissue from bacterial invasion [203, 204]. In this manner, SAA3 is an acute phase response protein with immunoprotective function. In this role, it also participates in the inhibition of neutrophil oxidative bursts, migration of neutrophils, monocytes, and T cells, and cell adhesion to components of the extracellular matrix [204]. The expression of the SAA3 gene is dependent upon IL-1 activation that is synergistically enhanced by other inflammatory cytokines such as TNFα and IL-6, which on their own have limited inductive properties [181, 205, 206]. IL-1 activates the transcription factors NFκB and NF-IL6 (a.k.a. C/EBPβ), which then bind to their response elements to stimulate SAA3 gene expression [181]. While these transcription factors are known to induce pro-inflammatory cytokine genes, perhaps more interestingly, they increase transcription of immunoprotective factors, such as SAA3. The fact that SAA3 gene expression peaked at 3.5 hours with the high dose suggests an attempt to prevent bacterial attachment and activation of a pro-inflammatory response. If we can exploit the protective nature of SAA3, we may prevent the bacterial invasion that triggers PTL. Clearly, low-level infection does not stimulate this extreme response. If the effectiveness of bacterial attachment is reduced

by increased mucin production and secretion by the endometrial epithelium the acute phase response may be averted. It appears a struggle between an acute phase response and an attempt to maintain uterine and fetal tissue integrity and prolong gestation is in play during infection-induced PTL.

Down-regulation of Genes in Infection-Induced Preterm Labour

We also detected the down-regulation of genes (Cluster A) accounting for approximately 20% of the genes deemed significant in our analysis. The three polypeptides (alpha, beta, and gamma) contributing equally to the hexameric structure of fibrinogen were moderately repressed at the moment of infection-induced PTL. Fibrinogen serves as a substrate for conversion to fibrin by thrombin. Fibrin, the insoluble molecule, is deposited at the site of blood clots. A suppression of transcription may be an attempt to prevent clot formation and resulting tissue damage due to hypoxia. Contrary to this, plasminogen activator inhibitor-1 (PAI-1) is induced moderately at the time just initial to and during infection-induced PTL (Cluster F). It is protective against fibrinolysis by preventing the conversion of plasminogen to plasmin, the factor responsible for lysing fibrin and fibrinogen [130]. This may be in compensation for reduced fibrinogen product; stabilizing the integrity of clots formed in preparation of placental detachment. Additionally, fibrinogen degradation products are inhibitors of thrombin activity [130]. Thrombin itself is described as an uterotonic agent with effective protein levels much lower than that found in clotting blood [131, 132]. This surprising sensitivity of the uterus to thrombin leads to myometrial contractions during clot formation. In fact, limited spotting during pregnancy can lead to spontaneous abortion. Reducing fibrinogen transcription and subsequently, limiting protein quantity could lead to a lessened inhibitory effect, facilitating thrombin stimulated uterine contractions. In our model of infection-induced PTL, changes in gene expression of its precursor prothrombin (coagulation factor 2 (F2)) do not reach statistical significance by our strict criteria. It does appear that the blood clotting and fibrolytic mechanisms are both antagonistic and complementary in their roles in the process of infection-induced PTL.

The gene most significantly suppressed with PTL was alpha fetoprotein (Afp) with a 17-fold reduction in expression relative to time-matched controls, low dose expression level and preceding 3.5 hour and 8 hour high dose time points. Afp is characterized as having anti-estrogenic properties, reaching peak expression during the final trimester of normal pregnancy [138]. Afp is capable of binding steroids in addition to estrogen, of being taken up by cells to interfere with nuclear receptor function, and of exerting immunosuppressive effects on lymphocytes [139]. Afp was reported to induce immunosuppressive cytokine production and to diminish the phagocytic activity of macrophages [207]. The sudden, specific, and significant drop in mRNA levels at the onset of infection-induced PTL may reflect a permissive effect on estrogen levels and the immune system. By day 15 of gestation when PTL is proceeding, uterine estrogen content has not increased significantly and bound to Afp, is rendered ineffective. A drop in Afp gene transcription could lead to a shift in the effective progesterone/estrogen balance favouring estrogen, consequently activating cFos and CAP genes, among others, ultimately resulting in uterine contractions and premature delivery. This is of course dependent upon a short half-life of the Afp. Additionally, a decrease would also relieve the immune protection imparted to the fetus, with T_h1 cytokine production increasing along with immune cell infiltration for the destruction of foreign fetal-derived tissue, leading to placental detachment. In murine parturition, the placenta is delivered with its fetus.

While not quite as dramatic a drop, the expression of apolipoprotein A1 (ApoA1) was significantly repressed with high dose infection. This repression, similar to that of Afp, was specific to the PTL time point with a greater than 9-fold suppression relative to the 8 hour control, a 7-fold reduction relative to the 8 hour low dose expression level and a dynamic change in expression from levels prior to parturition. ApoA1 glycoprotein is found at higher concentrations in serum of pregnant women than non-pregnant women and is associated with increased endothelial-dependent relaxation of myometrial arteries, likely through ApoA1 stimulation of endothelial prostacyclin secretion [142]. Preeclampsia results in part from an impaired relaxatory mechanism leading to myometrial vessel contraction and

vascular injury [142]. The down-regulation of this relaxation-promoting factor at normal labour, from high levels at term, may increase uterine tonicity and facilitate uterine stretch responses. An anti-inflammatory role is also assigned to ApoA1. It was demonstrated to directly interfere with T cell-macrophage contact thereby preventing the stimulation of the macrophage to produce inflammatory T_h1 cytokines IL-1β and TNFα, and promoting the production of immunomodulatory T_h2 cytokines [143, 144]. ApoA1 binds HDL-bound cholesterol and transports it to the liver for catabolism. During blastocyst implantation, lipid accumulation in the uterine endometrium increases, reflecting low ApoA1-mediated transport [146]. This accumulation leads to cell breakdown and tissue disruption, contributing to the uterine changes necessary at these times as well as immune system potentiation. Tissue remodelling during labour results in placental detachment. Postpartum, it returns the tissue to its non-pregnant state. ApoA1 is also capable of transporting cell and tissue damaging reactive oxygen species to the liver for detoxification. ApoA1 reduces cell death and subsequent immune activation. The drop in ApoA1 gene expression and subsequently protein levels would diminish the protection this "negative" acute phase protein imparts to maternal and fetal tissues, leading to a permissive state for a rejection response leading to parturition.

Progesterone Withdrawal

The question of the specific nature of the genes regulated during infection-induced PTL induced approximately 75% of the way through gestation brought us to investigate the genes expressed after induction of PTL in the absence of infection. We chose to induce labour by bilateral ovariectomy at this same stage of gestation. The loss of progesterone provided by the corpus luteum of pregnancy provides the hormonal cue for parturition. In this analysis we were able to better understand the changes responsible for PTL in the absence of infection, better characterize the importance of steroid influence on infection-induced PTL and perhaps highlight mechanisms similar to or involved in normal murine labour, also dependent upon luteolysis in the cascade of events.

With regard to infection-induced PTL, controversy over the involvement of a progesterone withdrawal has been highlighted in studies by Fidel Jr. et al. and by Hirsch and Muhle. Fidel and colleagues administered LPS by intraperitoneal injection for a systemic infection on day 15 of murine parturition. They observed by radioimmunoassay a dramatic drop in serum progesterone levels. They also induced PTL in rabbits by cervical deposition of *E.coli* resulting in an ascending bacterial infection of the uterus and came to the same conclusion: that a progesterone drop precedes infection-induced parturition [208]. This drop is likely accomplished by LPS induction of IL-1 and IL-6 all three of which are known to induce COX-2 expression during infection-induced PTL in mice. The transcriptional up-regulation and synthesis of this enzyme leads to increased prostaglandin (PG) production. $PGF_{2\alpha}$ in turn, induces luteolysis and a loss of the ovarian source of serum progesterone. Hirsch and Muhle also observed this drop in serum progesterone with intrauterine injection of heat killed bacterial (10^9/mouse) [209]. They observed a modest decline in progesterone levels (only a 28% drop), about half the loss recorded by Fidel Jr. et al. [208]. Supplementation with physiological levels of progesterone did not prolong gestation while pharmacological levels were successful. The question of progesterone relevance to infection-induced PTL requires further analysis. The use of our ovariectomy model may provide some answers while providing insight into otherwise idiopathic PTL.

Gene Expression Patterns in Ovariectomy-Induced Preterm Labour

Analysis of gene expression in our ovariectomy (Ovx) model by the murine Affymetrix oligonucleotide array U74Av2 indicated 79 probe sets were detecting a dynamic regulation of gene expression. These changes relative to sham operated control levels were observed either early after Ovx (5 hours), after approximately 20 hours when Ovx-induced PTL occurred, or were regulated early and remained so by the time of Ovx-induced PTL. While the number of genes detected and the magnitude of the expression changes were more moderate than that found for infection-induced PTL, this does not diminish the importance of these genes. The impact their protein

products have on the outcome of PTL may well exceed the influence of ovariectomy on their gene expression levels.

Genes Induced at Ovariectomy-Induced Preterm Labour

Gene probe sets assigned to Clusters A-D and for tumour necrosis factor induced protein 6 (Genbank# U83903) demonstrated an increased expression at the moment of Ovx-induced PTL. Most of these are induced during normal murine labour, infection-induced PTL or both conditions including the genes for cFos, COX-2, and connexin 43. Unique to Ovx-induced PTL is the gene for tumour necrosis factor induced protein 6 (Tnfip6), up-regulated 2.5-fold at PTL. This is surprising as its expression is induced by TNF-alpha, LPS, and IL-1 and its product possesses a strong anti-inflammatory activity [210]. It is well characterized in association with several inflammatory diseases [211]; however it did not reach statistically significant levels in our infection-induced PTL. It has been identified in the processes of ovulation where its expression in cumulus oocyte complexes is dependent on prostaglandin E_2 and COX-2 activity [178]. The Tnfip6 gene product is found in the cumulus extracellular matrix where it participates in the degradation of the matrix facilitating oocyte growth. In a similarly regulated manner, it participates in cervical ripening [178, 211]. This is the first description of uterine expression of Tnfip6. Perhaps the parturient uterus normally expresses this gene to suppress an immune response against the fetus and promote uterine growth and therefore levels are relatively unchanged with infection. With respect to Ovx-induced PTL, the induction of Tnfip6 may provide support for the growth of the fetus in the absence of progesterone in an attempt to avoid activating stretch-induced CAP genes and subsequent parturition.

During Ovx-induced PTL, Purkinje cell protein 4 (PCP4) was up-regulated. Pep-19, the rat homologue of murine PCP4 [157] is described as a neuron-specific transcript [158, 159]. Our study confirms the evidence of extraneural PCP4 gene expression provided by our study of normal labour (Chapter 2). PCP4/Pep-19 has been proposed to function in inhibiting calmodulin activity [160]. Calmodulin, in

complex with Ca^{2+}, activates myosin light chain kinase. PCP4 inhibits the complex from forming, resulting in the repression of myosin light chain kinase activity, inhibition of myosin light chain phosphorylation and preventing myometrial responsiveness and contractility. As myosin phosphorylation is essential to uterine force production and that alternate contractile pathways do not function in the myometrium [20], a role for PCP4 in modulating myometrial contraction during labour is proposed. The down-regulation observed during normal labour indicated an alleviation of this block to contraction. Pep19 has also been characterized to have an anti-apoptotic effect on neuronal cells [161]. This function is executed by inhibiting Ca^{2+}-mediated cell death. In the uterus, this block facilitates uterine growth during pregnancy. The induction of PCP4 gene expression during PTL may participate in the further growth of the uterus in an attempt to avoid uterine stretch and subsequent activation of connexin 43 and OTR gene expression. An induction of PCP4 gene expression at Ovx-induced PTL may be an attempt by the uterus to maintain quiescence in the presence of competing factors. The regulation of this gene in non-neural tissue clearly requires further examination. PCP4 may be an important factor to be exploited for therapeutic intervention during idiopathic preterm parturition and although not differentially regulated during infection-induced PTL, may provide a useful means to prevent uterine contractions under these and other delivery-inducing stimuli.

Genes Repressed Prior to Ovariectomy-Induced Preterm Labour

The probe sets for genes that were down-regulated by 5 hours post-ovariectomy fell mostly into Cluster G; ScyB2, follistatin, RIKEN cDNA for Mig-6, regulator of G-protein signalling 2 (RGS-2), and EST AW047643 round out this group. This list also includes the probe set for small inducible cytokine (Scy) A2 of the transition Cluster E and EST AI467657 of Cluster H. In the case of follistatin, RGS-2, and EST AI467657, the suppression of these genes extended to the time of Ovx-induced PTL. Follistatin is a negative regulator of activin function by competitive binding to the activin type II receptor [212]. Activin is implicated in the

decidualization of the endometrium, inhibiting angiogenesis, and participating in acute inflammatory responses. Follistatin mRNA, present in the decidua, reaches peak levels midway through gestation and is significantly reduced in the final trimester [213] while activin mRNA levels continue to rise [212]. In the ovary, follistatin blocks activin stimulation of progesterone synthesis and promotes estrogen synthesis by granulosa cells leading to follicle atresia [214]. Decidual follistatin may play a paracrine role in regulating progesterone synthesis. The drop in mRNA levels during late pregnancy would be permissive to increased progesterone production and maintenance of uterine quiescence. The decrease in follistatin gene expression early after Ovx reflects the loss of the target tissue and requirement for progesterone synthesis regulation and allows activin to contribute to an acute inflammatory response.

ScyA2 and ScyB2 were both down-regulated after 5 hours but were no longer statistically significant in their repression by 20 hours, when Ovx-induced PTL occurred. ScyA2/MCP-1 is a chemoattractant specific for monocytes while Scyb2/macrophage inflammatory protein 2 is a chemoattractant expressly for neutrophils. Both of these are dramatically induced during infection-induced PTL. The early repression of gene expression and subsequent alleviation of this repression during Ovx-induced PTL suggests an attempt to prevent the infiltration of immune cells and prevent the tissue destruction that accompanies an immune response and parturition. This appears to be an unsuccessful endeavour.

Genes Repressed Specifically at Ovariectomy-Induced Preterm Labour

Probe sets assigned to Clusters F and H detected the specific down-regulation of gene expression at the moment of PTL. Consistent with normal labour, connexin 26 gene repression is reported here. Down-regulation of this gap junction protein, important for maintaining uterine quiescence, is not unexpected as it is positively regulated by progesterone. Its expression pattern is reciprocal to the induction of connexin 43, a CAP important for communication between adjacent myometrial cells

to coordinate the transmission of contraction signals. This switch in connexin gene expression in the uterus at normal parturition is well documented [33].

Profilin 2 is repressed in the condition of Ovx-induced PTL and has not previously been described in uterine tissue. It is characterized as an actin-regulatory protein promoting the organization of actin cytoskeleton and significant to the development of smooth muscle force during contraction [215]. Its down-regulation at PTL suggests a further attempt by the uterine environment to maintain the pregnancy by compromising the ability of the myometrium to contract. In the presence of an up-regulation of COX-2 and connexin 43 genes at this time, the down-regulation of profilin 2 proves futile. In normal labour profilin 2 gene expression is induced postpartum where it may participate in uterine involution. Along with this mechanism is the finding that actin alpha cardiac and actin alpha 1 skeletal muscle genes are also suppressed at Ovx-induced PTL. Both of these actin genes were also suppressed in postpartum uteri in our normal labour model possibly contributing to tissue degeneration and remodelling. The use of profilin 2 antisense oligonucleotides or antibody therapy may be successful in abrogating uterine smooth muscle contractions during labour.

It appears that numerous countermeasures are underway but unsuccessful during Ovx-induced PTL. If we can augment their effectiveness, we may provide a means to blocking PTL.

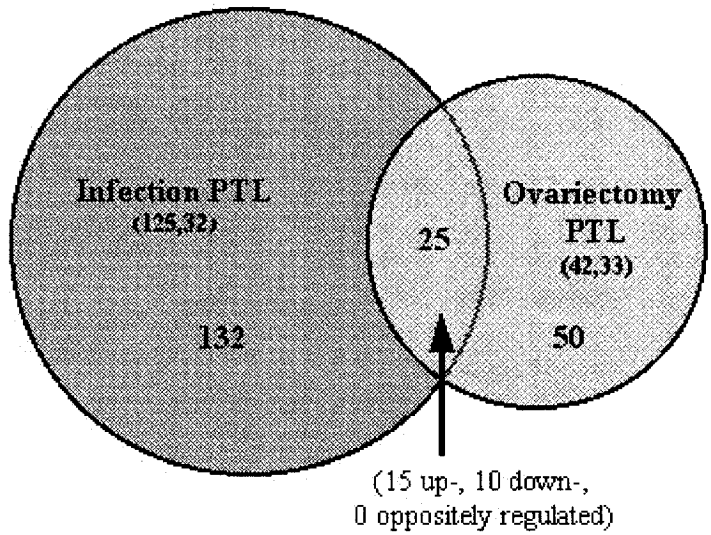


Figure 3.5 Venn diagram comparison of the numbers of probe sets for genes significantly expressed at PTL induced by infection, ovariectomy, and both conditions. Probe sets demonstrating significant changes in gene regulation (up-regulated, down-regulated) at PTL (PTL vs. C8; Ovx20-PTL vs. Sham20). Total numbers of unique and common probe sets are indicated. Of the 25 common to both time-point comparisons, all were similarly regulated.

In addition to those already indicated above, a variety of genes are common to the conditions of infection-and ovariectomy-induced PTL (Figure 3.5). At the moment PTL occurred there were 157 probe sets representing genes significantly changing with infection (125 up-regulated and 32 down-regulated) and 75 probe sets reflecting genes differentially regulated due to ovariectomy (42 induced, 33 repressed). Of these, 25 probe sets were found in both comparisons of PTL vs. their time-matched control. In each case, the genes were regulated in the same manner; 15 were up-regulated and 10 were down- regulated. In most cases, the regulation was to the same degree; COX-2 and PAI-1 genes were induced to a higher level of expression in response to infection. These common genes also include those for small proline rich proteins, each induced during these processes, transforming growth factor β2 (TGFβ2), macrophage colony stimulating factor (M-Csf /Csf1), and aquaporin 8, each

down-regulated during PTL, and RGS-2 and RGS-16, which show the same regulation (RGS-16 induced and RGS-2 repressed) during PTL.

Small proline rich protein 2 (Sprr2) family of genes are expressed in mouse epithelial tissues and are implicated in the differentiation of this tissue [216]. The high expression of Sprr2 genes in the uterus was described by Song et al. and we find a similar induction specific to PTL when sudden endometrial remodelling is triggered. In our models, we found Sprr2B, C, D, E, F, G and I to be up-regulated with high dose infection leading to PTL and Sprr2B, C, E, G, and I genes induced with Ovx-induced PTL. Sprr2A and Sprr2C were expressed less during normal labour than postpartum. The significance of the induction of this family of genes is unclear but may reflect the state of differentiation, perhaps a de-decidualization of the endometrial epithelium with the onset of labour and a return of the uterus to a non-pregnant, receptive state.

TGFβ2 is a T_h2 cytokine, having a suppressive effect on pro-inflammatory responses [113, 114]. Its repression in these conditions results in a decreased tolerance of fetal antigens and the mounting of an immune response, culminating in detachment of the placenta and activation of the uterus for expulsion of the fetus.

Csf1/M-Csf is synthesized by uterine epithelial and stromal cells promoting the proliferation of resident macrophages, which regulate the structure and function of the tissue [217]. M-Csf /Csf1 is a chemokine, attracting macrophages along the increasing concentration gradient to the site of expression promoting an inflammatory response. In an in vitro study on Csf1 stimulation of endometrial cells, this cytokine promoted the growth of these cells, suggestive of a positive feedback leading to increased tissue growth [218]. The effect is accomplished by binding to its cell surface receptor, which is also expressed in the endometrium. A down-regulation of the Csf1 gene was found in both PTL models. In infection-induced PTL other chemokines may compensate for its loss while the growth inhibition would facilitate a uterine stretch response as it may with Ovx-induced PTL. As this gene is positively regulated by both estrogen and by progesterone [219], this further supports a hormone component to infection-induced PTL. The drop in expression of Csf1 during Ovx-induced PTL is not surprising. During normal murine parturition, uterine Csf1 mRNA

was detected by day 3 and reached peak levels around days 14-15 [219], precisely when surgery for our PTL models took place. While a modulation of M-Csf /Csf1 gene expression during normal labour was not statistically significant at the time-points we measured, an increase in receptor transcript was found to be induced at term and during labour, functionally increasing the effectiveness of Csf1 levels present in these tissues. In normal labour, it likely recruits macrophages for the digestion of foreign fetal antigens while immune tolerance is diminishing, participating in the mounting of an immune response to the fetus and contributing to the culmination of labour.

The regulator of G protein signalling (RGS) family attenuate signalling through G protein coupled receptors (GPCRs). Ligand binding to its GPCR, such as receptors for cytokines and chemokines, leads to the exchange of GDP for GTP and dissociation of the G_α subunit of trimeric $G_{\alpha\beta\gamma}$ proteins. The $_\alpha$ subunit as well as the $_{\beta\gamma}$ dimer are then free to activate downstream effectors. RGS proteins promote the intrinsic GTPase activity of the G_α subunit resulting in the exchange of GTP for GDP by the activated G_α subunit and its re-association with the $_{\beta\gamma}$ dimer. This interaction arrests signalling. RGS-16 interferes with signalling reliant upon $G_{\alpha q}$ and $G_{\alpha i}$ proteins while RGS-2 GTPase activity is directed specifically at $G_{\alpha q}$, enabling the regulation of different signalling pathways. Both of these regulators of G-protein signalling are expressed in mononuclear myocytes and leukocytes.

T cell activation by pro-inflammatory cytokines leads to an increase in RGS-16 transcription and a decrease in RGS-2 mRNA levels [220]. The increase in RGS-16 would suppress excessive cytokine/chemokine signalling. RGS-2, in addition to restraining GPCR signalling promotes T cell proliferation and IL-2 production through the inhibition of cAMP production [221]. The effects of down-regulating RGS-2 gene (reducing T cell proliferation) are amplified by the up-regulation of RGS-16 (limiting T cell signalling) resulting in the muting of an immune response. We observed the same response as RGS-16 and RGS-2 were equally but oppositely regulated to the same extent in both PTL models.

LPS stimulation of cardiac myocytes also leads to an induction of RGS-16 [222]. RGS-4 transcription was induced by this stimulus as well and we recorded its up-regulation during infection-induced PTL. RGS-16 (and RGS-4) inhibits endothelin-1 stimulation of PLC and subsequent cardiac muscle contraction [222]. The up-regulation of RGS 16 (and RGS-4 with infection) during PTL could suppress the function of the uterotonin along with others thereby exerting a negative effect on the force of muscular contractions. Oxytocin stimulation of human myometrial cells induced the expression of RGS-2 [223]. The effect may be to attenuate OTR signalling and subsequently inhibit uterine contractions. Conversely, RGS-2, in addition to squelching GCPR signalling, also inhibits cAMP production by constraining certain isoforms of adenylate cyclase [221] and may contribute to a decrease in myometrial relaxation thereby facilitating contractions. Its role in uterine contractions requires clarification.

The expression and role of these RGS proteins is dependent upon tissue and stimulus. In T cells, they oppositely affect cell function and proliferation and their regulation in these models results in a cumulative suppression of T cell activation. If the inflammatory response is quelled by up-regulated RGS-16 (and RGS-4) and down-regulated RGS-2 levels, the uterus may be relieved of the negative inotropic effects of pro-inflammatory cytokines and therefore achieve contractions with enough force to expel the fetus. It appears that an acute infection initially dampens the force of uterine contractions however, in an attempt to reduce the magnitude of immune response, the uterine environment may actually promote its own contractions. The fact that this system is involved in Ovx-induced PTL suggests an immune response (against fetal antigens) is mounted. To correctly characterize the effects of these regulators on parturition it is important to dissect the cell types in the uterus expressing them.

3.6 CONCLUSION

Microarray technology has allowed us to follow the expression patterns of thousands of genes simultaneously while we monitored the progression to PTL. We investigated the changes in gene expression in response to an infection that lead to labour, an infection that did not, and an ovariectomy model that demonstrated changes leading to PTL in the absence of infection, reflecting the change in hormone status. We used these models to identify novel genes and characterize gene networks whose regulation participates in the process to PTL. Infection leads to a significant shift in T_h1/T_h2 cytokine balance, promoting a reduced tolerance to fetal antigens and inciting a rejection response. This T_h1-dominant environment also promoted COX-2 transcription necessary to the production of prostaglandins capable of triggering luteolysis and uterine contractions. Under the stimulus of bacterial infection, an immune response is primary but a secondary hormonal contribution collaborates in a struggle to prevent and to promote parturition. Ovx produced a primary hormonal response that mimics luteolysis required for normal parturition. In an attempt to resist the progression to PTL, Ovx caused the down-regulation of immune factors among others. We found several similarities of gene expression between the two studies suggesting a final common mechanism. If therapeutics can be developed to address these commonalities, we may provide a broad tool for preventing PTL regardless of cause. As well, we have highlighted individual components for specific intervention, either to be antagonized or to be exploited. We believe this body of evidence provides a springboard for future research and development of diagnostic or therapeutic interventions for premature or otherwise complicated labour

3.7 ACKNOWLEDGEMENTS

We thank Dr. Robert Sladek and Dr. Yoshihiko Nagai from the Montreal Genome Centre for their assistance in Affymetrix GeneChip processing and analysis support. We most gratefully acknowledge the financial support of this project by Dr. Roberto Romero and the National Institute of Health as well as by grants from the Canadian Institutes of Health Research.

Functional Classification

Cluster	GenBank	Gene Symbol	Title
DNA/Nucleotide Metabolism			
B	L32973	Tyki	thymidylate kinase family LPS-inducible member
B	AI853875	Zrfp1-pending	zinc ring finger protein 1
C	AA959954	9130009C22Rik	RIKEN cDNA: helicard
E	M27695	Uox	urate oxidase
E	L09737	Gch	GTP cyclohydrolase 1
Growth/Differentiation/Apoptosis			
A	AI854285	Nd1-pending	Nd1
A	U22399	Cdkn1c	cyclin-dependent kinase inhibitor 1C (P57)
A	X57413	Tgfb2	transforming growth factor beta 2
B	L24118	Tnfaip2	TNF D24 alpha-induced protein 2
B	AF099973	Slfn2	schlafen 2
B	U88908	Birc2	baculoviral IAP repeat-containing 2
B	AF099973	Slfn2	schlafen 2
B	AI849533	Clic4	chloride intracellular channel 4 (mitochondrial)
B	AI845237	Clic4	chloride intracellular channel 4 (mitochondrial)
C	AF099977	Slfn4	schlafen 4
C	AF076482	Pglyrp	peptidoglycan recognition protein
D	U44088	Tdag51	T-cell death associated gene 51
D	AV138783	Gadd45b	growth arrest and DNA-damage-inducible 45 beta
E	AV092014	Pglyrp	peptidoglycan recognition protein
E	AF099974	Slfn3	schlafen 3
E	AI852641	Nupr1	nuclear protein 1
E	U55060	Lgals9	lectin galactose binding soluble 9 (Galectin-9)
E	AV049898	Lgals9	lectin galactose binding soluble 9 (Galectin-9)
E	X87128	Tnfrsf1b	TNF receptor superfamily member 1b
F	J02872	Gzmg	granzyme G
F	M36901	Gzme	granzyme E
F	M22527	Gzmc	granzyme C
F	X56990	Gzmd	granzyme D
F	M95200	Vegfa	vascular endothelial growth factor A
F	M32490	Cyr61	cysteine rich protein 61 aka: IGFBP10
F	AF041054	Bnip3	BCL2/adenovirus E1B 19 kDa-interacting protein 1NIP3
F	M70642	Ctgf	connective tissue growth factor
Transcription regulation			
B	U57524	Nfkbia	nuclear factor of kappa light chain gene enhancer in B-cells inhibitor alpha

Cluster	GenBank	Gene Symbol	Title
B	AI642048	Nfkbia	nuclear factor of kappa light chain gene enhancer in B-cells inhibitor alpha
B	M12731	Nmyc1	neuroblastoma myc-related oncogene 1
B	AW122207	Dr1	down-regulator of transcription 1
B	Y15907	Hivep2	human immunodeficiency virus type I enhancer binding protein 2
B	M57999	Nfkb1	nuclear factor of kappa light chain gene enhancer in B-cells 1p105
B	AW047899	Nfkb2	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2p49/p100
B	X61800	Cebpd	CCAAT/enhancer binding protein (C/EBP) delta
C	U73037	Irf7	interferon regulatory factor 7
D	V00727	cFos	FBJ osteosarcoma oncogene
D	M61007	Cebpb	CCAAT/enhancer binding protein (C/EBP) beta
D	AI846152	Dscr1	Down syndrome critical region homolog 1 (human)
D	Y07836	Bhlhb2	basic helix-loop-helix domain containing class B2
D	M21065	Irf1	interferon regulatory factor 1
D	U20344	Klf4	Kruppel-like factor 4 (gut)
D	U19118	Atf3	activating transcription factor 3
D	M28845	Egr1	early growth response 1
D	X67863	Srst	simple repeat sequence-containing transcript
E	J03776	Trim30	tripartite motif protein 30
RNA/Protein Biosynthesis			
Protein Metabolism/Modification			
A	AI853714	Ctsb	cathepsin B
A	AI005782	Tmprss2	transmembrane protease serine 2
A	AI845735	Polr2e	polymerase (RNA) II (DNA directed) polypeptide E
A	AF084482	Wfs1	Wolfram syndrome 1 homolog (human)
A	X58196	H19	H19 fetal liver mRNA
C	U22033	Psmb8	proteosome subunit beta type 8 (large multifunctional protease 7)
E	D44456	Psmb9	proteosome subunit beta type 9 (large multifunctional protease 2)
E	M64085	Spi2-1	serine protease inhibitor 2-1
F	U96700	Spi6	serine protease inhibitor 6
Signalling			
A	AF009246	Rasd1	RAS dexamethasone-induced 1
A	AI840130	Srcasm	Src activating and signaling molecule
A	U67187	Rgs2	regulator of G-protein signaling 2
A	U02602	Tshr	thyroid stimulating hormone receptor
B	AJ007972	Gtpi-pending	interferon-g induced GTPase
C	AJ007970	Gbp2	guanylate nucleotide binding protein 2
C	AW047476	Gbp3	guanylate nucleotide binding protein 3
D	AB020886	Akap12	A kinase (PRKA) anchor protein (gravin) 12
D	AF071180	Fpr-rs2	formyl peptide receptor related sequence 2

Cluster	GenBank	Gene Symbol	Title
D	U53219	Igtp	interferon gamma induced GTPase
D	AI642662	3830417M17Rik	RIKEN cDNA: MAP kinase phosphatase-7
D	X61940	Ptpn16	protein tyrosine phosphatase non-receptor type 16
D	AA914345	Iigp-pending	interferon-inducible GTPase
E	L38444	Tgtp	T-cell specific GTPase
E	AJ007971	Iigp-pending	interferon-inducible GTPase
E	AI746846	2410004M09Rik	RIKEN cDNA: sorting nexin 10
F	U94828	Rgs16	regulator of G-protein signaling 16
F	AB004315	Rgs4	regulator of G-protein signaling 4
Calcium Binding/Transport			
D	AI844128	Ehd1	EH-domain containing 1
E	M83218	S100a8	S100 calcium binding protein A8 (calgranulin A)
E	M83219	S100a9	S100 calcium binding protein A9 (calgranulin B)
Intercellular adhesion/communication			
Extracellular Matrix		**Blood Clotting**	
A	AA986050	AI256424	EST: fibrinogen gamma polypeptide
A	AI196896	Fgb	fibrinogen B beta polypeptide
A	D50086	Nrp	neuropilin
A	AF026073	Sultn	N-sulfotransferase
A	AI876446	Fga	fibrinogen alpha polypeptide
B	AF019385	Hs3st1	heparan sulfate (glucosamine) 3-O-sulfotransferase 1
C	L36244	Mmp7	matrix metalloproteinase 7
D	X66473	Mmp13	matrix metalloproteinase 13
E	AF087825	Cldn7	claudin 7
F	M33960	Serpine1	serine proteinase inhibitor clade E1 (nexin plasminogen activator inhibitor type 1)
F	X66402	Mmp3	matrix metalloproteinase 3
F	M62470	Thbs1	thrombospondin 1
F	M63801	Gja1	gap junction membrane channel protein alpha 1 (connexin 43)
F	AV230686	Tnc	tenascin C
F	X56304	Tnc	tenascin C
Cytoskeleton		**Muscle Development**	
A	D88792	Csrp2	cysteine-rich protein 2
C	K02108	Krt2-6a	keratin complex 2 basic gene 6a
Lipid Binding/Transport/Metabolism			
A	U79573	Apoa1	apolipoprotein A-I
A	X13060	Alb1	albumin 1

Cluster	GenBank	Gene Symbol	Title
B	D78354	Plscr1	phospholipid scramblase 1
C	X81627	Lcn2	lipocalin 2
C	AV210775	Lcn2	lipocalin 2
E	M69260	Anxa1	annexin A1
Hormone Biosynthesis/Metabolism			
A	V00743	Afp	alpha fetoprotein
D	M88242	Ptgs2	prostaglandin-endoperoxide synthase 2 (COX-2)
D	AF059213	Ch25h	cholesterol 25-hydroxylase
Carbohydrate Biosynthesis/Metabolism			
Electron Transport -Energy			
A	AW125884	Acas2	acetyl-Coenzyme A synthetase 2
A	AI843448	Mgst3	microsomal glutathione S-transferase 3
A	AF009605	Pck1	phosphoenolpyruvate carboxykinase 1 cytosolic
C	M25944	Car2	carbonic anhydrase 2
E	X93035	Chi3l1	chitinase 3-like 1
F	M22998	Slc2a1	solute carrier family 2 (facilitated glucose transporter) member 1
Cell Defense/Immune Function			
A	AV373027	Scya6	small inducible cytokine A6
A	X66295	C1qc	complement component 1q subcomponent c polypeptide
A	X58861	C1qa	complement component 1q subcomponent alpha polypeptide
A	M21952	Csf1	colony stimulating factor 1 (macrophage)
A	M58004	Scya6	small inducible cytokine A6
B	L38281	Irg1	immunoresponsive gene 1
B	AI323667	Irg1	immunoresponsive gene 1
B	X53798	Scyb2	small inducible cytokine subfamily member 2
B	M33266	Scyb10	small inducible cytokine B subfamily (Cys-X-Cys) member 10
B	AA204579	Vig1-pending	EST: Viral hemorrhagic septicemia virus (VHSV) induced gene 1
B	U27267	Scyb5	small inducible cytokine B subfamily member 5
B	U43084	Ifit1	interferon-induced protein with tetratricopeptide repeats 1
B	J04596	Gro1	GRO1 oncogene
B	J04596	Gro1	GRO1 oncogene
B	M21038	Mx1	myxovirus (influenza virus) resistance 1
B	M84487	Vcam1	vascular cell adhesion molecule 1
B	M90551	Icam1	intercellular adhesion molecule
B	U23781	Bcl2a1d	B-cell leukemia/lymphoma 2 related protein A1d
B	M31419	Ifi204	interferon activated gene 204
B	M74123	Ifi205	interferon activated gene 205
B	X83601	Ptx3	pentaxin related gene

Cluster	GenBank	Gene Symbol	Title
B	AV229143	Ifi202a	EST: interferon activated gene 202A
B	AV374868	Cish3	cytokine inducible SH2-containing protein 3
B	AA960657	Ifi203	EST: interferon activated gene 203
C	X56602	Isg15	interferon-stimulated protein (15 kDa)
C	M31419	Ifi204	interferon activated gene 204
C	AW047653	Usp18	ubiquitin specific protease 18
C	X13333	Cd14	CD14 antigen
C	X03505	Saa3	serum amyloid A3
C	U12884	Vcam1	vascular cell adhesion molecule 1
D	X54542	Il6	interleukin 6
D	M19681	Scya2	small inducible cytokine A2
D	M34815	Scyb9	small inducible cytokine B subfamily (Cys-X-Cys) member 9
D	X70058	Scya7	small inducible cytokine A7
D	M13926	Csf3	colony stimulating factor 3 (granulocyte)
D	L32838	Il1rn	interleukin 1 receptor antagonist
D	M14639	Il1a	interleukin 1 alpha
D	AA614971	Mail-pending	molecule possessing ankyrin-repeats induced by lipopolysaccharide
D	M15131	Il1b	interleukin 1 beta
D	J04491	Scya3	small inducible cytokine A3
E	AF065947	Scya5	small inducible cytokine A5
E	AV152244	ISG15	RIKEN cDNA: interferon-stimulated protein (15 kDa)
E	U43086	Ifit3	interferon-induced protein with tetratricopeptide repeats 3
E	U43085	Ifit2	interferon-induced protein with tetratricopeptide repeats 2
E	L35528	Sod2	superoxide dismutase 2 mitochondrial
E	U12884	Vcam1	vascular cell adhesion molecule 1
E	AF002719	Slpi	secretory leukocyte protease inhibitor
E	M31418	Ifi202a	interferon activated gene 202A
E	AF022371	Ifi203	interferon activated gene 203
E	U19119	Ifi1	interferon inducible protein 1
E	M35247	H2-T17	histocompatibility 2T region locus 17
E	M35244	H2-T10	histocompatibility 2T region locus 10
E	Y00629	H2-T23	histocompatibility 2T region locus 23
E	U15635	Samhd1	SAM domain and HD domain1
F	AF030636	Scyb13	small inducible cytokine subfamily B (Cys-X-Cys) member 13
F	AV223216	Il1r2	interleukin 1 receptor type II
Transport			
A	X14061	Hbb-bh1	hemoglobin Z beta-like embryonic chain
A	V00726	Hbb-y	hemoglobin Y beta-like embryonic chain
A	AF018952	Aqp8	aquaporin 8
A	M60348	Abcb1b	ATP-binding cassette sub-family B (MDR/TAP) member 1B

Cluster	GenBank	Gene Symbol	Title
A	M13125	Hba-x	hemoglobin X alpha-like embryonic chain in Hba complex
C	AA591002	Slc5a1	solute carrier family 5 member 1
C	U49430	Cp	ceruloplasmin aka:Ferroxidase
D	AW124544	Ermelin-pending	endoplasmic reticulum membrane protein
E	J03298	Ltf	lactotransferrin
E	U60020	Abcb2	ATP-binding cassettesub-family B (MDR/TAP) member 2
OTHER: Circadian Rhythm Regulation			
D	AW047630	Ccr4	carbon catabolite repression 4 homolog (yeast)
UNKNOWN			
A	AW047237		EST
A	AI852838	Meg3	maternally expressed gene 3
A	AF110520	Rps28	ribosomal protein S28
A	AI843106	Pa26-pending	p53 regulated PA26 nuclear protein
A	AW124483	3110038L01Rik	RIKEN cDNA
A	AW047919	C78582	EST
A	AW061234	AW061234	EST
A	AI844396	3110038L01Rik	RIKEN cDNA
C	AJ005560	Sprr2b	small proline-rich protein 2B
C	AJ005561	Sprr2c	small proline-rich protein 2C
C	AJ005567	Sprr2i	small proline-rich protein 2I
C	AJ005563	Sprr2e	small proline-rich protein 2E
C	AJ005565	Sprr2g	small proline-rich protein 2G
C	AJ005564	Sprr2f	small proline-rich protein 2F
D	AW060549	1300007C21Rik	RIKEN cDNA: GAG homolog (mouse)
D	AW047207	1810037I17Rik	RIKEN cDNA 1810037I17 gene
E	AJ005562	Sprr2d	small proline-rich protein 2D
E	AI121305	1600029D21Rik	RIKEN cDNA
E	AV206059	Mtlrp-pending	motilin-related peptide
E	AA816121		EST
E	AA958903	AW112010	EST
F	AW123751	2310056P07Rik	RIKEN cDNA: growth and transformation-dependent (fragment) homolog (rat)
F	M32486	M32486	Mouse 19.5 mRNA

GenBank	Alternate Classifications		
L32973	Cell Defense/Immune Response		
AI853875	Transcription regulation		
AA959954			
M27695			
L09737	Signalling		
AI854285	Signalling		
U22399			
X57413	Cell Defense/Immune Response		
L24118	Cell Defense/Immune Response		
AF099973	Cell Defense/Immune Response		
U88908			
AF099973	Cell Defense/Immune Response		
AI849533	Intercellular adhesion/communication		
AI845237	Intercellular adhesion/communication		
AF099977	Cell Defense/Immune Response		
AF076482	Cell Defense/Immune Response	Signalling	
U44088			
AV138783	RNA/Protein Biosynthesis		
AV092014	Cell Defense/Immune Response		
AF099974	Cell Defense/Immune Response		
AI852641			
U55060	Cell Defense/Immune Response		
AV049898	Cell Defense/Immune Response		
X87128	Cell Defense/Immune Response		
J02872	Cell Defense/Immune Response	Protein Metabolism/Modification	
M36901	Cell Defense/Immune Response	Protein Metabolism/Modification	
M22527	Cell Defense/Immune Response	Protein Metabolism/Modification	
X56990	Cell Defense/Immune Response	Protein Metabolism/Modification	
M95200	Extracellular matrix		
M32490			
AF041054	Calcium Binding/Transport		
M70642	Extracellular matrix	DNA/Nucleotide Metabolism	
U57524	Growth/Differentiation/Apoptosis	Intercellular adhesion/communication	Cell Defense/Immune Response

GenBank	Alternate Classifications		
AI642048	Growth/Differentiation/Apoptosis	Intercellular adhesion/communication	Cell Defense/Immune Response
M12731	Growth/Differentiation/Apoptosis		
AW122207			
Y15907	Signalling		
M57999	Growth/Differentiation/Apoptosis	Signalling	Cell Defense/Immune Response
AW047899	Cell Defense/Immune Response		
X61800	Cell Defense/Immune Response		
U73037	Cell Defense/Immune Response		
V00727	Growth/Differentiation/Apoptosis		
M61007	Cell Defense/Immune Response	Signalling	
AI846152			
Y07836	Growth/Differentiation/Apoptosis		
M21065	Cell Defense/Immune Response		
U20344			
U19118			
M28845	Growth/Differentiation/Apoptosis		
X67863			
J03776	Cell Defense/Immune Response		
AI853714			
AI005782			
AI845735			
AF084482			
X58196			
U22033			
D44456			
M64085			
U96700			
AF009246			
AI840130	Protein Metabolism/Modification		
U67187	Growth/Differentiation/Apoptosis		
U02602			
AJ007972	Cell Defense/Immune Response		
AJ007970	Cell Defense/Immune Response		
AW047476			
AB020886			
AF071180	Cell Defense/Immune Response		

GenBank	Alternate Classifications	
U53219	Cell Defense/Immune Response	
AI642662	Growth/Differentiation/Apoptosis	Protein Metabolism/Modification
X61940	Growth/Differentiation/Apoptosis	Protein Metabolism/Modification
AA914345	Cell Defense/Immune Response	
L38444	Cell Defense/Immune Response	
AJ007971	Cell Defense/Immune Response	
AI746846	Protein Metabolism/Modification	
U94828	Growth/Differentiation/Apoptosis	
AB004315	Growth/Differentiation/Apoptosis	
AI844128	Transport	
M83218	Cell Defense/Immune Response	
M83219	Cell Defense/Immune Response	
AA986050		
AI196896		
D50086	Growth/Differentiation/Apoptosis	
AF026073	RNA/Protein Biosynthesis	
AI876446		
AF019385	Protein Metabolism/Modification	
L36244	Protein Metabolism/Modification	
X66473	Protein Metabolism/Modification	
AF087825		
M33960	Protein Metabolism/Modification	
X66402	Protein Metabolism/Modification	
M62470	Calcium Binding/Transport	
M63801	Cell Defense/Immune Response	
AV230686		
X56304		
D88792	Growth/Differentiation/Apoptosis	
K02108	Growth/Differentiation/Apoptosis	Blood Clotting
U79573	Hormone Biosynthesis/Metabolism	
X13060	Hormone Biosynthesis/Metabolism	

GenBank	Alternate Classifications		
D78354	Growth/Differentiation/Apoptosis	Blood Clotting	Cell Defense/Immune Response
X81627	Hormone Biosynthesis/Metabolism		
AV210775	Hormone Biosynthesis/Metabolism		
M69260	Hormone Biosynthesis/Metabolism	Calcium Binding/Transport	
V00743	Transport		
M88242	Cell Defense/Immune Response		
AF059213	Lipid Binding/Transport/Metabolism		
AW125884			
AI843448			
AF009605	Signalling		
M25944			
X93035	Extracellular matrix		
M22998			
AV373027	Signalling		
X66295			
X58861			
M21952	Lipid Binding/Transport/Metabolism		
M58004	Signalling		
L38281			
AI323667			
X53798	Signalling		
M33266			
AA204579			
U27267	Signalling		
U43084			
J04596	Growth/Differentiation/Apoptosis		
J04596	Growth/Differentiation/Apoptosis		
M21038	Signalling		
M84487	Intercellular adhesion/communication	Signalling	
M90551	Intercellular adhesion/communication	Signalling	
U23781	Growth/Differentiation/Apoptosis		
M31419	Transcription regulation	RNA/Protein Biosynthesis	
M74123			
X83601			

GenBank	Alternate Classifications	
AV229143	Transcription regulation	
AV374868	Signalling	Growth/Differentiation/Apoptosis
AA960657		
X56602	Protein Metabolism/Modification	
M31419	RNA/Protein Biosynthesis	Transcription regulation
AW047653	Growth/Differentiation/Apoptosis	Protein Metabolism/Modification
X13333	Growth/Differentiation/Apoptosis	Intercellular adhesion/communication
X03505	Lipid Binding/Transport/Metabolism	
U12884	Signalling	Intercellular adhesion/communication
X54542	Growth/Differentiation/Apoptosis	
M19681	Signalling	Growth/Differentiation/Apoptosis
M34815	Growth/Differentiation/Apoptosis	
X70058	Extracellular matrix	Growth/Differentiation/Apoptosis
M13926	Growth/Differentiation/Apoptosis	
L32838	Signalling	Growth/Differentiation/Apoptosis
M14639	Growth/Differentiation/Apoptosis	
AA614971		
M15131	Growth/Differentiation/Apoptosis	
J04491	Growth/Differentiation/Apoptosis	Extracellular matrix
AF065947	Signalling	
AV152244	Protein Metabolism/Modification	
U43086		
U43085		
L35528		
U12884	Signalling	Intercellular adhesion/communication
AF002719	Protein Metabolism/Modification	Extracellular matrix
M31418	Transcription regulation	
AF022371		
U19119	Signalling	
M35247		
M35244		
Y00629		
U15635	Signalling	
AF030636		
AV223216	Signalling	
X14061		
V00726		
AF018952		
M60348		

GenBank	Alternate Classifications
M13125	
AA591002	Carbohydrate Biosynthesis/Metabolism
U49430	
AW124544	
J03298	
U60020	Cell Defense/Immune Response
AW047630	
AW047237	
AI852838	
AF110520	
AI843106	
AW124483	
AW047919	
AW061234	
AI844396	
AJ005560	
AJ005561	
AJ005567	
AJ005563	
AJ005565	
AJ005564	
AW060549	
AW047207	
AJ005562	
AI121305	
AV206059	
AA816121	
AA958903	
AW123751	
M32486	

Table 3.5 Function-organized probe sets reflecting significant changes in gene expression levels due to intrauterine infection. Alternative functional classifications are indicated where applicable. EST, expressed sequence tag; RIKEN cDNA, Japanese consortium expressed sequence.

Functional Classification

Cluster	GenBank	Gene Symbol	Title
DNA/Nucleotide Metabolism			
H	U35374	Pnp	purine-nucleoside phosphorylase
H	M14223	Rrm2	ribonucleotide reductase M2
Growth/Differentiation/Apoptosis			
A	AF007769	Birc1a	baculoviral IAP repeat-containing 1a
C	U94331	Tnfrsf11b	TNF receptor superfamily member 11b (osteoprotegerin)
C	AI852641	Nupr1	nuclear protein 1
F	AF090140	Prlpc	prolactin-like protein C
G	AF077861	Idb2	inhibitor of DNA binding 2
H	X57413	Tgfb2	transforming growth factor beta 2
Transcription regulation			
A	X70298	Sox4	SRY-box containing gene 4
A	AW124153	Sox4	SRY-box containing gene 4
A	AF016294	Elf3	E74-like factor 3
B	AF041847	Crap	cardiac responsive adriamycin protein
B	U67840	Dlx5	distal-less homeobox 5
C	AI846152	Dscr1	Down syndrome critical region homolog 1 (human)
D	V00727	cFos	FBJ osteosarcoma oncogene
D	U19118	Atf3	activating transcription factor 3
H	AI553024	AI467657	EST: Highly similar to promyelocyte leukemia Zn finger protein (mouse)
RNA/Protein Biosynthesis			
Protein Metabolism/Modification			
A	D10049	Mela	melanoma antigen 80 kDa
A	AB013848	Pdi1	peptidyl arginine deiminase type I
B	U51805	Arg1	arginase 1 liver
C	AF096875	Dio2	deiodinaseiodothyronine type II
H	AI845735	Polr2e	polymerase (RNA) II (DNA directed) polypeptide E
H	AI853714	Ctsb	cathepsin B
H	AW122851	1110002O23Rik	RIKEN cDNA: FK506 binding protein precursor homolog (human)
H	AI851255	Ctsb	cathepsin B
Signalling			
D	U94828	Rgs16	regulator of G-protein signaling 16
D	X61940	Ptpn16	protein tyrosine phosphatase non-receptor type 16

Cluster	GenBank	Gene Symbol	Title
G	AI853531	Mig-6 homolog	RIKEN cDNA: Mitogen-inducible gene 6 protein homolog
G	U67187	Rgs2	regulator of G-protein signaling 2
Calcium Binding/Transport			
A	X17320	Pcp4	Purkinje cell protein 4
Intercellular adhesion/communication			
Extracellular Matrix		**Blood Clotting**	
A	L19932	Tgfbi	transforming growth factor beta induced 68 kDa
A	M77196	Ceacam1	CEA-related cell adhesion molecule 1
B	X56304	Tnc	tenascin C
C	M63801	Gja1	gap junction membrane channel protein alpha 1 (connexin 43)
C	M62470	Thbs1	thrombospondin 1
D	M33960	Serpine1	serine proteinase inhibitor E1 (nexin plasminogen activator inhibitor type 1)
E	U83903	Tnfip6	tumor necrosis factor induced protein 6
F	AI156095	Ceacam11	CEA-related cell adhesion molecule 11
H	AF026073	Sultn	N-sulfotransferase
H	M81445	Gjb2	gap junction membrane channel protein beta 2 (connexin 26)
H	M26071	F3	coagulation factor III
Cytoskeleton		**Muscle Development**	
A	AA606367	9030623C06Rik	RIKEN cDNA: keratin 21, type I, cytoskeletal homolog (rat)
B	AV260677	Enah	enabled homolog (Drosophila)
F	AW122536	Pfn2	profilin 2
H	M15501	Actc1	actin alpha cardiac
H	M12347	Acta1	actin alpha 1 skeletal muscle
Lipid Binding/Transport/Metabolism			
A	M63335	Lpl	lipoprotein lipase
A	AA726364	Lpl	lipoprotein lipase
H	AI846600	Mgll	monoglyceride lipase
H	AI317360	Elovl2	elongation of very long chain fatty acids (FEN1/Elo2SUR4/Elo3yeast)-like 2
Hormone Biosynthesis/Metabolism			
D	M88242	Ptgs2	prostaglandin-endoperoxide synthase 2 (COX-2)
G	Z29532	Fst	follistatin

Cluster	GenBank	Gene Symbol	Title
Carbohydrate Biosynthesis/Metabolism			
Electron Transport -Energy			
F	Y12657	Cyp26a1	cytochrome P450 26 retinoic acid A1
H	AW125884	Acas2	acetyl-Coenzyme A synthetase 2
H	AI648067	Hao3	hydroxyacid oxidase (glycolate oxidase) 3
Cell Defense/Immune Function			
B	L12029	Sdf1	stromal cell derived factor 1
E	M19681	Scya2	small inducible cytokine A2
G	X53798	Scyb2	small inducible cytokine subfamily member 2
H	D17444	Lifr	leukemia inhibitory factor receptor
H	M21952	Csf1	colony stimulating factor 1 (macrophage)
H	U62021	Nptx1	neuronal pentraxin 1
Transport			
A	M60493	Cftr	cystic fibrosis transmembrane conductance regulator homolog
H	AI846308	Sfxn1	sideroflexin 1
H	AF018952	Aqp8	aquaporin 8
UNKNOWN			
A	AJ005561	Sprr2c	small proline-rich protein 2C
A	AJ005567	Sprr2i	small proline-rich protein 2I
A	M26005	1300007C21Rik	RIKEN cDNA: GAG homolog (mouse)
A	AJ005563	Sprr2e	small proline-rich protein 2E
A	AJ005565	Sprr2g	small proline-rich protein 2G
B	AJ005560	Sprr2b	small proline-rich protein 2B
B	M17327		clone IMAGE:5053066 mRNA partial cds
B	M17327		clone IMAGE:5053066 mRNA partial cds
B	AI850090	5730469M10Rik	RIKEN cDNA
C	AW060549	1300007C21Rik	RIKEN cDNA: GAG homolog (mouse)
G	AW047643		EST
H	AA986114	Timd2	T-cell immunoglobulin and mucin domain containing 2
H	AI844396	3110038L01Rik	RIKEN cDNA
H	AW061234	AW061234	EST
H	AW047919	C78582	EST

GenBank	Alternate Classifications	
U35374 M14223		
AF007769 U94331 AI852641 AF090140 AF077861 X57413	Signalling	Extracellular matrix
X70298 AW124153 AF016294 AF041847 U67840 AI846152 V00727 U19118 AI553024	Growth/Differentiation/Apoptosis	
D10049 AB013848 U51805 AF096875 AI845735	Calcium Binding/Transport	
AI853714 AW122851 AI851255		
U94828	Growth/Differentiation/Apoptosis	
X61940	Growth/Differentiation/Apoptosis	

GenBank	Alternate Classifications
AI853531	Transcription regulation
U67187	Growth/Differentiation/Apoptosis
X17320	
L19932	Growth/Differentiation/Apoptosis
M77196	
X56304	
M63801	Cell Defense/Immune Response
M62470	Calcium Binding/Transport
M33960	Signalling
U83903	
AI156095	
AF026073	RNA/Protein Biosynthesis
M81445	
M26071	
AA606367	
AV260677	
AW122536	Signalling
M15501	
M12347	
M63335	Extracellular matrix
AA726364	Extracellular matrix
AI846600	
AI317360	
M88242	Cell Defense/Immune Response
Z29532	Growth/Differentiation/Apoptosis

GenBank	Alternate Classifications	
Y12657		
AW125884		
AI648067		
L12029		
M19681	Signalling	Growth/Differentiation/Apoptosis
X53798	Signalling	
D17444	Signalling	
M21952	Lipid Binding/Transport/Metabolism	
U62021	Growth/Differentiation/Apoptosis	
M60493		
AI846308		
AF018952		
AJ005561		
AJ005567		
M26005		
AJ005563		
AJ005565		
AJ005560		
M17327		
M17327		
AI850090		
AW060549		
AW047643		
AA986114		
AI844396		
AW061234		
AW047919		

Table 3.6 Function-organized probe sets reflecting significant changes in gene expression levels due to ovariectomy. Alternative functional classifications are indicated where applicable. EST, expressed sequence tag; RIKEN cDNA, Japanese consortium expressed sequence.

Chapter 4

Oxytocin Receptor Gene Expression in the Mouse Uterus during Normal and Premature Labour

4.1 ABSTRACT

The Affymetrix mouse oligonucleotide array U74Av2 used for our microarray studies (Chapters 2 and 3) does not include a probe set for the oxytocin receptor (OTR) gene. The expression analysis of this gene was instead performed by Northern blotting and by relative quantification of Real-Time RT-PCR products. In each of the experimental models (normal labour, infection-induced preterm labour (PTL), and ovariectomy-induced PTL), an increase in OTR gene expression was found just prior to and during uterine contractions. This substantiates the relevance of the OTR in the process of normal and PTL. The nature of its role in these various models requires further investigation.

4.2 INTRODUCTION

With the approach of parturition, increasing levels of OT are produced and secreted from magnocellular neurons of the hypothalamic supraoptic and paraventricular nuclei. Released in a pulsatile manner from the posterior pituitary, OT enters the circulation where it may then bind to endometrial OTRs. This interaction results in the stimulation of pulsatile secretion of $PGF_{2\alpha}$ by endometrium. Pulsatile $PGF_{2\alpha}$ reaches the ovary and causes regression of the corpus luteum of pregnancy. This facilitates a drop in progesterone production resulting in a positive feedback loop of increased OTR gene expression due to increased effective estrogen levels. OTR expression is further induced in the myometrium and endometrium by estrogen-stimulated cFos production and by the static stretch imparted by the growing fetus in an environment that is no longer accommodating [34]. The increase in OTR and OT production culminates in the activation of the uterus and stimulation of contractions.

Infection results in an increased synthesis of pro-inflammatory cytokines by T helper type 1 (T_h1) cells of the host environment. In the case of intrauterine infection during pregnancy, this leads to a shift in the balance from a T_h2 anti-inflammatory cytokine milieu necessary to maintain immune tolerance to the semi-allogeneic fetus, to one of T_h1 cytokines associated with transplant rejection. The outcome is the premature expulsion of the fetus. T_h1 cytokines include IL-1β, IL-6, TNFα, and IFNγ. The effects these cytokines have on OTR and OT gene expression suggest an involvement in the mechanism of infection-induced PTL. In cultured human smooth muscle cells, IL-1β down-regulated the OTR gene [224, 225]. The effect of IL-6 in these cells remains controversial. These effects may more accurately reflect the physiological state of the cultured uterine cells. Both IL-6 and IL-1β have been implicated in up-regulating the central and peripheral release of OT from hypothalamic neurons [226, 227] and it may be by this route that they positively affect OTR gene expression and function. The role these cytokines play on regulating OTR gene expression, in the otherwise quiescent uterus, during infection-induced PTL

clearly needs to be refined. The importance of other factors such as those indicated by microarray study is not to be overlooked.

In addition to clarification of the role of cytokines on the regulation of OTR gene expression is the need to further parse the role of hormones. The interplay between progesterone and estrogen during murine pregnancy and the effect on OTR gene expression requires further investigation. Ovariectomy during pregnancy mimics luteal regression normally observed just prior to parturition. In the pregnant rat uterus, ovariectomy significantly increased OTR gene expression, which peaked prior to PTL [103]. Addition of estrogen to these rats further up-regulated OTR transcription while progesterone abolished this increase. Preterm induction of intact rats by $PGF_{2\alpha}$ administration was also demonstrated but this effect was eliminated by tamoxifen, having anti-estrogenic effects. The possible influence on OTR gene expression by other mediators of ovariectomy-induced PTL, such as those indicated by microarray analysis may further delineate the regulation and importance of the OT-OTR system in parturition, particularly in light of the fact that the OT knock-out mouse shows no parturition defect [4, 5].

As the OTR gene is not represented on the Affymetrix murine oligonucleotide array U74Av2, we investigated the regulation of OTR transcription by Northern blot and real-time RT-PCR by LightCycler with relative quantification analysis on the samples prepared for microarray detection. Further clarification of the role of the OTR in normal and premature labour induced by intrauterine infection or by ovariectomy may provide additional support for the development of effective tocolytic therapies.

4.3 MATERIALS AND METHODS

mRNA Preparation

Poly A^+ mRNA was isolated from uterine total RNA samples used for microarray analysis. Based on the approximation of 5% of total RNA from uterus is mRNA, 800 µl of total RNA (1 µg/µl) was processed for approximately 40 µg of mRNA of each sample. Oligotex Midi kit purification columns (Qiagen Inc., Mississauga, ON) were used according to manufacturers instructions. Uterine mRNA samples were eluted in 100 µL volumes and stored at -70°C until needed.

Northern Blot

A standard 1.2% agarose gel containing 1.5 M formaldehyde was used to electophoretically separate mRNAs. An aliquot of RNA molecular weight ladder (Invitrogen, Burlington, ON) was loaded along side with the uterine samples for size estimation. Separated samples were transferred and cross-linked to nylon membrane (Hybond N, Amersham Pharmacia Biotech, Arlington Heights, IL). A probe homologous to the first 257 bp of OTR coding sequence (p257) was radioactively labelled by incorporating α^{32}P-dCTP using the Random Primed DNA Labelling Kit (Roche, Laval, QC). After pre-hybridizing the RNA blot with QuickHyb (Stratagene, La Jolla, CA) for 30 minutes at 65°C the labelled probe was added and hybridization proceeded for 1 hour at 65°C. The membrane was washed to remove non-specific radiolabel binding and then exposed to a phosphorimager screen (Typhoon, Amersham Pharmacia Biotech) overnight. The screen was scanned and the OTR specific signals were quantitated using ImageQuant phosphorimager software. The blot was stripped and re-probed with a labelled cDNA probe to HPRT prepared with PCR primers used for real-time RT-PCR (see Chapter 2, Table 1) and quantified. OTR gene expression was corrected to background pixel level and subsequently standardized relative to similarly corrected HPRT mRNA levels in the samples. Relative OTR transcript abundance was calculated.

Relative Quantification of Two Step Real-Time RT-PCR

Following the method outlined in the Materials and Methods of Chapters 2 and 3, relative quantification of OTR transcript levels was performed using the forward PCR primer F1C (5'GGACG TCAATGCGCCCAAAGAAG3'), and the reverse R8 (5'ACTCGAGCTGCAACGACT CA3') primer. These primers bridge the large third intron of the mouse OTR gene thus preventing the possibility of amplifying a product from contaminating genomic DNA. Comparison of the resulting 235 bp OTR amplicon quantities with HPRT control levels for the same sample was performed as previously described. Once again, each sample replicate was amplified in three separate runs for each experimental time-point.

4.4 RESULTS

Uterine OTR Gene Expression

Northern blot of poly A+ mRNA purified from total RNA samples revealed the 5 kb mouse uterine OTR transcript and subsequently, the 1.3 kb HPRT transcript. Measurements by phosphorimager scanning and ImageQuant analysis allowed comparison between OTR and HPRT transcript levels. In each case, the gene expression patterns observed by Northern blot analysis were corroborated by relative quantification of real-time RT-PCR. During normal labour, a peak expression was observed and this induction of gene expression was switched off postpartum (Fig.4.1, Panel A). Under the condition of infection, a significant change in OTR gene expression levels was demonstrated. This up-regulation was greatest for the high dose infection (10^{10} heat-killed bacteria) with expression rising from low levels at 3.5 hours to a peak found at the 8 hour time point and a sustained level at the time of PTL (Fig.4.1, Panel B). Ovariectomy stimulated an early increase in OTR transcript level compared with sham operated, time-matched control mice. This moderate increase was recorded at parturition (~20 hours) as well (Fig.4.1, Panel C) with no significant variation with the advance of parturition.

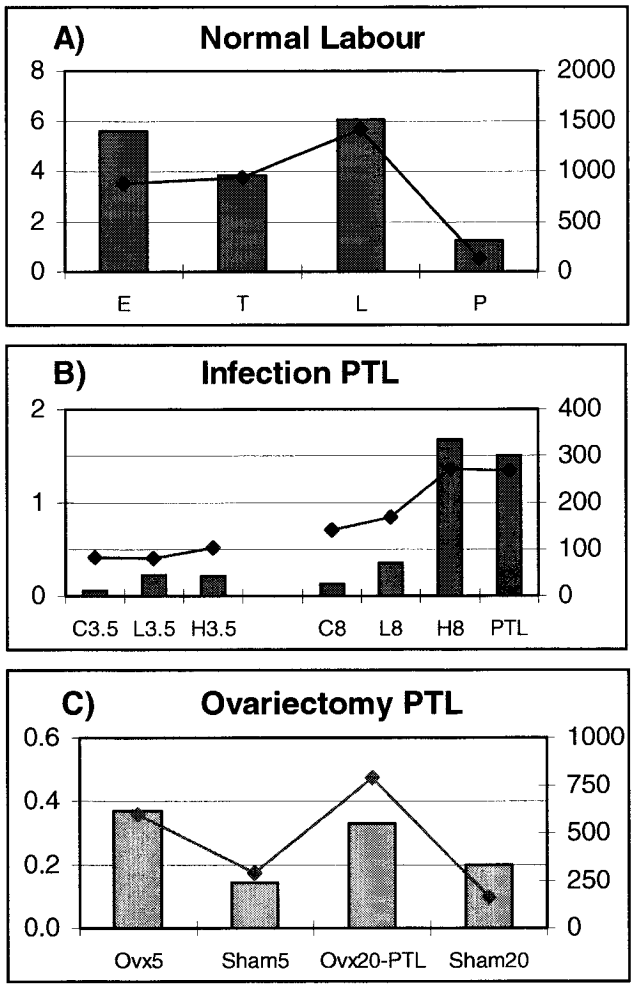


Figure 4.1 Determination of OTR mRNA levels using Northern blot and real-time RT-PCR in models of normal and preterm labour

OTR mRNA levels were detected by Northern blot using radiolabelled p257 probe and are indicated in bar graph form and correspond with the left axis values. Amplification of OTR cDNA by Real-Time RT-PCR and subsequent relative quantification resulted in a confirmation of OTR transcript levels across all microarray time-points investigated. Average values of triplicate RT-PCR amplification of the microarray replicate RNA samples (n=6) and the associated standard errors are plotted in line graph form relative to the right axis values. **Panel A**) E, estrus; T, term; L, labour; P, postpartum; **Panel B**) C, Luria Broth control; L, 10^7 –low dose infection; H, 10^{10}–high dose bacterial infection; 3.5 and 8 hours post inoculation; PTL, preterm labour by high dose infection. **Panel C**) Ovx5, 5 hours after ovariectomy; Ovx20-PTL, preterm labour occurs approximately 20 hours after ovariectomy; Sham, sham operated time controls of matched time-points.

4.5 DISCUSSION

Normal Labour

OTR transcription is up-regulated in the non-pregnant uterus to peak levels at proestrus and is somewhat diminished at estrus [51, 228]. Not surprisingly, we found OTR gene expression at estrus. Our particularly high levels at estrus may also reflect an up-regulation in response to the stimulus during vaginal cell flushing. The insertion of a plastic tube into the vagina is sufficient to mimic copulation and induce a pseudopregnant state [8]. The OTR-ligand interaction during mating may play a role in fertilization by promoting sperm transport up the uterine horns. Kubota et al. [49] demonstrated an absence of the 5 kb uterine OTR transcript by day 1 of murine gestation with increasing progesterone and decreasing estrogen levels. This repression is maintained throughout pregnancy, maintaining uterine quiescence. OTR gene transcription was later stimulated by increasing circulating estrogen levels at term, achieving a peak induction during labour. By 12 hours postpartum, the requirement for OTR-stimulated contractions has ceased and levels quickly returned to the low expression described during gestation. This significant down-regulation was observed independently by Kubota et al. and by Larcher et al. [49, 51]. This recapitulation of the known pattern of OTR gene expression increases the reliability of our model and tissue and RNA preparations.

Infection-Induced Preterm Labour

Several studies have shown that IL-1β suppresses OTR gene expression in human uterine smooth muscle cells [183, 224, 225]. The stimulation of these cells with IL-6 was met with varied responses ranging from up-regulating OTR gene expression [229], to having no effect [225], to having a suppressive effect [183]. In bovine cycling endometrial explants stimulated with IL-1α or IL-6, a suppression of OTR gene expression was observed during the late luteal phase while no effect was found by either of these in early luteolysis [230]. These *in vitro*/*ex vivo* experiments suggest a hormonal influence over OTR gene expression during infection-induced PTL as well as a pro-inflammatory cytokine regulation and highlight the importance

of *in vivo* experimentation in deciphering the actual combinatory influences over gene expression.

Our infection model demonstrated an induction of OTR gene expression with a high bacterial infection, peaking just prior to the onset of overt labour. In the pregnant mouse, both IL-1β and IL-1α induce PTL [179]. Microarray analysis demonstrated a moderate increase in IL-1α and IL-1β gene expression that was significantly greater with the high dose than the low, reaching these levels at 3.5 hours post-infection. As only the high dose leads to PTL, it therefore suggests this interleukin up-regulation to be important for labour, possibly by activation (rather than suppression observed in vitro) of OTR gene transcription. The OTR promoter contains an acute phase response element, an NFκB binding element and an IFNα binding site [49]. The presence of these promoter elements further substantiates a role for OTR in infection-induced PTL. The mouse OTR promoter region also contains an NF-IL6 binding motif [49]. IL-1 and IL-6 increase the expression of NF-IL6 transcription factor. C/EBPβ is identical to this transcription factor and in the microarray analysis C/EBPβ was significantly up-regulated 8 hours after the high dose infection and during PTL that followed. These suggest a positive regulation of OTR gene expression during infection. NF-IL6 also up-regulates cFos expression and this transcription factor could then induce OTR gene expression during infection-induced PTL.

While an increase in myometrial and decidual OTR numbers has been reported during PTL [231] the levels determined here were somewhat lower than that found at normal labour. This may reflect a lessened dependence on the OT-OTR system in infection-induced PTL, particularly in the presence of such a large induction of COX-2 transcription (28-fold), leading to a substantial increase in prostaglandin synthesis. As well, OTR gene expression in the uterus at 15 d.p.c is normally being suppressed by the progesterone:estrogen ratio. It seems a competition between gene inductive and suppressive factors for OTR gene regulation is underway. In the context of all these competing factors and the low levels of OTR relative to that found with normal labour, the question of relevance is raised, particularly in light of the limited effectiveness of the OTR antagonist atosiban in preventing PTL [1].

Ovariectomy-Induced Preterm Labour

Progesterone produced by the corpus luteum of pregnancy promotes uterine growth during fetal development and imparts a relaxant affect on the myometrium. The significant loss of progesterone by ovariectomy is followed by a decrease in uterine growth and an increased sensitivity to uterotonic agents. Accompanying this loss is a shift in the progesterone:estrogen ratio to an estrogen-dominant uterine environment. As a result, estrogen and uterine stretch lead to the induction of the transcription factor gene cFos and the contraction associated protein (CAP) genes including the OTR gene.

Compared with Sham controls, OTR mRNA levels increased in response to ovariectomy, approximately 2-fold. This induction was observed as early as 5 hours later and was sustained through PTL, approximately 20 hours after surgery. In ovariectomized day 18 pregnant rats, OTR mRNA expression reached significant levels 24 hours after surgery [103]. This pattern was observed with day 16 ovariectomized mice as well [64]. The levels measured here were significantly lower than found with normal labour, perhaps due to the correction against somewhat higher HPRT mRNA levels in this model or perhaps reflecting a suppressed response due to the stress of PTL [73]. Additionally, the higher induction of COX-2 and hence prostaglandin production than that found in normal labour may compensate for this level. As well, there may be an increased output of OT from hypothalamic neurons. Infection-induced IL-1β has been demonstrated to stimulate the release of OT from hypothalamic and supraoptic neurons [227]. The peripheral release of greater amounts of OT may precipitate a lessened requirement for receptor production to achieve parturition. A similar induction of OT synthesis by endocrine factors in response to ovariectomy may take place and account for the differences in OTR mRNA levels in each model. Other factors may also contribute to the culmination of ovariectomy-induced PTL.

The role OT may play in the function of the OTR during parturition requires further definition especially in light of the fact that OT knock-out mice do not experience impaired parturition [4, 5]. During normal murine labour OT synthesis in

the hypothalamus is increased and released via the portal system of the posterior pituitary, resulting in greater levels of circulating hormone. This elevated level of OT reaches the ovary where it exerts its effects on high levels of OTRs, leading to luteolysis and subsequently on the uterus where, in the absence of suppressive progesterone levels, uterine contraction occurs [53]. A positive feedback exists between the contracting uterus and the supraoptic nucleus of the hypothalamus via activation of neurons of the solitary tract nuclei to increase OT synthesis and output [73]. As the OTR gene is clearly up-regulated in each of these models, an involvement is indicated. The fact that atosiban, an OTR antagonistic ligand, suppresses PTL for only a short duration reveals that the OTR is not alone in stimulating the contractions. Prostaglandin receptor FP knock-out mice implicate $PGF_{2\alpha}$ in this mechanism [58]. The up-regulation of COX-2, cFos and CAP genes for the OTR and Cx43 during all three manners of labour reflect an association relevant to the development of preventative therapies.

4.6 CONCLUSION AND FURTHER ANALYSIS

An induction of uterine OTR gene expression was found in each model studied by Northern blot analysis. This suggests a participatory role in the mechanism for uterine contractions, however the dependence upon OTR function seems to vary from condition to condition. The exact nature of the role the OTR plays in each process of murine parturition still requires clarification. The effect of increased levels of central OT synthesis and secretion on parturition should also be addressed and therefore analysis of OTR gene expression should be extended to the brain. To study OTR gene expression in the mouse, we ventured to create an OTR knock-out mouse replacing the coding sequence of the OTR gene with sequence encoding the bacterial lacZ reporter gene. Histochemical detection of the lacZ gene product to demarcate the tissue specificity and expression pattern of OTR gene expression may provide us with a better understanding of the regulation of this gene and the importance of this receptor in mammalian reproduction.

Chapter 5

Mapping Oxytocin Receptor Gene Expression in the Mouse Brain and Mammary Gland Using an OTR-LacZ Reporter Mouse

Barbara R. Gould and Hans H. Zingg

Published in Neuroscience: Volume 122, Issue 1, p.155-168

5.1 ABSTRACT

The hypothalamic nonapeptide oxytocin (OT) has an established role as a circulating hormone but can also act as a neurotransmitter and as a neuromodulator by interacting with its central OT receptor (OTR). To understand the role of the OTR in the mouse brain we investigated the expression of the OTR gene at the cellular level. We targeted the lacZ reporter gene to the OTR gene locus downstream of the endogenous OTR regulatory elements. Using lactating mouse mammary gland as a control for OTR promoter directed specificity of lacZ gene expression, X-gal histochemistry on tissue sections confirmed that gene expression was restricted to the myoepithelial cells. We also identified for the first time in mice the expression of the OTR gene in neighbouring adipocytes. Further, investigation in the mouse brain identified numerous nuclei containing neurons expressing the OTR gene. While some of these regions had been described for rat or sheep, the OTR- LacZ reporter mouse enabled the identification of novel sites of central OTR gene expression. These regions include the accessory olfactory bulb, the medial septal nucleus, the posterolateral cortical amygdala nucleus, the posterior aspect of the basomedial amygdala nucleus, the medial part of the supramammillary nucleus, the dorsotuberomammillary nucleus, the medial and lateral entorhinal cortices, as well as specific dorsal tegmental, vestibular, spinal trigeminal, and solitary tract subnuclei. By mapping the distribution of OTR gene expression, depicted through histochemical detection of ß-galactosidase, we were able to identify single OTR gene expressing neurons and small neuron clusters that would have remained undetected by conventional approaches.

These novel sites of OTR gene expression suggest additional functions of the oxytocinergic system in the mouse. These results lay the foundation for future investigation into the neural role of the OTR and provide a useful model for further study of oxytocin functions in the mouse.

5.2 INTRODUCTION

Oxytocin (OT) is a nonapeptide hormone produced within hypothalamic neurons. It is transported to the posterior pituitary where it is released into the periphery to elicit its effects on target tissues such as the myometrium, stimulating uterine contractions, and the myoepithelial cells of the mammary gland, stimulating milk ejection. OT also influences the functions of the kidneys, ovaries, testis, heart, and other tissues [6, 232]. Centrally released OT has been demonstrated to affect neuroendocrine function, maternal, sexual and social behaviours as well as learning and memory [76, 232]. The many functions of OT are mediated by the oxytocin receptor (OTR). We endeavoured to generate an OTR knock-out - LacZ reporter mouse to investigate the importance of the OTR and to map the distribution pattern of OTR gene expression using X-gal histochemistry. In this pursuit, we created a mouse that retained the OTR gene while incorporating the lacZ gene under the control of the entire OTR gene promoter. This OTR-LacZ reporter mouse allowed us to study OTR gene expression patterns without having altered the phenotype of the animal.

Because of the remarkable variability of OTR expression between brains of different species, sexes, and under different hormonal influences [233, 234] we chose to characterize the OTR-promoter driven lacZ gene expression in this tissue. The female mouse brain has been partially characterized with respect to the distribution of OT binding sites [235-237] however; no information is available regarding the distribution of OTR mRNA in the brain. This study gives a thorough representation of OTR gene expression in the female mouse brain. The 12-hour post-parturient female mouse model was chosen to study OTR gene expression at this physiological stage. The oxytocinergic system has been shown to have little apparent influence on maternal behaviour in mice [79]. The neurological functions of this system in mice clearly require further study.

This study describes the development of the OTR-LacZ reporter mouse and demonstrates its utility by confirming recognized expression profiles within the mouse brain and mammary gland and more importantly, identifying new brain regions and cell types that express the OTR gene.

5.3 MATERIALS AND METHODS

Development of the OTR-LacZ Reporter Mouse

Overlapping mouse OTR genomic fragments 4 kb, 6 kb, and 6.5 kb in length were isolated from a λ-dash 129/Sv mouse genomic library and cloned into pBluescript. From these, sequences 5' of the ATG translation start site and 3', within the third intron [49], were subcloned into pGNA (a gift from Dr. Alan Peterson, Montreal, QC), a modified version of the pGN vector [238] having an additional poly-A sequence. The final targeting vector was denoted as 5'3'pGNA (Figure 5.1*a*). These sequences were selected and oriented to facilitate the homologous targeting and subsequent replacement of the genomic OTR coding region with the lacZ and neo^r genes of the pGNA vector. 129/Sv embryonic stem (ES) cells were electroporated with the vector and then grown in G418 containing media. Southern blotting allowed the selection of clones that had undergone site-specific homologous recombination. These ES cells were injected into developing Balb/c blastocysts and these new blastocysts were introduced into pseudopregnant Balb/c mice for further development. Chimeras were achieved and then backcrossed to Balb/c mice (Charles River, St. Constant, QC). Germline transmission was established and the mice were then bred to homozygosity for the lacZ allele. All animals were maintained under a 12-hour light/dark cycle with access to food and water *ad libitum*. All animal experiments were carried out in accordance with the Bioethics Committee of the Royal Victoria Hospital Research Institute. All efforts were made to minimize the number of animals used as well as their suffering.

Southern Blot

Genomic DNA was extracted from ES cells and eventually from mouse tail clippings by proteinase K cell lysis, phenol/chloroform extraction, and subsequent ethanol precipitation. After AccI digestion overnight, the DNA was electrophoresed through a 0.8% agarose gel. The DNA was then transferred to nylon membrane and α^{32}P-dCTP labelled mouse OTR and lacZ probes (see Figure 5.1*a*) were hybridized to the DNA. All probes were prepared using the Random Primed DNA Labelling Kit

(Roche, Laval, QC). A 1.7 kb probe (p1.7) isolated from the 6.5 kb OTR clone as an AccI-KpnI fragment was used as a probe external to the recombination. The wild-type OTR allele and recombinant lacZ allele produce 6.5 kb and 8.65 kb AccI fragments, respectively. A 580 bp lacZ probe (pLac) generated by PCR using the forward amplification primer Lac1 (5'GGCTTACGGCGGTGATTTTGG3') and the reverse primer Lac2 (5'GCGGCGGTCAGCAGTTGTTTTT3') was designed to detect the same 8.65 kb AccI fragment if specific incorporation had taken place. ES cells were genotyped as +/+ or +/z, and mice as +/+, +/z, and z/z, depending on the number of wild-type (+) and recombinant OTR-LacZ (z) alleles present. A probe homologous to the first 257 coding base pairs of the OTR (p257) generated by PCR using the forward primer F3 (5'ATGGAGGGCACGCCCGCAGCCAACTGG3') and the reverse primer R1 (5'AGGTCGGCGATGCTCAGGTGCTTCATG3') was used to determine the presence or absence of the OTR coding region.

Long PCR Determination of OTR-LacZ Gene Structure

The Expand Long Template PCR kit (Roche) system 1 was used to characterize the DNA extracted from the livers of +/+, +/z, and z/z mice. PCR primers that were used are indicated in Figure 5.1*a*. Primer sequences are as follows: forward primers - 5FA (5'GCC TCCCAAGTTCTGGGATTAAAGAAT3'), 5FB (5'TGGAGCTCCTGGCTCAGTCT3'), ZF3 (GGTCGGCTTACGGCGGTGATTT TGG3'), and reverse primers - R2D (5'GGCT CTTTAAAGTGTGGCACGT3'), ZR4 (5'GGGCGATCGGTGCGGGCCTCTTCGCTATTA3'), 5RA (5'GCAGGAGTTAC TAGAGGTAGGTGCTA3'). Both annealing and elongation temperatures were 68°C and the PCR products underwent 30 cycles of amplification. Primers 5FB and R2D produce a wild-type OTR gene product of 3.5 kb. Primers 5FA and ZR4 produce a 4.2 kb product indicating correct lacZ incorporation downstream of the entire endogenous OTR promoter. Primers ZF3 and 5RA should not produce a product at all if proper homologous recombination had occurred but would give a 7.8 kb product if a duplicate of 5'OTR sequence were downstream of the lacZ gene.

RNA Extraction and RT-PCR

Total RNA was isolated from uterine tissues collected from parturient control (+/+) mice, heterozygous (+/z), and homozygous (z/z) OTR-LacZ mice and an independent wild-type (wt) control strain. Uteri were placed into appropriate amounts of cold TRIzol™ Reagent (Invitrogen, Burlington, ON) for immediate processing. Total RNA was isolated according to the directions for the TRIzol™ Reagent and subsequently quantified and stored at -70°C for later use. Two micrograms of total RNA of each sample were reverse transcribed (RT) and the cDNAs amplified, using specific primers according to a standard RT-PCR protocol [239]. The OTR forward, F1C (5'GGACGTCAATGCGCCCAAAGAAG3'), and reverse, R8 (5'ACTCGAGCTGCAACGACTCA3') primer combination produces a 235 bp amplicon. These primers bridge the large third intron thus preventing the possibility of amplifying a product from contaminating genomic DNA (see Figure 5.1*a*). Glutaradehyde phosphate dehydrogenase (GAPDH) primers, forward F (5'CCCTTCA TTGACCTCAACTACATGGT3') and reverse R (5'GAGGGGCCATCCACAGTC TTCTG3'), produce a 470 bp amplicon. The annealing temperature for both these PCR reactions was 70°C. The OTR reactions underwent 35 cycles of amplification while the GAPDH reactions underwent 25 cycles. Samples were visualized by agarose gel electrophoresis along with a 1 kb DNA ladder (Invitrogen). Digital photographs were taken and PCR products were quantified using ImageQuant 5.1 analysis software by Molecular Dynamics (University of Virginia, Charlottesville, VA).

Mouse Tissue Fixation and Cryostat Sectioning

Control (+/+), +/z, and z/z mice approximately 12-hours post-parturition were anaesthetised with ~40 µl (100 mg/ml) Ketamine injected ip. Ice-cold phosphate buffer (PBS) was perfused through the mouse by injection into the left ventricle of the heart. The blood was flushed out through an incision in the right ventricle. Cold Webster's Fixative (1g paraformaldehyde and 10 ml 50% glutaraldehyde in PBS for a final volume of 200 ml and pH adjusted to 7.2) was then perfused through the mouse.

The tissues were dissected and immediately placed into tissue culture plate wells containing cold Webster's fixative. The tissues were incubated for 1 hour on ice. They were rinsed with cold PBS and then cryoprotected overnight at 4°C in a 30% sucrose-PBS solution. Tissues were washed with cold PBS, frozen in liquid N_2-cooled isopentane and then in liquid N_2, and finally stored at -70°C until sectioning. Cryostat sectioning of tissues (brains n=3, mammary glands n=3; each for control and lacZ tissue) took place below -22°C. Sections were cut and collected on poly-L-lysine coated microscope slides (Fisher Scientific, Nepean, ON).

LacZ Staining

Sections cut 10 μm thick were fixed by submersing the slides for 3 minutes in 1.5% glutaraldehyde-PBS and then washed in four consecutive baths of PBS at room temperature. The sections were stained overnight at 37°C with an X-gal solution (3.1 mM $K_4Fe(CN)_6$, 3.1 mM $K_3Fe(CN)_6$, 2 mM $MgCl_2$, 2 mM X-gal (Invitrogen) prepared as 40 mg/ml in N,N-dimethylformamide, PBS). The next day the slides were washed in 2 changes of 95% ethanol and then dehydrated in 100% ethanol. The slides were cleared in xylene for 3 minutes and allowed to dry. Cover slips were mounted using Permount (Fisher) and allowed to dry for at least 1 hour before viewing under a microscope. Localization and nomenclature of brain nuclei containing blue β-galactosidase expressing cells were determined according to Paxinos and Franklin [240].

α-Smooth Muscle Actin Staining

Mammary gland sections (5 μm) were stained following the protocol accompanying the α-smooth muscle actin immunohistochemistry kit (Sigma-Aldrich, St. Louis, MO) with modifications. Briefly, endogenous peroxide activity was quenched with 3% hydrogen peroxide for 5 minutes, washed with 0.2% TritonX-100 – PBS and incubated with 1% goat serum for 15 minutes to block. Excess serum was removed and slides were covered with monoclonal anti-α-smooth muscle actin

antibody for 1 hour. Negative control slides were instead treated with a 10^{-6} dilution of mouse $IgG_{2a\kappa}$ isotype control in PBS for the same length of time. Slides were treated sequentially with biotinylated goat anti-mouse antibody (20 min), ExtrAvidin®-conjugated peroxidase reagent (20 min), and the chromogenic substrate solution containing 3-amino-9-ethylcarbazole (AEC).

Oil Red O Lipid Staining

Mammary gland sections (5 μm) were stained with Oil Red O (Sigma) solution (2 mg/ml) for 10 minutes. The slides were rinsed with 60% isopropanol for 5 minutes to remove background and then counterstained with Harris hematoxylin (Fisher).

5.4 RESULTS

Generation of the OTR-LacZ Reporter Mouse.

Neomycin selection for ES cells which had undergone recombination resulted in the proliferation of nearly 500 resistant clones. These were screened for homologous recombination by Southern blotting using α^{32}P-dCTP randomly labelled DNA probes. Probe p1.7 was used to detect homologous recombination within the 5' OTR sequence. The wild-type OTR allele produced a 6.5 kb AccI fragment whereas the recombinant allele produced an 8.65 kb fragment. Re-screening of blots using a probe corresponding to lacZ gene sequence (pLac) allowed the exclusion of recombination positive ES cells which also had random and/or multiple incorporations of the targeting vector. Only those recombination positive ES cells that showed a single 8.65 kb AccI fragment with pLac, indicating a single and specific recombination event, were selected for injection into isolated, developing Balb/c blastocysts. Once germline transmission of the OTR-lacZ allele was established, the offspring were genotyped. Figure 5.1*b* depicts typical results of genotyping by Southern blot using p1.7.

Southern blotting of wild-type DNA with a probe homologous to the first 257 bp of the OTR coding region (p257) hybridized, as expected, to the 6.5 kb AccI digested DNA fragment (see Figure 5.1*c*, lane 1). In z/z mice, this probe detected a 5.5 kb fragment indicating that the OTR gene was still present (Figure 5.1*c*, lane 3). The size difference was hypothesized to have resulted from "rolling circle" insertion of non-linearized 5'3'pGNA vector within the homologous 5' OTR gene sequence, causing the downstream displacement of the OTR coding region including 4.2 kb of 5' sequence. This was instead of the actual replacement of OTR coding region sequence by linear 5'3'pGNA targeting vector. PCR genotyping of these mice with the primers indicated in Figure 5.1*a* demonstrated the presence of the lacZ gene in tandem with the OTR gene (Figure 5.1*d*), providing support for this hypothesis. Primer pair 5FB and R2D (lanes 1-3) amplify 3.5kb of 5' untranslated sequence and OTR coding sequence. This product should not be present if the OTR coding sequence had been knocked out, however lane 3 indicates its presence in the homozygous lacZ mouse.

Amplification of a 4.2 kb product by primers 5FA and ZR4 (lanes 4-6) indicates the site-specific incorporation of 5'3'pGNA into the 5' sequence of the OTR. No amplification from DNA of homozygous wild-type mice (+/+, lane 4) occurred. The 7.8 kb PCR product present in +/z (lane 8) and z/z (lane 9) mice using the ZF3 and 5RA primers (lanes 7-9) is only possible if the OTR gene is present and downstream of the integrated 5'3'pGNA targeting vector, as shown in Figure 1*a*. No PCR product is produced from DNA of homozygous wild-type mice (+/+, lane 7).

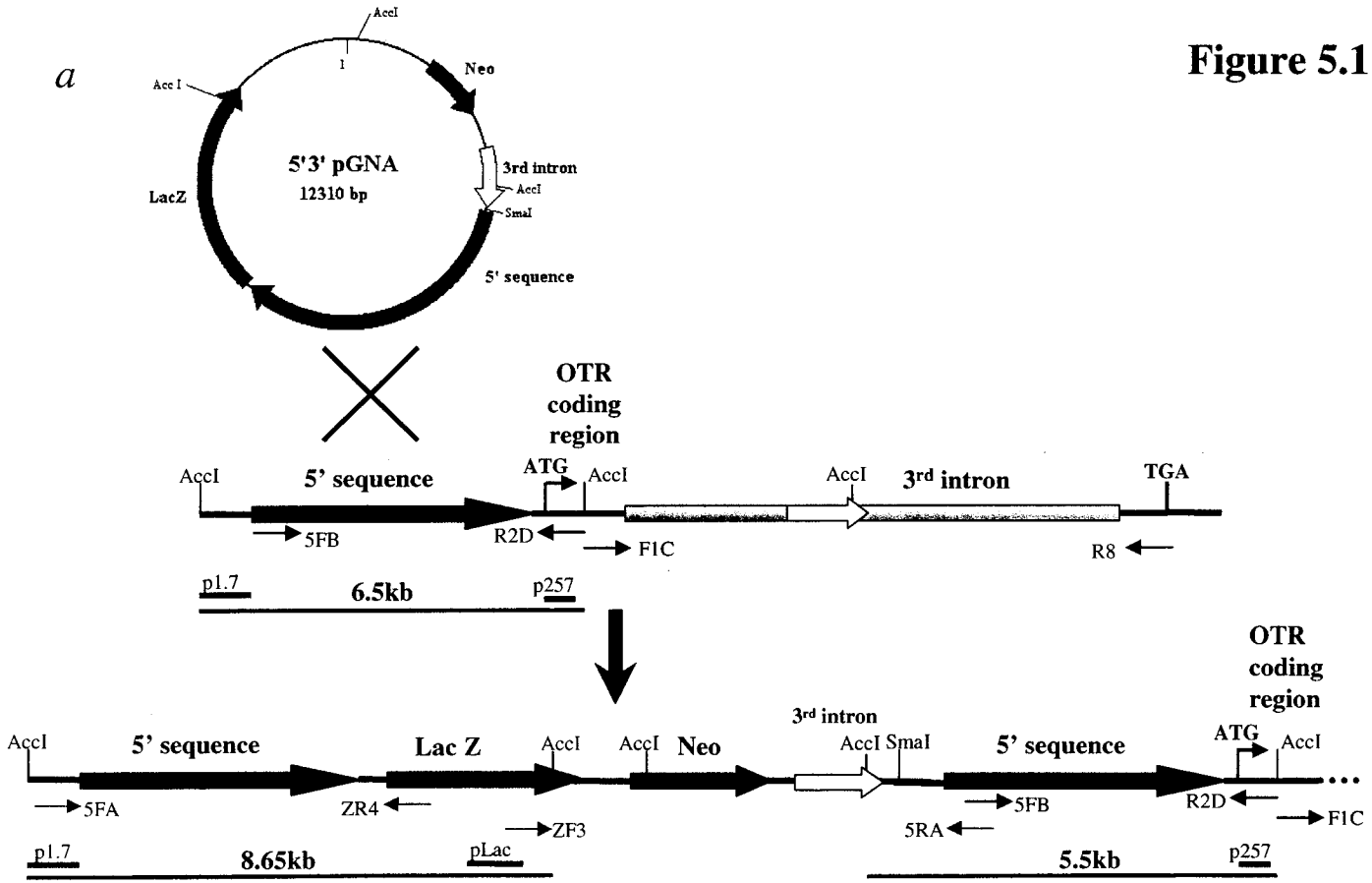
The insertion of 5'3'pGNA upstream of the OTR gene sequence resulted in a reduced level of OTR gene expression. This is demonstrated by RT-PCR of parturient uterus mRNA after 35 cycles of amplification (Figure 5.1*e*). OTR mRNA was detected using primers F1C and R8 (lanes 1-7). Lanes 1-3 are amplification controls. Lanes 4-7 indicate the expression of the OTR gene in parturient mouse uterus, even in z/z mice (lane 7). Quantification using ImageQuant 5.1 analysis software demonstrated approximately 75% of normal OTR gene expression levels in the heterozygote (+/z, lane 6) and approximately 50% of normal OTR gene expression levels in the homozygous mutant (z/z, lane 7). The +/+ mouse uterine tissue (lane 5) expressed OTR mRNA at levels equivalent to wild-type (lane 4), as expected. All comparisons were made after correction to individual GAPDH levels (lanes 8-11).

The diminished OTR gene expression seen in mice carrying the lacZ allele appears neither to affect normal reproductive and social behaviours, nor the general health of these animals. Mice engage in normal sexual behaviours including genital sniffing, the lordosis reflex, and mounting, leading to fertilization. The females successfully carry the pregnancy to full term (19 days) and deliver the pups within an appropriate time frame. Maternal behaviours are engaged immediately with mothers cleaning the newborns, retrieving the pups to the nest, and nursing their young in the typical crouching position. Animals experience a normal lifespan in the controlled environment.

The unexpected recombination event, demonstrated by Southern blot and PCR, resulted in a phenotypically normal mouse that expresses the lacZ gene under the control of the entire OTR gene promoter. It also expresses the OTR gene, as

evidenced by RT-PCR of parturient uterus mRNA. This mouse is referred to as the OTR-LacZ reporter mouse.

Figure 5.1


a
AccI
Acc I
Neo
5'3' pGNA
12310 bp
LacZ
3rd intron
AccI
SmaI
5' sequence
OTR coding region
AccI
5' sequence
ATG
AccI
AccI
3rd intron
TGA
5FB
R2D
F1C
R8
p1.7
6.5kb
p257
OTR coding region
3rd intron
AccI
5' sequence
Lac Z
AccI
AccI
Neo
AccI SmaI
5' sequence
ATG
AccI
5FA
ZR4
ZF3
5FB
R2D
F1C
5RA
p1.7
8.65kb
pLac
5.5kb
p257

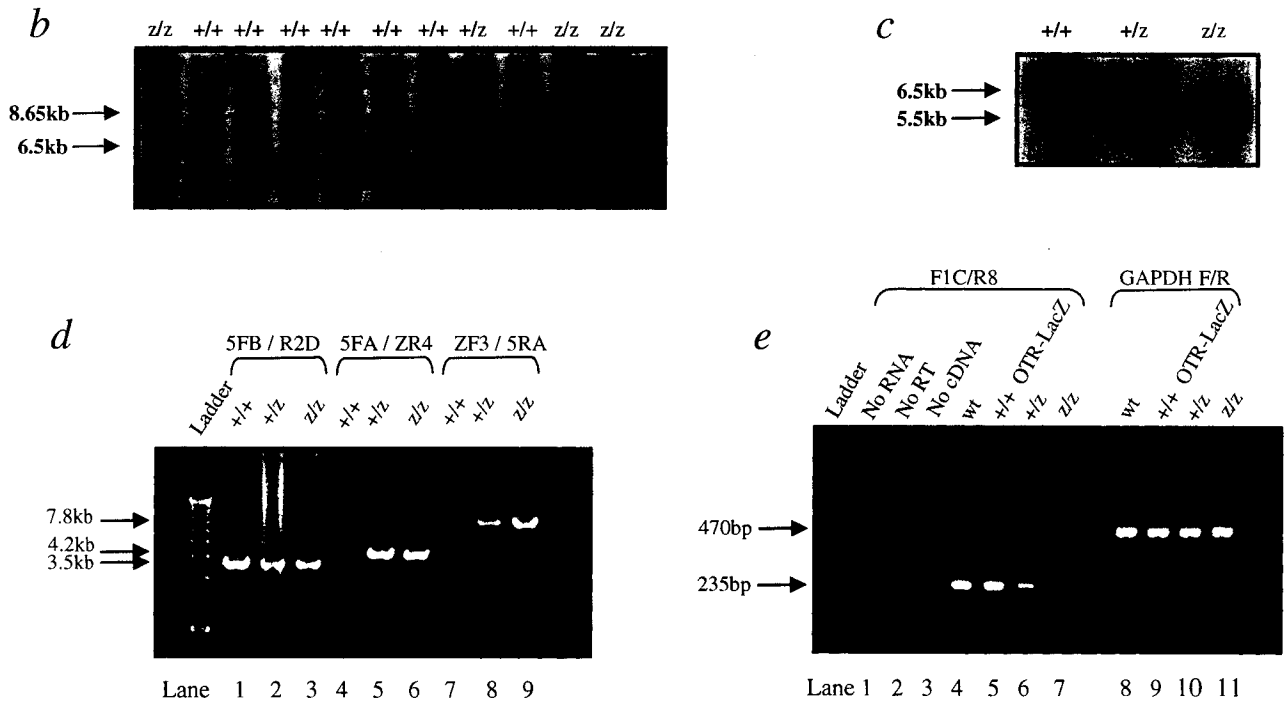

b
z/z +/+ +/+ +/+ +/+ +/+ +/+ +/z +/+ z/z z/z
8.65kb
6.5kb
c
+/+ +/z z/z
6.5kb
5.5kb
d
5FB / R2D 5FA / ZR4 ZF3 / 5RA
Ladder +/+ +/z z/z +/+ +/z z/z +/+ +/z z/z
7.8kb
4.2kb
3.5kb
Lane 1 2 3 4 5 6 7 8 9
e
F1C/R8 GAPDH F/R
Ladder No RNA No RT No cDNA wt +/+ OTR-LacZ +/z z/z wt +/+ OTR-LacZ +/z z/z
470bp
235bp
Lane 1 2 3 4 5 6 7 8 9 10 11

Figure 5.1 Generation of the OTR-LacZ reporter mouse. ***a***, Schematic of the pGNA targeting vector containing OTR gene sequences 5' to the coding region (5' sequence) and sequence downstream, within the 3rd intron (3^{rd} intron), inserted to direct homologous recombination. The OTR gene locus organization before and after site-specific plasmid insertion is depicted. Hybridization positions of probes for Southern blots (p1.7, p257, and pLac) are indicated by bars positioned over the sequence in the AccI digested DNA fragment detected by them. Positions of PCR primer hybridization are indicated by arrows in their 5' to 3' direction. ***b***, Mouse genotyping by Southern blot using p1.7 after AccI digestion. Arrows indicate the wild-type (**+**, 6.5 kb) and recombinant (**z**, 8.65 kb) alleles. ***c***, Southern blot using the p257 probe after AccI digestion. A 5.5 kb band was detected in +/z and z/z mice indicating that OTR coding sequence was still present (see ***a***). ***d***, PCR genotyping of DNA extracted from liver. The primer pairs used are indicated above the lanes. Sizes of amplification products are indicated at the left. The 3.5 kb band is indicative of an unmodified OTR gene structure. The 4.2 kb product in +/z and z/z samples indicates the correct insertion of the lacZ gene downstream of the OTR regulatory sequence. The 7.8 kb band seen in +/z and z/z samples demonstrates that the OTR coding sequence is present and downstream of the lacZ gene as depicted in ***a***. ***e***, RT-PCR detection of OTR mRNA in +/+, +/z, and z/z parturient mouse uteri. OTR primers F1C and R8 were used for detection of gene expression. The 235 bp amplicon confirms that coding sequence is present and intact OTR mRNA is produced at levels not significantly altered by the insertion of the 5'3'pGNA targeting vector. GAPDH primers F and R amplified a 470 bp product to which OTR gene expression levels were corrected.

LacZ Mapping of OTR Gene Expression in the Mammary Gland

LacZ expression was examined in the lactating mammary gland as a positive control to confirm OTR promoter regulated cell specificity of lacZ gene expression. Cryostat sections of mammary gland tissue from 12-hour post-partum +/+ and z/z mice stained with X-gal, a substrate for the β-galactosidase product of the lacZ gene, demonstrated blue crystal formation and hence lacZ expression in the myoepithelial cells of the ducts and alveoli of the z/z tissue (Figures 5.2*b*, *c*). This staining pattern corresponded to the well-established cell specific site of OTR gene expression in the mammary gland [241-243]. No X-gal staining was observed in wild-type controls (Figure 5.2*a*). Immunohistochemistry for smooth muscle actin on z/z mammary gland tissue further corroborated these findings (Figures 5.2*d-f*). These results confirm the OTR-LacZ reporter mouse model to be a faithful indicator of OTR gene expression.

Further, X-gal staining was observed in z/z mammary gland adipocytes, indicative of a novel site for OTR gene expression in mice (Figure 5.2*c*). This identification was confirmed by lipid staining with Oil Red O with nuclei indicated by hematoxylin counterstain (Figures 5.2*g-i*).

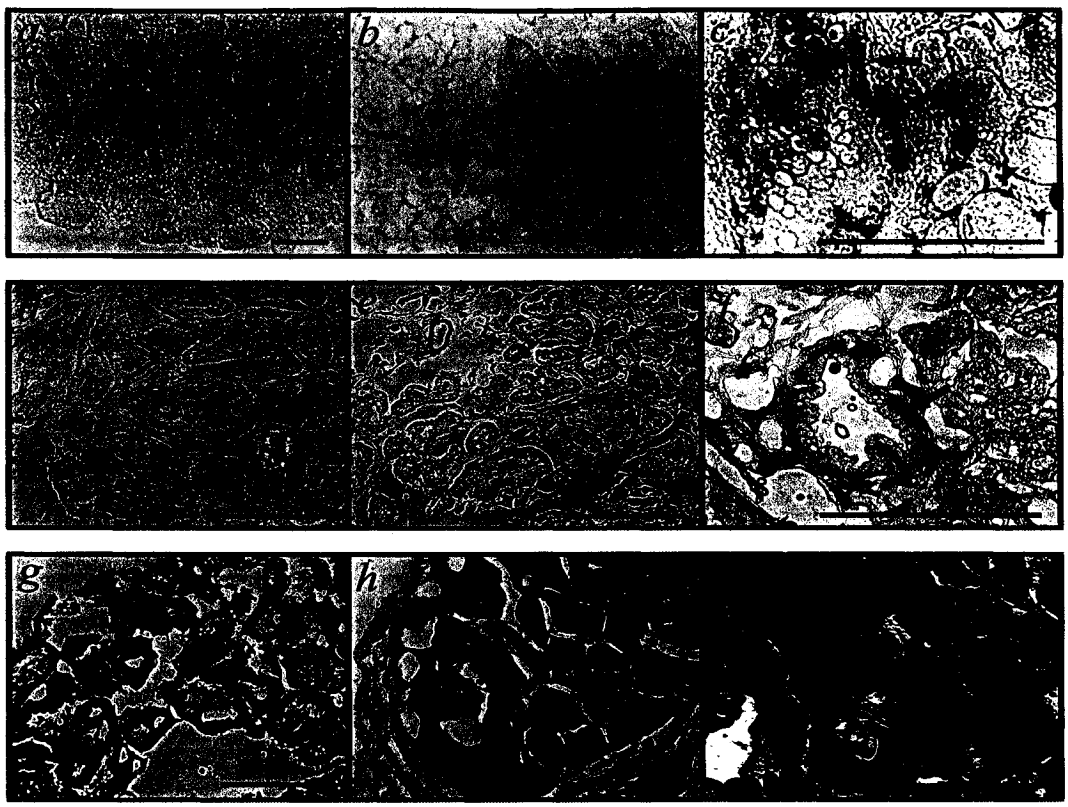


Figure 5.2 OTR gene expression in 12-hour post-parturient mammary gland tissue. ***a***, +/+ control; ***b*** - ***i***, z/z. Cryostat sections were 10μm thick for X-gal staining (***a-c***) and 5mm thick for smooth muscle actin (***d-f***) and Oil Red O staining (***g-i***). The arrows in ***c*** point to blue stained myoepithelial cells; the secreting glandular cells are unstained. Specific smooth muscle actin staining of myoepithelial cells is demonstrated (***e*** and ***f*** vs. isotype control, ***d***) and indicated by arrows in ***f***. The arrowhead in ***c*** indicates the stained cytoplasm of an adipocyte. Oil Red O stain for lipids demonstrates adipocytes (arrowheads in ***i***) amongst the ducts and alveoli of the mammary gland. Hematoxylin counterstain in *g-i* distinguishes these structures. Control tissue (***g***) was treated only with hematoxylin stain. Bars indicate approximately 250μm.

LacZ Mapping of OTR Gene Expression in the Brain

Sectioned brains of 12-hour post-parturient +/z and +/+ mice were stained with X-gal. Neurons demonstrating X-gal substrate conversion to blue crystals by the ß-galactosidase lacZ gene product and hence reflecting OTR gene expression in these nuclei were detected throughout various regions of the brain. Again, no staining was detected in the wild-type controls.

In the telencephalon, strong staining was observed in the accessory olfactory bulb and the anterior olfactory nucleus indicating high levels of OTR gene expression. The piriform cortex layers 2 and 3 also stained strongly (Figure 5.3*a*) and this level was maintained throughout its more caudal aspects as well. The inset photo in Figure 5.3*a* demonstrates that the ß-galactosidase is specifically expressed in neuronal cell bodies. No staining was observed in the control mouse (Figure 5.3*b*). In contrast to the intense staining of the piriform cortex, the medial septum and the ventral aspect of the lateral septum (Figure 5.3*c*) exhibited a few, specific, moderately stained neurons. More ventrally, the central region of the medial preoptic nucleus (Figure 5.3*d*) exhibited some staining. Within the amygdala, staining was moderate to strong. Nuclei in which neurons were stained in this region were the posterolateral and posteromedial cortical amygdala regions (Figure 5.3*e*) and the posterior aspect of the basomedial amygdala nucleus. More caudally, the parasubiculum, medial and lateral entorhinal cortices all stained for lacZ gene expression at high levels. No staining was detected in the hippocampus or the bed nucleus of the stria terminalis.

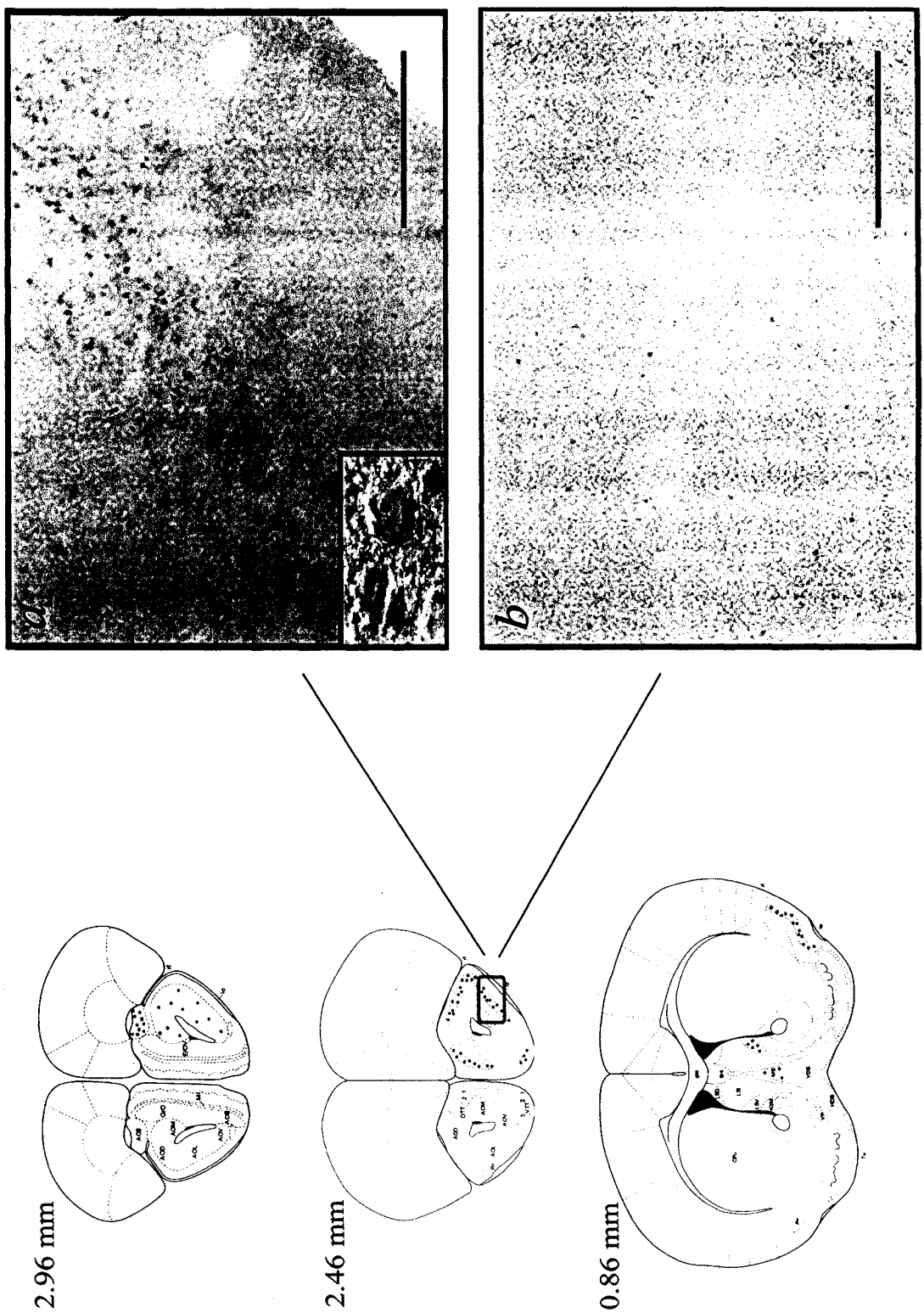
Several diencephalic regions demonstrated ß-galactosidase expression. Many hypothalamic nuclei were intensely stained. Of particular note, these were the dorsomedial and ventrolateral aspects of the ventromedial hypothalamic nucleus, as well as the compact subnucleus of the dorsomedial hypothalamic nucleus. Aspects of the mammillary nucleus were stained moderately, including the supramammillary nucleus medial area and the dorsotuberomammillary nucleus. There was no X-gal detection within the thalamus.

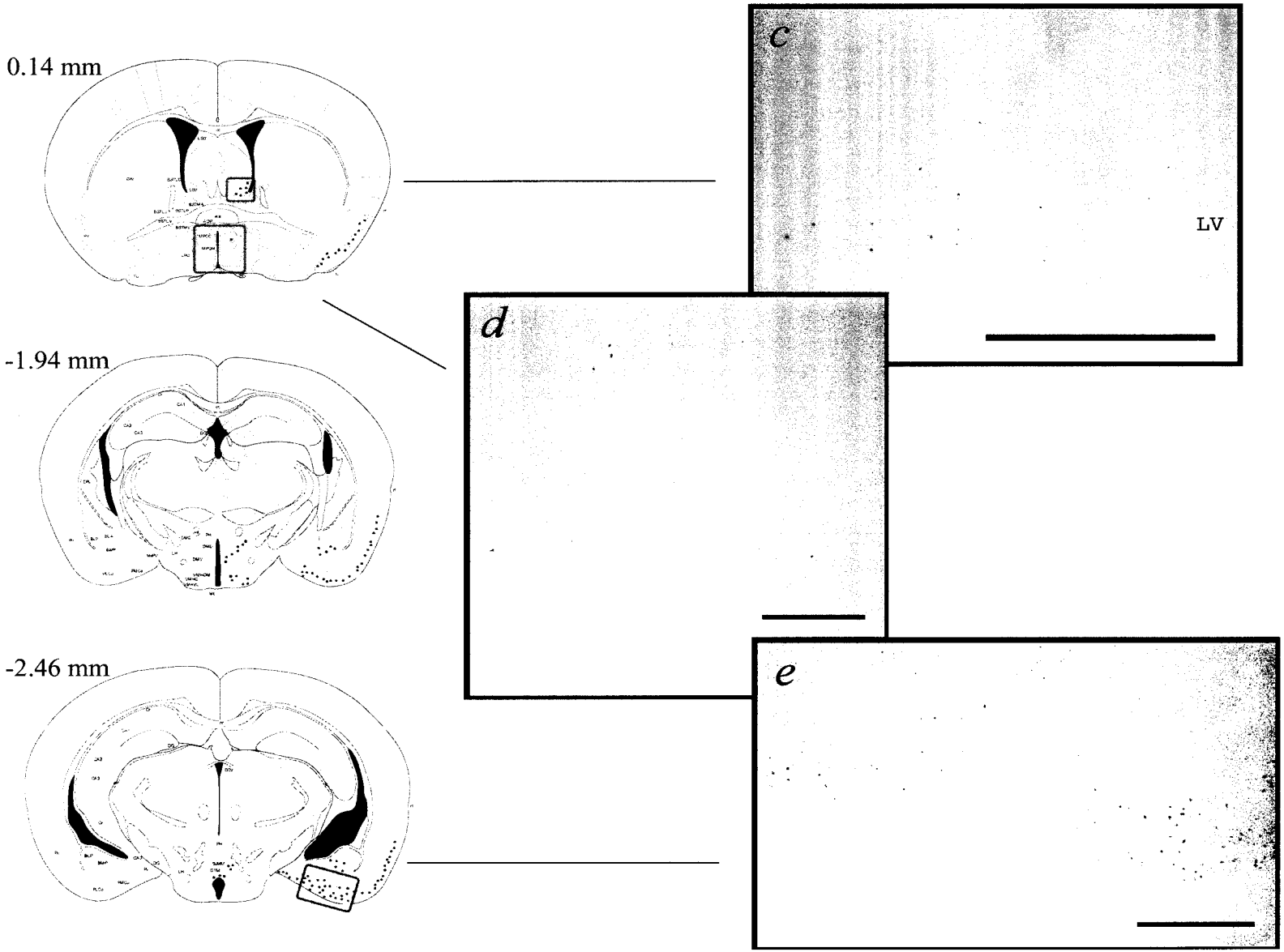
Numerous regions within the pons, medulla, and brainstem expressed the lacZ gene. Weak to moderate staining was detected in many areas including the

reticulotegmental nucleus (Figure 5.3*f*). This level of staining was also found in the laterodorsal tegmental nucleus, the central and pericentral regions of the dorsal tegmental nucleus, and the interfascicular area of the dorsal raphe nucleus (Figure 5.3*g*). The facial nucleus was strongly stained (Figure 5.3*h*) while neurons of the hypoglossal nucleus weakly expressed the lacZ gene. The vestibulocerebellar nucleus weakly expressed the gene but the medial and spinal vestibular nuclei were more intensely stained. Most subdivisions of the nucleus of the solitary tract exhibited strong X-gal labelling of neurons. These regions include the dorsolateral part of the solitary nucleus, the commissural, central, ventrolateral and ventral solitary nuclei (Figure 5.3*i*). The lateral reticular nucleus and the dorsal aspect of the medullary reticular nucleus both demonstrated high levels of β-galactosidase in many neurons (Figure 5.3*j*). This was true also of the dorsomedial spinal trigeminal (5) nucleus and the interpolar and caudal parts of the spinal trigeminal nucleus (Figure 5.3*j*).

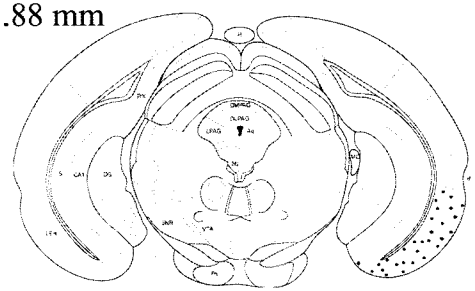
These results are summarized in the schematics of Figure 5.3 and in Table 5.1.

Figure 5.3

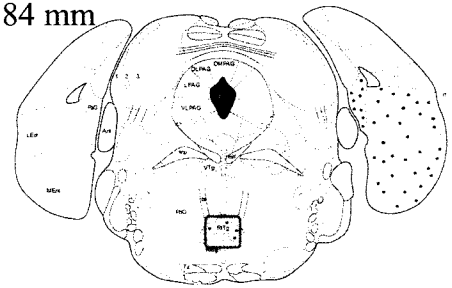



0.14 mm
-1.94 mm
-2.46 mm
c
LV
d
e

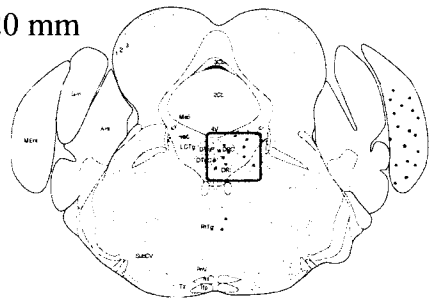
-3.88 mm

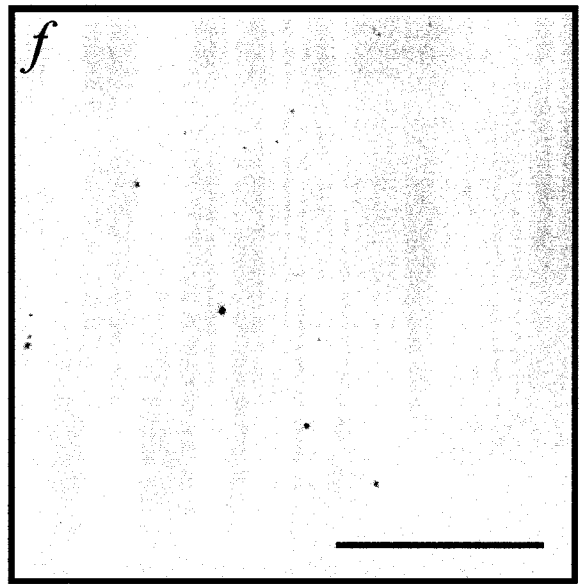


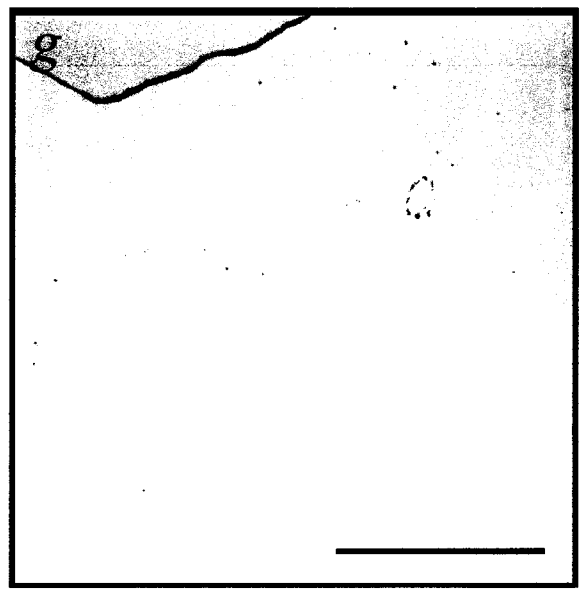
- 4.84 mm



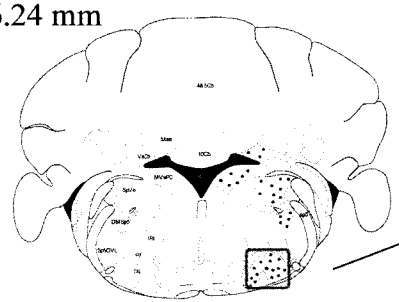
-5.20 mm



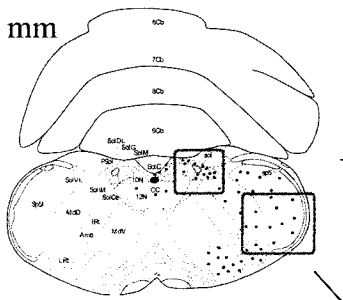




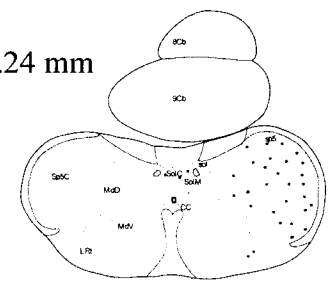
-6.24 mm

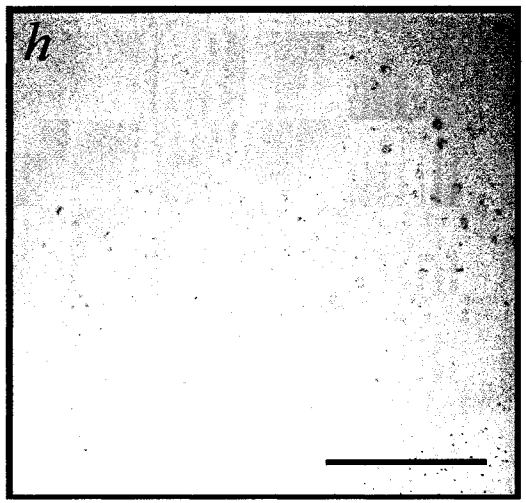


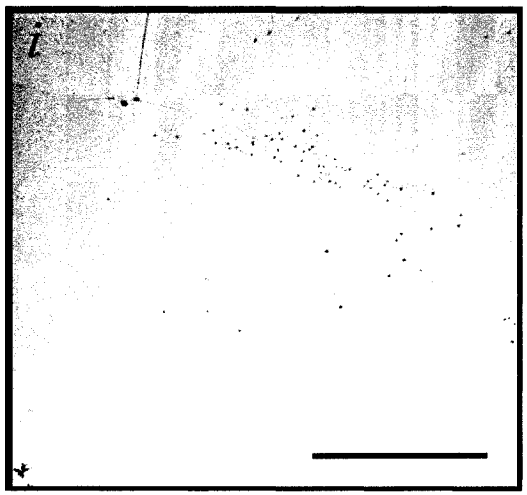
-7.48 mm



-8.24 mm







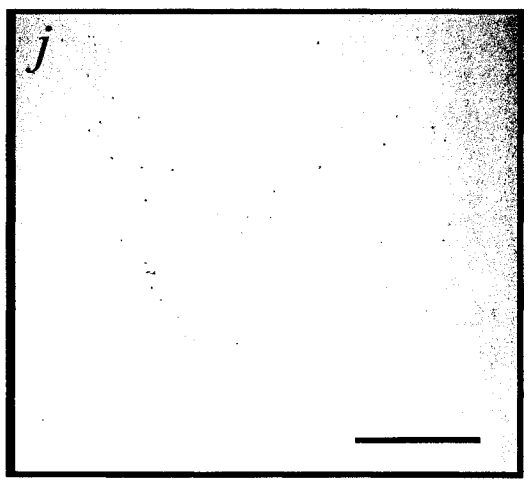


Figure 5.3 Mapping of OTR-promoter driven lacZ gene expression in neurons as detected by X-gal histochemical staining based on the atlas by Paxinos and Franklin (2001). Neurons demonstrating X-gal substrate conversion to blue crystals by the ß-galactosidase lacZ gene product are indicated by dots (•) in the schematics to the left. Abbreviations of stained regions are defined in Table1. Numbers at upper left of images indicate the anteroposterior distance of the section plane from the Bregma reference point. Microscopic images of selected regions are to the right. Boxes on schematics outline the corresponding areas. Cryostat sections were 10 µm thick. ***a***, piriform cortex layers 2 and 3, Bregma level 2.46 mm, inset photo demonstrates staining in neuronal cell bodies; ***b***, control mouse piriform cortex layers 2 and 3, Bregma level 2.46 mm, confirming specificity of lacZ gene expression; ***c***, lateral septal nucleus ventral aspect, Bregma level 0.14 mm; ***d***, medial preoptic nucleus central region, Bregma level 0.14 mm; ***e***, posterolateral and posteromedial cortical amygdala nuclei, Bregma level –2.46 mm; ***f***, reticulotegmental nucleus of pons, Bregma level –4.84 mm; ***g***, laterodorsal tegmental nucleus, dorsal tegmental nucleus central and pericentral regions, and the interfascicular part of the dorsal raphe nucleus, Bregma level –5.20 mm; ***h***, facial nucleus, Bregma level –6.24 mm; ***i***, solitary tract subnuclei: dorsolateral part of the solitary nucleus, the commissural, central, ventrolateral and ventral nuclei, Bregma level –7.48 mm; ***j***, spinal trigeminal (5) nucleus interpolar part, dorsal aspect of the medullary reticular nucleus, and the lateral reticular nucleus. Bars indicate approximately 250 µm. Digital microscopic photographs were taken and figures were produced by cropping and magnifying the respective areas and adjusting the contrast.

Table 5.1

Post-Parturient Mouse Brain Regions Expressing the OTR – LacZ Gene

	ABBREVIATION*	REGION	INTENSITY
Telencephalon			
Δ□	AOB	accessory olfactory bulb	+++
■ □	AON	anterior olfactory nucleus, all aspects	+++
■ □	Pir	piriform cortex, layers 2 and 3	+++
Δ	MS	medial septal nucleus	+
■ □	LSV	lateral septal nucleus, ventral	+
▲□	PLCo	posterolateral cortical amygdala nucleus	+++
■ □	PMCo	posteromedial cortical amygdala nucleus	++
▲	BMP	basomedial amygdala nucleus, posterior	++
Diencephalon			
▲	MPOC	medial preoptic nucleus, central	+
■ □	VMHVL	ventromedial hypothalamic nucleus, ventrolateral	+++
■	VMHDM	ventromedial hypothalamic nucleus, dorsomedial	++
■	DMC	dorsomedial hypothalamic nucleus, compact	+++
▲	SuMM	supramammillary nucleus, medial	++
▲	DTM	dorsotuberomammillary nucleus	++
■	PaS	parasubiculum	+++
▲	MEnt	medial entorhinal cortex	+++
▲	LEnt	lateral entorhinal cortex	+++
Mesencephalon			
Δ	LDTg	laterodorsal tegmental nucleus	++
Δ	DTgC	dorsal tegmental nucleus, central	+
Δ	DTgP	dorsal tegmental nucleus, pericent	+
Δ	RtTg	reticulotegmental nucleus of pons	+
▲	DRI	dorsal raphe nucleus, interfascicular	+

(cont...)

Table 1 (cont.)

	ABBREVIATION*	REGION	INTENSITY
	Pons, Medulla, and Brain Stem		
▲	VeCb	vestibulocerebellar nucleus	+
▲	MvePC	medial vestibular nucleus, parvicellular	++
■	MVe	medial vestibular nucleus	++
▲	SpVe	spinal vestibular nucleus	++
Δ	7N	facial nucleus	+++
▲	SolI	nucleus of solitary tract, interstitial	++
▲	PSol	parasolitary nucleus	++
▲	SolDL	solitary nucleus, dorsolateral part	+++
▲	SolC	nucleus of solitary tract, commissural	+++
▲	SolCe	nucleus of solitary tract, central	+++
▲	SolVL	nucleus of solitary tract, ventrolateral	+++
▲	SolV	solitary nucleus, ventral	+++
■	12N	hypoglossal nucleus	+
■	LRt	lateral reticular nucleus	+++
Δ	MdD	medullary reticular nucleus, dorsal	++
▲	DMSp5	dorsomedial spinal 5 nucleus	++
▲	Sp5I	spinal 5 nucleus, interpolar part	++
▲	Sp5C	spinal 5 nucleus, caudal part	+++

Table 1 Post-Parturient Mouse Brain Regions Expressing the OTR–LacZ Gene. The relative intensity of X-gal staining reflects a combination of the number of neurons stained in a region and the degree of blue crystal formation in the individual neurons. Relative intensity of staining: **+++** strong; **++** moderate; **+** weak. * According to Paxinos and Franklin (2001). Symbols to the left of the abbreviations indicate the following: Δ regions never previously shown to contain OTR mRNA expressing neurons; ▲ regions more accurately defined as containing OTR mRNA expressing neurons; ■ regions previously shown in rat or sheep to contain OTR mRNA expressing neurons; □ regions previously shown to contain OT binding sites in the mouse (male or female).

5.5 DISCUSSION

Development of the OTR-LacZ Reporter Mouse

Homologous recombination between the 5'3'pGNA targeting vector and the mouse OTR gene locus occurred without the deletion of the endogenous OTR gene. This targeted insertion placed the lacZ gene under the control of the entire and intact 5' flanking sequence of the OTR gene. Southern blot analysis using the probe to the OTR coding region (p257) as well as PCR genotyping results provided support for the hypothesis that "rolling circle" insertion of the non-linearized 5'3'pGNA plasmid within the 5'OTR sequence had occurred. This event was not detected within the ES cells prior to blastocysts injection.

The results of the RT-PCR analysis of parturient mouse uterus total RNA indicate that while the level of OTR expression from this displaced OTR gene in z/z mice is somewhat reduced compared to that of wild-type mice it is at a sufficient level for normal parturition to take place.

LacZ Mapping of OTR Gene Expression in the Mammary Gland

The development of OT knock-out mice [4, 5] has clearly demonstrated the imperative action of OT in the ejection of milk from the mammary gland. This action is mediated by the OTR. OTRs have been shown to be present on the surface of the myoepithelial cells [241-243] and the number of OTRs present in the mammary increase progressively during gestation and labour and are maintained throughout lactation at high levels [244, 245]. ß-galactosidase activity detected specifically in the myoepithelial cells of the mammary tissue of 12-hour post-parturient OTR-LacZ mice confirmed that lacZ gene expression was appropriately regulated by the OTR promoter.

In addition, the identification of ß-galactosidase activity within the cytoplasm of neighbouring adipocytes represents the first evidence of OTR gene expression in mouse adipocytes and adipocytes of the mammary gland. OTR expression has been shown in the murine preadipocyte 3T3-F442A cell line [246] and in numerous studies with rat adipocytes [247, 248]. The preponderance of evidence indicates that OT, like

insulin, stimulates glucose oxidation and lipogenesis in normal rat adipocytes involving a membrane-mediated protein kinase C (PKC) inhibition of hormone sensitive lipase [247].

LacZ Mapping of OTR Gene Expression in the Brain

Numerous studies of OTR receptor distribution within the brain have been performed in several species including rats [249-253], voles [254], humans [255], and meriones, a desert rodent [256]. The distribution of OT binding sites in mice has been investigated but primarily within forebrain regions [234-236, 257]. *In situ* hybridization studies of OTR mRNA distribution, however, have focussed predominantly on the rat brain [258-261]. The present study is the first investigation of the localization of cells within the mouse brain that express the OTR gene. The OTR-LacZ reporter mouse model provided confirmatory evidence of OTR gene expression in nuclei previously identified in the rat and sheep [262]. This model also allowed us to reveal several regions, many of them within the hindbrain, for which this is their first description as possessing OTR gene expressing neurons.

Areas previously revealed to contain OTR mRNA expressing neurons include aspects of the anterior olfactory nucleus, the piriform cortex layers 2 and 3. Neurons of the lateral septal nucleus ventral region, the posteromedial cortical amygdala, the ventrolateral and dorsomedial aspects of the ventromedial hypothalamus and the compact part of the dorsomedial hypothalamic nucleus were also determined to express the OTR gene in mice. This is in agreement with previous studies [258-262]. Confirmatory results were found for more caudal regions including the parasubiculum, medial vestibular nucleus, lateral reticular nucleus, and the hypoglossal nucleus.

More importantly, this mouse model allowed us to identify OTR gene expressing neurons in regions that have not been described previously. These new sites include the accessory olfactory nucleus, the medial septal nucleus, and the facial nucleus. Dorsal tegmental nuclei were also identified for the first time; the reticulotegmental, the laterodorsal, and central aspect of the dorsal tegmental nuclei

demonstrated blue neurons. The dorsal aspect of the medullary reticular nucleus was determined to be a novel site of OTR mRNA expressing neurons.

The specific and thorough demarcation of OTR gene expressing neurons afforded by this mouse model is further exemplified by the detection of blue staining within specific subnuclei of areas more generally described as possessing OTR gene expressing neurons. Greater accuracy of identification and cytoarchitectural localization was demonstrated in the amygdala where the posterolateral cortical region and the posterior aspect of the basomedial region were specifically indicated. Several diencephalic regions were more precisely identified as well. In the medial preoptic area, the OTR-LacZ reporter mouse enabled the demonstration of OTR expressing neurons specifically within its central aspect. Mammillary nuclei are known to express OTR mRNA; however, in the present study we were able to identify more precisely neurons within the medial region of the supramammillary nucleus and within the dorsotuberomammillary nucleus. OTR mRNA had been identified within the entorhinal cortex of male rats [259]. We have further localized OTR gene expression to neurons of the lateral and medial entorhinal cortices of this mouse. In the female rat, OTR mRNA was demonstrated within the dorsal raphe nucleus [260]. With the OTR-LacZ reporter mouse we were able to more accurately localize OTR gene expression to the interfascicular part of this nucleus.

Improved accuracy of identification of OTR gene expressing neurons was seen for numerous hindbrain regions. While the sheep *in situ* hybridization study indicated OTR mRNA expression within the vestibular region [262], this OTR-LacZ mouse enabled specific localization of this expression to be within neurons of the vestibulocerebellar nucleus, the spinal vestibular nucleus, and the parvicellular cells of the medial vestibular nucleus. These specific vestibular sites are in addition to the medial vestibular nucleus, previously identified in rats [259, 260]. Enhanced precision of mapping was also exemplified by the detection of blue staining within specific subnuclei of both the nucleus of the solitary tract and the spinal trigeminal nucleus. While the *in situ* hybridization study of female sheep brain OTR mRNA distribution suggested low levels of transcript in the nucleus of the solitary tract [262], we have

identified high levels of expression within distinct subnuclei. This delineation encompasses the parasolitary nucleus and the interstitial, dorsolateral, ventrolateral, ventral, central, and commissural divisions of the nucleus of the solitary tract. The rat did not exhibit any detectable levels of OTR mRNA within the nucleus of the solitary tract through *in situ* hybridization studies [259, 260]. The studies instead demonstrated low levels of mRNA for the vasopressin receptor, V1bR, which may mediate the functions of OT in this area of the brain. As well, in the rat mRNA studies, the spinal trigeminal nucleus was identified but with the OTR-LacZ mouse we were able to describe OTR gene expression specifically within the dorsomedial and the interpolar and caudal parts of this nucleus.

Several of these newly identified OTR gene expressing nuclei and subnuclei possessed only a few stained neurons. This supports the OTR-LacZ reporter mouse model as being advantageous over studies utilizing the traditional *in situ* hybridization technique. The absence of background staining and the readily identifiable blue chromogen allows the unambiguous identification of individual cells, even in isolation, with a precision that is not achievable with traditional *in situ* hybridization techniques. The specificity of the staining in individual OTR expressing cells defines a more accurate and comprehensive microstructure of the oxytocinergic system in the mouse brain.

There are nuclei that are notably unstained in this mouse model. Areas indicated as OTR mRNA expressing in rats yet not detected in the OTR-LacZ reporter mouse include the mitral cells of the olfactory nucleus, the olfactory tubercle, the thalamus, the hippocampal subfields CA1-CA4 and dentate gyrus, the bed nucleus of the stria terminalis, the central amygdala, and the ventral tegmental area. In this regard, the OTR gene expression distribution in the mouse more closely resembles that of the sheep [262]. This represents another example of the interspecies variation in the distribution of OTR mRNA expression. The absence of OTR mRNA in these areas, well known for their involvement in maternal behaviour in rats suggests an explanation as to why the oxytocinergic system has little apparent influence on maternal behaviour in mice [79]. It must also be reminded that this study focused on

the distribution within the brain of the post-parturient female mouse and that changes in gonadal steroid levels have been recognized to influence the pattern of OTR mRNA expression within the brain [76, 234, 250, 258, 263].

Concordance occurs between the distribution of the lacZ expressing neurons of the OTR-LacZ reporter mouse and documented OT binding sites of the female forebrain of a polygamous species of mouse (*P. maniculatus*) [235]. These regions of agreement are the anterior olfactory nucleus and the lateral septum. In addition, OTR binding sites provided by Tribollet et al. [236], from females of ICR and STR/N mouse strains, co-localize with OTR promoter-driven lacZ mRNA expression sites within the anterior olfactory nucleus, ventral part of the lateral septum, posterolateral cortical amygdala nucleus, and ventromedial hypothalamic nucleus.

Discrepancies are also evident between these studies, as we have not found lacZ gene expressing neurons in many regions described as possessing OTR binding sites. These regions include the olfactory tubercle, the hippocampus, and the central and lateral amygdala regions. These mouse OT binding sites are in agreement with rat mRNA and OT binding studies. This incongruity may result from differences in hormonal status between the virgin adult females used in the binding study and the post-partum OTR-LacZ reporter females as well as the differences between the mouse species, in general. This phenomenon of mRNA-protein discordance in localization has been documented previously [256, 260, 262]. It likely reflects the fact that mRNA is produced and remains within the neuronal cell body while the protein may be present either in the cell body, on distal dendrites, or may also be transported along the axons for presentation, and hence detection, in other regions of the brain. Little OTR binding information is available for the mouse brain caudal to Bregma –2.92 mm.

The exact role the OTR may play in the mouse brain requires further investigation. What is known about these regions may provide direction for these future studies. For example, OTR gene expression has been identified in neurons of olfactory areas, in the piriform cortex, in entorhinal cortices, and in the parasubiculum. Axonal connections between these regions transmit and process olfactory cues. These regions are important for memory processing. The entorhinal regions are specifically

involved in memory retention and recognition of prior experience. The presence of OTR gene expression in neurons involved in this system suggests a role for OT in the transmission, processing, storage, and retrieval of memory inputs.

Additional novel sites of OTR gene expression have been identified by this reporter mouse in several mesencephalic and hindbrain nuclei. These nuclei have been characterized as important for locomotor control. The dorsal tegmental nuclei have been implicated in playing an essential role in generating head direction signals necessary for processing sensory input regarding spatial navigation of the animal [264]. These inputs may collaborate with those processed by the vestibular nuclei, sensing cues regarding the movement and position of the head in space. This develops a sense of self-motion in the animal that allows the control of both reflex and voluntary movements [265]. Further locomotor responses are provided by signals from the lateral reticular nucleus [266]. The presence of OTR gene expression in neurons of the dorsal tegmental, vestibular, and lateral reticular nuclei suggests a contribution of the oxytocinergic system to the transmission of locomotor inputs or responses.

This investigation of OTR gene expression patterns in the post-parturient mother suggests new influences of OT on maternal behaviour in the mouse. OTR gene expression has been identified in neurons of the hypoglossal, facial, and spinal trigeminal nuclei. The hypoglossal nucleus is a collection of motor neurons whose function includes the control of movements of the anterior part of the tongue. In the post-partum mother, coordinated movement of the tongue is necessary for the licking/grooming behaviour associated with maternal behaviour. In rats, this behaviour stimulates increased estrogen sensitivity and OTR gene expression in areas important for the establishment of maternal behaviour [267, 268]. These neurons also coordinate with those of the facial nucleus through reciprocal neuronal projections to synchronize oro-facial control of chewing and swallowing as well as breathing and vocalization [269]. The trigeminal neurons participate in these vocalisations [270] and other functions including nociception. Trigeminal projections to the olfactory bulb also suggest a role in processing olfactory cues, in the post-parturient mouse, most

likely for the discrimination of foreign pups. A role for the OTR in maternal behaviours of grooming, care, and protection of pups as well as in the communication with pups is suggested and requires further investigation.

5.6 CONCLUSION

The role of the OTR in mediating social and sexual behaviours as well as other physiological processes is only partially understood. By relating the location of OTR gene expressing neurons (as depicted by lacZ gene expression) to the function of those neurons, one may further elucidate the role of the oxytocinergic system in the mouse brain.

The OTR-LacZ reporter mouse allowed the identification of individual OTR gene expressing cells. This is an advantageous tool over *in situ* hybridization techniques that may detect similar, yet not identical mRNA sequences and does not have the technical simplicity and resolution this reporter mouse affords. By studying mice under different hormonal, immunological, and other physiological states, one may easily observe the responsive changes in OTR gene expression using simple X-gal histochemistry. This OTR-LacZ reporter mouse provides valuable information regarding the contribution of the OTR in the mouse.

5.7 ACKNOWLEDGEMENTS

We would like to thank Dr. Alan Peterson (Laboratory of Molecular Oncology, Royal Victoria Hospital, McGill University) for the gift of the pGNA vector and his expert advice. We also wish to thank Dr. Michel Tremblay (McGill Cancer Centre), Dr. Naima Bachnou and Ms. Priscila Valera for their valuable assistance in the development of the OTR-LacZ reporter mouse. B.R.G. was supported by a CIHR Studentship Award and H.H.Z. is a holder of the Wyeth-Ayerst Chair in Reproductive Endocrinology and of a Senior Scientist Award from the Canadian Institutes of Health Research (CIHR). The research was supported by grants from the CIHR and Wyeth Pharmaceuticals, Canada.

5.8 APPENDIX

Construction of the Mouse OTR Gene Targeting Vector

Using a genomic probe from the rat OTR gene a λ-dash 129/Sv mouse genomic library (a gift from Dr. A. Peterson's laboratory) was screened and OTR sequence was isolated. A 6 kb SstI digest fragment was cloned into pBluescript K/S (pBS). This fragment extends from 5' flanking sequence through the coding region and more than 3 kb into the final intron. A 3.9 kb KpnI digest fragment consisting of > 2kb of promoter and of 5' untranslated (UTR) sequence was cloned separately into pBS. This work is detailed in the Master's thesis of Caterina Russo [271], from our lab.

An oxytocin receptor-targeting vector was constructed to replace the OTR coding region with a lacZ reporter gene. This was performed such that the lacZ gene would be under the control of the endogenous OTR promoter in an OTR knock-out mouse. This vector required OTR gene sequences both 5' and 3' of the coding region as sites for homologous recombination.

The 5' sequence was isolated and subcloned into pBS using both the 6 kb SstI/pBS and the 3.9 kb KpnI/pBS vectors. The 3.9 kb KpnI fragment was excised and reintroduced into pBS at the SmaI site through blunt end ligation. This destroyed both restriction sites. This 3.9 kb fragment was not sufficient for our purposes, as we wanted to include as much of the 5' UTR as possible, getting as close to the translation start site as sequence would allow. To do this a fragment from the 6 kb SstI/pBS plasmid was isolated by digestion with DraI and then BamHI to liberate a further 500 bp. The 3.9 kb KpnI /SmaI pBS was digested with XbaI and blunt ended (site destroyed) and subsequently digested with BamHI. This liberated a 3.7 kb linear plasmid with a BamHI sticky end and a blunt end, a 200 bp BamHI fragment, and a 12 bp BamHI-XbaI fragment. The 500 bp BamHI-DraI fragment was ligated to the linearized plasmid resulting in a 4.2 kb 5' UTR fragment in the SmaI -XbaI site of pBS.

As the SmaI site is a unique restriction site in the pBS and digestion of this site results in blunt ends it was chosen as the site for vector linearization to allow

homologous recombination between the 5' and 3' sequences. To this end the 4.2 kb insert in pBS had to be moved clockwise of the SmaI site. It was released from its vector by EcoRI/NotI digest, blunt ended and ligated into a new pBS at its blunted SpeI site. Orientation of the fragment was checked by SacII digestion, which produced a 1.3 kb fragment, and a 5.8 kb fragment if the orientation was correct.

Next, the 3' sequence needed to be subcloned into this 4.2 kb 5'pBS vector. Using the 6kb SstI/pBS vector EcoRI digestion liberated an 850 bp fragment that included 800 bp of sequence from the 3rd intron and 50 bp of pBS sequence. The 4.2 kb 5'pBS vector was digested with EcoRI and treated with calf intestine alkaline phosphatase to dephosphorylate the sticky ends and prevent self-ligation. The 850 bp 3' EcoRI fragment was ligated into the vector counter clockwise of the SmaI site and the correct orientation was confirmed using AccI digestion of this 5'3'pBS plasmid. A 600 bp AccI fragment, as hoped, resulted.

These 5' and 3' sequences, along with their dividing SmaI site were then inserted into a lacZ- and neo^r- containing vector. For this purpose the 7.3 kb pGNA vector (a gift from Alan Peterson's laboratory) was employed. XbaI/ApaI digestion of both the pGNA vector and the 5'3'pBS vector allowed directional insertion of the targeting and linearization sequences into the pGNA vector with the 5' promoter and untranslated sequence upstream of the lacZ gene, hopefully driving its expression. This digestion and ligation also removed the SmaI site of the pGNA vector. This left the insert SmaI site as the sole SmaI site in this new 5'3'pGNA vector. This site was to be used to linearize the vector to allow proper recombination to occur at the OTR locus. Unfortunately, in time, it was shown that the 5'3'pGNA vector was not linearized at this SmaI site before being electroporated into 129/Sv mouse ES cells.

This work was completed by technicians Levon Fendekian and John Tam prior to my undertaking of this project.

The 5'3'pGNA OTR targeting vector was given to Naima Bachnou of Dr. Peterson's laboratory for ES cell electroporation and subsequent growth of neomycin-resistant recombinant ES cells.

Chapter 6

General Discussion and Conclusions

Until recent years, analysis of gene regulation focussed predominantly on investigating one or a few genes implicated in a physiological phenomenon at a time. Major milestones in scientific exploration are being achieved with the near-completion of genome sequencing for numerous organisms and technical improvements in printing representative gene sequences on glass for hybridization detection of expressed gene sequences (DNA Microarray Chips). Under the influence of an experimental model, the expression of thousands of identified gene sequences can be simultaneously recorded and subsequently compared. These global genome expression analyses are hypothesis generating providing a springboard for further discovery, identifying candidates for further investigation by more traditional methodologies. The work described in this thesis reflects the investigation of gene expression under the stimulus of parturition, normally at term and with pathologic preterm labour (PTL) models. The oxytocin receptor (OTR), a known participant in the process of labour was not represented on the microarray and we therefore investigated the regulation of the OTR gene independently. Further investigation of the relevance of this receptor in the control of parturition and maternal behaviour was performed by generating a lacZ reporter mouse with which we mapped the distribution of OTR gene expression in the brain.

6.1 MICROARRAY ANALYSIS OF GENE EXPRESSION

We have applied microarray technology to identify novel participants in the processes of normal and PTL and provide support and clarification to current theories. By increasing our understanding of these processes we may better predict, manage, and prevent premature parturition, the leading cause of neonatal disability and death.

Normal Labour

Gene expression levels in uterine tissue at estrus, at term (not in labour), in labour, and approximately 12 hours postpartum were analysed and compared. By our strict analysis, the Affymetrix U74Av2 murine oligonucleotide array of probe sets for

more than 12,000 genes indicated that 342 gene probe sets were detecting differential expression during this process. The most numerous changes were observed when comparing term gene expression to that at estrus. There were 139 probe sets reflecting significant gene induction and 53 reflecting repression of transcription. Many of the genes represented demonstrated the greatest changes in expression levels found in our analysis.

With labour, fewer genes showed dynamic changes in expression relative to estrus expression, either positively or negatively. Most of these were regulated to a lessened extent than at term. Genes more significantly up-regulated at the moment of parturition included most of the genes in Cluster A, genes well described in the process of labour: cFos, cyclooxygenase (COX)-2, connexin 43, insulin-like growth factor binding protein (IGFBP)10, IGFBP2, along with many others assigned to Cluster E. We found this pattern of expression for the oxytocin receptor (OTR) gene as well by separate determination of expression using Northern blot and real-time RT-PCR (Chapter 4). Chloride channel calcium activated (Clca) 3 gene (Cluster H) exhibited the most dramatic down-regulation of gene expression at labour. Clca3 is suggested to be immunoprotective against bacterial infection by contributing to the secretion of uterine mucins [164]. Additionally, we propose that similar to Clca1, Clca3 possesses an anti-apoptotic effect. The gene suppression reported would contribute to a shift in the cytokine milieu and promote a rejection of the fetus while apoptosis would block cell proliferation and uterine growth leading to a stretch response and CAP gene activation. Together, the loss of the Clca3 gene product, and subsequently protein, would facilitate parturition.

Postpartum, fewer genes were being induced and more were being suppressed relative to expression levels found at estrus. Estrus is considered day 0 of pregnancy and a significant number of changes are taking place in the uterus in preparation for potential blastocyst implantation. The observed increase in the number of repressed genes postpartum reflects a return of the uterus to a non-pregnant state commensurate with changes in ovary, the resumption of the estrus cycle.

This technique allowed us to identify novel genes likely to mediate normal parturition, which require further, individual analysis. They present themselves as potential candidates or targets for diagnosis or therapeutic intervention of PTL. Our analysis identified numerous genes involved in blood clotting and fibrinolysis. The most significantly induced genes at term include fibrinogen alpha and beta polypeptides. A significant drop in the expression of these genes was found at labour and a complete return to estrus gene expression levels was found 12 hours postpartum. As thrombin is a known uterotonin [131, 132], the involvement of this pathway in parturition requires further investigation.

Following a similar expression pattern are the genes for alpha fetoprotein (Afp) and apolipoprotein A1 (ApoA1). Both of these can affect estrogen availability and possess immunomodulatory effects, promoting a tolerant uterine environment for the developing semi-allogeneic fetus. The dramatic drop in both of these at labour and further postpartum may reflect the necessity for their functions. Estrogen is produced and released to activate downstream gene targets such as cFos and CAP genes while immune tolerance of the fetus is no longer maintained. The significant induction of numerous cytokines, interferon-responsive genes, and other inflammatory mediators during the period studied highlights the importance of the immune system to normal parturition. The suppression of liver arginase 1 (Arg1) gene expression by estrogen [122], seen in our analysis, and its reported induction by anti-inflammatory T_h2 cytokines [147] further connects the hormonal and immune influences to normal parturition.

Infection-Induced Preterm Labour

Intrauterine infection accounts for a significant proportion of premature deliveries. We extended our microarray analysis to identify the genetic factors and elucidate their participation in this phenomenon. We injected pregnant mice with a PTL-inducing dose of heat killed bacterium (10^{10}), with a low dose (10^7) that does not trigger labour, or with Luria broth as a control. We followed the dynamic changes in gene expression from early (3.5 hours after injection) to late (8 hours after injection) to

PTL in the case of high dose infection. Comparing the levels of gene expression detected by probe sets on the Affymetrix DNA chip using stringent analysis 204 probe sets passed, depicting significant changes in gene expression between appropriate comparisons. A significant number of genes were induced with the high dose of bacteria. This was evident even at the 3.5 hour time-point. By 8 hours, the numbers of probe sets reflecting gene regulation had increased and this was further so at the time of parturition. As well, a significant number were experiencing a suppression compared to levels found both with control and low dose treated mice. Many of the genes induced early continued to be significantly expressed at 8 hours and at PTL. A significant number of these genes were found in our study of normal labour and will be discussed below.

Of no surprise is the fact that more than a third of the genes identified by this analysis play a role in immune function. Interleukin (IL) induction of transcription factors C/EBP and NFκB activates the expression of immune mediators including GRO1, intercellular adhesion molecule-1, VCAM-1 and SAA3 [181] and induce cFos, COX-2, and OTR genes [49, 182, 183]. All of these genes were induced in our infection model of PTL. OTR gene expression was monitored by Northern blot and real-time RT-PCR (Chapter 4). Many of the genes with immune function also possess uterine growth regulatory functions. Inhibition of uterine growth and resulting uterine stretch by the growing fetus leads to the stimulation of IL-6, amplifying its induction further, along with the activation of CAP gene expression. Cytokine induction of calgranulin A and B gene products leads to their heterodimerization and subsequent function as a chemoattractant referred to as calprotectin. The increased amount of this calprotectin complex, normally growth promoting, now functions in growth inhibition and apoptosis [195, 196]. It may also contribute to an increase in prostaglandin synthesis by providing the substrate arachidonic acid to COX-2 [197]. The use of lycoricidinol (narciclasine), a potent inhibitor of calprotectin's pro-inflammatory and growth inhibitory effects [195] may prove useful for suppressing the immune response leading to premature labour. Lactotransferrin and SAA3 genes produce products that suppress an acute inflammatory response by contributing to the mucosal barrier of the

uterus, preventing bacterial attachment and growth. The induction of these genes suggests an attempt by the uterus to resist the cascade of events leading to parturition. Promoting the expression of these genes or the functions of their products may also promote the maintenance of gestation. Preventing the shift in cytokine balance from an anti-inflammatory T_h2 environment to a pro-inflammatory T_h1 acute phase response to bacterial infection would attenuate the activation of T_h1 cytokine-responsive genes and maintain the uterus in a state of quiescence. An IL-1 receptor antagonist has been suggested in the prevention of PTL induced by IL-1 [179]. The level of antagonist induced by this model is itself insufficient. Supplementation could prove beneficial.

Ovariectomy-Induced Preterm Labour

In order to clarify whether a hormonal component to infection-induced PTL exists we performed microarray analysis on a model of PTL arising in the absence of any infection for comparison. Bilateral ovariectomy (Ovx) was performed to mimic the progesterone withdrawal due to luteolysis that precedes term parturition in mice. We collected uterine tissue 5 hours after surgery and at the time PTL commenced to fully define the genes important to the process. Analysis of microarray data revealed that 79 gene probe sets represented genes that were differentially regulated in response to Ovx of pregnant mice. All but four genes were transcriptionally regulated at PTL, each of these four were specifically repressed at the 5 hour time point. These were small inducible cytokine (Scy) A2, Scyb2, RIKEN cDNA for mitogen inducible gene 6 protein homologue, and an expressed sequence tag (EST) (Genbank ID# AW047643). This suppression of cytokine genes suggests an attempt to prevent the premature switch in T_h2/T_h1 cytokines that promote fetal tissue rejection.

Transcription of genes for follistatin and regulator of G protein signalling (RGS) -2 and EST AI467657 (Genbank ID# AI553024) was repressed at 5 hours and further so at PTL identifying them as early players in the mechanism of Ovx-induced PTL. Follistatin is a negative regulator of activin functions. In the uterus, activin A promotes endometrial decidualization, inhibits angiogenesis, and participates in an

acute inflammatory response [212, 272] while in the ovary activin A regulates steroid biosynthesis and represses follistatin production, maintaining the corpus luteum of pregnancy [214]. The repression of follistatin gene expression would be permissive towards an inflammatory response. Additionally, the loss of a potential paracrine effect on the corpus luteum during late gestation would support activin stimulation of progesterone, maintaining uterine quiescence and pregnancy. In the case of Ovx, control of ovarian progesterone production is no longer required and follistatin gene transcription is no longer induced.

Most of the genes experiencing dynamic changes in transcriptional regulation identified in this model were found to be significantly regulated in another of our labour models. We also found, by Northern blot and real-time RT-PCR, that OTR gene expression is induced as early as 5 hours after Ovx. This stimulation was sustained at PTL. This gene induction is in response to the loss of progesterone and the resulting estrogen-dominant environment. The OTR promoter is responsive to both steroids and steroid- and cytokine-induced transcription factors [6, 49].

Genes Common to Multiple Mechanisms of Labour

Comparison of the probe sets representing genes undergoing significant regulation at the moment parturition occurs (labour vs. estrus levels; infection-PTL vs. C8; Ovx20-PTL vs. Sham20) revealed surprisingly little overlap (Figure 6.1).

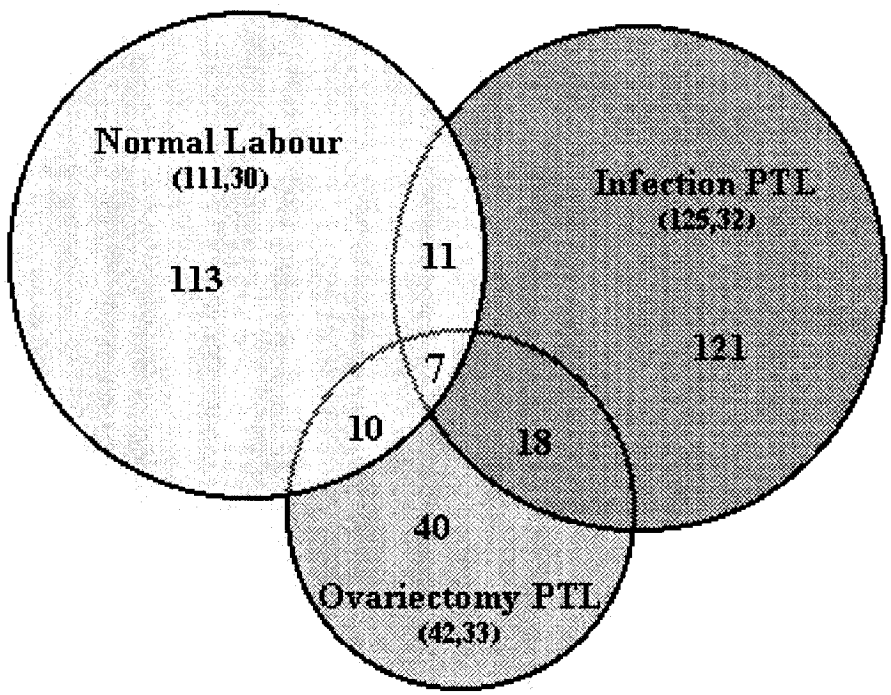


Figure 6.1 Comparison of the numbers of probe sets for genes significantly regulated in their expression at normal labour and PTL induced by infection and by ovariectomy. The number of probe sets demonstrating significant changes in gene regulation in each of the three conditions are indicated along with the number in each direction (up-regulated, down-regulated). Of the 141 probe sets depicting gene expression changes at normal labour 11 are common with infection-induced PTL, most of which appear to be oppositely regulated. There were 10 probe sets depicting gene expression changes common with Ovx-induced PTL. All were similarly regulated except for liver arginase 1 (Arg1) and Purkinje cell protein 4 (PCP4). Lipoprotein lipase was represented by two probe sets, both detecting gene changes in each condition. PTL by infection was accompanied by the regulation of genes represented by 157 probe sets, by Ovx, 75 probes represented gene regulation. Comparison of the probe sets at PTL by these two conditions revealed 18 to be unique to the mechanisms to PTL. Each probe set was similarly regulated in both models. There were 7 probe sets common to all three conditions of labour, all similarly regulated.

Common Genes Regulated at PTL Induced by Infection and Ovariectomy

The 18 probe sets representing significantly regulated genes common to PTL alone include many for genes of the small proline rich protein 2 family (Sprr2). Sprr2B, 2C, 2E, 2G, and 2I genes were up-regulated at PTL and all to the same extent relative to controls. The exact nature of the functions of these proteins is not clear. They are implicated in the differentiation of epithelial tissue [216]. In the context of PTL, these may function in uterine epithelial transformation during placental detachment and the return of the endothelium to a non-pregnant, receptive state. Sprr2A and 2C are expressed in normal parturition suggesting that different family members may accomplish the same goal. It is surprising that Sprr2C, a gene that is actually induced under all three conditions of labour should not produce a detectable protein [216]. The gene contains two in-frame non-sense mutations that would prematurely abort translation. While Song et al. reported an absence of transcript in uterine tissue the detection of expression by the microarray probe set indicates that this gene is transcribed, suggesting that its probe set is specific for 5' Sprr2C gene sequences. The importance of this gene and its family members in the process of parturition requires further investigation.

The genes for cytokines transforming growth factor (TGF) β and macrophage-colony stimulating factor (M-Csf/Csf1) were both down-regulated at PTL, as detected by their probe sets. TGBβ is an anti-inflammatory cytokine and the repression of its gene would promote an acute phase response. M-Csf/Csf1 attracts circulating macrophages to the site of inflammation and promotes the proliferation of resident macrophages in the uterine endometrium. It also exhibits a proliferative effect on the endometrium [218]. A significant suppression of M-Csf in the decidua is associated with spontaneous, recurrent abortion [17]. Its loss during PTL could contribute to diminished uterine growth. Increased stretch by the fetus would participate in the activation of CAP genes and hence uterine contraction in the presence of uterotonins. The induction of this gene by both estrogen and progesterone [219] further links the hormonal and inflammatory mechanisms in parturition.

The regulator of G protein signalling (RGS) genes -2 and -16 were also uniquely regulated in PTL. Inflammation induces RGS-16 gene transcription while suppressing RGS-2 gene expression [220]. The functions of their protein products on an inflammatory response appear to be at opposition; their similarly opposite regulation would contribute to the cumulative repression of an inflammatory response. RGS-16, along with pro-inflammatory cytokines exhibit inotropic effects on muscle contraction [222]. The role of RGS-2 in regulating muscle contraction is unclear as it is able to attenuate both cAMP production leading to a tonic state and a T cell proliferative environment [221]. As well, signalling through the OTR is prevented by RGS-2, blocking down-stream events necessary for contraction [223]. Together the effects of the RGS proteins appear to be the induction/permission of contractions, which are of insufficient force to expel the fetus. Identifying the uterine cell types expressing these genes and their products may allow the nature and importance of these factors in PTL to be clarified.

Aquaporin (Aqp) 8 gene suppression was also common to these models at the moment PTL occurs. Aquaporins are membrane channel proteins facilitating the transport of large quantities of water. Aqp8 has been identified in the endometrial stroma and in the myometrium. It is believed to function in re-distributing water accumulating in the myometrium in response to estrogen-stimulated edema, throughout the stroma [273]. The loss of this function by the suppression of Aqp8 gene expression could result in an increase in myometrial intracellular pressure and contribute to the activation of parturition via the stretch mechanism. In the process of normal labour Aqp4 is suppressed at term and at labour relative to estrus levels. While different aquaporin genes are being suppressed at the time of parturition it seems a common mechanism of decreased water transport from the uterus leads to swelling and stretch, promoting parturition.

Down syndrome candidate/critical region (Dscr) 1 gene is induced in the myometrium of spontaneous term labouring ewes; induction of PTL with betamethasone had the same inductive effect [274]. This induction is dependent upon COX-2 expression, is amplified by uterine stretch (which is ineffective on its own),

but is not affected by estrogen or progesterone, alone or in combination [274]. In our mouse model of normal labour the Dscr1 gene was not differentially regulated. This absence could reflect high Dscr1 mRNA levels at estrus and a lack of significant variation in mRNA levels between term, labour, and postpartum therefore negating any calculated increase with parturition. This requires further analysis and highlights the need for a variety of sample comparisons in microarray analysis of gene expression. Conversely, this discrepancy may reflect different transcriptional regulation between the species. At both infection- and Ovx-induced PTL Dscr1 gene transcription was reported as up-regulated by microarray analysis. The pattern of Dscr1 expression suggests it is involved in myometrial activation with a proposed classification as a CAP gene. It is also called myocyte-enriched calcineurin interacting protein 1 or Calcipressin and is described to function by inhibiting calcineurin-mediated transcription [113]. The nature of its involvement in activating the parturient uterus remains to be characterized.

Genes Regulated at Normal and Ovariectomy-Induced Preterm Labour

The 10 probe sets reflecting gene expression common to both normal labour and ovariectomy-induced PTL represent 9 unique genes; lipoprotein lipase gene is represented by two probe sets and both reflect an induction at parturition. Lipoprotein lipase is expressed on the endothelial surface of tissues where, as a key enzyme in the break down of triglycerides and uptake of free fatty acids by tissues, provides a source of energy and cellular lipids for cell maintenance and function [275]. It is also essential to the synthesis of apolipoproteins for the formation of HDL molecules and facilitating HDL-cholesterol complex uptake by the liver [276]. Its induction during normal and Ovx-induced PTL would provide an increased supply of energy to sustain uterine contractions. Its absence at infection-induced PTL could reflect a negative transcriptional regulation by cytokines as is found in adipose tissue [275].

The transcription factor SRY-box containing gene 4 (Sox4) was also induced at labour in both conditions. The regulation of Sox4 expression was demonstrated to be under the influence of both estrogen and progesterone. Several studies, however,

show conflicting responses. Uterine expression analysis of non-pregnant ovariectomized mice indicated estrogen or progesterone alone caused a decrease in Sox4 transcript levels while a combination treatment had a much lessened effect [277]. A study using virgin estrogen receptor α knock-out mice injected with estrogen, however, demonstrated a lack of alteration in uterine Sox4 mRNA levels [122]. An investigation of human SOX4 gene regulation in normal and malignant breast cells described its transcription to be rapidly induced by progesterone [278]. In human myometrial cells, SOX4 gene regulation was not significantly changed during parturition [118]. It appears that Sox4 gene regulation is dependent upon species, tissue type, and hormonal milieu.

In contrast to the observation by Bethin et al. of an absence of Sox4 expression in the non-gravid mouse uterus, Hunt and Clarke find the Sox4 gene to be moderately expressed at all stages, peaking at estrus when estrogen and progesterone levels are lowest [277]. We also found moderate levels of Sox4 gene expression at estrus. In the pregnant mouse uterus, we observed the expression of the Sox4 gene to be further up-regulated at labour and postpartum, with declining progesterone levels. The increase found here is similar to that found by Bethin et al. [118].

In ovariectomized pregnant mice we found a 3-fold increase in Sox4 expression at the time of PTL. As the corpus luteum of pregnancy is the significant source of progesterone necessary for the maintenance of pregnancy, removal of the ovary leads to a sudden drop in progesterone. As the ovary is also a source of estrogen, a drop in levels of this hormone is expected. Estrogen is supplemented by the placenta [19] and possibly the uterus itself (Chapter 2 discussion). As with normal parturition, we find the estrogen:progesterone balance shifting in favour of estrogen dominance. It therefore is not surprising to find the Sox4 gene to be similarly regulated at the moment of parturition. In the scenario of murine parturition, this supports a negative regulatory effect of progesterone on uterine Sox4 gene expression. An influence by estrogen remains to be established. Sox4 transcription factor is believed to activate the expression of CAP genes as the promoters of both the murine OTR and connexin 43 (Cx43) genes contain potential Sox4 binding sites [118]. As

the OTR and Cx43 genes can also be activated by estrogen, we suggest that estrogen also induced Sox4. Estrogen stimulation of cFos expression leads to increased transcription factor induction of target CAP genes ultimately leading to contractions. The expression pattern of Sox4 may implicate the transcription factor in activating the uterus and sustaining the uterine contractions necessary for uterine involution post-partum.

There is also evidence that Sox4 transcription factor is expressed in thymic T cells facilitating, but not essential to, the development of T cells [279]. Sox4 gene is expressed in pre-B cells in the bone marrow of mice; Sox4 homozygous knock-out mice experience a block in B cell development [277]. This gene is not significantly regulated during infection-induced PTL, likely since other factors responsible for increasing the production of these cells are abundant. In the context of labour without infection, the uterine tissue may heighten sensitivity to fetal antigens by the induction of Sox4 and increasing lymphocyte numbers or responsiveness. Sox4 may contribute to the rejection and expulsion of the fetus by multiple routes. The importance of this transcription factor in the process of parturition requires further investigation.

Connexin 26 expression is repressed under both conditions as expected since it is under the regulation of progesterone and imparts a relaxatory effect on the uterus during gestation [33, 34].

Liver arginase 1 (Arg1) and Purkinje cell protein 4 (PCP4) genes are oppositely regulated at parturition in these two models. Their expression is down-regulated at normal parturition but up-regulated with Ovx-induced PTL. Arg1 gene expression is negatively regulated by estrogen [122]; therefore, as estrogen levels rise to a crescendo at labour one would expect the down-regulation of Arg1 transcription, as we have found. The role of Arg1 appears to be one of immunomodulation as T_h2 cytokines up-regulate the gene and lead to a block in T-cell proliferation [147]. Immunotolerance is lifted with increased estrogen, leading to a fetal tissue rejection and expulsion of the offspring. Arg1 has also been attributed to stimulating proliferation of smooth muscle cells [148]. A suppression of Agr1 with increased estrogen levels would contribute to limiting uterine growth and facilitating the stretch

response. The induction of this at PTL by Ovx suggests a lessened estrogen inhibition. Ovx removes a source of circulating estrogen as well as a major source of progesterone. The net balance between them shifts to a greater influence by estrogen. Perhaps it is their ratio, rather than a direct influence by estrogen, which affects Arg1 expression. The induction of Arg1 gene expression at Ovx-PTL may be an attempt to quell a mounting rejection of fetal tissue and prevent a uterine stretch response from triggering uterine contraction. Arg1 absence from our infection study is not surprising as a T_h1 cytokine milieu dominates preventing the up-regulation by T_h2 cytokines.

PCP4 gene product is described as a neuron-specific transcript [158, 159] but we suggest otherwise. As an inhibitor of calmodulin activity [160], it may function to prevent calcium/calmodulin-dependent activation of myosin light chain kinase, altering myometrial contractility during gestation. We found significant suppression of gene expression during the final stages of normal labour relative to high levels at estrus. This would alleviate the block to a myometrial contractile response. The induction of PCP4 gene expression at PTL in response to Ovx above those levels we suggest to be present during gestation for myometrial non-responsiveness (to be determined experimentally) suggests an attempt to prevent uterine contractions stimulated by the altered hormone environment. A hormonal regulatory effect remains to be seen.

As PCP4 is described to have an anti-apoptotic effect on neuronal cells [161], the repression of this gene during normal parturition would restrict uterine cell proliferation, stimulating a stretch response and uterine activation. The stimulation of this gene at PTL in response to Ovx would inhibit calcium-mediated cell death, promoting uterine growth and again attempting to maintain gestation, rebelling against the effects of Ovx. The therapeutic possibilities of this gene product require analysis.

Genes Regulated at Normal and Infection-Induced Preterm Labour

There were 11 probe sets representing significantly regulated genes common only to normal and infection-induced PTL. Interferon-induced protein with tetratricopeptide repeats gene 2 (Ifit2)/glucocorticoid attenuated response gene 39

(GARG-39), serum amyloid A3 (SAA3) gene, and the insulin-like growth factor binding protein 10 (IGFBP10) gene were similarly induced at term parturition and infection-induced PTL relative to their controls. Ifit2/GARG-39 is induced by LPS and interferons to promote an inflammatory response [188] while SAA3, induced by pro-inflammatory cytokines increases mucin production, protecting the tissue from bacterial invasion while inhibiting neutrophil oxidative bursts, the migration of neutrophils, monocytes, and T cells, and cell adhesion to components of the extracellular matrix [204]. During infection both Ifit2/GARG-39 and SAA3 gene expression was up-regulated 3.5 hours after high dose treatment and was sustained to the time of PTL, seemingly in competition. During normal labour Ifit2/GARG-39 gene induction reached significant levels at labour and increased postpartum promoting fetal rejection and expulsion as well as contribution to uterine remodelling. SAA3 gene expression peaked at term and dropped nearly 50% at the time labour occurred and experienced a further suppression postpartum. The patterns of Ifit2/GARG-39 and SAA3 expression during normal labour suggest a permissive effect on an immune response leading to rejection. PTL, occurring at approximately 15 d.p.c., is not beneficial to the survival of the organism and therefore factors capable of suppressing an inflammatory response and prolonging gestation are called to action. In the case of a high dose of bacteria (10^{10}), this defence fails.

Some of the most significantly induced genes found in our normal labour study are the most significantly repressed genes in response to infection leading to PTL. Fibrinogen alpha (FbgA), alpha fetoprotein (Afp), apolipoprotein A1, and H19 mRNA genes were all induced to impressive levels at term. This was somewhat diminished at labour and was insignificantly different from estrus levels by 12 hours postpartum. These 4 genes were unaffected by high dose bacterial infection until the moment PTL commenced. At this time, each gene experienced a significant repression of transcription. While it would appear that they are oppositely regulated under these two conditions, they are in fact experiencing a repression of transcription at the time of labour relative to levels preceding parturition. Term labour occurs 4 days later than our induced PTL model. The levels of transcripts vary significantly at these different

stages of gestation; however, the change in regulation of these genes as parturition ensues is the same. This suggests these genes, their products having immunoprotective, growth promoting, and estrogen balance promoting effects, would contribute, through their loss, to fetal rejection and uterine remodelling, uterine stretch and CAP gene activation leading to contractions and delivery.

Cyclin dependent kinase inhibitor 1C (P57) gene experiences this same repression from high levels at term to moderate at labour to insignificantly different from that at estrus postpartum while experiencing a sustained repression with high dose infection as early as 3.5 hours after treatment to PTL. The product of this gene is a negative regulator of cell proliferation and its high levels at term might promote a stretch response, which is no longer necessary once labour has begun while a down-regulation with infection may be a compensatory mechanism trying to avert uterine stretch.

The absence of a significantly altered expression of these genes at Ovx-PTL suggests, in accordance with the little overlap between all conditions, that parturition can be achieved in response to various stimuli via divergent pathways. This raises the importance of elucidating the mechanism of each as separate entities. The common regulators of these mechanisms may provide a means to halt the progression of PTL regardless of stimulus.

Regulated Genes Common to All Three Manners of Parturition

Only seven probe sets representing genes that were dynamically expressed at parturition regardless of mechanism were found. These genes include COX-2, cFos, and Cx43. Northern blot and real-time RT-PCR confirms the OTR gene to be induced under each condition. The presence of increased numbers of these transcripts is of no surprise to normal labour but confirms a common mechanism of CAP gene activation by transcriptional activation as well as stretch and supports an involvement of estrogen even in infection-induced PTL.

The probe set for plasminogen activator inhibitor-1 (PAI-1) (a.k.a. Serpine1) gene is common to all models and the gene is induced most significantly at the time of parturition. PAI-1 is protective against blood clot dissolution by preventing the conversion of plasminogen to plasmin, the factor responsible for lysing fibrin and fibrinogen. Fibrinogen degradation products are inhibitors of thrombin uterotonic activity [130-132]. The laying down of blood clots at parturition leads to decreased blood flow to the fetus, triggering a hypoxic stress response in the fetus. This stress stimulates increased fetal production of cortisol and prostaglandins (among other factors beyond the scope of this study), which lead to the fetal promotion of uterine CAP gene expression and uterine activation. Wound healing promoted by clot formation activates the infiltration of leukocytes and increases cytokine production. Digestion of necrotic tissue takes place in an inflammatory environment leading to an acute rejection of the fetus. PAI-1 may be central to this common mechanism, which ties blood clotting, hormone biosynthesis, immune activation, and even a fetal contribution to the process of parturition.

The regulation of many of the genes identified and the functions they fulfill provide a link between hormonal influences and inflammatory effects in the course of normal and premature parturition.

Our discussion could not possibly cover the expression and function of each gene represented in our lists of probe sets reflecting gene regulation throughout the processes of parturition nor have we identified every regulated member of the transcriptome. The Affymetrix mouse oligonucleotide microarray U74Av2 does not

possess probe sets for every sequence transcribed. This is evidenced by the absence of and OTR gene probe set as well as an IL-8 probe set. Additionally, several genes we expected to find in our infection study, such as TGFβ and IFNγ, did not pass our criteria for significance. As suggested for IFNγ, this may reflect a defect in the microarray probe set for this gene. Multiple probe sets for a single gene occasionally experienced different levels or patterns of regulation and suggest cross hybridization of similar sequences or competitive hybridization between complimentary stretches of target cRNAs. Cluster assignment of probe sets by GeneCluster software [108] was not always consistent in grouping together those with similar expression patterns. Next generation clustering software tools may provide a better organization of genes similarly regulated and therefore facilitate the detection of expression patterns important to the process studied.

This draws to focus the importance of a reliable statistical model in analysing the data generated by microarray analysis. Currently, there are numerous tools available for processing and analysing the data ranging from ArrayStat to Signifigene, to Bioconductor, to GeneSpring, and the list goes on. The problem with this excessive selection is that the results arising from a single microarray data set using different analytical methods often are quite variable. Which is the correct analysis result? Should we use them all and only report the regulation of a gene as significant if its probe set data passes each analysis as significant? Where do we draw the line for statistical significance? We chose to use DNA-Chip Analyzer (dChip) version 1.2 developed by Li and Wong [105] and applied PM-only analysis [106]. We set a significance level of a 2-fold change between time-points analysed with a $p < 0.05$ for each replicate and compared the probe set lists for reproducible results. Each investigator determines their own method for analysis and reporting of results. This makes interpretation and comparisons difficult.

Increasing the difficulty of comparing results by different analysis methods is the problem of comparing the output from different microarray platforms. Platforms range from nylon membranes printed with cDNA, to glass chips printed with cDNA sequences, to glass chips printed with oligonucleotide sequences as used in our

analysis. Each has their benefit. Affymetrix oligonucleotide arrays contain probe sets to detect the expression of thousands of genes simultaneously increasing the discovery rate. Because of the different means of processing the RNA and measuring and reporting their abundance, comparison between the data is tenuous. In an attempt to translate experimental results across different microarray platforms statistical models are being developed [280]. Additionally, genes reported to be significantly regulated in response to the stimulus of choice require confirmation by traditional methodologies including Northern blot, RT-PCR, in situ hybridization, antisense knock-down, knock-out, and transgenic mice. The benefit of microarray technology is that it can provide a large number of candidates for further inspection and stimulate the generation of new hypotheses relevant to a phenomenon.

With regard to our animal models, comparison with results by Bethin et al. [118] and by Muhle et al. [2], highlight the necessity for valid replicates and multiple time-point comparisons to properly define the regulation pattern of a gene. Even the use of different strains of pathogenic E.coli, and animal manipulation techniques led to different progression rates to PTL (20 hours with infection, 27 hours by Ovx [2] vs. 8 hours and 20 hours, respectively, in our analysis) further confounding cross-study comparison.

In the face of difficulties to interpretation and comparison of microarray technology, the wealth of knowledge to be generated by them is enormous. In our quest for a better understanding of the role of known players in the process of parturition and elucidation of novel mediators we have focussed the spotlight on factors involved in hormone biosynthesis and function, immune modulators in the process of the rejection of semi-allogeneic fetal tissue, blood clotting and wound repair, and suggested that while the preterm uterus submits to the effectors of parturition in these models, it is not without a fight. Exploiting the gene products suggested to have an inhibitory effect on uterine contraction and parturition and developing inhibitors against those with stimulatory effects, the development of more effective therapeutics is possible. The recognition of gene expression patterns in

advance of parturition may allow additional screening measures and earlier detection of PTL, allowing earlier and more successful intervention therapy.

It is important, now, to further investigate the nature of the effects these candidates possess. Immunohistochemistry is currently being performed for several gene products to identify the uterine cell types in which they are expressed. Individual studies of therapeutic and diagnostic candidate genes and proteins should also be performed both in vitro and in vivo. ELISA analysis of serum obtained from each of the mice can also be used to clarify the influence of progesterone in each of these models to parturition. Together, these studies have identified numerous potential mediators in the process of normal and preterm parturition in response to bacterial infection and ovariectomy and suggest several means to preventing the deleterious outcome of PTL.

6.2 OTR GENE EXPRESSION IN THE MOUSE

In the pursuit of investigating the role of the OTR in parturition in light of the fact that the knock-out of its ligand, oxytocin (OT), does not lead to a parturition defect and yet is considered the most uterotonic agent known, and the fact that the OTR gene is not represented on the microarray we used, we analysed the expression of the OTR by more traditional techniques. Northern blot, real-time RT-PCR, and our development of an OTR-lacZ reporter mouse [281] allowed us to monitor the expression of this gene under the influence of parturition. Uterine gene expression was induced in advance of term parturition and PTL induced by infection and by Ovx. The OT knock-out mice exhibited a dependence on this hormone for milk ejection defect [4, 5]. In the mammary gland, by histochemistry, our lacZ reporter mouse confirmed faithful depiction of cell specific OTR gene expression and supporting the relationship between these partners. Our examination extended to the brain of postparturient mice to study the central regulation of parturition and influence on maternal behaviour necessary for the survival of the offspring. LacZ histochemistry of sectioned brain tissue demonstrated the expression of the OTR gene in regions previously defined in other species, or suggested by radioligand binding, in many

cases a more accurate delineation was provided. We identified numerous novel sites of OTR gene expression in the mouse brain and proposed, based on the known functions and interconnections of these nuclei, a role for the OTR in these. We propose the OTR, based on the neural distribution of gene expression, to participate in maternal behaviours such as scent recognition of pups, licking/grooming, and vocal communication, as well as supporting memory processing, retention, and retrieval, and the transmission of locomotor inputs.

While there was some incongruity between sites of expression previously described for the rodents, sheep, and humans, we suggest this is attributed to species, sex, and hormonal status differences between the models analysed. This variation further supports a regulation of this gene that is important across species. Histochemical staining of mouse brain sections allowed the precise identification of individual neurons expressing the lacZ gene, representing locations of OTR gene expression, supporting theories to a role in maternal, sexual, and social behaviours while suggesting novel roles for the oxytocinergic system. The OTR-lacZ reporter mouse is a useful tool in easily identifying novel sites of OTR gene expression. It can be exploited to measure gene expression changes in response to various stimuli further clarifying the role of this receptor in physiological processes.

Together, these studies have enabled the identification of novel mediators of parturition and presented clarification of the involvement of known mediators, as well as providing insight into the behaviours important for the survival of the species.

Claim of Original Contribution to Knowledge

This thesis has presented the following novel findings...

- ✧ We have identified novel genes demonstrating dynamic regulation during the process of parturition many of which had not previously been described to be expressed in the uterus at any time (i.e. PCP4).
- ✧ We characterized the timing and pattern of the induction and/or suppression of these genes.
- ✧ We identified common mechanisms to parturition regardless of stimulus, suggesting that hormone biosynthesis and availability, blood clotting and wound healing, inflammation and rejection all contribute to the activation of CAP gene expression for uterine activation and stimulation of uterine contractions for the expulsion of the fetus.
- ✧ We suggest several potential therapies to prevent or inhibit premature parturition.
- ✧ OTR gene transcription is up-regulated in advance of parturition and this is a common feature of normal and premature parturition induced by infection or by ovariectomy. Our data indicate a varying dependence upon OTR gene expression in the three models in light of other, unique mediators, providing explanation as to why the OTR antagonist atosiban has a limited inhibitory effect on premature labour.
- ✧ This is the first mapping of OTR gene expression in the mouse brain providing an accurate and comprehensive microstructure of the oxytocinergic system in the early postpartum maternal brain. We suggest novel roles for the OTR in several identified nuclei.
- ✧ We identified, for the first time in mice and in the mammary gland, the expression of the OTR gene in adipocytes.

References

1. Slatterly, M.M. and J.J. Morrison, *Preterm delivery.* The Lancet, 2002. **360**: p. 1489-1497.
2. Muhle, R.A., et al., *A high-throughput study of gene expression in preterm labor with a subtractive microarray approach.* American Journal of Obstetrics & Gynecology, 2001. **185**(4): p. 716-724.
3. Mussali, G.M., et al., *Inflammatory Cytokines in a Murine Model of Infection-Induced Preterm Labor: Cause or Effect?* Journal of the Society for Gynecologic Investigation, 1999. **6**(4): p. 188-195.
4. Nishimori, K., et al., *Oxytocin is required for nursing but is not essential for parturition or reproductive behavior.* Proceedings of the National Academy of Sciences of the United States of America, 1996. **93**: p. 11699-11704.
5. Young, W.S., et al., *Deficiency in mouse oxytocin prevents milk ejection, but not ferility or parturition.* Journal of Neuroendocrinology, 1996. **8**: p. 847-853.
6. Gimpl, G. and F. Fahrenholz, *The Oxytocin Receptor System: Structure, Function, and Regulation.* Physiological Reviews, 2001. **81**(2): p. 629-683.
7. Kimura, T., et al., *What knockout mice can tell us about parturition.* Reviews of Reproduction, 1999. **4**(2): p. 73-80.
8. Jackson Laboratory, *Biology of the Laboratory Mouse - by the Staff of the Jackson Laboratory.* second ed, ed. E.L. Green. 1966, New York: McGraw-Hill Book Company. 706.
9. Mahesh, V.B., *The dynamic interaction between steroids and gonadotropins in the mammalian ovulatory cycle.* Neuroscience and Biobehavioral Reviews, 1985. **9**(2): p. 245-260.
10. Telfer, E., R.G. Gosden, and M.J. Faddy, *Impact of exogenous progesterone on ovarian follicular dynamics and function in mice.* Journal of Reproduction & Fertility, 1991. **93**(2): p. 263-269.
11. Paria, B.C., et al., *Deciphering the Cross-Talk of Implantation: Advances and Challenges.* Science, 2002. **296**: p. 2185-2188.

12. Kao, L.C., et al., *Global Gene Profiling in Human Endometrium during the Window of Implantation.* Endocrinology, 2002. **143**(6): p. 2119-2138.
13. Kao, L.C., et al., *Expression Profiling of Endometrium from Women with Endometriosis Reveals Candidate Genes for Disease-Based Implantation Failure and Infertility.* Endocrinology, 2003. **144**(7): p. 2870-2881.
14. Yoshioka, K.-i., et al., *Determination of Genes Involved in the Process of Implantation: Application of GeneChip to Scan 6500 Genes.* Biochemical & Biophysical Research Communications, 2000. **272**: p. 531-538.
15. Reese, J., et al., *Global Gene Expression Analysis to Identify Molecular Markers of Uterine Receptivity and Embryo Implantation.* The Journal of Biological Chemistry, 2001. **276**(47): p. 44137-44145.
16. Thellin, O. and E. Heinen, *Pregnancy and the immune system: between tolerance and rejection.* Toxicology, 2003. **185**: p. 179-184.
17. Piccinni, M.-P., *T-cell Cytokines in Pregnancy.* American Journal of Reproductive Immunology, 2002. **47**: p. 289-294.
18. Steinborn, A., H. Gunes, and E. Halberstadt, *Signal for Term Parturition is of Trophoblast and therefore of Fetal Origin.* Prostaglandins, 1995. **50**: p. 237-252.
19. Challis, J.R.G., et al., *Endocrine and Paracrine Regulation of Birth at Term and Preterm.* Endocrine Reviews, 2000. **21**(5): p. 514-550.
20. Longbottom, E.R., et al., *The effects of inhibiting myosin light chain kinase on contraction and calcium signalling in human and rat myometrium.* Pflugers Archiv - European Journal of Physiology, 2000. **440**: p. 315-321.
21. Sanborn, B.M., *Uterine contractility symposium: The Litchfield Lecture. Hormones and calcium: mechanisms controlling uterine smooth muscle contractile activity.* Experimental Physiology, 2001. **86**(2): p. 223-237.
22. Mesiano, S., et al., *Progesterone withdrawal and estrogen activation in human parturition are coordinated by progesterone receptor A expression in the myometrium.* Journal of Clinical Endocrinology & Metabolism, 2002. **87**(6): p. 2924-2930.

23. Schumacher, M., et al., *Genomic and membrane actions of progesterone: implications for reproductive physiology and behavior.* Behavioural Brain Research, 1999. **105**(1): p. 37-52.

24. Salmi, A., M. Ammala, and E.M. Rutanen, *Proto-oncogenes c-jun and c-fos are down-regulated in human endometrium during pregnancy: relationship to oestrogen receptor status.* Molecular Human Reproduction, 1996. **2**(12): p. 979-984.

25. Weisz, A. and F. Bresciani, *Estrogen induces expression of c-fos and c-myc protooncogenes in rat uterus.* Molecular Endocrinology, 1988. **2**(9): p. 816-824.

26. Yamashita, S., A. Takayanagi, and N. Shimizu, *Temporal and cell-type specific expression of c-fos and c-jun protooncogenes in the mouse uterus after estrogen stimulation.* Endocrinology, 1996. **137**(12): p. 5468-5475.

27. Bani, D., et al., *Relaxin up-regulates the nitric oxide biosynthetic pathway in the mouse uterus: involvement in the inhibition of myometrial contractility.* Endocrinology, 1999. **140**(10): p. 4434-4441.

28. Ohleth, K.M., et al., *Relaxin increases insulin-like growth factors (IGFs) and IGF-binding proteins of the pig uterus in vivo.* Endocrinology, 1997. **138**(9): p. 3652-3658.

29. Hall, J.A., et al., *Influence of Ovarian Steriods on Relaxin-Induced Uterine Growth in Ovariectomized Gilts.* Endocrinology, 1992. **130**(6): p. 3159-3166.

30. Cerro, J.A. and J.E. Pintar, *Insulin-like Growth Factor Binding Protein Gene Expression in the Pregnant Rat Uterus and Placenta.* Developmental Biology, 1997. **184**: p. 278-295.

31. Lenhart, J.A., et al., *Expression of connexin-26, -32, and -43 gap junction proteins in the porcine cervix and uterus during pregnancy and relaxin-induced growth.* Biology of Reproduction, 1999. **61**(6): p. 1452-1459.

32. Ciray, H.N., et al., *Presence and localization of connexins 43 and 26 in cell cultures derived from myometrial tissues from nonpregnant and pregnant*

women and from leiomyomas. American Journal of Obstetrics and Gynecology, 2000. **182**(4): p. 926-930.

33. Orsino, A., C.V. Taylor, and S.J. Lye, *Connexin-26 and connexin-43 are differentially expressed and regulated in the rat myometrium throughout late pregnancy and with the onset of labor.* Endocrinology, 1996. **137**(5): p. 1545-1553.
34. Ou, C.W., A. Orsino, and S.J. Lye, *Expression of connexin-43 and connexin-26 in the rat myometrium during pregnancy and labor is differentially regulated by mechanical and hormonal signals.* Endocrinology, 1997. **138**(12): p. 5398-5407.
35. Grummer, R., O. Traub, and E. Winterhager, *Gap junction connexin genes cx26 and cx43 are differentially regulated by ovarian steroid hormones in rat endometrium.* Endocrinology, 1999. **140**(6): p. 2509-2516.
36. Geimonen, E., et al., *Elevated Connexin-43 Expression in Term Human Myometrium Correlates with Elevated c-Jun Expression and Is Independent of Myometrial Estrogen Receptors.* Journal of Clinical Endocrinology & Metabolism, 1998. **83**(4): p. 1177-1185.
37. Ku, C.-Y. and B.M. Sanborn, *Progesterone Prevents the Pregnancy-Related Decline in Protein Kinase A Association with Rat Myometrial Plasma Membrane and A-Kinase Anchoring Protein.* Biology of Reproduction, 2002. **67**: p. 605-609.
38. Kirkland, J.L., L. Murthy, and G.M. Stancel, *Progesterone inhibits the estrogen-induced expression of c-fos messenger ribonucleic acid in the uterus.* Endocrinology, 1992. **130**(6): p. 3223-3230.
39. Salmi, A. and F.-M. Rutanen, *C-fos and c-jun expression in human endometrium and myometrium.* Molecular & Cellular Endocrinology, 1996. **117**: p. 233-240.
40. Mitchell, J.A. and S.J. Lye, *Differential expression of activator protein-1 transcription factors in pregnant rat myometrium.* Biology of Reproduction, 2002. **67**(1): p. 240-246.

41. Shynlova, O.P., et al., *Regulation of c-fos expression by static stretch in rat myometrial smooth muscle cells.* American Journal of Obstetrics & Gynecology, 2002. **186**(6): p. 1358-1365.
42. Cook, J.L., et al., *Expression of Myometrial Activation and Stimulation Genes in a Mouse Model of Preterm Labor: Myometrial Activation, Stimulation, and Preterm Labour.* Endocrinology, 2000. **141**(5): p. 1718-1728.
43. Ou, C.W., et al., *Increased expression of the rat myometrial oxytocin receptor messenger ribonucleic acid during labor requires both mechanical and hormonal signals.* Biology of Reproduction, 1998. **59**: p. 1055-1061.
44. Lefebvre, D.L., et al., *Myometrial transcriptional regulation of the gap junction gene, connexin-43.* Reproduction, Fertility, & Development, 1995. **7**(3): p. 603-611.
45. Pierce, B.T., et al., *Connexin 43 expresion in normal versus dysfunctional labor.* American Journal of Obstetrics & Gynecology, 2002. **186**(3): p. 504-511.
46. Geimonen, E., et al., *Activation of protein kinase C in human uterine smooth muscle induces connexin-43 gene transcription through an AP-1 site in the promoter sequence.* Journal of Biological Chemistry, 1996. **271**(39): p. 23667-23674.
47. Mitchell, J.A. and S.J. Lye, *Regulation of connexin43 expression by c-fos and c-jun in myometrial cells.* Cell Communication and Adhesion, 2001. **8**(4-6): p. 299-302.
48. Rozen, F., et al., *Structure, characterization, and expression of the rat oxytocin receptor gene.* Proceedings of the National Academy of Sciences of the United States of America, 1995. **92**(1): p. 200-204.
49. Kubota, Y., et al., *Structure and expression of the mouse oxytocin receptor gene.* Molecular & Cellular Endocrinology, 1996. **124**(1-2): p. 25-32.
50. Mitchell, B.F. and B. Schmid, *Oxytocin and its receptor in the process of parturition.* Journal of the Society for Gynecologic Investigation, 2001. **8**(3): p. 122-133.

51. Larcher, A., et al., *Oxytocin Receptor Gene Expession in the Rat Uterus During Pregnancy and the Estrous Cycle and in Response to Gonadal Steroid Treatment.* Endocrinology, 1995. **136**: p. 5350-5356.

52. Grazzini, E., et al., *Inhibition of oxytocin receptor function by direct binding of progesterone.* Nature, 1998. **392**(6675): p. 509-512.

53. Imamura, T., et al., *Oxytocin modulates the onset of murine parturition by competing ovarian and uterine effects.* American Journal of Physiology - Regulatory Integrative & Comparative Physiology, 2000. **279**(3): p. R1061-R1067.

54. Blanks, A.M. and S. Thornton, *The role of oxytocin in parturition.* British Journal of Obstetrics and Gynaecology, 2003. **110**(Supplement 20): p. 46-51.

55. Slater, D.M., S. Zervou, and S. Thornton, *Prostaglandins and Prostanoid Receptors in Human Pregnancy and Parturition.* Journal of the Society for Gynecologic Investigation, 2002. **9**: p. 118-124.

56. Dong, Y.-L. and C. Yallampalli, *Pregnancy and Exogenous Steroid Treatments Modulate the Expression of Relaxant EP2 and Contractile FP Receptors in the Rat Uterus.* Biology of Reproduction, 2000. **62**: p. 533-539.

57. Lopez Bernal, A., *Uterine Contractility Symposium: Overview of current research in parturition.* Experimental Physiology, 2001. **86.2**: p. 213-222.

58. Sugimoto, Y., et al., *Failure of Parturition in Mice Lacking the Prostaglandin F Receptor.* Science, 1997. **277**: p. 681-683.

59. Kuby, J., *Immunology*. Second ed. 1994, New York: W. H. Freeman and Company. 678.

60. Bryant-Greenwood, G.D. and L.K. Millar, *Human Fetal Membranes: Their Preterm Premature Rupture.* Biology of Reproduction, 2000. **63**: p. 1575-1579.

61. Smith, R., *The Timing of Birth.* Scientific American, 1999(March): p. 68-75.

62. Reis, F.M., et al., *Putative Role of Placental Corticotropin-Releasing Factor in the Mechanisms of Human Parturition.* Journal of the Society for Gynecolgical Investigation, 1999. **6**: p. 109-119.

63. Jain, V., et al., *In vivo effects of corticotropin releasing factor in pregnant rats.* American Journal of Obstetrics & Gynecology, 1998. **178**(1 part 1): p. 186-191.

64. Cook, J.L., et al., *Mouse Placental Prostaglandins Are Associated with Uterine Activation and the Timing of Birth.* Biology of Reproduction, 2003. **68**: p. 579-587.

65. Hansen, W.R., et al., *Key enzymes of prostaglandin biosynthesis and metabolism. Coordinate regulation of expression by cytokines in gestational tissues: A review.* Prostaglandins & Other Lipid Mediators, 1999. **57**: p. 243-257.

66. Abulencia, J.P., et al., *Shear-induced cyclooxygenase-2 via a JNK2/c-Jun-dependent pathway regulates prostaglandin receptor expression in chondrocytic cells.* Journal of Biological Chemistry, 2003. **278**(31): p. 28388-28394.

67. Gross, G.A., et al., *Opposing actions of prostaglandins and oxytocin determine the onset of murine labor.* Proceedings of the National Academy of Sciences of the United States of America, 1998. **95**(20): p. 11875-11879.

68. Tsuboi, K., et al., *Role of Prostaglandin H2 Synthase 2 in Murine Parturition: Study on Ovariectomy-Induced Parturition in Prostaglandin F Receptor-Deficient Mice.* Biology of Reproduction, 2003. **69**: p. 195-201.

69. Zingg, H.H., *Neurohypophysial Hormones*, in *Encyclopedia of Reproduction.* 1999, Academic Press: San Diego. p. 384-390.

70. Zingg, H.H., et al., *Mechanisms of oxytocin gene regulation in neuronal and uterine epithelial cells*, in *Vasopressin*, P. Gross, D. Richter, and G.L. Robertson, Editors. 1993, John Libbey Eurotext. p. 111-118.

71. Ho, M.-Y. and D. Murphy, *A bovine transgene in mice: expression in the female reproductive organs and regulation during pregnancy, parturition and lactation.* Molecular & Cellular Endocrinology, 1997. **136**: p. 15-21.

72. Lefebvre, D.L., et al., *Uterine Oxytocin Gene Expression. II. Induction by Exogenous Steroid Administration.* Endocrinology, 1994. **134**(6): p. 2562-2566.

73. Douglas, A.J., G. Leng, and J.A. Russell, *The importance of oxytocin mechanisms in the control of mouse parturition.* Reproduction, 2002. **123**: p. 543-552.

74. Winslow, J.T., et al., *Infant vocalization, adult aggression, and fear behavior of an oxytocin null mutant mouse.* Hormones & Behavior, 2000. **37**(2): p. 145-155.

75. Theodosis, D.T. and D.A. Poulain, *Maternity leads to morphological synaptic plasticity in the oxytocin system.* Progress in Brain Research, 2001. **133**: p. 49-58.

76. Insel, T.R., L. Young, and Z. Wang, *Central oxytocin and reproductive behaviours.* Reviews of Reproduction, 1997. **2**: p. 28-37.

77. Francis, D.D., F.C. Champagne, and M.J. Meaney, *Variations in maternal behaviour are associated with differences in oxytocin receptor levels in the rat.* Journal of Neuroendocrinology, 2000. **12**: p. 1145-1148.

78. Pedersen, C., *Oxytocin Control of Maternal Behavior. Regulation by Sex Steroids and Offspring Stimuli.* Annals of the New York Academy of Sciences, 1997. **807**: p. 126-145.

79. Insel, T.R., B.S. Gingrich, and L.J. Young, *Oxytocin: who needs it?* Progress in Brain Research, 2001. **133**: p. 59-66.

80. Andrews, W.W., J.C. Hauth, and R.L. Goldenberg, *Infection and Preterm Birth.* American Journal of Perinatology, 2000. **17**(7): p. 357-365.

81. Challis, J.R., et al., *Understanding preterm labor.* Annals of the New York Academy of Sciences, 2001. **943**: p. 225-234.

82. Fortunato, S.J., R. Menon, and S.J. Lombardi, *Stromelysins in Placental Membranes with Premature Rupture of Membranes.* Obstetrics & Gynecology, 1999. **94**(3): p. 435-440.

83. Tashima, L.S., L.K. Millar, and G.D. Bryant-Greenwood, *Genes Upregulated in Human Fetal Membranes by Infection or Labor.* Obstetrics & Gynecology, 1999. **94**(3): p. 441-449.

84. Tashima, L.S., et al., *Decidual relaxins: Gene and protein up-regulation in preterm premature rupture of the membranes by complementary DNA arrays and quantitative immunocytochemistry.* American Journal of Obstetrics & Gynecology, 2002. **187**: p. 785-797.

85. Page, N.M., et al., *Placental peptides as markers of gestational disease.* Reproduction, 2002. **123**: p. 487-495.

86. Roberts, J.M., et al., *Summary of the NHLBI Working Group on Hypertension During Pregnancy.* Hypertension, 2003. **41**(3): p. 437-445.

87. Kanayama, N., *Trophoblastic injury: new etiological and pathological concept of preeclampsia.* Croatian Medical Journal, 2003. **44**(2): p. 148-156.

88. Marsal, K., *Intrauterine growth restriction.* Current Opinion in Obstetrics and Gynecology, 2002. **14**: p. 127-135.

89. Bergstrom, S., *Infection-Related Morbidities in the Mother, Fetus and Neonate.* Journal of Nutrition, 2003. **133**: p. 1656S-1660S.

90. Romero, R., et al., *Infection and prematurity and the role of preventive strategies.* Seminars in Neonatology, 2002. **7**(4): p. 259-274.

91. Dudley, D.J., *Pre-term labor: an intra-uterine inflammatory response syndrome?* Journal of Reproductive Immunology, 1997. **36**(1-2): p. 93-109.

92. Saji, F., et al., *Cytokine production in chorioamnionitis.* Journal of Reproductive Immunology, 2000. **47**: p. 185-196.

93. Nakanishi, Y., et al., *A model for local infection-induced preterm labor in the mouse: delivery of live pups before term following the intracervical administration of lipopoysaccharide.* Prenatal and Neonatal Medicine, 2001. **6**: p. 116-121.

94. Watari, M., et al., *Lipopolysaccharide induces expression of genes encoding pro-inflammatory cytokines and the elastin-degrading enzyme, cathepsin S, in*

human cervical smooth-muscle cells. Journal of the Society for Gynecologic Investigation, 2000. **7**(3): p. 190-198.

95. Goldenberg, R.L., *The management of preterm labor.* Obstetrics & Gynecology, 2002. **100**(5 Pt 1): p. 1020-1037.
96. Meis, P.J., et al., *Prevention of Recurrent Preterm Delivery by 17 Alpha-Hydroxyprogesterone Caproate.* The New England Journal of Medicine, 2003. **348**(24): p. 2379-2385.
97. Thorp, J.M.J., et al., *Antibiotic therapy for the treatment of preterm labor: A review of the evidence.* American Journal of Obstetrics & Gynecology, 2002. **186**: p. 587-592.
98. Murphy, D., *Gene Expression Studies Using Microarrays: Principles, Problems, and Prospects.* Advances in Physiology Education, 2002. **26**(4): p. 256-270.
99. Lockhart, D.J. and E.A. Winzeler, *Genomics, gene expression and DNA arrays.* Nature, 2000. **405**: p. 827-836.
100. Duggan, D.J., et al., *Expression profiling using cDNA microarrays.* Nature Genetics, 1999. **21**(supplement): p. 10-14.
101. Lipshutz, R.J., et al., *High density synthetic oligonucleotide arrays.* Nature Genetics, 1999. **21**(supplement): p. 20-24.
102. Sabatti, C. *Detecting Patterns in Gene Expression Data.* in *DNA Microarrays: The New Frontier in Gene Discovery and Gene Expression Analysis.* 2001. San Diego: Society for Neuroscience.
103. Murata, T., et al., *Oxytocin receptor gene expression in rat uterus: regulation by ovarian steroids.* Journal of Endocrinology, 2000. **166**: p. 45-52.
104. Novak, J.P., R. Sladek, and T.J. Hudson, *Characterization of Variablility in Large-Scale Gene Expression Data: Implications for Study Design.* Genomics, 2002. **79**(1): p. 104-113.
105. Li, C. and W.H. Wong, *Model-based analysis of oligonucleotide arrays: Expression index computation and outlier detection.* Proceedings of the

National Academy of Sciences of the United States of America, 2001. **98**(1): p. 31-36.

106. Li, C. and W.H. Wong, *Model-based analysis of oligonucleotide arrays: model validation, design issues and standard error application.* Genome Biology, 2001. **2**(8): p. research 0032.1-0032.11.

107. Steel, R.G.D. and J.H. Torrie, *Principles and Procedures of Statistics: A Biometrical Approach.* Second ed, ed. C. Napier and J.W. Maisel. 1980, Toronto: McGraw-Hill Book Company. 633.

108. Eisen, M.B., et al., *Cluster analysis and display of genome-wide expression patterns.* Proceedings of the National Academy of Sciences of the United States of America, 1998. **95**: p. 14863-14868.

109. Woessner, J.F.J., *Regulation of matrilysin in the rat uterus.* Biochemistry and Cell Biology, 1996. **74**(6): p. 777-784.

110. Chow, L. and S.J. Lye, *Expression of the gap junction protein connexin-43 is increased in the human myometrium toward term and with the onset of labor.* American Journal of Obstetrics and Gynecology, 1994. **170**(3): p. 788-795.

111. Winterhager, E., et al., *Expression of different connexin genes in rat uterus during decidualization and at term.* European Journal of Cell Biology, 1991. **55**(1): p. 133-142.

112. *http://www.affymetrix.com.*

113. *http://us.expasy.org/sprot.*

114. *http://www.ncbi.nlm.nih.gov.*

115. Tan, Y.F., et al., *Global gene profiling analysis of mouse uterus during the oestrus cycle.* Reproduction, 2003. **126**: p. 171-182.

116. Carson, D.D., et al., *Changes in gene expression during the early to mid-luteal (receptive phase) transition in human endometrium detected by high-density microarray screening.* Molecular Human Reproduction, 2002. **8**(9): p. 871-879.

117. Aronow, B.J., B.D. Richardson, and S. Handwerger, *Microarray analysis of trophoblast differentiation: gene expression reprogramming in key gene function categories.* Physiological Genomics, 2001. **6**(2): p. 105-116.

118. Bethin, K.E., et al., *Microarray Analysis of Uterine Gene Expression in Mouse and Human Pregnancy.* Molecular Endocrinology, 2003. **17**(8): p. 1454-1469.

119. Chan, E.C., et al., *Human Myometrial Genes are Differentially Expressed in Labor: A Suppression Subtractive Hybridization Study.* TheJournal of Clinical Endocrinology and Metabolism, 2002. **87**(6): p. 2435-2441.

120. Aguan, K., et al., *Application of a functional genomics approach to identify differentially expressed genes in human myometrium during pregnacy and labour.* Molecular Human Reproduction, 2000. **6**(12): p. 1141-1145.

121. Girotti, M. and H.H. Zingg, *Gene Expression Profiling of Rat Uterus at Different Stages of Parturition.* Endocrinology, 2003. **144**(6): p. 2254-2265.

122. Watanabe, H., et al., *Analysis of temporal changes in the expression of estrogen-regulated genes in the uterus.* Journal of Molecular Endocrinology, 2003. **30**: p. 347-358.

123. Weston, G.C., I. Haviv, and P.A.W. Rogers, *Microarray analysis of VEGF-responsive genes in myometrial endothelial cells.* Molecular Human Reproduction, 2002. **8**(9): p. 855-863.

124. Loukovaara, M., et al., *Serum insulin-like growth factor-I and insulin-like growth factor binding protein-3 in premature rupture of membranes.* Acta Obstetricia et Gynecologica Scandinavica, 2002. **81**(10): p. 905-908.

125. Badinga, L., et al., *Complex mediation of uterine endometrial epithelial cell growth by insulin-like growth factor-II (IGF-II) and IGF-ginding protein-2.* Journal of Molecular Endocrinology, 1999. **23**: p. 277-285.

126. Adriaenssens, E., et al., *Steriod hormones modulate H19 gene expression in both mammary gland and uterus.* Oncogene, 1999. **18**: p. 4460-4473.

127. Jones, J.I. and D.R. Clemmens, *Insulin-Like Growth Factors and Their Binding Proteins: Biological Actions.* Endocrine Reviews, 1995. **16**(1): p. 3-34.

128. Corkins, M.R., et al., *Growth stimulation by transfection of intestinal epithelial cells with an antisense insulin-like growth factor binding protein-2 construct.* Biochemical & Biophysical Research Communications, 1995. **211**(3): p. 707-713.

129. Huynh, H., *Suppression of uterine insulin-like growth factor binding protein 5 by estrogen is mediated in part by insulin-like growth factor 1.* International Journal of Oncology, 1998. **12**(2): p. 427-432.

130. Ganong, W.F., *Review of Medical Physiology.* 17 ed, ed. J. Dolan and C. Langan. 1995, Norwalk: Appleton and Lange. 781.

131. Elovitz, M.A., et al., *Effects of thrombin on myometrial contractions in vitro and in vivo.* American Journal of Obstetrics & Gynecology, 2000. **185**(4): p. 799-804.

132. Elovitz, M.A., J. Baron, and M. Phillippe, *The role of thrombin in preterm parturition.* American Journal of Obstetrics & Gynecology, 2001. **185**(5): p. 1059-1063.

133. Alfaidy, N., et al., *Prostaglandin F2alpha Potentiates Cortisol Production by Stimulating 11beta-Hydroxysteroind Dehydrogenase 1: a Novel Feedback Loop That May Contribute to Human Labor.* The Journal of Clinical Endocrinology and Metabolism, 2001. **86**: p. 5585-5592.

134. Waddell, B.J. and P.J. Burton, *Full induction of rat myometrial 11beta-hydroxysteroid dehydrogenase type 1 in late pregnancy is dependent on intrauterine occupancy.* Biology of Reproduction, 2000. **62**(4): p. 1005-1009.

135. Burton, P.J., et al., *Induction of Myometrial 11beta-Hydroxysteroid Dehydrogenase Type 1 Messenger Ribonucleic Acid and Protein Expession Late in Rat Pregnancy.* Endocrinology, 1996. **137**(12): p. 5700-5706.

136. Simpson, E.R. and S.R. Davis, *Minireview: aromatase and the regulation of estrogen biosynthesis--some new perspectives.* Endocrinology, 2001. **142**(11): p. 4589-4594.

137. Hukkanen, J., et al., *Expression of cytochrome P450 genes encoding enzymes active in the metabolism of tamoxifen in human uterine endometrium.* Pharmacology and Toxicology, 1998. **82**(2): p. 93-97.

138. Lambe, M., et al., *Ethnic Differences in Breast Cancer Rish: A Possible Role for Pregnancy Levels of Alpha-Fetoprotein?* Epidemiology, 2003. **14**: p. 85-89.

139. Gabant, P., et al., *Alpha-fetoprotein, the major fetal serum protein, is not essential for embryonic development but is required for female fertility.* Proceedings of the National Academy of Sciences of the United States of America, 2002. **99**(20): p. 12865-12870.

140. Huerta-Enochan, G., V. Katz, and S. Erfurth, *The association of abnormal alpha-fetoprotein and adverse pregnancy outcome: does increased fetal surveillance affect pregnancy outcome?* American Journal of Obstetrics & Gynecology, 2001. **184**(7): p. 1549-1553 and 1553-1555.

141. Rymer, J., et al., *Serum lipoprotein (A) and apolipoproteins during pregnancy and postpartum in normal women.* Journal of Obstetrics and Gynaecology, 2002. **22**(3): p. 256-259.

142. Hayman, R.G., et al., *Relationship between myometrial resistance artery behavior and circulating lipid composition.* American Journal of Obstetrics & Gynecology, 1999. **180**: p. 381-386.

143. Hyka, N., et al., *Apolipoprotein A-I inhibits the production of interleukin-1beta and tumor necrosis factor-alpha by blocking contact-mediated activation of monocytes by T lymphocytes.* Blood, 2001. **97**: p. 2381-2389.

144. Burger, D. and J.-M. Dayer, *Cytokines, Acuter-Phase Proteins, and Hormones.* Annals of the New York Academy of Sciences, 2002. **966**: p. 464-473.

145. Tikkanen, M.J., et al., *Lipophilic oestrogen derivatives contained in lipoprotein particles.* Acta Physiologica Scandinavica, 2002. **176**: p. 117-121.

146. Hall, K., *Lipids in the mouse uterus during early pregnancy.* Journal of Endocrinology, 1975. **65**(2): p. 233-243.

147. Bronte, V., et al., *L-arginine metablism in myeloid cells controls T-lymphocyte functions.* Trends in Immunology, 2003. **24**(6): p. 302-306.

148. Wei, L.H., et al., *Elevated arginase I expression in rat aortic smooth muscle cells increases cell proliferation.* Proceedings of the National Academy of Sciences of the United States of America, 2001. **98**(16): p. 9260-9264.

149. Ma, X., W.X. Wu, and P.W. Nathanielsz, *Differential regulation of prostaglandin EP and FP receptors in pregnant sheep myometrium and endometrium during spontaneous term labor.* Biology of Reproduction, 1999. **61**(5): p. 1281-1286.

150. Carbillon, L., N. Seince, and M. Uzan, *Myometrial maturation and labour.* Annals of Medicine, 2001. **33**(9): p. 571-578.

151. Katsuyama, M., et al., *'Distinct cellular localization' of the messenger ribonucleic acid for prostaglandin E receptor subtypes in the mouse uterus during pseudopregnancy.* Endocrinology, 1997. **138**(1): p. 344-350.

152. Szekeres-Bartho, J., *Immunological relationship between the mother and the fetus.* International Reviews in Immunology, 2002. **21**(6): p. 471-495.

153. Kvirkvelia, N., et al., *Placentally derived prostaglandin E2 acts via the EP4 receptor to inhibit IL-2-dependent proliferation of CTLL-2 T cells.* Clinical and Experimental Immunology, 2002. **127**(2): p. 263-269.

154. Fletcher, B.H., et al., *The murine chaperonin 10 gene family contains an intronless, putative gene for early pregnancy factor, Cpn10-rs.* Mammalian Genome, 2001. **12**(2): p. 133-140.

155. Morton, H., et al., *Production of a recombinant form of early prenancy factor that can prolong allogeneic skin graft survival time in rats.* Immunology and Cell Biology, 2000. **78**: p. 603-607.

156. Cavanagh, A.C., *Identification of early pregnancy factor as chaperonin 10: implications for understanding its role.* Reviews of Reproduction, 1996. **1**: p. 28-32.

157. Chen, S.L. and H.T. Orr, *Sequence of a murine cDNA, pcp-4, that encodes the homolog of the rat brain-specific antigen PEP-19.* Nucleic Acids Research, 1990. **18**: p. 1304.

158. Ziai, R., et al., *Isolation, sequence, and developmental profile of a brain-specific polypepide, PEP-19.* Proceedings of the National Academy of Sciences of the United States of America, 1986. **83**: p. 8420-8423.

159. Sangameswaran, L., J. Hempstead, and J.I. Morgan, *Molecular Cloning of a Neuron-Specific Transcript and its Regulation during Normal and Aberrant Cerebellar Development.* Proceedings of the National Academy of Sciences of the United States of America, 1989. **86**(14): p. 5651-5655.

160. Johanson, R.A., et al., *Calmodulin-Binding Peptide PEP-19 Modulates Activation of Calmodulin Kinase II In Situ.* The Journal of Neuroscience, 2000. **20**(8): p. 2860-2866.

161. Erhardt, J.A., et al., *Expression of PEP-19 inhibits apoptosis in PC12 cells.* Neuroreport, 2000. **11**(17): p. 3719-3723.

162. Leverkoehne, I. and A.D. Gruber, *The Murine mCLCA3 (Alias gob-5) Protein Is Located in the Mucin Granule Membranes of Intestinal, Respiratory, and Uterine Goblet Cells.* The Journal of Histochemistry and Cytochemistry, 2002. **50**(6): p. 829-838.

163. Zhou, Y., et al., *A calcium-activated chloride channel blocker inhibits goblet cell metaplasia and mucus overproduction.* Novartis Foundation Symposium, 2002. **248**: p. 150-165.

164. Leverkoehne, I., et al., *Real-time RT-PCR quantitation of mCLCA1 and mCLCA2 reveals differentially regulated expression in pre- and postnatal murine tissue.* Histochemical Cell Biology, 2002. **118**: p. 11-17.

165. Young, A., et al., *Immunolocalization of Proinflammatory Cytokines in Myometrium, Cervix, and Fetal Membranes During Human Parturition at Term.* Biology of Reproduction, 2002. **66**: p. 445-449.

166. Melendez, J.A., et al., *Localization and regulation of IL-1alpha in rat myometrium during late pregnancy and the postpartum period.* American

Journal of Physiology Regulatory Integrative Comparative Physiology, 2001. **280**: p. R879-R888.

167. Hirsch, E., R. Blanchard, and S.P. Mehta, *Differential fetal and maternal contributions to the cytokine milieu in a murine model of infection-induced preterm birth.* American Journal of Obstetrics & Gynecology, 1999. **180**: p. 429-434.

168. Murtha, A.P., et al., *Maternal serum interleukin-6 concentration as a marker for impending preterm delivery.* Obstetrics & Gynecology, 1998. **91**(2): p. 161-164.

169. Dudley, D.J., *Immunoendocrinology of preterm labor: the link between corticotropin-releasing hormone and inflammation.* American Journal of Obstetrics & Gynecology, 1999. **180**(1 Pt 3): p. S251-S256.

170. Hirsch, E., I. Saotome, and D. Hirsh, *A model of intrauterine infection and preterm delivery in mice.* American Journal of Obstetrics & Gynecology, 1995. **172**(5): p. 1598-1603.

171. Hirsch, E., et al., *Bacterially induced preterm labor in the mouse does not require maternal interleukin-1 signalling.* American Journal of Obstetrics & Gynecology, 2002. **186**(3): p. 523-530.

172. Swaisgood, C.M., et al., *Coordinate expression of inducible nitric oxide synthase and cyclooxygenase-2 genes in uterine tissues of endotoxin-treated pregnant mice.* American Journal of Obstetrics & Gynecology, 1997. **177**(5): p. 1253-1262.

173. Gross, G., et al., *Inhibition of cyclooxygenase-2 prevents inflammation-mediated preterm labor in the mouse.* American Journal of Physiology Regulatory Integrative Comparative Physiology, 2000. **278**: p. R1415-R1423.

174. Ausubel, F.M., et al., *Current protocols in molecular biology.* Vol. 1. 1989, New York: Greene Publishing and Associates and Wiley-Interscience.

175. Allen, M.A. and M. Nilsen-Hamilton, *Granzymes D, E, F, and G are regulated trhough pregnancy and by IL-2 and I-15 in Granulated Metrial Gland Cells.* The Journal of Immunology, 1998. **161**: p. 2772-2779.

176. Garcia-Sanz, J.A., et al., *Cell specificity of granzyme gene expression.* The Journal of Immunology, 1990. **145**(9): p. 3111-3118.

177. Trapani, J.A., *Granzymes: a family of lymphocyte granule serine proteases.* Genome Biology, 2001. **2**(12): p. REVIEWS3014.

178. Oschsner, S.A., et al., *Decreased Expression of Tumor Necrosis Factor-alpha-Stimulated Gene 6 in Cumulus Cells of the Cyclooxygenase-2 and EP2 Null Mice.* Endocrinology, 2003. **114**(3): p. 1008-1019.

179. Romero, R. and B. Tartakovsky, *The natural interleukin-1 receptor antagonist prevents interleukin-1-induced preterm delivery in mice.* American Journal of Obstetrics & Gynecology, 1992. **167**(4 pt 1): p. 1041-1045.

180. Kobayashi, S., et al., *Stretch-induced IL-6 secretion from endothelial cells requires NFkappaB activation.* Biochemical & Biophysical Research Communications, 2003. **308**: p. 306-312.

181. Bing, Z., J.H. Huang, and W.S.-L. Liao, *NFkB Interacts with Serum Amyloid A3 Enhancer Factor to Synergistically Activate Mouse Serum Amyloid A3 Gene Transcription.* The Journal of Biological Chemistry, 2000. **275**(41): p. 31616-31623.

182. Tamura, M., et al., *Induction of cyclooxygenase-2 in human endometrial stromal cells by malignant endometrial epithelial cells: evidence fo the involvement of extracellularly regulated kinases and CCAAT/enhancer binding proteins.* Journal of Molecular Endocrinology, 2003. **31**: p. 95-104.

183. Schmid, B., S. Wong, and B.F. Mitchell, *Transcriptional Regulation of Oxytocin Receptor by Interleukin-1beta and Interleukin-6.* Endocrinology, 2001. **142**: p. 1380-1385.

184. Deschamps, S., et al., *The mouse Ifi200 gene cluster: genomic sequence, analysis, and comparison with the human HIN-200 gene cluster.* Genomics, 2003. **43**: p. 34-46.

185. Wang, H., et al., *Characteristics of Three Homologous 202 Genes (Ifi202a, Ifi202b, and Ifi202c) from the Murine Interferon-Activatable Gene 200 Cluster.* Genomics, 1999. **60**: p. 281-294.

186. Liu, C.-J., et al., *The MyoD-Inducible p204 Protein Overcomes the Inhibition of Myoblast Differentiation by Id Proteins.* Molecular and Cellular Biology, 2002. **22**(9): p. 2893-2905.

187. Gribaudoa, G., et al., *The antiproliferative activity of the murine interferon-inducible Ifi 200 proteins depends on the presence of two 200 amino acid domains.* FEBS Letters, 1999. **456**(1): p. 31-36.

188. Smith, J.B. and H.R. Herschman, *The Glucocorticoid Attenuated Response Genes GARG-16, GARG-39, and GARG-49/IRG2 Encode Inducible Proteins Containing Multiple Tetratricopeptide Repeat Domains.* Archives of Biochemistry & Biophysics, 1996. **330**(2): p. 290-300.

189. Ong, V.H., et al., *Monocyte chemoattractant protein 3 as a mediator of fibrosis: Overexpression in systemic sclerosis and the type 1 tight-skin mouse.* Arthritis and Rheumatism, 2003. **48**(7): p. 1979-1991.

190. Park, M.K., et al., *The CXC Chemokine Murine Monokine Induced by IFN-gamma (CXC Chemokine Ligand 9) Is Made by APCs, Targets Lymphocytes Including Activated B Cells, and Supports Antibody Responses to a Bacterial Pathogen In Vivo.* The Journal of Immunology, 2002. **169**: p. 1433-1443.

191. Kallapur, S.G., et al., *Increased IP-10 and MIG expression after intra-amniotic endotoxin in preterm lamb lung.* American Journal of Respiratory and Critical Care Medicine, 2003. **167**(5): p. 779-786.

192. Cheon, Y.P., et al., *Immune-Responsive Gene 1 (Irg1) is a Novel Target of Progesterone Receptor and Plays a Critical Role during Implantation in the Mouse.* Endocrinology, 2003. **144**(12): p. 5623-5630.

193. Recklies, A.D., C. White, and H. Ling, *The chitinase 3-like protein human cartilage glycoprotein 39 (HC-gp39) stimulates proliferation of human connective-tissue cells and activates both extracelluar signal-regulated kinase- and protein kinase B- mediated signalling pathways.* Biochemical Journal, 2002. **365**: p. 119-126.

194. Mork, G., et al., *Proinflammatory cytokines upregulated expression of calprotecting (L1 protein, MRP-8/MRP-14) in cultured human keratinocytes.* British Journal of Dermatology, 2003. **149**: p. 484-491.

195. Yui, S., Y. Nakatani, and M. Mikami, *Calprotectin (S100A8/S100A9), an Inflammatory Protein Complex from Neutrophils with a Broad Apoptosis-Inducing Activity.* Biological and Pharmaceutical Bulletin, 2003. **26**(6): p. 753-760.

196. Seeliger, S., et al., *Expression of Calcium-Binding Proteins MRP8 and MRP14 in Inflammatory Muscle Diseases.* American Journal of Pathology, 2003. **163**(3): p. 947-956.

197. Sopalla, C., et al., *Evidence for the Involvement of the Unique C-Tail of S100A9 in the Binding of Arachadonic Acid to the Heterocomplex S100A8/A9.* Biological Chemistry, 2002. **383**: p. 1895-1905.

198. Kanyshkova, T.G., et al., *Multiple enzymic activities of human milk lactoferrin.* European Journal of Biochemistry, 2003. **270**(16): p. 3353-3361.

199. Okutomi, T., et al., *Anti-Candida activity of calprotectin in combination with neutrophils or lactoferrin.* Microbiology and Immunology, 1998. **42**(11): p. 789-793.

200. Teng, C.T., C. Beard, and W. Gladwell, *Differential Expression and Estrogen Response to Lactoferrin Gene in the Female Reproductive Tract of Mouse, Rat, and Hamster.* Biology of Reproduction, 2002. **67**: p. 1439-1449.

201. Mitsuhashi, Y., et al., *Effect of lactoferrin on lipopolysaccharide (LPS) induced preterm delivery in mice.* Acta Obstetricia et Gynecologica Scandinavica, 2000. **79**(5): p. 355-358.

202. Pacora, P., et al., *Lactoferrin in intrauterine infection, human parturition, and rupture of fetal membranes.* American Journal of Obstetrics & Gynecology, 2000. **183**(4): p. 904-910.

203. Larson, M.A., et al., *Human serum amyloid A3 peptide enhances intstinal MUC3 expression and inhibits EPEC adherence.* Biochemical & Biophysical Research Communications, 2003. **300**: p. 531-540.

204. Larson, M.A., et al., *Induction of human mammary-associated serum amyloid A3 expression by prolactin and lipopolysaccharide.* Biochemical & Biophysical Research Communications, 2003. **301**: p. 1030-1037.

205. Thorn, C.F. and A.S. Whitehead, *Differential transcription of the mouse acute phase serum amyloid A genes in respons to pro-inflammatory cytokines.* Amyloid, 2002. **9**(4): p. 229-236.

206. Shimizu, H. and K. Yamamoto, *NF-kB and C/EBP transcription factor families synergistically function in mouse serum amyloid A gene expression induced by inflammatory cytokines.* Gene, 1994. **149**: p. 305-310.

207. Atemezem, A., et al., *Human alpha-fetoprotein binds to primary macrophages.* Biochemical and Biophysical Research Communications, 2002. **296**: p. 507-514.

208. Fidel Jr., P.L., et al., *Bacteria-Induced or Bacterial Product-Induced Preterm Parturition in Mice and Rabbits Is Preceded by a Significant Fall in Serum Progesterone Concentrations.* The Journal of Maternal-Fetal Medicine, 1998. **7**: p. 222-226.

209. Hirsch, E. and R. Muhle, *Intrauterine Bacterial Inoculation Induces Labor in the Mouse by Mechanisms Other than Progesterone Withdrawal.* Biology of Reproduction, 2002. **67**: p. 1337-1341.

210. Wisniewski, H.-G. and J. Vilcek, *TSG-6: An IL-1/TNF-Inducible Protein with Anti-Inflammatory Activity.* Cytokine & Growth Factor Reviews, 1997. **8**(2): p. 143-156.

211. Milner, C.M. and A.J. Day, *TSG-6: a multifunctional protein associated with inflammation.* Journal of Cell Science, 2003. **116**(10): p. 1863-1873.

212. Jones, R.L., L.A. Salamonsen, and J.K. Findlay, *Potential roles for endometrial inhibins, activins and follistatin during human embryo implantation and early pregnancy.* Trends in Endocrinology & Metabolism, 2002. **13**(4): p. 144-150.

213. Arai, K.Y., et al., *Characterization of Rat Follistatin-Related Gene: Effects on Estrous Cycle Stage and Pregnancy on Its Messenger RNA Expression in Rat Reproductive Tissues.* Biology of Reproduction, 2003. **68**: p. 199-206.

214. Lin, S.-Y., et al., *Regulation of ovarian function by the TGF-beta superfamily and follistatin.* Reproduction, 2003. **126**: p. 133-148.

215. Tang, D.D. and J. Tan, *Downregulation of profilin with antisense oligodeoxynucleotides inhibits force development during stimulation of smooth muscle.* American Journal of Physiology Heart and Circulatory Physiology, 2003. **285**: p. H1528-H1536.

216. Song, H.-J., et al., *Mouse Sprr2 Genes: A Clustered Family of Genes Showing Differential Expression in Epithelial Tissues.* Genomics, 1999. **55**: p. 28-42.

217. Cohen, P.E., et al., *Macrophages: important accessory cells for reproductive function.* Journal of Leukocyte Biology, 1999. **66**: p. 765-772.

218. Gill, K., et al., *Regulation of colony stimulating factor-1 (Csf-1) in endometrial cells: glucocorticoids and oxidative stress regulate the expresion of CSF-1 and its receptor c-fms in endometrial cells.* Fertility & Sterility, 2001. **76**(5): p. 1005-1011.

219. Gorivodsky, M., et al., *Colony-stimulating factor-1 (CSF-1) expression in the uteroplacental unit of mices with spontaneous and induced pregnancy loss.* Clinical and Experimental Immunology, 1999. **117**: p. 540-549.

220. Giorelli, M., et al., *Interferon beta-1a counteracts effects of activation on the expression of G-protein-coupled receptor kinases 2 and 3, beta-arrestin-1, and regulators of G-protein signalling 2 and 16 in human mononuclear leukocytes.* Cellular Signalling, 2002. **14**: p. 673-678.

221. Kehrl, J.H. and S. Sinnarajah, *RGS2: a multifunctional regulator of G-protein signaling.* The International Journal of Biochemistry and Cell Biology, 2002. **34**: p. 432-438.

222. Patten, M., et al., *Interleukin-1beta mediates endotoxin- and tumor necrosis factor alpha-induced RGS16 protein expression in cultured cardiac myocytes.* Naunyn-Schmiedeberg's Archives of Pharmacology, 2003. **368**(5): p. 360-365.

223. Park, E.S., et al., *Oxytocin stimulation of RGS2 mRNA expression in cultured human myometrial cells.* American Journal of Physiology - Endocrinology & Metabolism, 2002. **282**(3): p. E580-E584.

224. Rauk, P.N. and U. Friebe-Hoffmann, *Interleukin-1 beta down-regulates the oxytocin receptor in cultured uterine smooth muscle cells.* American Journal of Reproductive Immunology, 2000. **43**(2): p. 85-91.

225. Helmer, H., et al., *Production of oxytocin receptor and cytokines in primary uterine smooth muscle cells cultivated under inflammatory conditions.* Journal of the Society for Gynecologic Investigation, 2002. **9**(1): p. 15-21.

226. Yasin, S.A., et al., *Interleukin-1 beta and interleukin-6 stimulate neruohypophysial hormone release in vitro.* Journal of Neuroendocrinology, 1994. **6**(2): p. 179-184.

227. Landgraf, R., et al., *Interleukin-1 beta stimulates both central and peripheral release of vasopressin and oxytocin in the rat.* European Journal of Neuroscience, 1995. **7**(4): p. 592-598.

228. Murata, T., et al., *Changes of receptor mRNAs for oxytocin and estrogen during the estrous cycle in rat uterus.* Journal of Veterinary Medical Science, 2003. **65**(6): p. 707-712.

229. Rauk, P.N., et al., *Interleukin-6 up-regulates the oxytocin receptor in cultured uterine smooth muscle cells.* American Journal of Reproductive Immunology, 2001. **45**(3): p. 148-153.

230. Leung, L.T., et al., *The effects of lipopolysaccharide and interleukins-1alpha, -2 and -6 on oxytocin receptor expression and prostaglandin production in bovine endometrium.* Journal of Endocrinology, 2001. **168**: p. 497-508.

231. Rauk, P.N. and J.P. Chiao, *Oxytocin Signaling in Human Myometrium Is Impaired by Prolonged Exposure to Interleukin-1.* Biology of Reproduction, 2000. **63**: p. 846-850.

232. Zingg, H.H., *Oxytocin*, in *Hormones, brain and behavior*, D. Pfaff, et al., Editors. 2002, Academic Press: San Diego. p. 779-803.

233. Tribollet, E., et al., *Oxytocin receptors in the central nervous system. Distribution, development, and species differences. Review.* Annals of the New York Academy of Sciences, 1992. **652**: p. 29-38.

234. Insel, T.R., Young, L., Witt, D.M., Crews, D., *Gonadal steroids have paradoxical effects on brain oxytocin receptors.* Journal of Neuroendocrinology, 1993. **5**: p. 619-628.

235. Insel, T.R., R. Gelhard, and L.E. Shapiro, *The comparative distribution of forebrain receptors for neurohypophyseal peptides in monogamous and polygamous mice.* Neuroscience, 1991. **43**(2/3): p. 623-630.

236. Tribollet, E., et al., *Up-regulation of vasopressin and angiotensin II receptors in the thalamus and brainstem of inbred polydipsic mice.* Neuroendocrinology, 2002. **75**(2): p. 113-23.

237. Whitten, P.L., H.B. Patisaul, and L.J. Young, *Neurobehavioral actions of coumestrol and related isoflavonoids in rodents.* Neurotoxicology & Teratology, 2002. **24**(1): p. 47-54.

238. Le Mouellic, H., Y. Lallemand, and P. Brulet, *Targeted replacement of the homeobox gene Hox-3.1 by the Escherichia coli lacZ in mouse chimeric embryos.* Proceedings of the National Academy of Sciences of the United States of America, 1990. **87**: p. 4712-4716.

239. Breton, C., et al., *Oxytocin Receptor Messenger Ribonucleic Acid: Characterization, Regulation, and Cellular Localization in the Rat Pituitary Gland.* Endocrinology, 1995. **136**(7): p. 2928-2963.

240. Paxinos, G. and K.B.J. Franklin, *The Mouse Brain in Stereotaxic Coordinates.* Second ed. 2001, San Diego: Academic Press. 350.

241. Adan, R.A., et al., *Rat oxytocin receptor in brain, pituitary, mammary gland, and uterus: partial sequence and immunocytochemical localization.* Endocrinology, 1995. **136**(9): p. 4022-4028.

242. Pettibone, D.J., C.J. Woyden, and J.A. Totaro, *Identification of functional oxytocin receptors in lactating rat mammary gland in vitro.* European Journal of Pharmacology, 1990. **188**(4-5): p. 235-241.

243. Soloff, M.S., M.A. Fernstrom, and M.J. Fernstrom, *Vasopressin and oxytocin receptors on plasma membranes from rat mammary gland. Demonstration of vasopressin receptors by stimulation of inositol phosphate formation, and oxytocin receptors by binding of a specific 125I-labeled oxytocin antagonist, d(CH2)5(1)[Tyr(Me)2, Thr4,Tyr-NH2(9)]OVT.* Biochemistry & Cell Biology, 1989. **67**(2-3): p. 152-162.

244. Sala, N.L. and F. Freire, *Relationship between ultrastructure and response to oxytocin of the mammary myoepithelium throughout pregnancy and lactation: effect of estrogen and progesterone.* Biology of Reproduction, 1974. **11**(1): p. 7-17.

245. Soloff, M.S. and M.H. Wieder, *Oxytocin receptors in rat involuting mammary gland.* Canadian Journal of Biochemical and Cell Biology, 1983. **61**(7): p. 631-635.

246. Wilson, E.J. and M.D. Hollenberg, *Effects of oxytocin and vasopressin on the preadipocyte 3T3-F442A cell line.* Biochemistry & Cell Biology, 1987. **65**(3): p. 211-218.

247. Egan, J.J., et al., *Insulin, oxytocin, and vasopressin stimulate protein kinase C activity in adipocyte plasma membranes.* Proceedings of the National Academy of Sciences of the United States of America, 1990. **87**(3): p. 1052-1056.

248. Augert, G. and J.H. Exton, *Insulin and oxytocin effects on phosphoinositide metabolism in adipocytes.* Journal of Biological Chemistry, 1988. **263**(8): p. 3600-3609.

249. Tribollet, E., et al., *Localization and pharmacological characterization of high affinity binding sites for vasopressin and oxytocin in the rat brain by light microscopic autoradiography.* Brain Research, 1988. **442**(1): p. 105-118.

250. Tribollet, E., et al., *Gonadal steroids regulate oxytocin receptors but not vasopressin receptors in the brain of male and female rats. An autoradiographical study.* Brain Research, 1990. **511**(1): p. 129-140.

251. Shelat, S.G., S.J. Fluharty, and L.M. Flanagan-Cato, *Adrenal steroid regulation of central angiotensin II receptor subtypes and oxytocin receptors in the rat brain.* Brain Research, 1998. **807**: p. 135-146.

252. Freund-Mercier, M.J., et al., *Pharmacological characteristics and anatomical distribution of [3H]oxytocin-binding sites in the wistar rat brain studied by autoradiography.* Neuroscience, 1987. **20**(2): p. 599-614.

253. Condes-Lara, M., et al., *Correlation between oxytocin neuronal sensitivity and oxytocin-binding sites in the amygdala of the rat: electrophysiological and histoautoradiographic study.* Brain Research, 1994. **637**: p. 277-286.

254. Wang, Z., Young, L.J., *Ontogeny of oxytocin and vasopressin receptor binding in the lateral septum in prairie and montane voles.* Developmental Brain Research, 1997. **104**: p. 191-195.

255. Loup, F., et al., *Localization of high-affinity binding sites for oxytocin and vasopressin in the human brain. An autoradiographic study.* Brain Research, 1991. **555**(2): p. 220-232.

256. Rabhi, M., et al., *Historadioautographic localisation of oxytocin and vasopressin binding sites in the central nervous system of the merione (Meriones shawi).* Brain Research Bulletin, 1999. **48**(2): p. 147-63.

257. Paban, V., et al., *The behavioral effect of vasopressin in the ventral hippocampus is antagonized by an oxytocin receptor antagonist.* European Journal of Pharmacology, 1998. **361**: p. 165-173.

258. Ostrowski, N.L., *Oxytocin receptor mRNA expression in rat brain: implications for behavioral integration and reproductive success.* Psychoneuroendocrinology, 1998. **23**(8): p. 989-1004.

259. Vaccari, C., S.J. Lolait, and N.L. Ostrowski, *Comparative Distribution of Vasopressin V1b and Oxytocin Receptor Messenger Ribonucleic Acids in Brain.* Endocrinology, 1998. **139**(12): p. 5015-5033.

260. Yoshimura, K., et al., *Localization of oxytocin receptor messenger ribonucleic acid in the rat brain.* Endocrinology, 1993. **133**(3): p. 1239-1246.

261. Yoshimura, R., et al., *Differential expression of oxytocin receptor mRNA in the developing rat brain.* Neuroscience Research, 1996. **24**: p. 291-304.

262. Broad, K.D., et al., *Previous maternal experience potentiates the effect of parturition on oxytocin receptor mRNA expression in the paraventricular nucleus.* European Journal of Neuroscience, 1999. **11**: p. 3725-3737.

263. Insel, T.R., *Postpartum increases in brain oxytocin binding.* Neuroendocrinology, 1986. **44**: p. 515-518.

264. Sharpe, P.E., A. Tinkelman, and J. Cho, *Angular velocity and head direction signals recorded from the dorsal tegmental nucleus of gudden in the rat: implications for path integration in the head direction cell circuit.* Behavioral Neuroscience, 2001. **115**(3): p. 517-588.

265. McCrea, R., G. Gdowski, and H. Luan, *Current concepts of vestibular nucleus function: transformation of vestibular signals in the vestibular nuclei.* Annals of the New York Academy of Sciences, 2001. **942**: p. 328-344.

266. Grottel, K., P. Krutki, and W. Mrowczynski, *Bidirectional neurones in the cervical enlargement of the cat spinal cord with axons descending to sacral segments and ascending to the cerebellum and the lateral reticular nucleus.* Experimental Physiology, 1999. **84**(6): p. 1059-1071.

267. Champagne, F., et al., *Naturally occurring variations in maternal behavior in the rat are associated with differences in estrogen-inducible central oxytocin receptors.* Proceedings of the National Academy of Sciences of the United States of America, 2001. **98**(22): p. 12736-12741.

268. Champagne, F. and M.J. Meaney, *Like mother, like daughter: evidence for non-genomic transmission of parental behavior and stress responsivity.* Progress in Brain Research, 2001. **133**: p. 287-302.

269. Popratiloff, A.S., et al., *Hypoglossal and reticular interneurons involved in oreo-facial coordination in the rat.* Journal of Comparative Neurology, 2001. **433**(3): p. 364-379.

270. Jurgens, U., *Neural pathways underlying vocal control.* Neuroscience and Biobehavioral Reviews, 2002. **26**(2): p. 235-258.

271. Russo, C., *Partial Sturcture and Characterization of the Mouse Oxytocin Receptor Gene*, in *Chemistry/Biochemistry*. 2001, Concordia University: Montreal. p. 69.

272. Jones, R.L., L.A. Salamonsen, and J.K. Findlay, *Activin A Promotes Human Endometrial Stromal Cell Decidualization In Vitro.* The Journal of Clinical Endocrinology and Metabolism, 2002. **87**(8): p. 4001-4004.

273. Jablonski, E.M., et al., *Estrogen Regulation of Aquaporins in the Mouse Uterus: Potential Roles in Uterine Water Movement.* Biology of Reproduction, 2003. **69**: p. 1481–1487.

274. Wu, W.X., et al., *Characterization of two labor-induced genes, DSCR1 and TCTE1L, in the pregnant ovine myometrium.* Journal of Endocrinology, 2003. **178**(1): p. 117-126.

275. Zhang, Y., et al., *Regulation of Lipoprotein Lipase by the Oxysterol Receptors, LXRalpha and LXRbeta.* The Journal of Biological Chemistry, 2001. **276**(46): p. 43018-43024.

276. Merkel, M., R.H. Eckel, and I.J. Goldberg, *Lipoprotein lipase: genetics, lipid uptake, and regulation.* Journal of Lipid Research, 2002. **43**: p. 1997-2006.

277. Hunt, S.M. and C.L. Clarke, *Expression and hormonal regulation of the Sox4 gene in mouse female reproductive tissues.* Biology of Reproduction, 1999. **61**(2): p. 476-481.

278. Graham, J.D., et al., *Regulation of the expression and activity by progestins of a member of the SOX gene family of transcriptional modulators.* Journal of Molecular Endocrinology, 1999. **22**: p. 295-304.

279. Schilham, M.W., et al., *Sox-4 facilitates thymocyte differentiation.* European Journal of Immunology, 1997. **27**(5): p. 1292-1295.

280. Ferl, G.Z., J.M. Timmerman, and O.N. Witte, *Extending the utility of gene profiling data by bridging microarray platforms.* Proceedings of the National Academy of Sciences of the United States of America, 2003. **100**(19): p. 10585-10587.

281. Gould, B.R. and H.H. Zingg, *Mapping Oxytocin Receptor Gene Expression in the Mouse Brain and Mammary Gland using an Oxytocin Receptor-lacZ Reporter Mouse.* Neuroscience, 2003. **122**(1): p. 155-168.

Neuroscience 122 (2003) 155–168

Appendix

MAPPING OXYTOCIN RECEPTOR GENE EXPRESSION IN THE MOUSE BRAIN AND MAMMARY GLAND USING AN OXYTOCIN RECEPTOR–lacZ REPORTER MOUSE

B. R. GOULD AND H. H. ZINGG*

Laboratory of Molecular Endocrinology, Royal Victoria Hospital, McGill University, 687 Pine Avenue West, Montreal, Quebec, Canada H3A 1A1

Abstract—The hypothalamic nonapeptide oxytocin (OT) has an established role as a circulating hormone but can also act as a neurotransmitter and as a neuromodulator by interacting with its central OT receptor (OTR). To understand the role of the OTR in the mouse brain we investigated the expression of the OTR gene at the cellular level. We targeted the lacZ reporter gene to the OTR gene locus downstream of the endogenous OTR regulatory elements. Using lactating mouse mammary gland as a control for OTR promoter directed specificity of lacZ gene expression, X-gal histochemistry on tissue sections confirmed that gene expression was restricted to the myoepithelial cells. We also identified for the first time in mice the expression of the OTR gene in neighbouring adipocytes. Further, investigation in the mouse brain identified numerous nuclei containing neurons expressing the OTR gene. Whilst some of these regions had been described for rat or sheep, the OTR-LacZ reporter mouse enabled the identification of novel sites of central OTR gene expression. These regions include the accessory olfactory bulb, the medial septal nucleus, the posterolateral cortical amygdala nucleus, the posterior aspect of the basomedial amygdala nucleus, the medial part of the supramammillary nucleus, the dorsotuberomammillary nucleus, the medial and lateral entorhinal cortices, as well as specific dorsal tegmental, vestibular, spinal trigeminal, and solitary tract subnuclei. By mapping the distribution of OTR gene expression, depicted through histochemical detection of β-galactosidase, we were able to identify single OTR gene expressing neurons and small neuron clusters that would have remained undetected by conventional approaches.

These novel sites of OTR gene expression suggest additional functions of the oxytocinergic system in the mouse. These results lay the foundation for future investigation into the neural role of the OTR and provide a useful model for further study of oxytocin functions in the mouse.

Key words: β-galactosidase, gene targeting, gene expression, histochemistry, post-parturient.

Oxytocin (OT) is a nonapeptide hormone produced within hypothalamic neurons. It is transported to the posterior pituitary where it is released into the periphery to elicit its effects on target tissues such as the myometrium, stimulating uterine contractions, and the myoepithelial cells of the mammary gland, stimulating milk ejection. OT also influences the functions of the kidneys, ovaries, testis, heart, and other tissues (Gimpl and Fahrenholz, 2001; Zingg, 2002). Centrally released OT has been demonstrated to affect neuroendocrine function, maternal, sexual and social behaviours as well as learning and memory (Insel et al., 1997; Zingg, 2002). The many functions of OT are mediated by the OT receptor (OTR). We endeavoured to generate an OTR knock out–LacZ reporter mouse to investigate the importance of the OTR and to map the distribution pattern of OTR gene expression using X-gal histochemistry. In this pursuit, we created a mouse that retained the OTR gene whilst incorporating the lacZ gene under the control of the entire OTR gene promoter. This OTR-LacZ reporter mouse allowed us to study OTR gene expression patterns without having altered the phenotype of the animal.

Because of the remarkable variability of OTR expression between brains of different species, sexes, and under different hormonal influences (Insel et al., 1993; Tribollet et al., 1992) we chose to characterise the OTR-promoter driven lacZ gene expression in this tissue. The female mouse brain has been partially characterised with respect to the distribution of OT binding sites (Insel et al., 1991; Tribollet et al., 2002; Whitten et al., 2002) however; no information is available regarding the distribution of OTR mRNA in the brain. This study gives a thorough representation of OTR gene expression in the female mouse brain. The 12-h post-parturient female mouse model was chosen to study OTR gene expression at this physiological stage. The oxytocinergic system has been shown to have little apparent influence on maternal behaviour in mice (Insel et al., 2001). The neurological functions of this system in mice clearly require further study.

This study describes the development of the OTR-LacZ reporter mouse and demonstrates its utility by confirming recognised expression profiles within the mouse brain and mammary gland and more importantly, identifying new brain regions and cell types that express the OTR gene.

EXPERIMENTAL PROCEDURES

Development of the OTR-LacZ reporter mouse

Overlapping mouse OTR genomic fragments 4 kb, 6 kb, and 6.5 kb in length were isolated from a λ-dash 129/Sv mouse genomic library and cloned into pBluescript. From these, sequences 5' of

*Corresponding author. Tel: +1-514-843-1553; fax: +1-514-982-0893.
E-mail address: hans.zingg@mcgill.ca (H. H. Zingg).

Abbreviations: ES, cells, embryonic stem cells; OT, oxytocin; OTR, oxytocin receptor; PBS, phosphate-buffered saline; RT, reverse transcribed.

0306-4522/03$30.00+0.00
doi:10.1016/S0306-4522(03)00283-5

the ATG translation start site and 3', within the third intron (Kubota et al., 1996), were subcloned into pGNA (a gift from Dr. Alan Peterson, Montreal, QC, Canada), a modified version of the pGN vector (Le Mouellic et al., 1990) having an additional poly-A sequence. The final targeting vector was denoted as 5'3'pGNA (Fig. 1a). These sequences were selected and oriented to facilitate the homologous targeting and subsequent replacement of the genomic OTR coding region with the lacZ and neo^r genes of the pGNA vector. 129/Sv embryonic stem (ES) cells were electroporated with the vector and then grown in G418 containing media. Southern blotting allowed the selection of clones that had undergone site-specific homologous recombination. These ES cells were injected into developing Balb/c blastocysts and these new blastocysts were introduced into pseudopregnant Balb/c mice for further development. Chimeras were achieved and then backcrossed to Balb/c mice (Charles River, St. Constant, QC, Canada). Germline transmission was established and the mice were then bred to homozygosity for the lacZ allele. All animals were maintained under a 12-h light/dark cycle with access to food and water *ad libitum*. All animal experiments were carried out in accordance with the Bioethics Committee of the Royal Victoria Hospital Research Institute. All efforts were made to minimise the number of animals used as well as their suffering.

Southern blot

Genomic DNA was extracted from ES cells and eventually from mouse tail clippings by proteinase K cell lysis, phenol/chloroform extraction, and subsequent ethanol precipitation. After *Acc*I digestion overnight, the DNA was electrophoresed through a 0.8% agarose gel. The DNA was then transferred to nylon membrane and α^{32}P-dCTP labelled mouse OTR and lacZ probes (see Fig. 1a) were hybridised to the DNA. All probes were prepared using the Random Primed DNA Labelling Kit (Roche, Laval, QC, Canada). A 1.7 kb probe (p1.7) isolated from the 6.5 kb OTR clone as an *Acc*I-*Kpn*I fragment was used as a probe external to the recombination. The wild-type OTR allele and recombinant lacZ allele produce 6.5 kb and 8.65 kb *Acc*I fragments, respectively. A 580 bp lacZ probe (pLac) generated by PCR using the forward amplification primer Lac1 (5'GGCTTACGGCGGTGATTTTGG3') and the reverse primer Lac2 (5'GCGGCGGTCAGCAGTTGTTTTT3') was designed to detect the same 8.65 kb *Acc*I fragment if specific incorporation had taken place. ES cells were genotyped as +/+ or +/z, and mice as +/+, +/z, and z/z, depending on the number of wild-type (+) and recombinant OTR-LacZ (z) alleles present. A probe homologous to the first 257 coding base pairs of the OTR (p257) generated by PCR using the forward primer F3 (5'ATGGAGGGCACGCCCGCAGCCAACTGG3') and the reverse primer R1 (5'AGGTCGGCGATGCTCAGGTGCTTCATG3') was used to determine the presence or absence of the OTR coding region.

Long PCR determination of OTR-LacZ gene structure

The Expand Long Template PCR kit (Roche) system 1 was used to characterise the DNA extracted from the livers of +/+, +/z, and z/z mice. PCR primers that were used are indicated in Fig. 1a. Primer sequences are as follows: forward primers, 5FA (5'GCC TCCCAAGTTCTGGGATTAAAGAAT3'), 5FB (5'TGGAGCTCCTGGCTCAGTCT3'), ZF3 (GGTCGGCTTACGGCGGTGATTTTGG3'), and reverse primers, R2D (5'GGCT CTTTAAAGTGTGGCACGT3'), ZR4 (5'GGGCGATCGGTGCGGGCCTCTTCGCTATTA3'), 5RA (5'GCAGGAGTTACTAGAGGTAGGTGCTA3'). Both annealing and elongation temperatures were 68 °C and the PCR products underwent 30 cycles of amplification. Primers 5FB and R2D produce a wild-type OTR gene product of 3.5 kb. Primers 5FA and ZR4 produce a 4.2 kb product indicating correct lacZ incorporation downstream of the entire endogenous OTR promoter. Primers ZF3 and 5RA should not produce a product at all if proper homologous recombination had occurred but would give a 7.8 kb product if a duplicate of 5'OTR sequence were downstream of the lacZ gene.

RNA extraction and RT-PCR

Total RNA was isolated from uterine tissues collected from parturient control (+/+) mice, heterozygous (+/z), and homozygous (z/z) OTR-LacZ mice and an independent wild-type (wt) control strain. Uteri were placed into appropriate amounts of cold TRIzol Reagent (Invitrogen, Burlington, ON, Canada) for immediate processing. Total RNA was isolated according to the directions for the TRIzol Reagent and subsequently quantitated and stored at −70 °C for later use. Two micrograms of total RNA of each sample were reverse transcribed (RT) and the cDNAs amplified, using specific primers according to a standard RT-PCR protocol (Breton et al., 1995). The OTR forward, F1C (5'GGACGTCAATGCGCCCAAAGAAG3'), and reverse, R8 (5'ACTCGAGCTGCAACGACTCA3') primer combination produces a 235 bp amplicon. These primers bridge the large third intron thusly preventing the possibility of amplifying a product from contaminating genomic DNA (see Fig. 1a). GAPDH primers, forward F (5'CCCTTCATTGACCTCAACTACATGGT3') and reverse R (5'GAGGGGCCATCCACAGTCTTCTG3'), produce a 470 bp amplicon. The annealing temperature for both these PCR reactions was 70 °C. The OTR reactions underwent 35 cycles of amplification whilst the GAPDH reactions underwent 25 cycles. Samples were visualised by agarose gel electrophoresis along with a 1 kb DNA ladder (Invitrogen). Digital photographs were taken and PCR products were quantitated using ImageQuant 5.1 analysis software by Molecular Dynamics (University of Virginia, Charlottesville, VA, USA).

Mouse tissue fixation and cryostat sectioning

Control (+/+), +/z, and z/z mice approximately 12 h post-parturition were anaesthetised with approximately 40 μL (100 mg/ml) ketamine injected i.p. Ice-cold phosphate-buffered saline (PBS) was perfused through the mouse by injection into the left ventricle of the heart. The blood was flushed out through an incision in the right ventricle. Cold Webster's Fixative (1 g paraformaldehyde and 10 ml 50% glutaraldehyde in PBS for a final volume of 200 ml and pH adjusted to 7.2) was then perfused through the mouse. The tissues were dissected and immediately placed into tissue culture plate wells containing cold Webster's fixative. The tissues were incubated for 1 h on ice. They were rinsed with cold PBS and then cryoprotected overnight at 4 °C in a 30% sucrose–PBS solution. Tissues were washed with cold PBS, frozen in liquid N_2-cooled isopentane and then in liquid N_2, and finally stored at −70 °C until sectioning. Cryostat sectioning of tissues (brains n=3, mammary glands n=3; each for control and lacZ tissue) took place below −22 °C. Sections were cut and collected on poly-L-lysine coated microscope slides (Fisher Scientific, Nepean, ON, Canada).

LacZ staining

Sections cut 10 μm thick were fixed by submersing the slides for 3 min in 1.5% glutaraldehyde–PBS and then washed in four consecutive baths of PBS at room temperature. The sections were stained overnight at 37 °C with an X-gal solution (3.1 mM $K_4Fe(CN)_6$, 3.1 mM $K_3Fe(CN)_6$, 2 mM $MgCl_2$, 2 mM X-gal; Invitrogen) prepared as 40mg/ml in *N,N*-dimethylformamide, PBS). The next day the slides were washed in two changes of 95% ethanol and then dehydrated in 100% ethanol. The slides were cleared in xylene for 3 min and allowed to dry. Coverslips were mounted using Permount (Fisher) and allowed to dry for at least 1 h before viewing under a microscope. Localisation and nomenclature of brain nuclei containing blue β-galactosidase expressing cells were determined according to Paxinos and Franklin (2001).

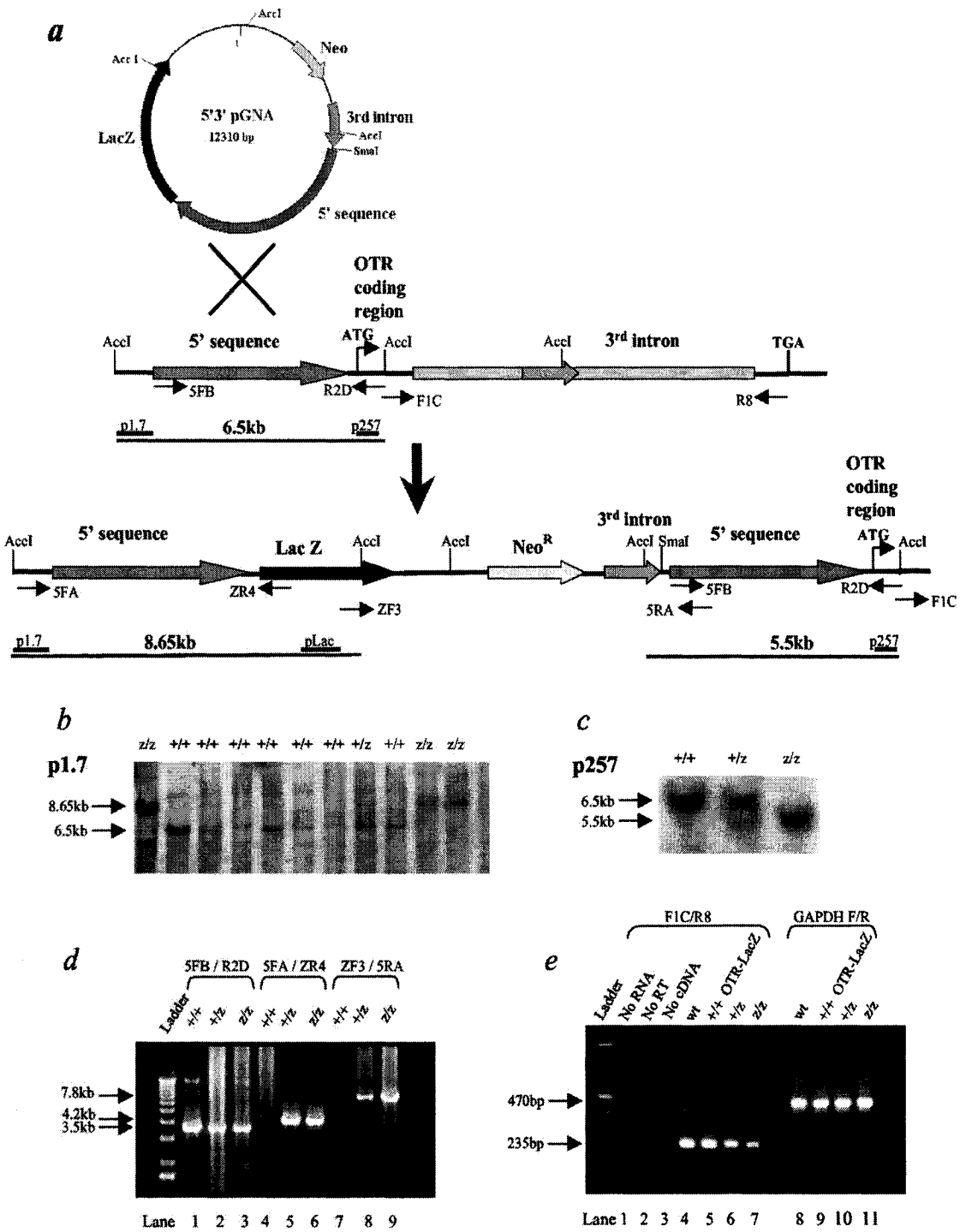


Fig. 1. Generation of the OTR-LacZ reporter mouse. (*a*) Schematic of the pGNA targeting vector containing OTR gene sequences 5' to the coding region (5' sequence) and sequence downstream, within the 3rd intron (3rd intron), inserted to direct homologous recombination. The OTR gene locus organisation before and after site-specific plasmid insertion is depicted. Hybridisation positions of probes for Southern blots (p1.7, p257, and pLac) are indicated by bars positioned over the sequence in the *AccI* digested DNA fragment detected by them. Positions of PCR primer hybridisation are indicated by arrows in their 5' to 3' direction. (*b*) Mouse genotyping by Southern blot using p1.7 after *AccI* digestion. Arrows indicate the wild-type (+,

α-Smooth muscle actin staining

Mammary gland sections (5 μm) were stained following the protocol accompanying the α-smooth muscle actin immunohistochemistry kit (Sigma-Aldrich, St. Louis, MO, USA) with modifications. Briefly, endogenous peroxide activity was quenched with 3% hydrogen peroxide for 5 min, washed with 0.2% TritonX-100–PBS and incubated with 1% goat serum for 15 min to block. Excess serum was removed and slides were covered with monoclonal anti-α-smooth muscle actin antibody for 1 h. Negative control slides were instead treated with a 10^{-6} dilution of mouse $IgG_{2a\kappa}$ isotype control in PBS for the same length of time. Slides were treated sequentially with biotinylated goat anti-mouse antibody (20 min), ExtrAvidin-conjugated peroxidase reagent (20 min), and the chromogenic substrate solution containing 3-amino-9-ethylcarbazole.

Oil Red O lipid staining

Mammary gland sections (5 μm) were stained with Oil Red O (Sigma) solution (2 mg/ml) for 10 min. The slides were rinsed with 60% isopropanol for 5 min to remove background and then counterstained with Harris haematoxylin (Fisher).

RESULTS

Generation of the OTR-LacZ reporter mouse

Neomycin selection for ES cells which had undergone recombination resulted in the proliferation of nearly 500 resistant clones. These were screened for homologous recombination by Southern blotting using α^{32}P-dCTP randomly labelled DNA probes. Probe p1.7 was used to detect homologous recombination within the 5' OTR sequence. The wild-type OTR allele produced a 6.5 kb *Acc*I fragment whereas the recombinant allele produced an 8.65 kb fragment. Re-screening of blots using a probe corresponding to lacZ gene sequence (pLac) allowed the exclusion of recombination positive ES cells which also had random and/or multiple incorporations of the targeting vector. Only those recombination positive ES cells that showed a single 8.65 kb *Acc*I fragment with pLac, indicating a single and specific recombination event, were selected for injection into isolated, developing Balb/c blastocysts. Once germline transmission of the OTR-lacZ allele was established, the offspring were genotyped. Fig. 1*b* depicts typical results of genotyping by Southern blot using p1.7.

Southern blotting of wild-type DNA with a probe homologous to the first 257 bp of the OTR coding region (p257) hybridised, as expected, to the 6.5 kb *Acc*I digested DNA fragment (see Fig. 1*c*, lane 1). In z/z mice, this probe detected a 5.5 kb fragment indicating that the OTR gene was still present (Fig. 1*c*, lane 3). The size difference was hypothesised to have resulted from "rolling circle" insertion of non-linearised 5'3'pGNA vector within the homologous 5' OTR gene sequence, causing the downstream displacement of the OTR coding region including 4.2 kb of 5' sequence. This was instead of the actual replacement of OTR coding region sequence by linear 5'3'pGNA targeting vector. PCR genotyping of these mice with the primers indicated in Fig. 1*a* demonstrated the presence of the lacZ gene in tandem with the OTR gene (Fig. 1*d*), providing support for this hypothesis. Primer pair 5FB and R2D (lanes 1–3) amplify 3.5 kb of 5' untranslated sequence and OTR coding sequence. This product should not be present if the OTR coding sequence had been knocked out; however, lane three indicates its presence in the homozygous lacZ mouse. Amplification of a 4.2 kb product by primers 5FA and ZR4 (lanes 4–6) indicates the site-specific incorporation of 5'3'pGNA into the 5' sequence of the OTR. No amplification from DNA of homozygous wild-type mice (+/+, lane 4) occurred. The 7.8 kb PCR product present in +/z (lane 8) and z/z (lane 9) mice using the ZF3 and 5RA primers (lanes 7–9) is only possible if the OTR gene is present and downstream of the integrated 5'3'pGNA targeting vector, as shown in Fig. 1*a*. No PCR product is produced from DNA of homozygous wild-type mice (+/+, lane 7).

The insertion of 5'3'pGNA upstream of the OTR gene sequence resulted in a reduced level of OTR gene expression. This is demonstrated by RT-PCR of parturient uterus mRNA after 35 cycles of amplification (Fig. 1*e*). OTR mRNA was detected using primers F1C and R8 (lanes 1–7). Lanes 1–3 are amplification controls. Lanes 4–7 indicate the expression of the OTR gene in parturient mouse uterus, even in z/z mice (lane 7). Quantitation using ImageQuant 5.1 analysis software demonstrated approximately 75% of normal OTR gene expression levels in the heterozygote (+/z, lane 6) and approximately 50% of normal OTR gene expression levels in the homozygous mutant (z/z, lane 7). The +/+ mouse uterine tissue (lane 5) expressed OTR mRNA at levels equivalent to wild-type (lane 4), as expected. All comparisons were made after correction to individual GAPDH levels (lanes 8–11).

The diminished OTR gene expression seen in mice carrying the lacZ allele appears neither to affect normal reproductive and social behaviours, nor the general health of these animals. Mice engage in normal sexual behaviours including genital sniffing, the lordosis reflex, and mounting, leading to fertilization. The females successfully carry the pregnancy to full term (19 days) and deliver the pups within an appropriate time frame. Maternal behaviours are engaged immediately with mothers cleaning the newborns, retrieving the pups to the nest, and nursing their

6.5 kb) and recombinant (z, 8.65 kb) alleles. (*c*) Southern blot using the p257 probe after *Acc*I digestion. A 5.5 kb band was detected in +/z and z/z mice indicating that OTR coding sequence was still present (see *a*). (*d*) PCR genotyping of DNA extracted from liver. The primer pairs used are indicated above the lanes. Sizes of amplification products are indicated at the left. The 3.5 kb band is indicative of an unmodified OTR gene structure. The 4.2 kb product in +/z and z/z samples indicates the correct insertion of the lacZ gene downstream of the OTR regulatory sequence. The 7.8 kb band seen in +/z and z/z samples demonstrates that the OTR coding sequence is present and downstream of the lacZ gene as depicted in *a*. (*e*) RT-PCR detection of OTR mRNA in +/+, +/z, and z/z parturient mouse uteri. OTR primers F1C and R8 were used for detection of gene expression. The 235 bp amplicon confirms that coding sequence is present and intact OTR mRNA is produced at levels not significantly altered by the insertion of the 5'3'pGNA targeting vector. GAPDH primers F and R amplified a 470 bp product to which OTR gene expression levels were corrected.

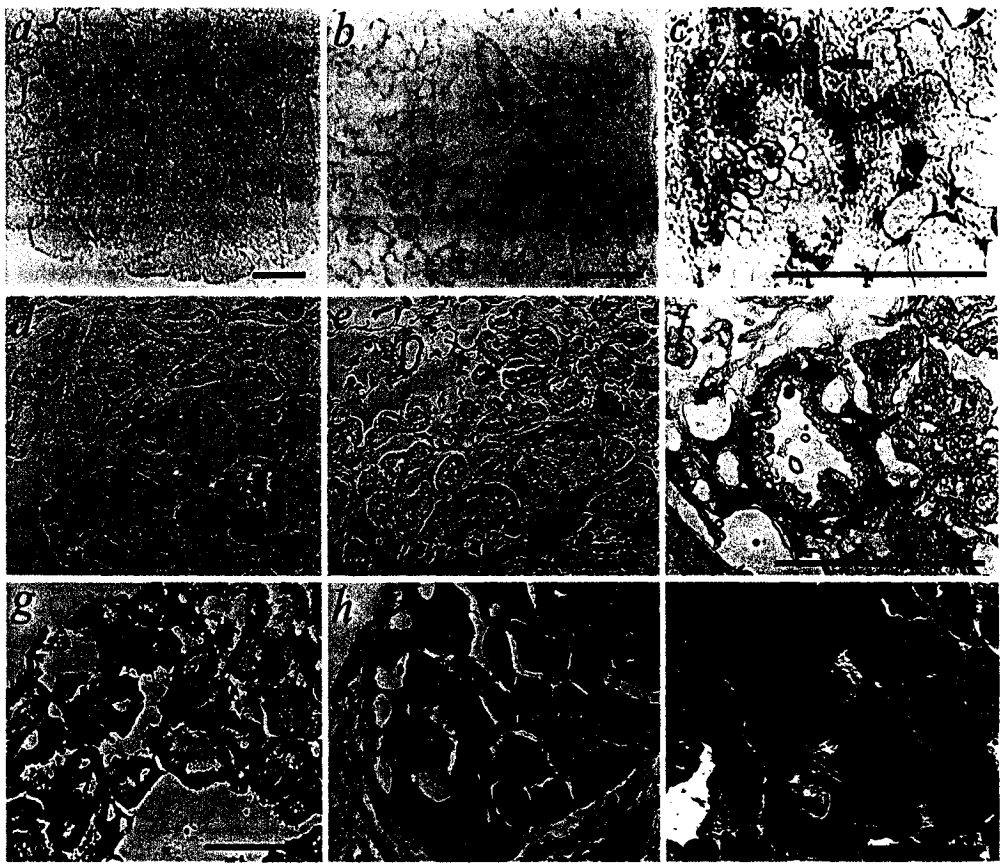


Fig. 2. OTR gene expression in 12-h post-parturient mammary gland tissue. *a*, +/+ control; *b–i*, z/z. Cryostat sections were 10 μm thick for X-gal staining (*a–c*) and 5 μm thick for smooth muscle actin (*d–f*) and Oil Red O staining (*g–i*). The arrows in *c* point to blue-stained myoepithelial cells; the secreting glandular cells are unstained. Specific smooth muscle actin staining of myoepithelial cells is demonstrated (*e* and *f* versus isotype control, *d*) and indicated by arrows in *f*. The arrowhead in *c* indicates the stained cytoplasm of an adipocyte. Oil Red O stain for lipids demonstrates adipocytes (arrowheads in *i*) amongst the ducts and alveoli of the mammary gland. Haematoxylin counterstain in *g–i* distinguishes these structures. Control tissue (*g*) was treated only with haematoxylin stain. Bars=approximately 250 μm.

young in the typical crouching position. Animals experience a normal lifespan in the controlled environment.

The unexpected recombination event, demonstrated by Southern blot and PCR, resulted in a phenotypically normal mouse that expresses the lacZ gene under the control of the entire OTR gene promoter. It also expresses the OTR gene, as evidenced by RT-PCR of parturient uterus mRNA. This mouse is referred to as the OTR-LacZ reporter mouse.

LacZ mapping of OTR gene expression in the mammary gland

LacZ expression was examined in the lactating mammary gland as a positive control to confirm OTR promoter regulated cell specificity of lacZ gene expression. Cryostat sections of mammary gland tissue from 12-h post-partum +/+ and z/z mice stained with X-gal, a substrate for the β-galactosidase product of the lacZ gene, demonstrated blue crystal formation and hence lacZ expression in the myoepithelial cells of the ducts and alveoli of the z/z tissue (Fig. 2*b*,*c*). This staining pattern corresponded to the well-established cell specific site of OTR gene expression in the mammary gland (Adan et al., 1995; Pettibone et al., 1990; Soloff et al., 1989). No X-gal staining was observed in wild-type controls (Fig. 2*a*). Immunohisto-chemistry for smooth muscle actin on z/z mammary gland tissue further corroborated these findings (Fig. 2*d–f*). These results confirm the OTR-LacZ reporter mouse model to be a faithful indicator of OTR gene expression.

Further, X-gal staining was observed in z/z mammary gland adipocytes, indicative of a novel site for OTR gene expression in mice (Fig. 2*c*). This identification was confirmed by lipid staining with Oil Red O with nuclei indicated by haematoxylin counterstain (Fig. 2*g–i*).

LacZ mapping of OTR gene expression in the brain

Sectioned brains of 12-h post-parturient +/z and +/+ mice were stained with X-gal. Neurons demonstrating X-gal substrate conversion to blue crystals by the β-galactosidase lacZ gene product and hence reflecting OTR gene

expression in these nuclei were detected throughout various regions of the brain. Again, no staining was detected in the wild-type controls.

In the telencephalon, strong staining was observed in the accessory olfactory bulb and the anterior olfactory nucleus indicating high levels of OTR gene expression. The piriform cortex layers 2 and 3 also stained strongly (Fig. 3*a*) and this level was maintained throughout its more caudal aspects as well. The inset photo in Fig. 3*a* demonstrates that the β-galactosidase is specifically expressed in neuronal cell bodies. No staining was observed in the control mouse (Fig. 3*b*). In contrast to the intense staining of the piriform cortex, the medial septum and the ventral aspect of the lateral septum (Fig. 3*c*) exhibited a few, specific, moderately stained neurons. More ventrally, the central region of the medial preoptic nucleus (Fig. 3*d*) exhibited some staining. Within the amygdala, staining was moderate to strong. Nuclei in which neurons were stained in this region were the posterolateral and posteromedial cortical amygdala regions (Fig. 3*e*) and the posterior aspect of the basomedial amygdala nucleus. More caudally, the parasubiculum, medial and lateral entorhinal cortices all stained for lacZ gene expression at high levels. No staining was detected in the hippocampus or the bed nucleus of the stria terminalis.

Several diencephalic regions demonstrated β-galactosidase expression. Many hypothalamic nuclei were intensely stained. Of particular note, these were the dorsomedial and ventrolateral aspects of the ventromedial hypothalamic nucleus, as well as the compact subnucleus of the dorsomedial hypothalamic nucleus. Aspects of the mammillary nucleus were stained moderately, including the supramammillary nucleus medial area and the dorsotuberomammillary nucleus. There was no X-gal detection within the thalamus.

Numerous regions within the pons, medulla, and brainstem expressed the lacZ gene. Weak to moderate staining was detected in many areas including the reticulotegmental nucleus (Fig. 3*f*). This level of staining was also found in the laterodorsal tegmental nucleus, the central and pericentral regions of the dorsal tegmental nucleus, and the interfascicular area of the dorsal raphe nucleus (Fig. 3*g*). The facial nucleus was strongly stained (Fig. 3*h*) whilst neurons of the hypoglossal nucleus weakly expressed the lacZ gene. The vestibulocerebellar nucleus weakly expressed the gene but the medial and spinal vestibular nuclei were more intensely stained. Most subdivisions of the nucleus of the solitary tract exhibited strong X-gal labelling of neurons. These regions include the dorsolateral part of the solitary nucleus, the commissural, central, ventrolateral and ventral solitary nuclei (Fig. 3*i*). The lateral reticular nucleus and the dorsal aspect of the medullary reticular nucleus both demonstrated high levels of β-galactosidase in many neurons (Fig. 3*j*). This was true also of the dorsomedial spinal trigeminal (5) nucleus and the interpolar and caudal parts of the spinal trigeminal nucleus (Fig. 3*j*).

These results are summarised in the schematics of Fig. 3 and in Table 1.

DISCUSSION

Development of the OTR-LacZ reporter mouse

Homologous recombination between the 5'3'pGNA targeting vector and the mouse OTR gene locus occurred without the deletion of the endogenous OTR gene. This targeted insertion placed the lacZ gene under the control of the entire and intact 5' flanking sequence of the OTR gene. Southern blot analysis using the probe to the OTR coding region (p257) as well as PCR genotyping results provided support for the hypothesis that "rolling circle" insertion of the non-linearised 5'3'pGNA plasmid within the 5'OTR sequence had occurred. This event was not detected within the ES cells prior to blastocysts injection.

The results of the RT-PCR analysis of parturient mouse uterus total RNA indicate that whilst the level of OTR expression from this displaced OTR gene in z/z mice is somewhat reduced compared with that of wild-type mice it is at a sufficient level for normal parturition to take place.

LacZ mapping of OTR gene expression in the mammary gland

The development of OT knock out mice (Nishimori et al., 1996; Young et al., 1996) has clearly demonstrated the imperative action of OT in the ejection of milk from the mammary gland. This action is mediated by the OTR. OTRs have been shown to be present on the surface of the myoepithelial cells (Adan et al., 1995; Pettibone et al., 1990; Soloff et al., 1989) and the number of OTRs present in the mammary increase progressively during gestation and labour and are maintained throughout lactation at high levels (Sala and Freire, 1974; Soloff and Wieder, 1983). β-galactosidase activity detected specifically in the myoepithelial cells of the mammary tissue of 12-h post-parturient OTR-LacZ mice confirmed that lacZ gene expression was appropriately regulated by the OTR promoter.

In addition, the identification of β-galactosidase activity within the cytoplasm of neighbouring adipocytes represents the first evidence of OTR gene expression in mouse adipocytes and adipocytes of the mammary gland. OTR expression has been shown in the murine preadipocyte 3T3-F442A cell line (Wilson and Hollenberg, 1987) and in numerous studies with rat adipocytes (Augert and Exton, 1988; Egan et al., 1990). The preponderance of evidence indicates that OT, like insulin, stimulates glucose oxidation and lipogenesis in normal rat adipocytes involving a membrane-mediated protein kinase C inhibition of hormone sensitive lipase (Egan et al., 1990).

LacZ mapping of OTR gene expression in the brain

Numerous studies of OTR receptor distribution within the brain have been performed in several species including rats (Condes-Lara et al., 1994; Freund-Mercier et al., 1987; Shelat et al., 1998; Tribollet et al., 1988, 1990), voles (Wang and Young, 1997), humans (Loup et al., 1991), and meriones, a desert rodent (Rabhi et al., 1999). The distribution of OT binding sites in mice has been investigated

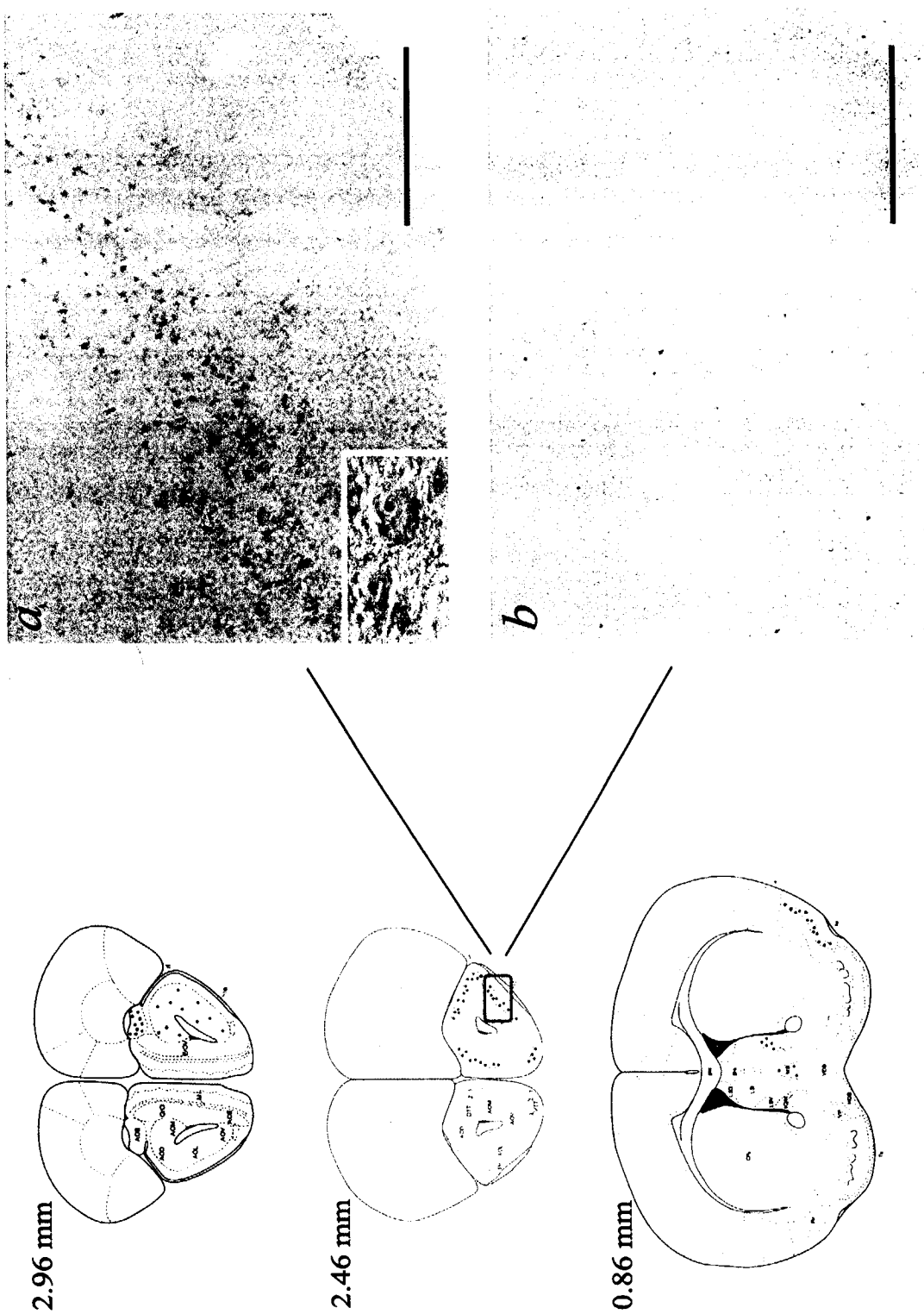


Fig. 3. (*Caption overleaf*).

but primarily within forebrain regions (Insel et al., 1991; Insel, 1993; Paban et al., 1998; Tribollet et al., 2002; Whitten et al., 2002). *In situ* hybridisation studies of OTR mRNA distribution, however, have focussed predominantly on the rat brain (Ostrowski, 1998; Vaccari et al., 1998; Yoshimura et al., 1993, 1996). The present study is the first investigation of the localisation of cells within the mouse brain that express the OTR gene. The OTR-LacZ reporter mouse model provided confirmatory evidence of OTR gene expression in nuclei previously identified in the rat and sheep (Broad et al., 1999). This model also allowed us to reveal several regions, many of them within the hindbrain, for which this is their first description as possessing OTR gene expressing neurons.

Areas previously revealed to contain OTR mRNA expressing neurons include aspects of the anterior olfactory nucleus, the piriform cortex layers 2 and 3. Neurons of the lateral septal nucleus ventral region, the posteromedial cortical amygdala, the ventrolateral and dorsomedial aspects of the ventromedial hypothalamus and the compact part of the dorsomedial hypothalamic nucleus were also determined to express the OTR gene in mice. This is in agreement with previous studies (Broad et al., 1999; Ostrowski, 1998; Vaccari et al., 1998; Yoshimura et al., 1993, 1996). Confirmatory results were found for more caudal regions including the parasubiculum, medial vestibular nucleus, lateral reticular nucleus, and the hypoglossal nucleus.

More importantly, this mouse model allowed us to identify OTR gene expressing neurons in regions that have not been described previously. These new sites include the accessory olfactory nucleus, the medial septal nucleus, and the facial nucleus. Dorsal tegmental nuclei were also identified for the first time; the reticulotegmental, the laterodorsal, and central aspect of the dorsal tegmental nuclei demonstrated blue neurons. The dorsal aspect of the medullary reticular nucleus was determined to be a novel site of OTR mRNA expressing neurons.

The specific and thorough demarcation of OTR gene expressing neurons afforded by this mouse model is further exemplified by the detection of blue staining within specific subnuclei of areas more generally described as possessing OTR gene expressing neurons. Greater accuracy of identification and cytoarchitectural localisation was demonstrated in the amygdala where the posterolateral cortical region and the posterior aspect of the basomedial region were specifically indicated. Several diencephalic regions were more precisely identified as well. In the medial preoptic area, the OTR-LacZ reporter mouse enabled the demonstration of OTR expressing neurons specifically within its central aspect. Mammillary nuclei are known to express OTR mRNA; however, in the present study we were able to identify more precisely neurons within the medial region of the supramammillary nucleus and within the dorsotuberomammillary nucleus. OTR mRNA had been identified within the entorhinal cortex of male rats (Vaccari et al., 1998). We have further localised OTR gene expression to neurons of the lateral and medial entorhinal cortices of this mouse. In the female rat, OTR mRNA was demonstrated within the dorsal raphe nucleus (Yoshimura et al., 1993). With the OTR-LacZ reporter mouse we were able to more accurately localise OTR gene expression to the interfascicular part of this nucleus.

Improved accuracy of identification of OTR gene expressing neurons was seen for numerous hindbrain regions. Whilst the sheep *in situ* hybridisation study indicated OTR mRNA expression within the vestibular region (Broad et al., 1999), this OTR-LacZ mouse enabled specific localisation of this expression to be within neurons of the vestibulocerebellar nucleus, the spinal vestibular nucleus, and the parvicellular cells of the medial vestibular nucleus. These specific vestibular sites are in addition to the medial vestibular nucleus, previously identified in rats (Vaccari et al., 1998; Yoshimura et al., 1993). Enhanced precision of mapping was also exemplified by the detection of blue staining within specific subnuclei of both the nucleus of the solitary tract and the spinal trigeminal nucleus. Whilst the *in situ* hybridisation study of female sheep brain OTR mRNA distribution suggested low levels of transcript in the nucleus of the solitary tract (Broad et al., 1999), we have identified high levels of expression within distinct subnuclei. This delineation encompasses the parasolitary nucleus and the interstitial, dorsolateral, ventrolateral, ventral, central, and commissural divisions of the nucleus of the solitary tract. The rat did not exhibit any detectable levels of OTR mRNA within the nucleus of the solitary tract through *in situ* hybridisation studies (Vaccari et al., 1998; Yoshimura et al., 1993). The studies instead demonstrated low levels of mRNA for the vasopressin receptor, V1bR, which may mediate the functions of OT in this area of the brain. As well, in the rat mRNA studies, the spinal trigeminal nucleus was identified but with the OTR-LacZ mouse

Fig. 3. Mapping of OTR-promoter driven lacZ gene expression in neurons as detected by X-gal histochemical staining based on the atlas by Paxinos and Franklin (2001). Neurons demonstrating X-gal substrate conversion to blue crystals by the β-galactosidase lacZ gene product are indicated by dots (●) in the schematics to the left. Abbreviations of stained regions are defined in Table 1. Numbers at upper left of images indicate the anteroposterior distance of the section plane from the Bregma reference point. Microscopic images of selected regions are to the right. Boxes on schematics outline the corresponding areas. Cryostat sections were 10 μm thick. (*a*) Piriform cortex layers 2 and 3, Bregma level 2.46 mm, inset photo demonstrates staining in neuronal cell bodies; *b*, control mouse piriform cortex layers 2 and 3, Bregma level 2.46 mm, confirming specificity of lacZ gene expression; *c*, lateral septal nucleus ventral aspect, Bregma level 0.14 mm; *d*, medial preoptic nucleus central region, Bregma level 0.14 mm; *e*, posterolateral and posteromedial cortical amygdala nuclei, Bregma level −2.46 mm; *f*, reticulotegmental nucleus of pons, Bregma level −4.84 mm; *g*, laterodorsal tegmental nucleus, dorsal tegmental nucleus central and pericentral regions, and the interfascicular part of the dorsal raphe nucleus, Bregma level −5.20 mm; *h*, facial nucleus, Bregma level −6.24 mm; *i*, solitary tract subnuclei: dorsolateral part of the solitary nucleus, the commissural, central, ventrolateral and ventral nuclei, Bregma level −7.48 mm; *j*, spinal trigeminal (5) nucleus interpolar part, dorsal aspect of the medullary reticular nucleus, and the lateral reticular nucleus. Bars=approximately 250 μm. Digital microscopic photographs were taken and figures were produced by cropping and magnifying the respective areas and adjusting the contrast.

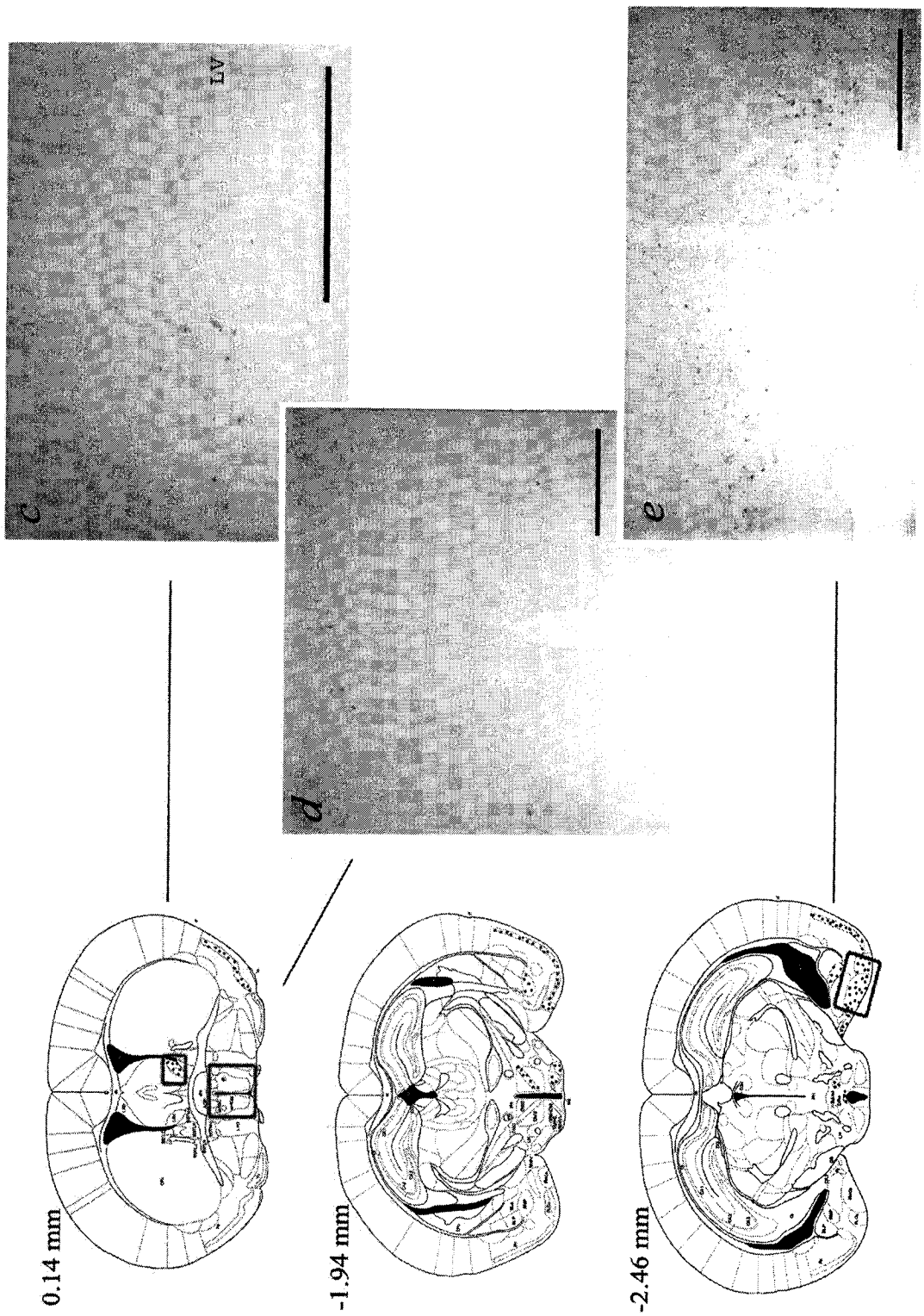


Fig. 3. (*c–e*).

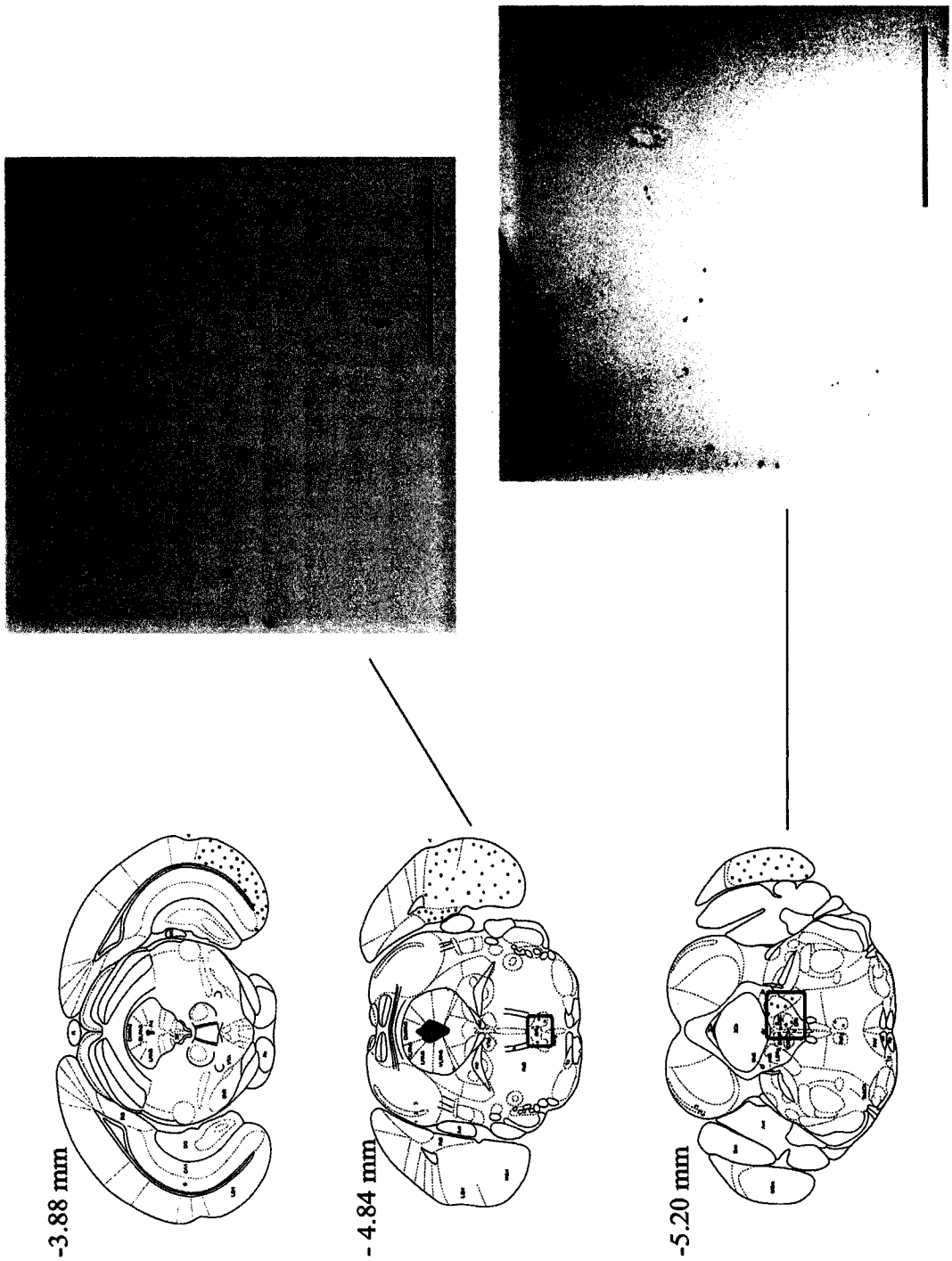


Fig. 3. (*f–g*).

we were able to describe OTR gene expression specifically within the dorsomedial and the interpolar and caudal parts of this nucleus.

Several of these newly identified OTR gene expressing nuclei and subnuclei possessed only a few stained neurons. This supports the OTR-LacZ reporter mouse model

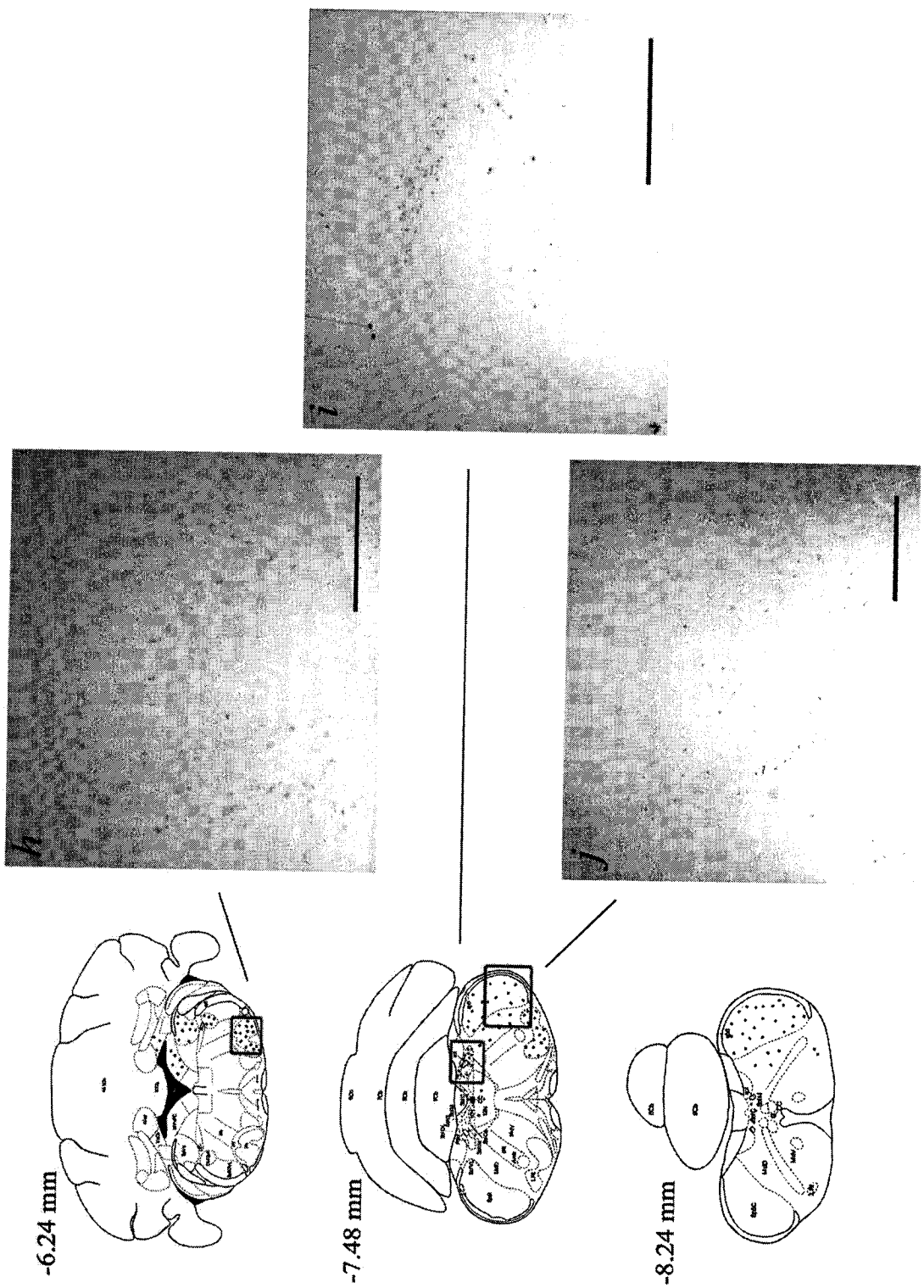


Fig. 3. (*h–j*).

Table 1. Post-parturient mouse brain regions expressing the OTR-LacZ gene

Abbreviation*		Region	Intensity
Telencephalon			
△□	AOB	Accessory olfactory bulb	+++
■□	AON	Anterior olfactory nucleus, all aspects	+++
■□	Pir	Piriform cortex, layers 2 and 3	+++
△	MS	Medial septal nucleus	+
■□	LSV	Lateral septal nucleus, ventral	+
▲□	PLCo	Posterolateral cortical amygdala nucleus	+++
■□	PMCo	Posteromedial cortical amygdala nucleus	++
▲	BMP	Basomedial amygdala nucleus, posterior	++
Diencephalon			
▲	MPOC	Medial preoptic nucleus, central	+
■□	VMHVL	Ventromedial hypothalamic nucleus, ventrolateral	+++
■	VMHDM	Ventromedial hypothalamic nucleus, dorsomedial	++
■	DMC	Dorsomedial hypothalamic nucleus, compact	+++
▲	SuMM	Supramammillary nucleus, medial	++
▲	DTM	Dorsotuberomammillary nucleus	++
■	PaS	Parasubiculum	+++
▲	MEnt	Medial entorhinal cortex	+++
▲	LEnt	Lateral entorhinal cortex	+++
Mesencephalon			
△	LDTg	Laterodorsal tegmental nucleus	++
△	DTgC	Dorsal tegmental nucleus, central	+
△	DTgP	Dorsal tegmental nucleus, pericent	+
△	RtTg	Reticulotegmental nucleus of pons	+
▲	DRI	Dorsal raphe nucleus, interfascicular	+
Pons, medulla, and brain stem			
▲	VeCb	Vestibulocerebellar nucleus	+
▲	MvePC	Medial vestibular nucleus, parvicellular	++
■	MVe	Medial vestibular nucleus	++
▲	SpVe	Spinal vestibular nucleus	++
△	7N	Facial nucleus	+++
▲	SolI	Nucleus of solitary tract, interstitial	++
▲	PSol	Parasolitary nucleus	++
▲	SolDL	Solitary nucleus, dorsolateral part	+++
▲	SolC	Nucleus of solitary tract, commissural	+++
▲	SolCe	Nucleus of solitary tract, central	+++
▲	SolVL	Nucleus of solitary tract, ventrolateral	+++
▲	SolV	Solitary nucleus, ventral	+++
■	12N	Hypoglossal nucleus	+
■	LRt	Lateral reticular nucleus	+++
△	MdD	Medullary reticular nucleus, dorsal	++
▲	DMSp5	Dorsomedial spinal 5 nucleus	++
▲	Sp5I	Spinal 5 nucleus, interpolar part	++
▲	Sp5C	Spinal 5 nucleus, caudal part	+++

The relative intensity of X-gal staining reflects a combination of the number of neurons stained in a region and the degree of blue crystal formation in the individual neurons. Relative intensity of staining: +++ strong; ++ moderate; + weak. * According to Paxinos and Franklin (2001). Symbols to the left of the abbreviations indicate the following: △ regions never previously shown to contain OTR mRNA expressing neurons: ▲ regions more accurately defined as containing OTR mRNA expressing neurons; ■ regions previously shown in rat or sheep to contain OTR mRNA expressing neurons; □ regions previously shown to contain OT binding sites in the mouse (male or female).

as being advantageous over studies utilising the traditional *in situ* hybridisation technique. The absence of background staining and the readily identifiable blue chromogen allows the unambiguous identification of individual cells, even in isolation, with a precision that is not achievable with traditional *in situ* hybridisation techniques. The specificity of the staining in individual OTR expressing cells defines a more accurate and comprehensive microstructure of the oxytocinergic system in the mouse brain.

There are nuclei that are notably unstained in this mouse model. Areas indicated as OTR mRNA expressing in rats yet not detected in the OTR-LacZ reporter mouse include the mitral cells of the olfactory nucleus, the olfactory tubercle, the thalamus, the hippocampal subfields CA1–CA4 and dentate gyrus, the bed nucleus of the stria terminalis, the central amygdala, and the ventral tegmental area. In this regard, the OTR gene expression distribution in the mouse more closely resembles that of the sheep (Broad et al., 1999). This represents another example of the interspecies variation in the distribution of OTR mRNA expression. The absence of OTR mRNA in these areas, well known for their involvement in maternal behaviour in rats suggests an explanation as to why the oxytocinergic system has little apparent influence on maternal behaviour in mice (Insel et al., 2001). It must also be reminded that this study focussed on the distribution within the brain of the post-parturient female mouse and that changes in gonadal steroid levels have been recognised to influence the pattern of OTR mRNA expression within the brain (Insel, 1986, 1993; Insel et al., 1997; Ostrowski, 1998; Tribollet et al., 1990).

Concordance occurs between the distribution of the lacZ expressing neurons of the OTR-LacZ reporter mouse and documented OT binding sites of the female forebrain of a polygamous species of mouse (*P. maniculatus*; Insel et al., 1991). These regions of agreement are the anterior olfactory nucleus and the lateral septum. In addition, OTR binding sites provided by Tribollet et al. (2002), from females of ICR and STR/N mouse strains, co-localise with OTR promoter-driven lacZ mRNA expression sites within the anterior olfactory nucleus, ventral part of the lateral septum, posterolateral cortical amygdala nucleus, and ventromedial hypothalamic nucleus.

Discrepancies are also evident between these studies, as we have not found lacZ gene expressing neurons in many regions described as possessing OTR binding sites. These regions include the olfactory tubercle, the hippocampus, and the central and lateral amygdala regions. These mouse OT binding sites are in agreement with rat mRNA and OT binding studies. This incongruity may result from differences in hormonal status between the virgin adult females used in the binding study and the post-partum OTR-LacZ reporter females as well as the differences between the mouse species, in general. This phenomenon of mRNA-protein discordance in localisation has been documented previously (Broad et al., 1999; Rabhi et al., 1999; Yoshimura et al., 1993). It likely reflects the fact that mRNA is produced and remains within the neuronal cell body whilst the protein may be present either in the cell

body, on distal dendrites, or may also be transported along the axons for presentation, and hence detection, in other regions of the brain. Little OTR binding information is available for the mouse brain caudal to Bregma −2.92 mm.

The exact role the OTR may play in the mouse brain requires further investigation. What is known about these regions may provide direction for these future studies. For example, OTR gene expression has been identified in neurons of olfactory areas, in the piriform cortex, in entorhinal cortices, and in the parasubiculum. Axonal connexions between these regions transmit and process olfactory cues. These regions are important for memory processing. The entorhinal regions are specifically involved in memory retention and recognition of prior experience. The presence of OTR gene expression in neurons involved in this system suggests a role for OT in the transmission, processing, storage, and retrieval of memory inputs.

Additional novel sites of OTR gene expression have been identified by this reporter mouse in several mesencephalic and hindbrain nuclei. These nuclei have been characterised as important for locomotor control. The dorsal tegmental nuclei have been implicated in playing an essential role in generating head direction signals necessary for processing sensory input regarding spatial navigation of the animal (Sharpe et al., 2001). These inputs may collaborate with those processed by the vestibular nuclei, sensing cues regarding the movement and position of the head in space. This develops a sense of self-motion in the animal that allows the control of both reflex and voluntary movements (McCrea et al., 2001). Further locomotor responses are provided by signals from the lateral reticular nucleus (Grottel et al., 1999). The presence of OTR gene expression in neurons of the dorsal tegmental, vestibular, and lateral reticular nuclei suggests a contribution of the oxytocinergic system to the transmission of locomotor inputs or responses.

This investigation of OTR gene expression patterns in the post-parturient mother suggests new influences of OT on maternal behaviour in the mouse. OTR gene expression has been identified in neurons of the hypoglossal, facial, and spinal trigeminal nuclei. The hypoglossal nucleus is a collection of motor neurons whose function includes the control of movements of the anterior part of the tongue. In the post-partum mother, coordinated movement of the tongue is necessary for the licking/grooming behaviour associated with maternal behaviour. In rats, this behaviour stimulates increased oestrogen sensitivity and OTR gene expression in areas important for the establishment of maternal behaviour (Champagne et al., 2001; Champagne and Meaney, 2001). These neurons also coordinate with those of the facial nucleus through reciprocal neuronal projections to synchronise oro-facial control of chewing and swallowing as well as breathing and vocalization (Popratiloff et al., 2001). The trigeminal neurons participate in these vocalisations (Jurgens, 2002) and other functions including nociception. Trigeminal projections to the olfactory bulb also suggest a role in processing olfactory cues, in the post-parturient mouse, most likely for the discrimination of foreign pups. A role for the OTR in maternal behaviours of grooming, care, and protection of pups as well as in the communication with pups is suggested and requires further investigation.

The role of the OTR in mediating social and sexual behaviours as well as other physiological processes is only partially understood. By relating the location of OTR gene expressing neurons (as depicted by lacZ gene expression) to the function of those neurons, one may further elucidate the role of the oxytocinergic system in the mouse brain.

The OTR-LacZ reporter mouse allowed the identification of individual OTR gene expressing cells. This is an advantageous tool over *in situ* hybridisation techniques that may detect similar, yet not identical mRNA sequences and does not have the technical simplicity and resolution this reporter mouse affords. By studying mice under different hormonal, immunological, and other physiological states, one may easily observe the responsive changes in OTR gene expression using simple X-gal histochemistry. This OTR-LacZ reporter mouse provides valuable information regarding the contribution of the OTR in the mouse.

Acknowledgements—We would like to thank Dr. Alan Peterson (Laboratory of Molecular Oncology, Royal Victoria Hospital, McGill University) for the gift of the pGNA vector and his expert advice. We also wish to thank Dr. Michel Tremblay (McGill Cancer Centre), Dr. Naima Bachnou and Ms. Priscila Valera for their valuable assistance in the development of the OTR-LacZ reporter mouse. B.R.G. was supported by a CIHR Studentship Award and H.H.Z. is a holder of the Wyeth-Ayerst Chair in Reproductive Endocrinology and of a Senior Scientist Award from the Canadian Institutes of Health Research (CIHR). The research was supported by grants from the CIHR and Wyeth Pharmaceuticals, Canada.

REFERENCES

Adan RA, van Leeuwen FW, Sonnemans MA, Brouns M, Hoffman G, Verbalis JG, Burbach JP (1995) Rat oxytocin receptor in brain, pituitary, mammary gland, and uterus: partial sequence and immunocytochemical localization. Endocrinology 136:4022–4028.

Augert G, Exton JH (1988) Insulin and oxytocin effects on phosphoinositide metabolism in adipocytes. J Biol Chem 263:3600–3609.

Breton C, Pechoux C, Morel G, Zingg H (1995) Oxytocin receptor messenger ribonucleic acid: characterization, regulation, and cellular localization in the rat pituitary gland endocrinology 136:2928–2963.

Broad KD, Levy F, Evans G, Kimura T, Keverne EB, Kendrick KM (1999) Previous maternal experience potentiates the effect of parturition on oxytocin receptor mRNA expression in the paraventricular nucleus. Eur J Neurosci 11:3725–3737.

Champagne F, Diorio J, Sharma S, Meaney MJ (2001) Naturally occurring variations in maternal behavior in the rat are associated with differences in estrogen-inducible central oxytocin receptors. Proc Natl Acad Sci USA 98:12736–12741.

Champagne F, Meaney MJ (2001) Like mother, like daughter: evidence for non-genomic transmission of parental behavior and stress responsivity. Prog Brain Res 133:287–302.

Condes-Lara M, Veinante P, Rabai M, Freund-Mercier MJ (1994) Correlation between oxytocin neuronal sensitivity and oxytocin-binding sites in the amygdala of the rat: electrophysiological and histoautoradiographic study. Brain Res 637:277–286.

Egan JJ, Saltis J, Wek SA, Simpson IA, Londos C (1990) Insulin, oxytocin, and vasopressin stimulate protein kinase C activity in adipocyte plasma membranes. Proc Natl Acad Sci USA 87:1052–1056.

Freund-Mercier MJ, Stoeckel ME, Palacios JM, Pazos A, Reichhart

JM, Porte A, Richard P (1987) Pharmacological characteristics and anatomical distribution of [3H]oxytocin-binding sites in the Wistar rat brain studied by autoradiography. Neuroscience 20:599–614.
Gimpl G, Fahrenholz F (2001) The oxytocin receptor system: structure, function, and regulation. Physiol Rev 81:629–683.
Grottel K, Krutki P, Mrowczynski W (1999) Bidirectional neurones in the cervical enlargement of the cat spinal cord with axons descending to sacral segments and ascending to the cerebellum and the lateral reticular nucleus. Exp Physiol 84:1059–1071.
Insel TR (1986) Postpartum increases in brain oxytocin binding. Neuroendocrinology 44:515–518.
Insel TR, Gelhard R, Shapiro LE (1991) The comparative distribution of forebrain receptors for neurohypophyseal peptides in monogamous and polygamous mice. Neuroscience 43:623–630.
Insel TR, Gingrich BS, Young LJ (2001) Oxytocin: who needs it? Prog Brain Res 133:59–66.
Insel TR, Young L, Wang Z (1997) Central oxytocin and reproductive behaviours. Rev Reprod 2:28–37.
Insel TR, Young L, Witt DM, Crews D (1993) Gonadal steroids have paradoxical effects on brain oxytocin receptors. J Neuroendocrinol 5:619–628.
Jurgens U (2002) Neural pathways underlying vocal control. Neurosci Biobehav Rev 26:235–258.
Kubota Y, Kimura T, Hashimoto K, Tokugawa Y, Nobunaga K, Azuma C, Saji F, Murata Y (1996) Structure and expression of the mouse oxytocin receptor gene. Mol Cell Endocrinol 124:25–32.
Le Mouellic H, Lallemand Y, Brulet P (1990) Targeted replacement of the homeobox gene Hox-3.1 by the *Escherichia coli* lacZ in mouse chimeric embryos. Proc Natl Acad Sci USA 87:4712–4716.
Loup F, Tribollet E, Dubois-Dauphin M, Dreifuss JJ (1991) Localization of high-affinity binding sites for oxytocin and vasopressin in the human brain: an autoradiographic study. Brain Res 555:220–232.
McCrea R, Gdowski G, Luan H (2001) Current concepts of vestibular nucleus function: transformation of vestibular signals in the vestibular nuclei. Ann NY Acad Sci 942:328–344.
Nishimori K, Young LJ, Guo Q, Wang Z, Insel TR, Matzuk MM (1996) Oxytocin is required for nursing but is not essential for parturition or reproductive behavior. Proc Natl Acad Sci USA 93:11699–11704.
Ostrowski NL (1998) Oxytocin receptor mRNA expression in rat brain: implications for behavioral integration and reproductive success. Psychoneuroendocrinology 23:989–1004.
Paban V, Alescio-Lautier B, Devigne C, Soumireu-Mourat B (1998) The behavioral effect of vasopressin in the ventral hippocampus is antagonized by an oxytocin receptor antagonist. Eur J Pharmacol 361:165–173.
Paxinos G, Franklin KBJ (2001) The mouse brain in stereotaxic coordinates, 2nd edition. San Diego: Academic Press.
Pettibone DJ, Woyden CJ, Totaro JA (1990) Identification of functional oxytocin receptors in lactating rat mammary gland in vitro. Eur J Pharmacol 188:235–241.
Popratiloff AS, Streppel M, Gruart A, Guntinas-Lichius O, Angelov DN, Stennert E, Delgado-Garcia JM, Neiss WF (2001) Hypoglossal and reticular interneurons involved in oro-facial coordination in the rat. J Comp Neurol 433:364–379.
Rabhi M, Stoeckel ME, Calas A, Freund-Mercier MJ (1999) Historadioautographic localisation of oxytocin and vasopressin binding sites in the central nervous system of the merione (*Meriones shawi*). Brain Res Bull 48:147–163.
Sala NL, Freire F (1974) Relationship between ultrastructure and response to oxytocin of the mammary myoepithelium throughout pregnancy and lactation: effect of estrogen and progesterone. Biol Reprod 11:7–17.
Sharpe PE, Tinkelman A, Cho J (2001) Angular velocity and head direction signals recorded from the dorsal tegmental nucleus of gudden in the rat: implications for path integration in the head direction cell circuit. Behav Neurosci 115:517–588.
Shelat SG, Fluharty SJ, Flanagan-Cato LM (1998) Adrenal steroid regulation of central angiotensin II receptor subtypes and oxytocin receptors in the rat brain. Brain Res 807:135–146.
Soloff MS, Fernstrom MA, Fernstrom MJ (1989) Vasopressin and oxytocin receptors on plasma membranes from rat mammary gland: demonstration of vasopressin receptors by stimulation of inositol phosphate formation, and oxytocin receptors by binding of a specific 125I-labeled oxytocin antagonist, d(CH2)5(1)[Tyr(Me)2, Thr4, Tyr-NH2(9)]OVT. Biochem Cell Biol 67:152–162.
Soloff MS, Wieder MH (1983) Oxytocin receptors in rat involuting mammary gland. Can J Biochem Cell Biol 61:631–635.
Tribollet E, Audigier S, Dubois-Dauphin M, Dreifuss JJ (1990) Gonadal steroids regulate oxytocin receptors but not vasopressin receptors in the brain of male and female rats: an autoradiographical study. Brain Res 511:129–140.
Tribollet E, Barberis C, Jard S, Dubois-Dauphin M, Dreifuss JJ (1988) Localization and pharmacological characterization of high affinity binding sites for vasopressin and oxytocin in the rat brain by light microscopic autoradiography. Brain Res 442:105–118.
Tribollet E, Dubois-Dauphin M, Dreifuss JJ, Barberis C, Jard S (1992) Oxytocin receptors in the central nervous system: distribution, development, and species differences. Rev Ann NY Acad Sci 652: 29–38.
Tribollet E, Ueta Y, Heitz F, Marguerat A, Koizumi K, Yamashita H (2002) Up-regulation of vasopressin and angiotensin II receptors in the thalamus and brainstem of inbred polydipsic mice. Neuroendocrinology 75:113–123.
Vaccari C, Lolait SJ, Ostrowski NL (1998) Comparative distribution of vasopressin V1b and oxytocin receptor messenger ribonucleic acids in brain. Endocrinology 139:5015–5033.
Wang Z, Young LJ (1997) Ontogeny of oxytocin and vasopressin receptor binding in the lateral septum in prairie and montane voles. Dev Brain Res 104:191–195.
Whitten PL, Patisaul HB, Young LJ (2002) Neurobehavioral actions of coumestrol and related isoflavonoids in rodents. Neurotoxicol Teratol 24:47–54.
Wilson EJ, Hollenberg MD (1987) Effects of oxytocin and vasopressin on the preadipocyte 3T3-F442A cell line. Biochem Cell Biol 65:211–218.
Yoshimura K, Kiyama H, Kimura T, Araki T, Maeno H, Tanizawa O, Tohyama M (1993) Localization of oxytocin receptor messenger ribonucleic acid in the rat brain. Endocrinology 133:1239–1246.
Yoshimura R, Kimura T, Watanabe D, Kiyama H (1996) Differential expression of oxytocin receptor mRNA in the developing rat brain. Neurosci Res 24:291–304.
Young WS, Shepard E, Amico J, Hennighousen L, Wagner KU, LaMarca ME, McKinney C, Ginns EI (1996) Deficiency in mouse oxytocin prevents milk ejection, but not fertility or parturition. J Neuroendocrinol 8:847–853.
Zingg HH (2002) Oxytocin. In: Hormones, brain and behavior, vol. 3 (Pfaff D, Arnold AP, Etgen AM, Fahrbach SE, Rubin RT, eds), pp 779–803. San Diego: Academic Press.

(Accepted 31 December 2002)

) Description of Animals

	Sp/strain 1	Sp/strain 2	Sp/strain 3	Sp/strain 4	Sp/strain 5	Sp/strain 6
ɔecies	Rat (pregnant)	Rat (lactating with litter)	Rat (ovariectomized)	Mice (male & female)	Mice (male & female)	
ıpplier/Source	Charles River (Canada)	Charles River (Canada)	Charles River (Canada)	Charles River (Canada)	Charles River (Canada)	
rain	Sprague-Dawley	Sprague-Dawley	Sprague-Dawley	C57 Black 6	129 SV OTR b-gal	
:x	Female	Female	Female	Male & Female	Male & Female	
ge/Wt	Adult	Adult	Adult	Weaning & Adult	Weaning & Adult	
To be purchased	60	40	60	25	- - - -	
Produced by in-use breeding	- - - -	- - - -	- - - -	- - - -	50	
Other g.field studies)	Wt 200g	Wt 250g	Wt 250g	Wt 25g	Wt 25g	
ieeded at one time	6	6	6	5	5	
per cage	2	1	2	2	2	
OTAL# /YEAR	60	40	60	25	50	

ıality Control Assurance: To prevent introduction of infectious diseases into animal facilities, a health status report or veterinary ıpection certificate may be required prior to receiving animals from all non-commercial sources or from commercial sources whose imal health status is unknown or questionable. Quarantine and further testing may be required for these animals.

Justification of Animal Usage

Please justify the number of animals requested for each species described in the table 6c above, BASED ON THE CPERIMENTAL OBJECTIVES OF THE PROJECT. Include information on experimental and control groups, # per oup, and failure rates. Also justify in terms of statistical requirements, product yield, etc. For breeding, specify how any adults are used, number of offspring produced, and how many offspring are used in experimental procedures. ıe numbers of animals are for one year only, not the length of funding. Use the table below when applicable (space ll expand as needed).

60 pregnant rats:
l DNAse hypersensitivity assays: 6-10 nuclei are needed for one reaction. One rat uterus yields 2-10 nuclei. The 6-10 clei yield 100 ug DNA following DNAse digestion, restriction enzyme digestion and purification. Therefore, 3 rats are eded per one single experiment. A total of 4 different probe/DNA target combinations will be assessed. To ensure roducibility a 2x repeat is necessary. In addition, 8 rats are needed for pilot experiments. Total: 32 rats.

! Gel shift experiments: We get 40 ug of nuclear protein per rat uterus after purification. We need 4-10 ug protein gel shift ıction, i.e. 4 reactions per uterus. For each oligonucleotide, homologous and heterologous displacement reactions are needed tal of 4). We want to assess 10 different oligonucleotide probes representing 10 different DNA elements. Total: 10 imals.

} Footprint analysis: We need up to 10 ug of nuclear proteins per reaction. To analyze 8 different DNA targets, with repeat :tor of 3 and the above outlined yield of nuclear proteins, we need 6 animals.

} 12 timed pregnant animals for uterine genome expression analysis using gene array techonology.
Using: 4 rats at term and labor.
4 rats at term and not in labor.
4 rats at day 20 of pregnancy.

40 lactating rats: DNAse hypersensitivity, footprint and gel shift analysis is carried out as above, but a reduced scale, based the results obtained in the uterus: 20 animals. The remaining animals are used for in situ hybridization studies (5) and munocytochemistry studies (5). In addition, Northern blot, RT-PCR studies and primer extension studies are performed (10 imals).

60 ovariectomized rats: For Northern blot and RT-PCR analysis in different brain regions (needs pools of 3-4/experiment), mammary gland, uterus and pituitary. We need 6 different steroid treatment conditions and we need to have a n>3 for ıtistical analysis (54 animals). In addition, we need 6 animals for pilot experiments.

Mice: 129 SV OTR b-gal mice for mapping of b-gal expression, in females, at different stages of pregnancy and lactation d in males.
ınimals each at four different stages of pregnancy = 20 animals.
nale mice = 5 animals.
ıtal: 25 animals.
me amount of animals for in situ hybridization studies; Northern blot analysis studies for comparison with expression of the dogenous OTR gene (25 animals).
;7 black 6 mice: for controls of 129 SV PTR b-gal mice females at four different stages of pregnancy and 5 males. Total of animals (5 each).

st Agents or ocedures 2 Drugs	# of Animals and Species Per Group e.g. 6 rats	Dosage and/or Route o Administration e.g. .03, .05 mg/kg – IM, IP (4 variables)	# of endpoints e.g. 1, 7, 10 days (3 variables)	Other variables (i.e. sex, weight, genotypes,etc.) e.g. Male, Female groups (2 variables)	Total number of animals per year e.g. 2 x 6 x 4 x 3 x 2 = 288

For the above table, enter the first agent/procedure, press 'enter', then the 2nd agent... complete the first column, then the 2nd, en the 3rd...

Please justify the need for live animals versus alternate methods (e.g. tissue culture, computer simulation).

udy of tissue-specific expression patterns and the effect of pregnancy cannot be studied in tissue culture.

Describe the characteristics of the animal species selected that justifies its use in the proposed study (consider aracteristics such as body size, species, strain, data from previous studies or unique anatomic/physiological features)

he rat has been classically used in all our previous studies on uterine oxytocin receptor gene expression. The mouse is the ly species in which the routine production of transgenic animals has been established.

Animal Husbandry and Care

Special cages	NO ☒	YES ☐	**Specify:**
Special diet	NO ☒	YES ☐	**Specify:**

Special handling NO ☒ YES ☐ **Specify:**

Is there any component to the proposed procedures which will result in immunosuppression or decreased immune action (e.g. stress, radiation, steroids, chemotherapeutics, genetic modification of the immune system)?

) ☒ YES ☐ **Specify:**

ultiple institution facility housing: NO ☒ YES ☐

dicate all facilities where animals will be housed: **Building:** Royal Victoria Hospital **Room No:** H3

dicate area(s) where animal use procedures will be nducted: **Building:** Royal Victoria Hospital **Room No:** H7.90

animal housing and animal use are in different locations, briefly describe procedures for transporting animals:
ıe two floors (H3 and H7) ar connected through an internal elevator.

Standard Operating Procedures (SOPs)

)mplete this section if you plan to use any of the UACC SOPs listed below. IT IS UACC POLICY THAT THESE)PS BE USED WHEN APPLICABLE. Any proposed variation of the SOPs must be described and justified. The andard Operating Procedures can be found at the UACC website at www.mcgill.ca/rgo/animal. The completed and med SOP form must be attached to the protocol.

ıeck all SOPs that will be used:

ood Collection (UACC#1)	☐	**Production of Monoclonal Antibodies (UACC#7)**	☐
ıaesthesia (rodents) (UACC#2)	☐	**Production of Polyclonal Antibodies(UACC#8)**	☐
ıalgesia (rodents/larger species) (UACC#3)	☐	**Collection of Amphibian Oocytes (UACC#9)**	☐
ɾeeding (transgenics/knockouts) (UACC#4)	☒	**Rodent Surgery (UACC#10)**	☐
ɾansgenic Generation (UACC#5)	☐	**Neonatal Rodent Anaesthesia and Euthanasia (UACC#11)**	☐
nockout/in Generation (UACC#6)	☐	(UACC #13)	☒

l. Description of Procedures

FOR EACH EXPERIMENTAL GROUP, DESCRIBE ALL PROCEDURES AND TECHNIQUES IN THE ORDER IN WHICH THEY WILL BE PERFORMED - surgical procedures, immunizations, behavioural tests, immobilization and restraint, food/water deprivation, requirements for post-operative care, sample collection, substance administration, special monitoring, etc. IF A PROCEDURE IS COVERED BY AN SOP, WRITE "AS PER SOP", NO FURTHER DETAIL IS REQUIRED. Appendix 2 of the Guidelines provides a sample list of points that should be addressed in this section.

Decapitation following C02 anesthesia at age 2-3 months. Uterine, mammary glands, pituitary and brain tissues will be removed as per SOP#13 and used for the following purposes:

1) Analysis of gene transcription rates and of protein/DNA interactions of the oxytocin receptor gene at different stages of pregnancy and lactation.

2) Determination of the structure of the different oxytocin receptor mRNAs by RACE-PCR, S1 analysis and cDNA cloning.

3) In vitro organ cultures of uterine tissue slices for the determination of the factors regulating oxytocin gene expression. The factors to be tested include gonadal steroids, cytokines, prostaglandins and different growth factos.

4) Some animals will be injected (s.c.) with estradiol (0.1 mg/kg) or progesterone (10 mg/kg) in 0.1 ml of 90% oil 10% ethanol once per day during 3 days prior to decapitation.

5) A breeding colony of a mice strain in which the oxytocin receptor gene locus has been altered is also maintained and b-gal expression pattern is analyzed on tissue sections (OTR-b-gal mice). Mice are killed at ages 8-14 weeks.

Standard operating procedure for maintenance of breeding colony of 129 SV OTR b-gal mice: Genotyping has been performed earlier by extracting DNA from a small piece of tail following by Southern blot analysis. Currently, only a homozygous breeder colony is maintained without the need of genotyping (except for occasional controls and after backcrossing to balb-c mice). Timed pregnant animals are generated by putting a breeding pair in one cage overnight and checking for a vaginal plug the following morning. The colony currently maintained does not exceed 60 animals at any one time.

Field Studies – Provide all relevant details. Procedures to be conducted (e.g. surgery, blood collection, tagging etc.) ıuld be described above.

:thod of capture/restraint, duration of captivity, potential injury/mortality, monitoring frequency:

ansportation and /or housing of animals in the field:

ecial handling required:

pture of non-target species, potential injury/mortality:

ill captured animals be released at or near the capture site YES ☐ NO ☒
not, specify if they will be relocated to other locations and/or populations.

scribe any potential ecological disruption this study may cause:

is the responsibility of the investigator to obtain all necessary permits for work with wildlife. Copies of these permits ıst be forwarded to the Research Ethics Officer (Animal Studies) when they are obtained.

) Pre-Anaesthetic/Anaesthetic/Analgesic Agents: List all drugs that will be used to minimize pain, distress or comfort. Table will expand as needed. (*complete 1st column pressing 'enter' after each species, then 2nd column...)

ıecies	Agent	Dosage (mg/kg)	Total volume(ml) per administration	Route	Frequency

Administration of non-anaesthetic substances: List all non-anaesthetic agents under study in the experimental mponent of the protocol, including but not limited to drugs, infectious agents, viruses (table will expand as needed). complete 1st column pressing 'enter' after each species, then 2nd column...)

ecies	Agent	Dosage (mg/kg)	Total volume (ml) per administration	Route	Frequency
Rats	Estradiol	0.1 mg/kg	0.1 ml	s.c.	Daily for 3 days
	Progesterone	10 mg/kg			

Endpoints : 1) Experimental – for each experimental group indicate survival time .
2) Clinical - describe the conditions, complications, and criteria (e.g. >20% wt.loss, tumour size, calizing, lack of grooming) that would lead to euthanasia of an animal before the expected completion of the periment (specify per species and project if multiple projects involved).

Experimental: For injection of gonadal steroids: max. survival time is 3 days.

Clinical: The injected hormone doses are within physiological range and there is no conceivable risk for the animal. ıwever, any animal that displays any signs of distress, either by vocalizing, >20% weight loss, lack of grooming of lack or :k of mobility will be euthanized.

ecify person(s) who will be responsible for animal monitoring and post-operative care
ıme: Phone#:

Method of Euthanasia – According to CCAC guidelines, justification must be provided for use of any physical thod of euthanasia without prior use of anaesthesia (justify here):

ecify Species

	☐ anaesthetic overdose, list agent/dose/route:
	☐ exsanguination with anaesthesia, list agent/dose/route:
ts	☐ decapitation without anaesthesia ☒ decapitation with anaesthesia,list agent/dose/route: CO2 chamber
	☐ cervical dislocation
ce and rats	☒ CO_2 chamber
	☐ other (specify)
	☐ not applicable (explain)

. Category of Invasiveness: B ☒ C ☐ D ☐ E ☐

tegories of Invasiveness (from the CCAC *Categories of Invasiveness in Animal Experiments*). Please refer to this document for a ıre detailed description of categories.

tegory A: Studies or experiments on most invertebrates or no entire living material.

tegory B: Studies or experiments causing little or no discomfort or stress. *These might include holding animals captive, injection, rcutaneous blood sampling, accepted euthanasia for tissue harvest, acute non-survival experiments in which the animals are completely aesthetized.*

tegory C: Studies or experiments involving minor stress or pain of short duration. *These might include cannulation or heterizations of blood vessels or body cavities under anaesthesia, minor surgery under anaesthesia, such as biopsy; short periods of straint, overnight food and/or water deprivation which exceed periods of abstinence in nature; behavioural experiments on conscious imals that involve short-term stressful restraint.*

tegory D: Studies or experiments that involve moderate to severe distress or discomfort. *These might include major surgery under aesthesia with subsequent recovery, prolonged (several hours or more) periods of physical restraint; induction of behavioural stresses, munization with complete Freund's adjuvant, application of noxious stimuli, procedures that produce pain, production of transgenics accordance with University policy).*

tegory E: Procedures that involve inflicting severe pain, near, at or above the pain threshold of unanaesthetized, conscious imals. *Not confined to but may include exposure to noxious stimuli or agents whose effects are unknown; exposure to drugs or emicals at levels that (may) markedly impair physiological systems and which cause death, severe pain or extreme distress or physical uma on unanaesthetized animals.* According to University policy, E level studies are not permitted.

. Potential Hazards to Personnel and Animals It is the responsibility of the investigator to obtain the necessary ohazard and/or Radiation Safety permits before this protocol is submitted for review. A copy of these certificates ust be attached, if applicable.

) hazardous materials will be used in this study: ☒

Indicate which of the following will be used in animals:
Toxic chemicals ☐Radioisotopes ☐Carcinogens ☐Infectious agents ☐Transplantable tumours

Complete the following table for each agent to be used (use additional page as required).

ent			
sage			
ute of administration			
equency of administration			
ıration of administration			
ımber of animals involved			
rvival time after administration			

After administration the animals will be housed in: ☐ the animal care facility

☐ laboratory under supervision of laboratory personnel

Please note that cages must be appropriately labeled at all times.

Describe potential health risk (s) to humans or animals:

Describe measures that will be used to reduce risk to the environment and all project and animal facility personnel:

. Reviewer's Modifications (to be completed by ACC only): The Animal Care Committee has made the following odification(s) to this protocol during the review process. Please make these changes to your copy. You must comply h the recommended changes as a condition of approval.

3.IDENTIFICATION OF PUPS BY TOE CLIPPING

- Can only be performed in mouse pups that are up to 14 days old.
- Using a pair of clean, sharp scissors or scalpel, remove the very last digit. Ensure that there is no blood left on the foot as this may trigger cannabalism by the mother
- No anaesthesia required

4.TAIL SNIPPING FOR GENOTYPING

- For all rodents, the **maximum** tail length that can be obtained during a single animal's lifetime is 1.0 cm, with a maximum of two transections.
- If hemorrhaging persists despite manual compression, apply a silver nitrate stick to the tip of the tail. Ensure that there is no blood left on the foot as this may trigger cannabalism by the mother.

4. 1. Mice less than THREE (3) weeks of age

- No anaesthesia is required.
- Place the tail between two wrapped blocks of ice, which will cause vasoconstriction and offer mild analgesia. Use a fresh scalpel blade to transect tail at a 90 degree angle.

4. 2. Mice older than THREE (3) weeks of age

- Anaesthesia is required. Same procedure as above.
- Analgesia usually not required however, based on individual needs. See UACC SOP #2 (General Anaesthesia in Adult Rodents) and UACC SOP #3 (Analgesia in Rabbit and Rodents) for appropriate drugs, doses etc.

UACC SOP#4
Approved April, 1999
Revised April, 2002

3. EUTHANASIA TECHNIQUES IN ADULT AND NEONATAL RODENTS

- Euthanasia techniques can be classified as either chemical (drugs) or physical.
- All personnel performing any of these techniques **must** be properly trained and qualified to carry out the procedure.
- **Neonate** refers to an animal less than 14 days of age.
- Euthanasia can be accomplished by using chemical or physical methods.
- **N.B.** All physical means of euthanasia **without** prior general anaesthesia require scientific justification and approval by the local Animal Care Committee.

3.1 MOST ACEPTABLE EUTHANASIA TECHNIQUES IN RODENTS

TECHNIQUE	ADULT	NEONATE
Carbon dioxide	Yes	Not in animals less than 10 days old
Barbituate (pentobarbital *) overdose IP, IV at 3X anaesthetic dose	Yes	Yes (IV access difficult in neonates)
General anaesthesia preceding physical euthanasia (cervical dislocation, decapitation, exsanguinations)	Yes	Yes (Cervical dislocation can be difficult in neonates) Cervical dislocation prohibited in older juvenile and adult rats
General anaesthesia preceding perfusion or IV KCl infusion	Yes	Yes (IV access difficult in neonates)
Gas anaesthesia overdose	Yes	Not in animals less than 10 days old

* Pentobarbital is a controlled drug. For information on its acquisition and use, please refer to SOP UACC #2 Section 3.1.

3.2 UNACCEPTABLE EUTHANASIA TECHNIQUES IN ALL RODENTS

- Decompression
- Asphyxiation
- Air embolism
- Rapid freezing
- Carbon monoxide
- Methoxyflurane
- Nitrogen
- Nitrous oxide
- Ether
- Chloral hydrate
- Chloroform
- Poisons (strychnine, cyanide)
- Household products and solvents (acetone, alcohol)

WHAT RIGHTS DO I RETAIN AS AN AUTHOR?

As an author, you retain rights for large number of author uses, including use by your employing institute or company. These rights are retained and permitted without the need to obtain specific permission from Elsevier. These include:

- Patent and trademark rights or rights to any process or procedure described in the article
- The right to photocopy or make single electronic copies of the article for your own personal use, including for your own classroom use, or for the personal use of colleagues, provided those copies are not offered for sale and are not distributed in a systematic way outside of your employing institution
- The right to post the article on a secure network (not accessible to the public) within the employer's institution
- The right to retain a preprint version of the article on a public electronic server such as the World Wide Web. Elsevier does not require that authors remove from publicly accessible servers versions of their paper that differ from the version as published by Elsevier. See also our information on electronic preprints for a more detailed discussion on these points.
- Posting of the article as published on a public server can only be done with Elsevier's specific written permission
- The right, subsequent to publication, to use this article or any part thereof free of charge in a printed compilation of works of your own, such as collected writings or lecture notes
- If the article is a "work for hire" made within the scope of your employment, the employer may use all or part of the information in this article for intra-company use
- The right to include the article in a thesis or dissertation provided that this is not to be published commercially
- The right to present the paper at a meeting or conference and to hand copies of the paper to the delegates attending the meeting
- The right to publish a different or extended version of the paper so long as it is sufficiently new to be considered a new work
- The right to expand the article into book-length form for publication
- The right to re-use parts of the paper in other works, provided that the new work is not to be published commercially.

Other uses by authors should be authorized by Elsevier through the **Global Rights Department** (for addresses see Obtaining Permissions), and authors are encouraged to let Elsevier know of any particular needs or requirements.