Deciphering the mechanisms involved in testicular gonocyte differentiation

By

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Abstract

Spermatogenesis is a complex process that results in the formation of spermatozoa throughout the lifetime of males. Not only is this process essential for the survival of the species, but it also allows for genetic material to be transferred from one generation to the next. Spermatogenesis relies on the existence of a spermatogonial stem cell (SSC) pool, acting as a reservoir of cells from which sperm can continuously be formed through a succession of phases, involving proliferation, migration, differentiation, meiosis and apoptosis. SSCs arise from the differentiation of their precursor cells, the gonocytes. Gonocytes, which differentiate from primordial germ cells, include prenatal and neonatal germ cells. Postnatal gonocytes undergo phases of proliferation, migration to the basement membrane of the seminiferous cords, and differentiation. Gonocyte development is not well understood, despite the importance of these cells as precursors of the germline stem cells. Gene expression studies and morphological analysis have suggested that improper gonocyte development could be at the origin of testicular germ cell tumor (TGCT) formation. The incidence of TGCTs has been steadily increasing for the past decades for reasons that remain unknown. Thus, the overall objective of this work was to determine the genes as well as molecular and cellular mechanisms that are involved in gonocyte differentiation and potentially linked to TGCT formation. In previous studies, we had shown that gonocyte differentiation was induced by retinoic acid (RA). In the present work, we found that *in vitro* RA-induced gonocyte differentiation required the activation of platelet-derived growth factor receptor (PDGFR) kinase activity, consecutive with the increased expression of a truncated form of PDGFRα. Furthermore, signaling pathway analysis demonstrated that RA-induced gonocyte differentiation also involved the activation of a SRC-related kinase, alongside JAK2 and STAT5. Because gonocytes express pluripotency markers similar to embryonic stem cells (ESCs), we compared their RA-induced differentiation process to that of F9 mouse embryonal carcinoma cells, a model commonly used as a surrogate for ESCs. The results highlighted interesting similarities and differences between the two cell types. Secondly, given that proper gonocyte development necessitates multiple processes occurring within a short period of time and a significant amount of protein turnover, degradation, and cellular remodeling, we hypothesized that the ubiquitin proteasome system would be involved in gonocyte differentiation. Indeed, our findings demonstrated that this was the case. Gene expression analysis and subsequent validation by quantitative real time PCR (qPCR) and protein analysis

identified the E3 ubiquitin ligase RNF149 as a protein actively regulated during gonocyte differentiation. Finally, in order to better understand how improper gonocyte development might lead to TGCT formation, we performed gene array analyses in which genes preferentially expressed in rat gonocytes in comparison to spermatogonia were compared to genes significantly upregulated in seminomas (the most common type of TGCTs) using normal human testicular tissues as controls. Functional pathway analysis of these upregulated genes indicated that cell adhesion molecules, in particular members of the claudin family, were highly expressed in gonocytes and seminoma. These data, further validated by qPCR and protein analyses, suggested that claudins play a role in gonocyte development, but also that their dysregulation in gonocytes might be linked to the formation of testicular tumors. Collectively, these studies have provided a better insight into the various genes and mechanisms involved in gonocyte differentiation and identified candidate genes potentially involved in TGCT formation.

Résumé

La spermatogenèse est un processus complexe menant à la formation de spermatozoïdes au cours de la vie des mâles. Ce processus n'est pas seulement essentiel à la survie des espèces, mais il permet aussi de transférer le matériel génétique d'une génération à l'autre. La spermatogénèse dépend de l'existence d'un réservoir de cellules souches spermatogéniques (SSCs) à partir duquel les spermatozoïdes peuvent être continuellement produits, à travers une succession d'étapes qui incluent des phases de prolifération, migration, différenciation, méiose, et apoptose. Les SSCs sont formées par la différenciation de leur précurseurs, les gonocytes. Les gonocytes, qui différentient à partir des cellules germinales primordiales, incluent des cellules germinales prénatal et néonatales. Le développement des gonocytes néonataux inclue des phases de prolifération, migration jusqu'à la membrane basale des cordes séminifères, et différenciation. Les mécanismes régulant ces processus sont peu connus, malgré l'importance de ces cellules en tant que précurseurs des cellules souches germinales. Des études morphologiques et d'expression génique ont suggèré qu'une perturbation du développement gonocytaire pourrait être à l'origine de la formation des tumeurs de cellules germinales testiculaires (TGCT). Au cours des dernières décennies, l'incidence des TGCTs a graduellement augmenté pour des raisons qui restent inconnues. Par conséquent, l'objectif global de ce projet était de déterminer les gènes, ainsi que les mécanismes moléculaires et cellulaires impliqués dans le processus de différenciation des gonocytes, et potentiellement liés à la formation des TGCTs. Dans des études préalables, nous avons montré que la différenciation des gonocytes était induite par l'acide rétinoïque (RA). Dans l'étude présente, nous avons trouvé que l'induction de la différenciation par RA nécessite l'activité kinase du récepteur au platelet derived growth factor (PDGFR), ainsi que de l'activation d'une protéine de la famille SRC et de JAK2 et STAT5. Compte tenu du fait que les gonocytes expriment certains marqueurs de pluripotence communs avec les cellules souches embronnaires (ESCs), nous avons performé une étude comparative de leurs mécanismes de différenciation avec ceux de la lignée F9 de carcinome embronnaire murin, un model fréquemment utilisé comme substitut des SSCs. Des similitudes et différences intéressantes entre les mécanismes induits par RA entre les deux types cellulaires ont été révélées par ces comparaisons. Notre second objectif était d'examiner l'hypothèse que le système protéasomal de l'ubiquitine (UPS) est impliqué dans la différenciation gonocytaire induite par RA, puisque ce processus nécessite de nombreux changements d'expression de protéines à des périodes

spécifiques et de courtes durées, et un remodelage cellulaire extensif. En effet, nos résultats ont confirmé que UPS participe au processus de différenciation. De plus, des analyses de réseaux de gènes sur puce, suivies de validation par PCR en temps réel quantitatif (qPCR) et analyses protéiques nous ont permis d'identifier l'E3 ubiquitin ligase RNF149 comme étant activement régulée pendant la différenciation des gonocytes. Enfin, afin de mieux comprendre comment des erreurs de développement des gonocytes pourraient mener à la formation de TGCTs, nous avons réalisé des analyses de réseaux de gènes sur puce, dans lesquelles les gènes plus fortement exprimés dans les gonocytes que dans les spermatogonies ont été comparés à la liste de gènes surexprimés dans les séminomes (le plus commun des TGCTs) comparés aux niveaux présents dans des testicules humains non tumoraux. L'analyse de voies d'interactions fonctionnelles par rapport aux profils d'expression des gènes surexprimés dans les gonocytes et les séminomes ont mis en évidence la forte expression de certaines molécules d'adhésion cellulaires, telles que la famille des claudins. Ces résultats, après validation par qPCR et analyses protéiques, suggèrent que des claudins jouent un rôle dans le développement des gonocytes, mais aussi que leur dérégulation pourrait être impliquée dans la formation des TGCTs. Collectivement, ces études ont apporté de nouvelles connaissances sur les gènes et mécanismes participant à la différenciation des gonocytes néonataux et ont permis d'identifier des gènes potentiellement impliqués dans la formation des tumeurs testiculaires.

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List of Abbreviations

A_{al}	A aligned
A_d	A dark
ADAM	A disintegrin and metalloproteinase
ADH	Alcohol dehydrogenase
A_p	A pale
AP2γ	Activating enhancer binding protein 2 gamma
A_{pr}	A paired
AR	Androgen receptor
A_s	A single
ATP	Adenosine triphosphate
BAX	BCL2-associated X protein
BCE	Belancanda chinensis extract
BCL	B-cell lymphoma
BEP	Bleomycin, etoposide, and cisplatin
BLK	B lymphoid tyrosine kinase
BMP	Bone morphogenic protein
BPA	Bisphenol A
BRDU	Bromodeoxyuridine
BSA	Bovine serum albumin
BTB	Blood testis barrier
cAMP	Cyclic adenosine monophosphate
CCN	Cyclin
CD	Cluster of differentiation
CDH	Cadherin
cDNA	Complementary deoxyribonucleic acid
CIS	Carcinoma in-situ
CML	Chronic myeloid leukemia
CNTF	Ciliary neurotrophic factor
CPE	Clostidium perfringens enterotoxin
CRABP	Cellular retinoic acid binding protein
CRBP	Cellular retinol binding protein
C_t	Threshold cycle
CUL	Cullin
CXCL	Chemokine (C-X-C motif) ligand
DAX	Dosage-sensitive sex reversal, adrenal hypoplasia critical region, chromosome X
DAZ	Deleted in azoospermia
DBD	DNA binding domain
DBP	Di (n-butyl) phthalate
DES	Diethylstilbesterol
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNMT	DNA (cytosine-5-)-methyltransferase
DPC	Days post coitum
DPPA	Dipeptide transporter

DTT	Dithiothreitol
DUB	Deubiquitinating enzyme
E	Embryonic day
EC	Embryonal carcinoma
ECM	Extra cellular matrix
EGF	Epidermal growth factor
ER	Estrogen receptor
ERK	Extracellular-signal-regulated kinase
ESC	Embryonic stem cell
FBS	Fetal bovine serum
FBXO	F-box protein
FGF	Fibroblast growth factor
FGR	Feline Gardner-Rasheed sarcoma viral oncogene
FSH	Follicle-stimulating hormone
FTS	Farnesylthiosalicylic acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GD	Gestational day
GDF	Growth and differentiation factor
GDNF	Glial cell-derived neurotrophic factor
GFRα1	GDNF family receptor alpha 1
GNRH	Gonadotropin releasing hormone
HCK	Hemopoietic cell kinase
hMSC	Human bone marrow-derived mesenchymal stromal cell
HRP	Horseradish peroxidase
HSP	Heat shock protein
ICC	Immunocytochemistry
ICSI	Intracytoplasmic sperm injection
IGF	Insulin growth factor
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IN	Intermediate
IPA	Ingenuity Pathway Analysis
IVF	In vitro fertilization
JAK	Janus kinase
JAM	Junctional adhesion molecule
kDa	Kilo-dalton
KEGG	Kyoto Encyclopedia of Genes and Genomes
LBD	Ligand binding domain
LCK	Lymphocyte-specific protein tyrosine kinase
LH	Luteinizing hormone
LIF	Leukemia inhibitory factor
MEHP	Mono-(2-ethylhexyl) phthalate
MgCl	Magnesium chloride
MIN	Minute
MIS	Mullerian inhibiting substance
MMP	Matrix metallopeptidase

NaCl	Sodium chloride
NEDD	Neural precursor cell expressed developmentally downregulated
NGF	Nerve growth factor
NGN	Neurogenin
NIH	National institutes of health
NPY	Neuropeptide Y
NSC	Neural stem cell
OCLN	Occludin
OCT4/POU5F1	Octamer-binding transcription factor 4/ POU class 5 homeobox 1
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PGC	Primordial germ cell
PH	Pleckstrin homology
PK	Protein kinase
PLAP	Placenta alkaline phosphatase
PLC	Phospholipase C
PLZF/ZBTB16	Promyelocytic leukemia zinc finger/ Zinc finger, BTB domain-containing protein
PMC	Peritubular myoid cell
PND	Post natal day
PPAR	Peroxisome proliferator-activated receptor
PTB	Phosphotyrosine binding
PTPR	Protein tyrosine phosphatase receptor
PVDF	Polyvinylidene fluoride
qPCR	Quantitative polymerase chain reaction
RA	Retinoic acid
RALDH	Retinaldehyde dehydrogenase
RAR	Retinoic acid receptor
RARE	Retinoic acid response element
RETALDH	Retinaldehyde
RNA	Ribonucleic acid
RNF	Ring finger protein
ROL	Retinol
RPMI	Roswell Park Memorial Institute medium
rRNA	Ribosomal RNA
RT-PCR	Reverse transcriptase polymerase chain reaction
RXR	Retinoid X receptor
SCF	Stem cell factor
SD	Sprague Dawley
SEC	Second
SEL	Selectin
SEM	Standard error of mean
SH2	SRC homology 2
SOX	SRY (sex determining region Y)-box

SPATA	Spermatogenesis associated
SPP	Secreted phosphoprotein
SRC	SRC v-src avian sarcoma (Schmidt-Ruppin A-2) viral oncogene
SRY	Sex-determining region Y
SSC	Spermatogonia stem cell
STAT	Signal Transducer and Activator of Transcription
STRA8	Stimulated by retinoic acid 8
STRING	Search Tool for the Retrieval of Interacting Genes/Proteins
TC	Testicular cancer
TDS	Testicular dysgenesis syndrome
TEX	Testis expressed
TFM	Testicular feminizing mice
TGCT	Testicular germ cell tumor
TGF	Transforming growth factor
THR	Thyroid hormone receptor
TJP	Tight junction protein
TNFR	Tumor necrosis factor receptor
TRIM	Tripartite motif containing proteins
UBQLN	Ubiquilin
UCHL	Ubiquitin carboxy-terminal hydrolase
UPS	Ubiquitin proteasome system
USP	Ubiquitin specific protease
VAD	Vitamin A deficient
VDR	Vitamin D receptor
VEGF	Vascular endothelial cell growth factor
VEGFR	Vascular endothelial growth factor receptor
YES1	Yamaguchi sarcoma viral (v-yes) oncogene homolog 1
ZO	Zonula occludens

Format of the Thesis

This is a manuscript-based thesis, which conforms with the "Guidelines for Thesis Preparation" of the Faculty of Graduate Studies and Research at McGill University. This thesis is comprised of five chapters, of which three chapters are in the form in which they were submitted or will be submitted for publication. Chapter 1 is a general introduction, which provides the relevant background material for this thesis. Chapters 2 and 4 are manuscripts in preparation for submission. Chapter 3 is a manuscript that has already been published in the journal 'Biology of Reproduction' (2012; 87(2): 44, 1-18). Connecting text between chapters 2-3 and chapters 3-4 is used to ensure continuity of the thesis. Chapter 5 contains a general overview of the findings from this thesis and discusses them in greater detail, alongside possible future experiments. References are provided at the end of each chapter. Appendices contain supplemental data that are available on the journal site for the original published chapter.

List of Original Publications (included in this thesis)

This thesis is based on the following original publications. It comprises five chapters, three of which are manuscripts that are written in the format they were, or will be submitted. Chapter 3, already published, was reproduced with the permission from its copyright holder.

(1) **Manku G**, Wang Y, Merkbaoui V, Boisvert A, Blonder J, and Culty M. Role of Retinoic Acid and Platelet-Derived Growth Factor Receptor crosstalk in the regulation of neonatal gonocyte and embryonal carcinoma cell differentiation. *(Manuscript In Preparation)*

(2) **Manku G**, Wing S, and Culty M. Expression of the Ubiquitin Proteasome System in rat gonocytes and spermatogonia: Role in gonocyte differentiation. (*Biology of Reproduction 2012; 87(2): 44, 1-18)*

(3) **Manku G**, Hueso A, Kaylor P, Brimo F, Chan P, and Culty M. Identification of genes overexpressed in testicular seminoma tumors and downregulated during gonocyte differentiation as potential players in the origins of testicular cancer. *(Manuscript In Preparation)*

List of Original Publications (not included in this thesis)

The following is a list of original publications that have contributed to the general understanding of germ cell development, in particular gonocytes, but were not included in this thesis.

(1) **Manku G**, Wang Y, Thuillier R, Rhodes C, and Culty M. Developmental expression of TSPO in testicular germ cells. Current Molecular Medicine 2012; 12(4): 467-475.

(2) **Manku G**, Mazer M and Culty M. Neonatal testicular gonocytes isolation and processing for immunocytochemical analysis. Methods in Molecular Biology 2012; 825: 17-29.

(3) Thuillier R, Mazer M, **Manku G**, Boivert A, Wang Y, and Culty M. Interdependence of platelet-derived growth factor and estrogen-signaling pathways in inducing neonatal rat testicular gonocytes proliferation. Biology of Reproduction 2010; 82(5): 825-836.

(4) Thuillier R, **Manku G**, Wang Y, and Culty M. Changes in MAPK pathway in neonatal and adult testis following fetal estrogen exposure and effects on rat testicular cells. Microscopy Research and Technique 2009; 72(11): 773-786.

(5) Bose R, **Manku G**, Culty M, and Wing S. Ubiquitin proteasome system in spermatogenesis. Book review. Posttranslational Protein Modifications in the Reproductive System. Editor: Dr Peter Sutovsky. Springer Publishing 2014. *(In Press)*

(6) Sarkar O, **Manku G**, and Culty M. Role of a variant PDGF receptor in the progression of rat testicular transitional gonocytes toward differentiation. *(Manuscript in preparation)*

(7) **Manku G** and Culty M. Unraveling genes and signaling pathways involved in gonocyte and spermatogonia differentiation. *(Working title, Manuscript in preparation)*

(8) **Manku G**, Papadopoulos P, and Culty M. Acetaminophen versus Ibuprofen: Effects on neonatal testicular gonocyte development. *(Working title, Manuscript in preparation)*

Contribution of Authors

This thesis is based on the following three original publications.

Chapter 2

Role of Retinoic Acid and Platelet-Derived Growth Factor Receptor crosstalk in the regulation of neonatal gonocyte and embryonal carcinoma cell differentiation.

Gurpreet Manku, Yan Wang, Vanessa Merkbaoui, Annie Boisvert, Josip Blonder, and Martine Culty.

Manuscript In Preparation.

I was responsible for carrying out the experiments detailed in this manuscript, except for the data in Figures 2.1 (A-E), 2.2 (A-D), 2.3 (D), and 2.7 (A) which was produced by Dr. Yan Wang. Vanessa Merkbaoui contributed to the quantitative PCR analysis described in Supplemental Figure 2.1 (B and C). Annie Boisvert performed the western blot in Figure 2.4 (A). Dr. Josip Blonder performed the mass spectrometry analysis presented in Figure 2.5 (C). I wrote the first draft and collaborated with Dr. Culty for the final version of the manuscript.

Chapter 3

Expression of the Ubiquitin Proteasome System in rat gonocytes and spermatogonia: Role in gonocyte differentiation.

Gurpreet Manku, Simon S. Wing, and Martine Culty

Biology of Reproduction 2012; 87(2): 44, 1-18.

I was reponsible for carrying out all of the experiments detailed in this manuscript. Dr. Wing provided us with the antibodies used in Figure 3.2 and participated to the discussion of the project. The first draft was written by Dr. Culty and we collaborated for the final version along with Dr. Wing.

Chapter 4

Identification of genes overexpressed in testicular seminoma tumors and downregulated during gonocyte differentiation as potential players in the origins of testicular cancer.

Gurpreet Manku, Aurelie Hueso, Paul Kaylor, Fadi Brimo, Peter Chan, and Martine Culty.

Manuscript In Preparation.

I was reponsible for carrying out the experiments detailed in this manuscript, except for the following data: Aurelie Hueso contributed to the immunohistochemistry experiments shown in Figure 4.7 (B and C). Paul Kaylor contributed to the immunocytochemical analysis shown in Figure 4.2 (D) and the RT-PCR analysis shown in Figure 4.3. Dr. Fadi Brimo provided the testicular tumor microscope slides analyzed in Figures 4.1 (B) and 4.7 (B and C), aided in the microscopic analysis of these stainings, and also provided invaluable explanations of the various testicular pathologies. Dr. Peter Chan provided the normal and tumoral testicular tissue used in Figure 4.1 (A) and in the human gene array analysis described within this manuscript. I wrote the first draft and collaborated with Dr. Culty for the final version of the manuscript.

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environment in which to work, and in this group, I have not only found great scientists, but have formed friendships that will last a lifetime. Here, I would like to especially thank Dr. Daniel Martinez for his invaluable scientific help throughout the years.

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Chapter 1

Introduction

Chapter 1: Introduction

Preface

Spermatogenesis is a complex process that is essential for the survival of the species. Resulting in the formation of spermatozoa, spermatogenesis requires phases of proliferation, migration, differentiation, and apoptosis, and relies on strict regulatory and feedback mechanisms for proper sperm formation. Spermatozoa arise from a reservoir of resident spermatogonial stem cells (SSCs) that must be maintained. Once formed, spermatozoa continue their maturation in the epididymis and become motile. Spermatozoa acquire the ability to fertilize an egg once in the oviduct by undergoing further processing such as capacitation. Spermatogenesis as a general process is very thoroughly studied. Numerous studies have added to the ever-growing understanding of the underlying mechanisms involved in normal sperm development. However, most often, these studies have focused on the development of sperm from the later stages of spermatogenesis to the fertilization of an egg and subsequent embryonic development. In the last years, more attention has been given to the study of the mechanisms regulating SSC self-renewal and differentiation. Unfortunately, the earlier phases leading to the formation of the SSCs are often over-looked, and even more rarely investigated.

The establishment of the SSC reservoir is an integral part of spermatogenesis, without which sperm formation would not be possible, and it arises from the differentiation of precursor cells known as gonocytes. Gonocytes are the germ cell type that results from the differentiation of primordial germ cells and exists during the perinatal phases of spermatogenesis, or "pre-spermatogenesis". Although gonocytes play an essential role in SSC pool formation, the process of gonocyte development is poorly understood. It has been suggested that improper gonocyte development can lead to the formation of carcinoma in-situ, a precursor of testicular germ cell tumors. Given the steady incline in testicular germ cell tumor rates over the past few decades, gonocytes represent a cell type that needs to be more thoroughly studied. The focus of this thesis is to gain a better overall understanding of the molecular mechanisms involved in gonocyte development and to understand which of these mechanisms could likely be involved in testicular germ cell tumor formation.

1.1 Male Reproductive System

1.1.1 The Origins of the Testis

During embryonic development, the gonad is known to originate as an indifferent tissue that can develop into one of two distinct pathways (1). It is at this time that a crucial decision regarding sex determination must occur and as a result, the developing gonad will either become an ovary in the female, or a male testis (2). Whereas differentiation towards the female is considered to be the default pathway, it is mainly due to the testis-determining SRY gene that differentiation towards a male testis can occur (1, 3, 4). Studies have shown that testis development does not occur in a linear manner but instead, is the result of a complex network of gene interactions that depends highly on expression levels and proper timing (1). In contrast to the importance of SRY, other genes such as DAX1 have been shown to be downregulated at the same time in development, indicating the possible necessity of low levels of such genes in order to maintain normal testis development (1). Upon sex determination, the gonads will further secrete factors that will promote male sexual development in the embryo (1).

1.1.2 Testis Structure and Function

The testis is the male gonad and is central to the male reproductive system. Although the testis only serves two functions in mammals, these two functions are essential for the survival of the species. The testis is essential for synthesis and release of male hormones important for male sexual differentiation, such as testosterone; and for the formation of spermatozoa through the process of spermatogenesis (5-8).

In rodents and humans, the testes are present in pairs, are oval-shaped organs that lie inside the scrotum, and are suspended by the spermatic cord (9, 10). This position is essential in order to provide the ideal temperature, 2-3 degrees lower than body temperature, that is necessary for proper testicular development to occur (11).

Morphologically, each individual testis is enveloped by a dense, fibrous layer of connective tissue known as the tunica albuginea (12). The testis is made up of two main compartments, the interstitium and the seminiferous tubules (12). These two compartments are not only physically separated but are also distinct in their function.

1.1.2.1 The Testicular Interstitium

The interstitial area mainly contains vasculature, macrophages, lymphatic vessels, peritubular myoid cells, and the Leydig cells (5, 7, 8, 13, 14).

1.1.2.1.1 The Leydig Cell

Leydig cells are important for growth and hormonal regulation of germ cell development and their main function is being the major source of testosterone (15). In rats and mice, Leydig cells exist as two distinct populations that do not arise from the same precursor cell lineage; fetal Leydig cells and adult Leydig cells (16). Fetal Leydig cells first appear at 12.5dpc (days post coitum), begin to mature a few days later while producing testosterone, and functioning in a gonadotropin independent manner until gestation day (GD) 18 where they become responsive to gonadotropins. Interestingly, these fetal cells begin to atrophy during the first two weeks after birth, at which time, Leydig stem cells of mesenchymal origin begin to develop. These stem cells then produce progenitor Leydig cells, which will eventually lead to the formation of immature, then adult Leydig cells by post natal day (PND) 56 (16). Adult Leydig cells can respond to LH via their LH receptors and produce testosterone (16). Interestingly, studies have found that testosterone is also vital during spermatogenesis and that a loss of testosterone can lead to a lack of spermatogenesis and fertility (17, 18). Testosterone is also known to be essential in the development of the male phenotype including masculinization of the brain, sexual behaviour, and differentiation of the male sex organs (17).

1.1.2.2 The Seminiferous Tubules

The seminiferous tubules are a group of well organized, long convoluted tubules which connect at both ends to the rete testis, which is important in transporting the sperm from the seminiferous tubules to the efferent duct, the initial part of the epididymis (9, 10). Studies have shown that the seminiferous tubules coordinate with Leydig cells and the brain via the hypothalamic-pituitary-testicular axis to regulate spermatogenesis. This confirms the importance of testosterone (produced by Leydig cells), LH (luteinizing hormone produced by the pituitary gland), FSH (follicle-stimulating hormone produced by the pituitary gland), and GnRH (gonadotropin releasing hormone produced by the hypothalamus) in spermatogenesis (19). Interestingly, as the later stages of spermatogenesis begin to occur, the relative volume of the

interstitial tissue is reduced to make way for the growing seminiferous tubules (20). The seminiferous tubules make up the majority of the testicular weight and are the site of spermatogenesis (5).

In the rat testis, these tubules are surrounded by a single layer of peritubular myoid cells, whereas in the human, there are multiple peritubular myoid cell layers (21, 22). These myoid cells are involved in supporting the transport of spermatozoa and testicular fluids from the testis to the efferent ducts due to their contractile motion (23). Although this contractile motion has been shown to possibly be affected by oxytocin, TGFβ, and prostaglandins, the exact mechanism of the actual contractility remains unknown (23). Peritubular myoid cells have the ability to secrete various components of the extracellular matrix (including fibronectin and collagen) and growth factors (including TGFβ and IGF-I) (22). Although the exact role of peritubular myoid cells remains unknown, the findings that they have the ability to affect Sertoli cell function (24), are involved in retinol processing (24), and contain androgen receptors (25) indicate that they likely play a role not only in the structural aspects of the seminiferous tubules, but also in the regulation of spermatogenesis.

The seminiferous tubules themselves are made up of two different types of cells. These include the supporting Sertoli cells and the germ cells (26). The germ cells represent a highly specialized type of cell in development and are present throughout spermatogenesis, from the fetal primordial germ cells to spermatozoa. Contrary to the Sertoli cells which are referred to as Sertoli cells at all ages, the germ cell names change according to the phases of spermatogenesis. Germ cells include primordial germ cells, gonocytes, spermatogonial stem cells, undifferentiated, differentiating and differentiated spermatogonia, primary and secondary spermatocytes, round and elongated spermatids, and spermatozoa (27). The final maturation of spermatozoa, in which they acquire motility and the ability to fertilize an ovum, occurs following their exit from the testes, during their travel through the epididymis (28).

1.1.2.2.1 The Sertoli Cells

Sertoli cells are essential for proper spermatogenesis to occur. First described by Enrico Sertoli in 1865, Sertoli cells are somatic cells that are the predominant cell type in the seminiferous tubule until puberty (29, 30). Morphologically, these cells are characterized as

having an asymmetrical appearance, abundant smooth endoplasmic reticulum, a well developed Golgi complex, lysosomes, mitochondria, and a rich cytoskeleton (30). In the fetus, Sertoli cells actually surround the germ cells, but as spermatogenesis occurs, germ cells are held in the spaces between adjacent Sertoli cells or in areas close to the luminal surface (12, 15).

Sertoli cells are also part of the blood-testis-barrier (BTB). The BTB is formed by tight junctions between adjacent Sertoli cells in adults and separates the epithelium into basal and adluminal components, where the basal compartment contains the spermatogonia and early spermatocytes, and the adluminal compartment contains the late spermatocytes, spermatids, and spermatozoa (15, 31, 32). This barrier functions to limit the entry of foreign substances from the blood and the interstitial extracellular fluid into the seminiferous tubules. By limiting the flow of substances, the BTB also helps maintain the microenvironment necessary in order for proper germ cell development to occur (33). In rats, this barrier does not occur before puberty (34).

Although the formation of the BTB is essential for spermatogenesis to occur, the main function of Sertoli cells is that of a supporting cell. Sertoli cells provide the nutrients and structural support needed for germ cells during their development by providing growth factors and other proteins necessary for their development (34-36). However, because the Sertoli cells are only able to support a finite number of germ cells through spermatogenesis, it is the number of Sertoli cells that limits the number of sperm that will eventually be produced (12, 15, 37). All in all, due to their importance in providing nutrients and structural support to their neighbouring germ cells, Sertoli cells play an essential role in spermatogenesis.

1.2 Spermatogenesis

Spermatogenesis is the process by which haploid spermatozoa are formed from diploid spermatogonia and SSCs (See Figure 1.1). In humans, spermatogenesis takes 64 days, while in rats it takes 50 days and in mice it only takes 35 days (38). These changes occur in waves through the length of each seminiferous tubule, determining specific associations of different germ cell types that have been classified as stages by Clermont and Leblond in the 1950s (6). While the human spermatogenic cycle includes only six stages, rats include 14 and mice include 12. Although there are differences in the number of days it takes for a spermatogenic cycle and

in the numbers of stages per cycle between species, the process itself is similar between humans and rodents, and thus, rodents are commonly used models to understand spermatogenesis (38).

Spermatogenesis is a dynamic process that requires multiple coordinated events to take place within a short period of time, requiring major protein turnover and cellular remodeling (39). The ubiquitin proteasome system (UPS), a major player in the regulation of processes such as protein degradation (40) has been shown to be involved in spermatogenesis (41). Simply, a ubiquitin molecule is activated and attached to a substrate protein via an E1 activating enzyme, E2 conjugating enzyme, and E3 ligase. The ubiquitin chain then acts as a signal recognized by the 26S proteasome, resulting in protein degradation (42, 43). Several studies have shown that UPS enzymes play major roles spermatogenesis from pre-pubertal to adult mice. For example, knockdown models of the E2 conjugating enzyme UBC4 resulted in mice with a delayed spermatogenic cycle (44). Mice lacking deubiquitinating enzyme USP2 did not have proper spermatozoa motility and had a severe fertilization defect (45). To add, knockdown models of E3 ligase RNF17 presented male sterility and an arrest in round spermatids (46). Thus, the UPS is essential for proper spermatogenesis to occur.

1.2.1 The Three Main Phases of Spermatogenesis in the rat

Although a complex process, spermatogenesis can be divided into three main parts: the mitotic phase in which spermatogonial stem cells undergo proliferation and differentiation to maintain an essential stem cell reservoir pool, the lengthy meiotic phase in which germ cells become haploid, and the post meiotic phase, spermiogenesis, in which the spermatids undergo dramatic morphological changes to become spermatozoa that will further transform into mature and functional spermatozoa once in the epididymis (15, 27). It is the continuation of this process that makes spermatogenesis a dynamic and massive process; producing, in humans, millions of mature sperm per gram of testis weight each day (15, 47).

In the first step of spermatogenesis, spermatogonia, the least differentiated spermatogenic cell, are located in the basal compartment of the seminiferous tubule and are surrounded by Sertoli cells (31). In order to ensure that a sufficient number of germ cells are primed to enter meiosis, at this stage, mitosis of the spermatogonia occurs (31). In the 1950's, Leblond and Clermont first reported that in rats there were six successive generations of differentiating

spermatogonia occurring during spermatogenesis: types A_1, A_2, A_3, A_4, intermediate (In), and B (48). They also reported that as these cells matured, there was a gain in heterochromatin and decreased nuclear size. Further work by this group proposed that it was type A_4 spermatogonia that were the stem cells and that at each cycle turnover, A_4 gave rise to A_1 (self-renewal) and In spermatogonia (differentiation) while a reserved population of stem cells, called A_0, underwent self-renewal only if needed due to damage (49, 50, 51). Although this model was reported early on, there was another model that was soon proposed, and is now more commonly accepted (10, 52). According to this more accepted model in rodents, first described by Huckins and Oatberg, there are four main populations of spermatogonia: undifferentiated type A spermatogonia (A single (A_s), A paired (A_{pr}), and A aligned (A_{al})), differentiating type A spermatogonia (A_1, A_2, A_3, A_4), intermediate (In) spermatogonia, and differentiated type B spermatogonia (53, 54). In humans, only type A pale (A_p) and type A dark (A_d) spermatogonia exist before type B spermatogonia (55). In rodents, A_s are considered to be the SSCs because they either undergo self-renewal to maintain a reservoir pool of these stem cells or they differentiate into A_{pr} (which are connected by an intercellular bridge due to incomplete cytokinesis) (56, 57). It is believed that these A_s will either replenish the SSC population by producing two daughter cells or will differentiate in an asymmetric manner by which one cell will remain a stem cell while the other continues to differentiate (57). Although evidence for this asymmetric division is not abundantly available, there are some studies that have provided better insight into the gene profiles associated with this theory (58). To continue, A_{pr} then leads to the formation of A_{al}, and eventually into differentiating spermatogonia A_1. Until the cells have reached the A_{al} phase, they are considered to be undifferentiated. A_1 will undergo mitosis six times and go through A_2, A_3, A_4, and In, until eventually, type B spermatogonia are formed (52).

At the end of the first stage of spermatogenesis, the resulting type B spermatogonia undergoes a mitotic division where every one type B spermatogonia becomes two preleptotene spermatocytes, pre-meiotic cells in which DNA duplicates (15). The subsequent meiotic prophase is divided into 4 main stages: leptotene, zygotene, pachytene, and diplotene (15). Diplotene spermatocytes undergo a first round of meiosis (meiosis I) to become secondary spermatocytes. Once the secondary spermatocytes have been formed, these cells quickly undergo meiosis again (meiosis II) to become haploid round spermatids (15), which mark the end of the second step of spermatogenesis.

Finally, in order for sperm to be produced, the round spermatids must undergo a process known as spermiogenesis (31). Spermiogenesis in itself is a complex process that involves nineteen steps in rats and leads to the formation and release of sperm from the seminiferous tubules (59). In brief, during this process, there is a multitude of morphological and biochemical changes that occur to the maturing sperm including structural modifications to the shape of their nucleus, compaction of the nuclear chromatin, acrosome formation, development of a flagellum, and shedding of the cytoplasm from the germ cell (resulting in residual bodies) (10, 59). Afterwards, spermiation occurs in which spermatozoa are released from the seminiferous tubules into the tubule lumen. The spermatozoa are transported into the rete testis, and then, through the efferent ducts into the epididymis (59). It is in the epididymis that these spermatozoa gain motility and become one step closer to being able to fertilize an egg (31). The epididymis itself is a highly complex structure made up of five main parts (the initial segment, the intermediate zone, the head (caput), the body (corpus), and the tail (cauda)) which all function together in order to make sure not only that the resulting sperm is motile, but also provides sperm storage, protection, and transport until further processing occurs within the oviduct (31, 60).

1.2.2 Spermatogonial Stem Cells

In the human body, there are multiple types of adult somatic stem cells which, although rare, ensure the maintenance and homeostasis of tissues such as skin, hair, and the blood (61). In the testis, the maintenance of a stem cell pool is critical to ensure the production of sperm throughout the life time of a male (57). Similar to these other adult somatic stem cells, SSCs are stem cells that need to undergo both self-renewal and differentiation in order to be a true stem cell (57, 61). In the seminiferous tubules, SSCs (a fraction of type A_s spermatogonia) and the cells that they will give rise to (A_{pr} and A_{al}) are located in the areas of the tubules that border the interstitial area, thus creating a SSC niche microenvironment characteristic of these stem cells (62, 63). The SSC niche provides the stem cells with the microenvironment that they require in order for proper self-renewal and for differentiation to occur, including Sertoli cell produced factors that can assist in stimulating both of these processes (63).

In normal spermatogenesis, the ratio of SSC self-renewal to differentiation is 1:1, which is sufficient to maintain spermatogenesis. If there was more differentiation occurring than self-renewal, this would result in a depletion of the SSC pool but if, on the other hand, the rates of

self-renewal were higher, there would not be an adequate amount of sperm being formed. However, this ratio can be altered if necessary in response to cell loss caused by irradiation or other toxic substances (57).

Although the periodic renewal of type A_s spermatogonia was recognized in the last century (64), the process by which the self-renewal occurs is complex and has only begun to be understood (10). Indeed, the exact mechanisms driving either the self-renewal or differentiation of SSCs are not that well known. Among known SSC regulators, GDNF (glial cell-derived neurotrophic factor) and FGF2 (fibroblast growth factor 2) were shown to promote SSC self-renewal (62, 63, 65, 66). The interest in better understanding how SSCs function has become greater over the past decades because following cytotoxic injury, SSCs are the only germ cells that would be able to regenerate spermatogenesis due to their ability to both self-renew and undergo differentiation to form spermatozoa (67). Thus, a better understanding of SSC development and maintenance is essential as these cells are at the base of our species' survival.

1.2.3 Early germ cell development

As previously mentioned, although the development of sperm from the SSC reservoir population onwards has been relatively well described, the development leading up to the SSC reservoir pool formation is less well understood. Germ cells are unique when compared to the other cells in an organism because they not only go through mitosis but are also able to go through meiosis to give rise to haploid gametes (67). Furthermore, germ cells are also unique as they are able to combine with a germ cell of the opposite sex and reproduce; thus creating a future generation of any given species.

1.2.3.1 Primordial Germ Cells

Although the Sertoli cells, Leydig cells, peritubular myoid cells, and other testicular cells derive from the somatic cells present in the differentiating gonad, germ cells have a different origin (67). Germ cells develop in both males and females before gender specification has occurred as primordial germ cells (PGCs) (68).

PGCs are pluripotent cells of the proximal epiblast (near to the extra-embryonic ectoderm) that migrate through the extra embryonic yolk sac mesoderm then through the hindgut and the

dorsal mesentery to reach the genital ridges within the embryo (68, 69). In mice, PGC precursor cells are first detected around 5.5 dpc in the proximal epiblast, where they can become true PGCs under the signaling influence of various bone morphogenic proteins (BMP4 and BMP8b) from cells of the extraembryonic ectoderm (39, 70, 71). At 7.5dpc in rodents (3 weeks gestation in humans), the few PGCs that are present can be identified by their strong positive expression of PLAP (placental-like alkaline phosphatase) as well as their retention of OCT-4 (POU5F1) transcription factor expression, which is originally expressed in all totipotent embryonic cells but later becomes restricted to expression only in PGCs (72, 73). At this point, the PGCs will migrate inside the extra-embryonic mesoderm at the base of the allantois towards the genital ridge (under the guidance of a cKIT tyrosine kinase receptor ligand, steel), where they become resident germ cells (39, 74, 75, 76).

While migrating, PGCs continue to undergo proliferation but do not differentiate (77). It is during the PGC phase that the parental DNA methylation marks are erased, in preparation for the establishment of a new DNA methylation pattern that will be retained until fertilization and zygote development (39). Once they have arrived at the genital ridge in the embryo, the sexually bipotential PGCs acquire male or female fates between 10.5-11.5dpc in mice, driven by SRY expression in male. In females, the germ cell enters meiosis and DNA methylation is delayed (78). To add, there is an arrest in prophase I of meiosis I until puberty (78). In males, these PGCs become enclosed by Sertoli cells, thus forming the testicular cords in males at 12.5dpc in rodents (4-5 weeks gestation in humans) (39, 73, 79, 80). Studies have found that even without the presence of germ cells, testicular cord formation can still occur (81). It is at this point that sex determination is decided, based on the expression of the SRY gene on the Y chromosome of the somatic cells that will lead to the formation of the fetal Sertoli cells from coelomic epithelium precursor cells. Furthermore, it has been reported that SRY and SOX9 (SRY-related HMG box family of transcription factors member) induce Sertoli cells to undergo differentiation and to secrete Mullerian Inhibiting Substance (MIS) in order to suppress the development of the female reproductive tract (4, 82-85). Once the testicular cords have formed, the germ cells present in the cord are no longer referred to as PGCs but instead, are called gonocytes (39, 73, 86).

Although the term gonocyte was originally proposed by Clermont and Perey in 1957 to describe a fetal germ cell after it had become resident in the developing gonad (86), other terms

such as pre- and pro-spermatogonia have been used to designate later developmental phases of this cell type (39). Interestingly, although the term ‘gonocytes’ is the most commonly used to describe these cells, the use of this term is still debated by those few who believe that pre- or pro-spermatogonia are terms that better describe the cells (39, 87, 88). In this thesis, the term gonocyte is used to identify germ cells starting when the cells become resident in the developing gonad to the time when they migrate to the basement membrane of the seminiferous cord to differentiate into spermatogonia, covering from fetal to neonatal periods.

1.3 Gonocytes

Morphologically, there are not many differences between gonocytes and their precursor PGCs (39, 89). However, studies have shown that PGCs can be cultured *in vitro* with any type of somatic cell whereas gonocytes can only be cultured with Sertoli cells (51). These germ cells now represent a cell population that, for the first time in development, has restricted potential and is committed only to further development into the germline lineage (51, 90). Another major difference distinguishing the two phases of development is that global DNA demethylation occurs in PGCs, constituting the "erasure" phase that removes DNA methylation patterns of parental origin. By contrast, gonocytes are the site of active DNA remethylation (91).

Electron microscopy analysis has shown that gonocytes are large cells (12-15μm) that can be distinguished from their surrounding Sertoli cells in the seminiferous tubule due to their circular morphology, prominent spherical euchromatic nucleus, and one or two nucleoli (92, 93). Gonocytes are surrounded by Sertoli cell cytoplasmic extensions and are separated from the seminiferous tubule basement membrane by three or four layers of Sertoli cells (87). Although the blood testis barrier has not been formed at the time when gonocytes exist, gonocytes and Sertoli cells are known to interact with each other using gap junctions and desmosomes (94).

Fetal gonocytes in the rat will proliferate for a few days until 17.5dpc where they will then become arrested in the G_0/G_1 phase of the cell cycle until 3 days after birth (1, 39). This represents one of the two active periods of proliferation that gonocytes undergo (the fetal phase and the neonatal phase), which are separated by this quiescent period (39, 86, 95, 96). Although there are two proliferative phases in rats and mice, the available data for human gonocyte development suggests that humans only have one phase in which gonocytes are proliferative,

between months 3-6 of gestation which is then followed by a quiescent phase and a not well understood transitional phase, that exists up until a few weeks after birth, at which time they differentiate to SSCs (39). In rats, at PND3, gonocytes will re-enter the mitotic phase and undergo proliferation, migration to the seminiferous tubule basement membrane, and differentiation into SSCs by PND6-8 (See Figure 1.2) (96). Gonocytes that have failed to migrate and differentiate will be eliminated by apoptosis between PND7-14 (97).

1.3.1 Gonocyte Proliferation and Quiescence

In brief, gonocytes undergo two proliferative phases. During fetal development, gonocytes undergo proliferation once they become the resident germ cells within the developing testis. After a quiescent phase that lasts from 17.5dpc to a few days after birth in rats, gonocytes then enter their second proliferative phase, the neonatal phase (39).

1.3.1.1 Fetal Gonocyte Proliferation

For years, few studies had been done on the fetal mitotic phase of gonocyte development. However, although still scarce, more findings have recently been reported, providing us with a better understanding of a few key elements involved in this developmental process.

For example, it has been reported that retinoic acid (RA) has an effect on fetal gonocyte proliferation (98). Interestingly, as will be described below, RA is also involved in neonatal gonocyte differentiation (99, 100). In regards to fetal gonocyte proliferation, studies have reported that upon RA stimulation, there is a slight increase in gonocyte proliferation and significant induction of apoptosis, leading to an overall reduction in the number of fetal gonocytes (98). This study further reported that RA allowing for both an increase in proliferation and apoptosis was possibly a mechanism by which the germ cells that were not undergoing proper development could undergo apoptosis and be eliminated (98, 101).

To continue, the activator of protein kinase C, phorbol ester PMA, has been shown to reduce fetal gonocyte proliferation (96). Furthermore, more recent studies done in mice indicated that there is a necessary balance of activin A needed for Sertoli cell and germ cell proliferation in the fetal period (102). Activin A is one of the many ligands for the TGFβ (transforming growth factor receptor) superfamily alongside bone morphogenic proteins (BMPs) and growth and

differentiation factors (GDFs) (103). To add, knockout models of activin beta A subunit (lacking activin A; Inhba-/-) were shown to have a significantly reduced number of Sertoli cells and twice the number of gonocytes at birth when compared to normal mice (102). Thus, activin A was playing an important inhibitory role in fetal gonocyte proliferation. Given that activin A is likely playing a role as a negative regulator of fetal gonocyte proliferation, it was not surprising to find that TGFβ itself has also been shown to be a negative regulator in this process (104).

It has also been reported that androgens are likely playing a direct role in inhibiting fetal gonocyte proliferation (105). These studies were done using testicular feminizing mice (TFM) which contain a mutation that inactivates the androgen receptor. In these studies, they found that TFM mice had higher levels of fetal gonocyte proliferation at 17.5dpc when compared to normal mice (105).

Although RA, activin A, and androgens have been shown to be involved in the regulation of fetal gonocyte proliferation, the exact mechanism by which this process occurs remains to be determined.

1.3.1.2 Gonocyte Quiescence

In rats, from 17.5dpc to PND3, gonocytes are quiescent. Although not much is known about the quiescent phase, some studies have attempted to better understand its underlying mechanisms.

For example, mutant mice with a constitutively active Notch1 intracellular binding domain in Sertoli cells resulted in gonocytes having an abnormally quick exit from quiescence, migrating towards the basement membrane of the seminiferous tubules, and undergoing differentiation, all before birth (106). Alongside apoptosis, by PND2, there were no gonocytes remaining in these tubules. This indicated that Notch1 regulation was essential in Sertoli cells to help gonocytes develop in a timely manner (106, 107).

Furthermore, studies have shown that TGFβ is not only a negative regulator of fetal gonocyte proliferation but also of fetal gonocyte apoptosis, and thus, as a result, is likely involved in regulation of the gonocyte quiescence phase (104).

To add, di (n-butyl) phthalate (DBP) has been shown to delay entry of proliferating gonocytes into quiescence and thus, postnatally, the gonocytes will then also be delayed in their exit from quiescence (108). Phthalates are a class of endocrine disruptor compounds that interfere with the normal function of various endocrine organs (109). Negative effects due to phthalates have been attributed to both female and male reproduction and thus, it is not surprising that these types of compounds also have an effect on gonocyte development, especially considering that the increase in testicular germ cell tumor incidence rates are commonly linked to increases in endocrine disruptor compounds in our environment (109, 110).

Finally, although it would seem that the period of quiescence would not be majorly important for gonocyte development, it is considered to be essential as major epigenetic reprogramming occurs during this period (111). In rats, the quiescence phase is completed at PND3, when gonocytes re-enter the mitotic phase, whereas in human, as previously mentioned, gonocytes do not appear to have a second proliferative phase (39).

1.3.1.3 Neonatal Gonocyte Proliferation

In rats, at PND3, gonocytes re-enter mitosis for the second mitotic phase of gonocyte development. For many years, it was not known how this mitotic phase was regulated. In the studies confirming the importance of RA in fetal gonocyte proliferation (98), the same effects were not seen in neonatal gonocyte proliferation which suggested that likely, different factors were regulating these two different mitotic phases.

Initial studies conducted in the 1990's indicated that factors such as thyroid hormone, thymulin, and Mullerian inhibiting substance (MIS) promoted neonatal gonocyte proliferation (112-114). Furthermore, studies reported that fibroblast growth factor-2 (FGF2) had a proliferative effect on gonocytes also while leukemia inhibitory factor (LIF) and ciliary neurotrophic factor (CNTF) had a survival effect on gonocytes (115, 116). However, although these factors were shown to play an important role in gonocyte proliferation, there was a slight problem on how this data was obtained. These studies had been done in either co-cultured cells including both gonocytes and Sertoli cells or using whole testis organ cultures. As previously mentioned, the seminiferous tubule is made up of two main cell types, Sertoli cells and germ cells, which at PND3 in the rat, are gonocytes. *In vivo*, Sertoli cells provide the nourishment and support that is needed for

gonocytes to develop adequately (35, 36). Thus, when studies are done using gonocytes co-culture with Sertoli cells (or whole testis organ cultures), it is not clear whether the tested factors (for example, FGF2) have a direct effect on gonocytes or if their effect is occurring via an indirect effect first occurring on Sertoli cells, which then in turn, are acting upon gonocytes to promote proliferation. Because many earlier studies performed to better understand gonocyte development were done in such co-cultured conditions, they could not resolve the question of what exactly was directly causing neonatal gonocyte proliferation.

As a result, our lab began to isolate gonocytes from PND3 rats, and studied gonocyte proliferation using isolated gonocytes. This approach allowed us to identify both platelet-derived growth factor (PDGF-bb) and 17β-estradiol as necessary regulators of neonatal gonocyte proliferation, where they acted in a concentration-dependent and non-additive manner, and to show that either factor present alone in the absence of serum was not able to induce proliferation (117). Our analysis indicated that stimulating isolated gonocytes with other growth factors, including NGF, FGF2, and EGF, did not significantly stimulate gonocyte proliferation (117). Furthermore, our study showed that inhibition of either the PDGFR receptor (PDGFR) or the estrogen receptor (ER) prevented gonocyte proliferation from occurring (118). This indicated that the PDGF ligand and estradiol both needed to bind to their respective receptors in order to induce gonocyte proliferation, identifying a crosstalk between the two pathways (118).

When analyzing the PDGFR pathway activation in more depth, we found that once the PDGFR pathway was activated, it was the activation of the downstream MEK/ERK 1/2 pathway that was necessary in order to induce proliferation of these cells (118). Here, because the cells were treated as isolated cells, and not in a co-culture Sertoli cell system, we demonstrated that both PDGF and 17β-estradiol were having a direct effect on gonocyte proliferation. Both PDGF and estradiol can be produced by Sertoli cells *in vivo*, and thus, a co-culture system would have prevented us from determining whether these factors had a direct effect on proliferation or whether, as previously mentioned, it was an indirect effect via the Sertoli cells.

We have also reported that PND3 gonocytes express both receptor forms of PDGFR α and β, the PDGFRβ form being predominant in gonocytes, while surrounding somatic cells mainly express PDGFRα (119). Interestingly, a variant form of PDGFRβ, termed V1-PDGFRβ, was first described by our lab where we found that it was preferentially expressed in the developing

gonads and gonocytes, but only at specific ages in fetal brain and kidneys, and not in other tissues such as the liver or heart (99). We then sequenced the V1-PDGFRβ and found that it did not contain the PDGF ligand binding site and thus, it could not possibly be involved in any process requiring direct binding of the PDGF ligand (99). Since the induction of gonocyte proliferation requires PDGF-bb and 17β-estradiol interaction with their receptors, it must be the full length receptors that are activated during proliferation, and not the V1-PDGFRβ variant. The PDGF-bb ligand can bind to PDGFRαα, αβ, and ββ dimers (120). However, in our study, although we found that PDGFRβ was involved in proliferation, we did not distinguish between the αβ and ββ receptor dimers. Another group later confirmed that PDGFRβ expression was involved in neonatal gonocyte proliferation in mice but again, could not distinguish if it was the full length or variant form that was involved, or whether it was the αβ or ββ receptor dimer that was necessarily activated (121).

Thus, although not many studies have analyzed neonatal gonocyte proliferation, the work of our group has been instrumental in showing that both PDGF-bb and 17β estradiol stimulate neonatal gonocyte proliferation via crosstalk between PDGFR and ER pathways, involving MEK/ERK 1/2 pathway activation.

1.3.2 Gonocyte Migration

Gonocytes undergo proliferation and migration towards the basement membrane of the seminiferous cords and then undergo differentiation. Interestingly, although the progression of events surrounding gonocyte development (proliferation, migration, and differentiation) are often listed as one occurring after the other, that is not actually the case. Gonocyte development, like spermatogenesis in general, does not occur in a synchronous manner and thus, although at PND3, the majority of gonocytes will be undergoing proliferation by re-entering the mitotic phase, there will be gonocytes that have already begun to migrate while others will have not yet re-entered mitosis (39). Gonocyte migration is necessary in order for differentiation to occur and gonocytes that remain at the centre of the seminiferous tubules at PND5 or later undergo apoptosis and are eliminated (121-123).

Although migration would likely require neonatal gonocytes to disassociate themselves from their surrounding Sertoli cells and translocate between the Sertoli cells to reach the seminiferous

cords basement membrane, there are reports indicating that no total dissociation is needed but that instead, gonocytes need to remain adherent to the Sertoli cells which would provide a needed substrate for this translocation (87, 93, 124). Given these opposite hypotheses, it seems likely that a balance of both cell adhesion and spacio-temporal dissolution of cell-cell junctions is required in order for gonocyte migration to occur. Indeed, this appears to be a very dynamic process of junction remodeling involving proteases and cell adhesion molecules (123). However, neither of these hypotheses has been experimentally validated as of yet.

Furthermore, studies have shown that in order for gonocytes to migrate to the periphery of the seminiferous cords, they do not have to obligatorily undergo proliferation (125). This was shown by labeling all proliferating gonocytes using ^{3}H-thymidine at PND2 for 48 hours. Once the incubation period was complete, the testis organ cultures were processed for autoradiography. Here, it was determined that although there were many gonocytes at the basement membrane that were labeled, there were still many gonocytes that had not been labeled, indicating that these gonocytes had not undergone mitosis but had still translocated to the basement membrane (125). Thus, the fact that not all gonocytes must undergo proliferation in order to undergo translocation to the basement membrane would indicate that these two processes are independent.

A few studies have been done to determine the factors that are likely involved in gonocyte migration. Organ culture microscopic analysis has shown that, when ready to migrate, gonocytes develop processes (similar to pseudopods) and will use these processes to orient themselves to migrate towards the basement membrane of the seminiferous tubules (93, 124). Co-culture studies have also shown that pseudopod containing gonocytes were positive for cKIT (a tyrosine kinase receptor) expression (126). cKIT also likely plays a role in migration as it is expressed in gonocytes at times that they are about to undergo migration, whereas quiescent gonocytes are cKIT negative (126). Other studies have determined that gonocytes with pseudopods were likely destined to undergo migration and differentiation whereas gonocytes without these pseudopods, round gonocytes, were destined to undergo apoptosis and be eliminated (127).

Another group of proteins likely involved in gonocyte migration includes an ADAM-Integrin-Tetraspanin complex (39, 123). These complexes consist of ADAMs 1 and 2 (A Disintegrin and A Metalloprotease), integrins α3β1 and α6β1, and the tetraspanins

(transmembrane-4 superfamily proteins) CD9 and CD81 (123). ADAMs are known to combine both cell adhesion and proteolytic properties (123). Both the ADAMs and the CD molecules can bind integrins, which in turn can bind laminin, a main component in the basement membrane of the seminiferous tubules (123).This protein complex forms a network of microdomains known as the tetraspanin web, and interestingly, cells primed to undergo apoptosis do not express these complexes (123). Given the positive expression of these proteins at a time when gonocytes are ready for migration, it seems likely that they are involved in this translocation (39, 123).

Previously, the importance of PDGFRβ activation in gonocyte proliferation has been described. Studies have also shown that PDGFRβ plays a role in gonocyte migration. However, again, it is not clear whether this migration is due to the full length receptor or to the previously described V1-PDGFRβ (121). In these studies, imatinib (a tyrosine kinase inhibitor commonly used for chronic myeloid leukemia and gastrointestinal stromal tumors) was used to inhibit PDGFR activity (128). Although imatinib can inhibit both PDGFR and cKIT, at the time-frame which was studied (up to PND5) most gonocytes do not express cKIT, thus avoiding dual inhibition (121, 129-131). Using this model, it was reported that upon PDGFRβ inhibition, gonocytes were not able to undergo efficient migration (121). Interestingly, cKIT has also been shown to be involved in gonocyte migration and both PDGFRβ and cKIT are tyrosine kinase receptors (121). Thus, the few studies that have analyzed gonocyte migration have concluded that tyrosine kinase receptors cKIT and PDGFRβ, alongside a complex formed of ADAMs, integrins, and tetraspanin proteins are likely involved in gonocyte translocation from the centre to the basement membrane of the seminiferous tubules.

1.3.3 Gonocyte Survival

The survival of an adequate number of gonocytes is necessary to ensure the production of a proper SSC reservoir. Studies have shown that there are a few key factors involved in gonocyte survival. Leukemia inhibitory factor (LIF) and ciliary neurotrophic factor (CNTF) have been shown to promote gonocyte survival (116). Using a co-culture system of Sertoli cells and gonocytes, it was shown that LIF increased the levels of proliferation after three days in culture when starting with PND1 gonocytes but not when the originating gonocytes were from PND3 rats, indicating a role for survival, and not proliferation, in PND3 gonocytes (116). FGF-2 (fibroblast growth factor) has not only been reported to induce proliferation when analyzed using

co-culture systems, but has been shown to promote gonocyte survival as displayed by a difference in the percentage of gonocytes undergoing proliferation and total number of gonocytes, indicating that FGF-2 was not only playing a proliferative role likely through Sertoli cell primary action, but also a role in cell survival (115). Furthermore, germ cells in DAZL (deleted in azoospermia-like; a commonly used germ cell marker) knockout mice did not survive past 19.5dpc, depicting its importance in gonocyte survival during the quiescent phase (132).

In addition, we have found that the previously described V1-PDGFRβ variant receptor form can promote F9 cell survival (99). F9 cells, described below in more detail, are an embryonic teratocarcinoma cell line model representing cells at a stage prior to that of gonocytes during development and are commonly used to study signaling pathways in embryonic stem cell differentiation (133). Studies previously done in our laboratory showed that overexpression of this receptor variant form led to increased F9 cell survival (99). However, if V1-PDGFRβ also has a similar affect during gonocyte development remains to be determined. All in all, it appears that factors such as LIF, CNTF, FGF2, DAZL, and V1-PDGFRβ may be involved in gonocyte survival.

In terms of survival, gonocyte location also plays an important role. As previously mentioned, gonocytes must undergo migration before undergoing differentiation. However, cells that failed to migrate by PND5-8 will undergo apoptosis and be eliminated (121-123). This would indicate that either Sertoli cells are programed to produce pro-apoptotic factors locally to induce apoptosis of these un-translocated gonocytes, or that once gonocytes have migrated to the seminiferous tubule basement membrane, they are no longer able to respond to pro-apoptotic signals (39). The exact mechanisms involved in governing gonocyte survival remain to be determined.

1.3.4 Gonocyte Apoptosis

Apoptosis is a cellular process that is not only involved in gonocyte development but is essential to ensure proper spermatogenesis in the male. As previously mentioned, the finite number of nourishment- and support-providing Sertoli cells can only support a certain number of germ cells during spermatogenesis, and thus, any excess of germ cells must undergo apoptosis. During gonocyte development, cells that have not migrated to the basement membrane will

undergo apoptosis and be eliminated. In view of published studies and our own unpublished observations, the precise time-line of this process appears to be between PND5 to 8 in Sprague Dawley (SD) rats. Apoptosis of un-translocated gonocytes is necessary as improper gonocyte development (including inadequate apoptosis) is hypothesized as being at the base of testicular germ cell tumor development (134).

In the fetal testis, gonocytes are the only cells that are able to undergo apoptosis (135). Throughout development, rat testicular gonocytes undergo two main phases of apoptosis, mainly during fetal and late neonatal development (101). The exact reason for which gonocytes must undergo apoptosis is not clearly understood. Some believe that gonocytes must undergo apoptosis in order to maintain a ratio of Sertoli cells to germ cells by which Sertoli cells can actually provide the necessary microenvironment and nourishment needed by the germ cells (16, 136). Others believe that apoptosis is used as a type of 'self-preservation' mechanism, by which improper developing, chromosomally abnormal gonocytes are eliminated (137, 138).

Although studies are limited, some key factors have been shown to be involved in gonocyte apoptosis. TGFβ has been shown to induce apoptosis in fetal gonocytes and in pre-pubertal germ cells, whereas the effect of TGFβ on PND3 gonocyte apoptosis is minimal (139-141). Furthermore, FAS, a transmembrane receptor also known as APO-1 belonging to the TNFR (tumor necrosis factor receptor) family, is present in gonocytes and the ligand, FASL, is secreted by Sertoli cells (99, 142, 143). FAS, known to be involved in apoptosis, has been shown to induce apoptosis in germ cells upon activation (143, 144).

To continue, overexpression of anti-apoptotic factor Bcl-2 in mice shows an inhibition of germ cell apoptosis in young mice, whereas in older mice, Bcl-2 overexpression leads to an increase in germ cell apoptosis and sterility (39, 145, 146). Studies have shown that Bcl-2 inactivation does not have any drastic affect on spermatogenesis, indicating that in order for the necessary apoptosis to occur, Bcl-2 does not act alone, and thus, the system is redundant, or that the effects seen due to Bcl-2 overexpression are not a direct effect and that Bcl-2 is not directly involved in germ cell apoptosis (101, 147). Effects seen with Bcl-2 overexpression are also seen with Bcl-XL overexpression, which is another anti-apoptotic gene (39). Abnormal spermatogenesis resulting from Bcl-XL or Bcl-2 overexpression is similar to what is also seen in BAX knockout models (39). BAX, a pro-apoptotic factor, deficiency in mice led to decreased

germ cell apoptosis at PND5 and PND15, and BAX inactivation results in an accumulation of premeiotic germ cells (148).

Other commonly activated components during programmed cell death and regulators of apoptosis include caspases, which are cysteine aspartate specific proteases (149). Not much is known about caspases and their effect on germ cell development. However, studies have shown that inactivation of caspase 1 does not alter spermatogenesis (101). Caspase 3 and caspase 9 inactivation results in death in utero and as a result, no spermatogenesis-related analysis can be done (149, 150). Finally, although caspase 2 inactivation has been shown to result in an accumulation of germ cells in the ovary, no effects on male germ cells have yet been reported (151). Furthermore, RA, which was previously shown to have a positive effect on fetal gonocyte proliferation, also induces fetal (but not neonatal) rat and human gonocyte apoptosis (95, 98).

In summary, although not much is known about apoptosis and its progression during gonocyte development, reports have indicated that TGFβ, FAS, Bcl-2, Bcl-XL, BAX, and RA likely play a regulatory role in germ cell apoptosis.

1.3.5 Gonocyte Differentiation

Of all of the processes involved in gonocyte development, both in the fetal and neonatal periods, gonocyte differentiation is the least well studied. This is surprising in light of the fact that gonocytes differentiate into SSCs, which are essential for the production of spermatozoa throughout the lifetime of a male. The reason for the lack of knowledge available on the differentiation process in gonocytes may be due in part to the lack of proper markers of differentiation, up until recently (39).

Morphologically, gonocytes and spermatogonia do not look similar. Gonocytes, while proliferating, are round cells with prominent nuclei located at the centre of the seminiferous cords, whereas spermatogonia have half-moon shapes and are located at the basement membrane of the seminiferous tubules (39). However, despite being easily distinguished from each other due to their morphology, location within the tubule, and presence at different ages, it is during transitional phases such as the PND5 to 6 period, when some perfectly round gonocytes still have not yet undergone differentiation but are already located at the basement membrane, that confusion can occur (39). At such times, morphology and location within the tubule alone is not

sufficient to characterize the cells as still being gonocytes and not already transitioned to spermatogonia (39). Thus, due to the lack of synchronization in gonocyte development, and the absence of a clear time frame at which gonocyte differentiation has ended and spermatogonial self-renewal has started, gonocyte differentiation as a process has been a challenging topic not well studied. However, recently over the years, various markers have been characterized that can be used to identify these different processes more clearly.

1.3.5.1 Markers for Gonocyte Differentiation

One marker for gonocyte differentiation is STRA8 (stimulated by retinoic acid 8). This gene was first identified as a target of all-trans RA in P19 mouse embryonal carcinoma cells, and later, in F9 mouse embryonal teratocarcinoma cells (152, 153). STRA8 is a cytosolic protein rich in glutamic acid that can be phosphorylated by kinases activated upon either all-trans RA or 9-cis RA interactions with their receptors (39, 153). STRA8 is important for germ cell maturation and STRA8 knockout animal models are infertile due to the lack of proper germ cell meiosis (154). Male mice lacking STRA8 do not have any sperm production because the spermatogenic cells that should be undergoing meiosis are not able to do so and instead, undergo apoptosis (154). Interestingly, STRA8 is not only important in male germ cell meiosis but also in the female, as knockout models for females are also infertile (154). In females, STRA8 is necessary for meiotic initiation of embryonic ovarian germ cells (154). Thus, in both males and females, entry into meiosis is mainly due to RA-induced expression of STRA8 (154). STRA8 is not only important during meiosis, but is also commonly used as a marker for differentiating spermatogonia (155). Several groups, including ours, have used STRA8, alongside cKIT (another marker of differentiating spermatogonia), as a marker for differentiating gonocytes (99, 100).

1.3.5.2 Retinoic Acid

1.3.5.2.1 Importance of Vitamin A for Proper Development

Gonocyte differentiation has been shown to be induced by retinoic acid (RA) (See Figure 1.3). RA is an active metabolite of vitamin A (retinol) (156). Vitamin A is a lipid soluble vitamin that is not only important in gonocyte differentiation but is also important in the formation and maintenance of other tissues such as bone, skin, and vasculature (157). More importantly, vitamin A is considered to be essential for life as it plays an important role during embryonic

development and organogenesis (157, 158). It is involved in cellular proliferation, differentiation, tissue homeostasis, and apoptosis (157).

Vitamin A deficiency is a global health concern as it is seen in over 120 countries worldwide and can result in blindness, improper spermatogenesis, and decreased resistance to infection (159). Interestingly, vitamin A excess is also a health concern in the general population as it can lead to liver toxicity, central nervous system toxicity, and skin problems (157, 159). Thus, maintaining a balance of vitamin A is important for normal development.

1.3.5.2.2 Sources of Vitamin A

The main source of vitamin A in animals is from their diet as vitamin A cannot be synthesized de novo (157). Plants and some bacteria and fungi produce carotenoid compounds which can be converted to vitamin A in animals. There are more than 600 identified carotenoid compounds but only 10% of these are precursors for vitamin A, mainly β-carotenoid (160). Carotenoid rich vegetables are easily identifiable due to their yellow, orange, and red colors (161). Another source of vitamin A in animals is provided by the ingestion of tissues from other animals that have already converted these carotenoid compounds into retinoids (159). Retinyl esters can also accumulate in the livers of mammals, birds, and fish, and thus, these retinyl esters add to the dietary intake of retinoid compounds (159). Retinyl esters are also found in other foods due to fortification processes including margarine, milk, and cereal (159). These RA precursors are converted to active RA forms in the liver, the gut, and other target tissues (159).

1.3.5.2.3 Vitamin A Transport and Metabolism to Retinoic Acid

In order to be transported in the plasma, retinol binds to a retinol-binding protein (RBP) (162, 163). It is believed that under physiological conditions, less than 1% of the RA present in the testis comes from circulation (164). Instead, it appears that RA needed in the testis is produced in situ (157). Although all-trans RA and 9-cis are the most common RA metabolites, all-trans RA is the major active cellular metabolite responsible for gonocyte differentiation and 9-cis levels remain insignificant in many vertebrates (165).

RA is produced from retinol (vitamin A) via a two-step process (159). In this case, vitamin A either comes from the blood (bound to RBP) or from the retinyl ester stores. Retinol is first

oxidized into retinaldehyde using alcohol dehydrogenases (ADHs) (159). ADH1, ADH3, and ADH4 are responsible for this oxidation and in the mouse testis, it has been reported that ADH3 is ubiquitously expressed whereas ADH1 is in Sertoli cells, and ADH4 is in late spermatids (166, 167). Retinaldehyde is then oxidized into retinoic acid via four retinaldehyde dehydrogenases (RALDH1-4, also known as ALDH1a1, ALDH1a2, ALDH1a3, and ALDH8a1) (168, 169). Studies have shown that RALDH1 is mainly involved in the catabolism of excess retinol and no major effects are seen in RALDH1 knockout models (157). RALDH2 is needed for retinoic acid production in a multitude of cell types during embryonic development, which is not surprising as knockout RALDH2 models die in utero due to cardiac defects (157, 170). RALDH3 is also important in retinoic acid production and is mainly expressed in mouse retina, surface ectoderm over the developing forebrain, and olfactory pit (170). The importance of RALDH3 is also seen as knock out models of RALDH3 die within a few hours after birth (170). Finally, RALDH4 is expressed in the liver and the kidney in mice and is involved in the biosynthesis of 9-cis RA specifically (171). Of these, RALDH1 and RALDH2 are present in the rodent testis (172, 173). β-carotene can also be converted into retinaldehyde using β-carotene 15,15'-monoxygenase present in the testis (157). To continue, RA levels must be balanced, and thus excess RA must be eliminated. Enzymes responsible for retinoic acid elimination include cytochrome P450 hydroxylases CYP26A1, B1, and C1 which hydroxylate RA until it becomes water-soluble 4-oxo and 4-hydroxy forms (169, 174).

Inside the cell, retinol can bind to cellular retinol binding proteins (CRBP1 and CRBP2) and retinoic acid can bind to cellular RA binding proteins (CRABP1 and CRABP2) (157, 175). CRBP1 and 2 are involved in storage and CRBP1 has been shown to be localized to Sertoli cells (176, 177). CRABP1 is localized to spermatogonia and CRABP2 is localized to Sertoli and Leydig cells (169, 178).

In order to induce differentiation, retinol is delivered to the gonocyte via the RBP from Sertoli cells or the serum (179). This retinol is then internalized due to a membrane receptor, STRA6 (stimulated by retinoic acid 6) (179, 180). Inside the cell, retinol then undergoes the two step oxidation process in order to become retinoic acid (157). It is also possible that RA is directly transported to the gonocytes from the serum (via the vasculature) or Sertoli cells (179). Although spermatids and testicular and epididymal sperm are known to be able to store retinoids,

Sertoli cells are the main site of RA synthesis in the testis which is then passed on to the germ cells (181).

1.3.5.2.4 Retinoic Acid Receptor Activation and Signaling

RA has its effects by binding to retinoic acid receptors (RARs) and retinoid x receptors (RXRs), which belong to the nuclear receptor family (169). Each of these receptor types has three main isoforms, α, β, and γ (169). These receptors contain a DNA binding domain (DBD) and a ligand binding domain (LBD) (157, 182). RARs bind to both all-trans RA and 9-cis RA and RXRs can bind only 9-cis RA (183). Although homodimers can exist *in vitro*, heterodimer formation is necessary for proper *in vivo* signaling transduction to occur (169, 184). RARs can form heterodimers with RXR whereas RXR can form heterodimers with RAR and other receptors such as VDR (vitamin D receptor), PPAR (peroxisome proliferator-activated receptor), and thyroid hormone receptor (THR) (185).

RAR/RXR heterodimer formation occurs on the LBD and DBD interface where the DBD interface can recognize the DNA response elements described below (157). When the RA ligand is unbound, RAR/RXR heterodimers are bound to co-repressor complexes that are associated with histone deacetylases and result in chromatin condensation and transcriptional silencing (186). However, upon RA ligand binding, there is a conformational change that occurs, allowing the co-repressors to be removed and for co-activator complexes to bind, which allows for chromatin decondensation and activation of gene transcription (186). Upon conformation changes, the transcriptional machinery will be recruited and the heterodimer will bind to retinoic acid response elements (RAREs) (154). RAREs consist of two or more copies of a particular sequence ((A/G)G(G/T)TCA or (A/G)G(G/T)(G/T)(G/C)A) arranged as direct repeats or palindromes, normally separated by a nucleotide spacer of 1, 2, or 5 nucleotides, known as direct repeat (DR) 1, 2, or 5 (187, 188). Furthermore, RARs are able to integrate signaling from other pathways due to their many phosphorylation sites on both the LBD and DBD (159, 169). These sites undergo phosphorylation due to molecules such as protein kinase A (PKA) and protein kinase C (PKC) (159). This phosphorylation is not only important for transcriptional activation of various domains and receptor interactions but is also essential for the ubiquitination process by which these receptors will eventually get degraded (189).

Both RARs and RXRs have three main isoforms (α, β, γ). Each of these receptor isoforms has distinct characteristics. RARα null mice are sterile because of seminiferous epithelium degeneration, have growth deficiencies, and other congenital malformations (190). RARβ is likely involved in morphogenesis and knockout models have locomoter defects and an abnormal vitreous humor in the eye (191). RARγ is highly expressed in the skin, and like RARα, RARγ null mice defects can be corrected upon RA treatment (192, 193). It is important to note that individually, each isoform knockout model has not shown any drastic effects on retinoid function, indicating redundancy within the system. However, double knockout models of these RAR isoforms are known to be embryonic lethal or die soon after birth (157, 190). RXRα inactivation is embryonic lethal due to improper heart and eye development (190, 194). RXRβ knockout animals are embryonic lethal 50% of the time and of the 50% that survive past birth, the males are sterile (195). RXRγ knock out mice are the least affected and the only reported problem is that these animals become resistant to the thyroid hormone (196, 197). Unlike RARs, where double knockouts are embryonic lethal, double knockouts of RXR have provided evidence that RXRα alone can perform the majority of the roles of the RXRs (196).

As previously described, upon RA binding to the RAR/RXR heterodimer, there is a conformational change which leads to transcriptional machinery recruitment and binding of this complex to the RAREs to initiate a response. This is considered to be the classic RAR activation pathway. RARs can also act independently of RXR via activator protein 1 (AP-1) transcription complexes that contain heterodimers of jun- and fos-related proteins to regulate gene expression (198). More interestingly, there is growing evidence that RA can indirectly target a plethora of genes, including PDGFRα, without having to bind to the RAREs or even the RAR/RXR heterodimers, in a non-classical pathway (157, 169, 199). Although it is known that this indirect activation of target genes is occurring independently of RAREs (and in some cases, independently of RA binding to RARs), the exact mechanism of how this is actually happening remains to be elucidated. However, it is likely that this indirect activation is occurring via a transcriptional intermediate, independent of RARs and RAREs (157, 169, 199).

1.3.5.3 Retinoic Acid and Spermatogenesis

The importance of vitamin A and RA in spermatogenesis has been known for many years due to the use of a vitamin A-deficient mouse model (VAD-mice). Studies have shown that in

these VAD adult mice, all differentiated germ cells are lost from the seminiferous tubules and the only cells remaining are the undifferentiated type A spermatogonia and Sertoli cells (200, 201). However, once these rats are administered vitamin A, normal spermatogenesis can be rescued (202, 203). Interestingly, once retinol is administered, the various stages of spermatogenesis become synchronized, unlike the usual unsynchronized manner by which spermatogenesis occurs (201, 202, 204). Although synchronized stages of spermatogenesis are not natural, it does provide researchers with an abundance of one particular stage of spermatogenesis at any given time, which makes it easier to study each stage individually (203).

At first, studies showed that normal spermatogenesis could only resume if vitamin A (retinol) was given to these deficient mice and that the same results were not seen upon RA administration (205, 206). The finding that RA alone could not help resume spermatogenesis confirmed the importance of the supporting Sertoli cells as this meant that un-metabolized retinol needed to become oxidized by the supporting Sertoli cells into RA to then have an effect on the germ cells. However, studies done thereafter have shown that RA can also stimulate normal spermatogenesis in these deficient mice, but that the doses required for such activity are much higher than retinol (207).

1.3.5.4 Retinoic Acid and Gonocyte Differentiation

Although RA induces differentiation in neonatal gonocytes, as previously mentioned, RA is known to reduce the overall number of fetal gonocytes by affecting their proliferation and apoptosis levels. RA has also been shown to have an effect on Sertoli cells, where RA can affect the organization of the Sertoli cells into forming seminiferous cords and increase the volume of Sertoli cells with or without altering their mitotic activity depending on the age (96). LH-stimulated testosterone secretion by Leydig cells is also reduced by RA in cultured 14.5 dpc testes (96). Thus, RA also has an effect on the non-germinal, supporting cells within the testis.

In general, it is not known whether, *in vivo*, RA induces differentiation directly on the germ cells or if it is through an indirect path using the Sertoli cells, as both cell types express RAR and RXRs at varying levels (56, 208). It has been shown that at PND3, gonocytes express varying levels of all isoforms of RAR and RXRs but mainly, gonocytes express high levels of RARα and RXRα, whereas Sertoli cells mainly express RXRγ (209). Other groups have reported that PND3

gonocytes do not express RARα and that this receptor isoform, at PND3, is expressed solely in Sertoli cells (169). Likely, the differences seen in expression levels can be attributed simply to the different antibodies used for detection. Furthermore, studies have shown that the phenotype resulting from removing all three RAR isoforms in Sertoli cells is not the same if all three isoforms of RXR are removed, and thus, RARs can exert functions *in vivo* that are independent of RXRs (210).

1.3.5.4.1 The First Wave of Spermatogenesis

During gonocyte development, RA induces gonocytes to differentiate into SSCs. The importance of a SSC reservoir pool has been previously described as it is necessary to ensure life-long production of sperm. However, there is a small subset of gonocytes (negative for basic helix loop helix transcription factor NGN3) that differentiates into $NGN3^-$ spermatogonia, and not $NGN3^+$ SSCs (39, 211). Known as the first wave of spermatogenesis, this round of spermatogenesis is less efficient than future rounds of spermatogenesis in the adult, has large amounts of apoptosis, and it is likely that the mature sperm resulting from this first round may not be fertile (211, 212, 213).

1.3.5.4.2 Cell-line Model to Better Study RA-induced Differentiation

RA has been known to induce differentiation in a multitude of cell types and thus, general RA-induced differentiation is a relatively well studied process. One method of better understanding the underlying mechanisms by which RA can induce differentiation is by using the commonly studied F9 mouse embryonal teratocarcinoma cell line (214). F9 cells resemble the pluripotent stem cells of the inner cell mass of the blastocyst (214, 215). Embryonic carcinoma cells (EC-cells) are the stem cells of teratocarcinomas, a type of testicular tumor, that have retained their ability to differentiate into derivatives of the various embryonic layers (216). F9 cells cannot undergo differentiation spontaneously but upon various treatments (RA with or without cAMP) and culture conditions, can differentiate into the primitive, parietal, or visceral endoderm (215, 216).

The F9 cell line model was first isolated in 1973, deriving from a teratocarcinoma that was established by implanting a six-day old embryo in the testis of a 129/J mouse (217). Upon RA treatment, these cells differentiate into the primitive endoderm, which can then be modulated

into the parietal or visceral endoderms. These primitive endoderm cells can be grouped into two main categories based on their morphologies: one population retains the original polygonal morphology where the cells remain grouped together, whereas the other population has large cytoplasmic processes and are more separated from each other (218). Furthermore, F9 cells are commonly used as a model to better study embryonic stem cells (ES-cells). EC-cells and ES-cells have many similarities including their morphology, growth behavior, and gene expression (215). Both of these cell types are also pluripotent, and thus, have the ability to differentiate into cells belonging to all three germ layers (215). The main differences between EC-cells and ES-cells are that ES-cells are involved in embryogenesis and able to differentiate into germ cells, whereas EC-cells are not (215).

There are several rationales for using F9 mouse embryonal teratocarcinoma cells in this study with gonocytes (chapter 2): 1) this is a commonly used cell line model to study the molecular mechanisms involved in the RA-induced differentiation of ES cells, 2) gonocytes share a number of pluripotency markers with ES-cells and F9 cells, 3) both gonocytes and F9 cells express Stra8 in response to RA, suggesting common regulatory mechanisms between the 2 models, 4) F9 cells are testicular embryonal teratocarcinoma cells, precisely the type of tumor that could arise from abnormal gonocyte development, starting from a CIS precursor, progressing to embryonal carcinoma and then teratocarcinoma.

1.3.6 Studying Gonocyte Development

Better understanding the development of early testicular germ cells is difficult for a number of reasons. Mainly, these cell types cannot be easily isolated due to the lack of synchronization in their development resulting in overlapping groups with different phenotypes or functions (e.g. co-existence of quiescent and proliferative PND3 gonocytes), and the lack of specific markers for each individual subset of cells. Even if they can be isolated, the low cell number obtained limits the amount and type of studies that can be done with these cells. In order to study gonocyte development, gonocytes must be isolated and studied as primary cultures. Although there is no available cell line model to study gonocyte development, the generation and use of a stable gonocyte cell line model would surely change how these cells underwent proliferation and differentiation, and as a result, the true mechanisms that are physiologically involved in these processes would likely not be determined (56).

In order to study gonocytes in primary culture, there are a few different methods that can be used. Organ culture methods are commonly used for studying gonocyte development because they allow the testis to stay relatively intact, preserving the architecture of gonocytes and the surrounding supporting cells (101, 219). Thus, this method can provide an *in vitro* environment that is quite similar to that seen *in vivo*. Another commonly used method is to use enriched populations of gonocytes cultured alongside Sertoli cells so that again, the culture system can better mimic what is happening in nature (115, 209). Although these are two commonly used methods that have provided a wealth of knowledge on better understanding gonocyte development within the testicular environment, they have limitations.

Mainly, in both of these techniques, gonocytes remain closely associated with their surrounding, supporting Sertoli cells. As previously mentioned, when gonocytes and Sertoli cells are cultured together and the cells are treated in order to determine the effect of various compounds on gonocytes specifically, the intended treatment target is not clear (101). Although the exogenously added compound may have an effect on an overall endpoint (e.g. differentiation), the co-cultured system does not permit to determine whether an observed effect was due to a direct action on germ cells or if the effects seen were the result of an indirect effect on Sertoli cells, subsequently affecting gonocytes (101). Sertoli cells are known to regulate gonocyte development through the secretion of various factors and also through gap junctions (94). Thus, this information would not indicate what factor was directly acting on gonocytes or on their support cells. However, these culture techniques are of use when studying the relationship between Sertoli cells and germ cells (e.g. cell junctions and cell migration) or when the identity of the primary target cell is not of concern.

In order to study what affects gonocytes, or germ cells, directly, these cells must be isolated and cultured alone. An efficient method by which to isolate gonocytes from PND3 testes has previously been described in our lab and includes sequential enzymatic digestion of the testes, then the seminiferous tubules, resulting in a Sertoli cell, myoid cell, and gonocyte cell suspension after which differential overnight plating is carried out to remove adherent somatic cells, and the non-adherent germ cells are further separated using a bovine serum albumin (BSA) gradient (See Figure 1.4) (117, 220). Studies in our lab have shown that once isolated, these cells can easily be cultured without having any support system for a few days, in order to determine

what factors directly affect gonocyte development (99, 117-119, 221). On the other hand, one cannot rule out that isolated gonocytes in the absence of their native environment may act a little differently from the *in vivo* settings. Thus, the choice of using isolated gonocytes versus gonocyte/Sertoli cell co-cultures or testis organ cultures depends highly on the purpose of the study and should be chosen with careful consideration. In the work presented in this thesis, the majority of experiments have been done using isolated gonocytes as to determine what regulates gonocyte development directly, without the interference of Sertoli cells.

1.4 The Importance of Platelet-Derived Growth Factors and Receptors in Spermatogenesis

Available data on gonocyte development, including our own, have identified platelet derived growth factors and their receptors as molecules involved in both neonatal gonocyte proliferation and migration (99, 117, 121).

1.4.1 Platelet-Derived Growth Factors

Platelet derived growth factors (PDGFs) were first discovered when it was found that the serum growth factor activity originally seen during chicken fibroblast proliferation was due to factors secreted from the platelets into the serum (222, 223). Originally, two forms of PDGFs were identified, A and B, but more recently, forms C and D have also been discovered (224, 225). PDGFs contain two disulfide bridges that force the molecules to undergo a conformational change, which leads to hydrophobic regions being exposed to hydrophilic environments (225). As a result, the PDGFs form homodimers (AA, BB, CC, and DD) or heterodimers (AB) (225). PDGF A, B, C, and D are located on chromosomes 7, 22, 4, and 11 respectively, in humans and while PDGF A, B, and C have six exons, PDGF-D has seven exons (226). Although these PDGF chains share high levels of homology within themselves, they also share similarities with vascular endothelial cell growth factors (VEGFs), mainly a cysteine knot structure containing cysteines necessary for dimer formation and intrachain disulfide bonds (227). PDGFs are synthesized by many different cell types and are known to play important roles in embryogenesis and adult maintenance (225, 227).

1.4.2 Platelet-Derived Growth Factor Receptors

The four PDGFs are inactive until they undergo dimerization, at which point they can bind to receptors which will, in turn, also undergo dimerization (225). There are two types of PDGF receptors: α and β. PDGFRα and β are located on chromosomes 4 and 5 respectively in humans (226). PDGFRs are receptor tyrosine kinases and each receptor has five immunoglobulin repeats in the extracellular ligand-binding domain and a tyrosine kinase domain in the cytoplasmic region which contains a characteristic insert sequence not a part of the kinase domain (228).

1.4.2.1 Platelet-Derived Growth Factor Receptor Activation

PDGF-AA can only bind to PDGFRαα, PDGF-AB can bind to PDGFRαα and αβ, PDGF-BB can bind to PDGFRαα, αβ, and ββ, PDGF-CC can bind to PDGFRαα and αβ, and PDGF-DD can bind PDGFRαβ and ββ (228). Once the ligand has bound to the receptor and receptor dimerization has occurred, the tyrosine kinase domain is then activated and autophosphorylation can take place (228). Autophosphorylation within the kinase domain (tyrosine 849 in PDGFRα and tyrosine 857 in PDGFRβ) increases the catalytic efficiency of the kinase, whereas phosphorylation outside of the kinase domain provides docking sites for other downstream molecules to bind and become activated (228). Usually, these docking sites are created for signal transduction molecules containing various domains such as the SH2 domain (227). The SH2 domain is a conserved motif that binds to a phosphorylated tyrosine and can then be the docking site used for other molecules to bind to the activated PDGFR (such as SRC, PI3K, and PLCγ) (227). Although the most common, SH2 is not the only conserved motif used for this purpose, and motifs including the SH3 domains, PTB (phosphotyrosine binding) domains, PH (pleckstrin homology) domains, and PDZ domains are also known adaptor proteins (228).

Upon PDGFR pathway activation, there is a multitude of downstream pathways that can be activated including the RAS/MEK/ERK pathway, the SRC pathway, the JAK/STAT pathway, the PI3K pathway, and the PLCγ pathway (See Figure 1.5) (227, 228). Each of these downstream pathways has been shown to be involved in many processes. The PDGFR pathway, in general, is known to be involved in various cellular processes including proliferation, migration, differentiation, apoptosis, and more specifically, the importance of PDGF and its receptors in neural and vascular cell development, central nervous system development, embryonic

hematopoietic cell migration, organogenesis, wound healing, and skeletal patterning has been well described (228). As a result, aberrations in PDGF and PDGFR signaling are also seen in a variety of different pathologies including oncogenesis, atherosclerosis, lung fibrosis, and kidney fibrosis (227, 228).

1.4.2.2 Platelet-Derived Growth Factor and Receptor Knockout Models

PDGFRα and β are quite similar but their differences can be seen by differences in the phenotype observed in knockout mice models of these receptors (229). PDGF-A knockout mice models will die before embryonic day (E) 10, but if they are able to survive past E10, they will survive until birth and then die after 6 weeks (230, 231). The PDGF-A knockouts are smaller than normal animals, have defects in their hair follicles, have a deficiency in oligodendrocytes, and lack both alveolar smooth muscle cells and testicular Leydig cells (230, 231). PDGFRα knockout mice are embryonic lethal and die between E8-16 (231, 232). Problems due to the PDGFRα knockout include cleft face, spina bifida, skeletal defects, and vascular defects (231, 232). Inactivation of PDGF-B and PDGFRβ leads to defective kidney development, defective blood vessel development, and cardiac muscle hypotrophy (231, 233, 234). PDGFRβ knockout mice are embryonic lethal and die around E16-19 (231). The importance of PDGF and PDGFR signaling can be seen, as both PDGFRα and PDGFRβ knockout models are embryonic lethal.

1.4.3 Platelet-Derived Growth Factors in the Testis

PDGF and its receptors are important players in a variety of different cellular processes and at various stages of development, and testicular development is no different. PDGFs and PDGFRs are produced within the testis both pre- and post-natally in a time-dependent manner (235). Studies have shown that PDGF-A and B and both receptors are first seen at E18 and persist until PND5 at which point the transcript levels of these factors and receptors begin to decrease in expression (236, 237). We have previously shown that before birth, PDGF-A and B are mainly produced by the Sertoli cells (117). Furthermore, we have shown that some gonocytes (in both whole testis and isolated gonocyte analysis) express PDGFRs up until PND5 (117). The fact that only some gonocytes positively express PDGFRs is further confirmation that these cells do not develop in a synchronized manner and that as the gonocytes grow older, the expression levels of PDGFRs are transiently increasing, to further decline after PND5.

In animals close to puberty, PDGF-A and B are mainly seen in the Sertoli cells whereas the receptors are seen in PMCs. In the adult rat, the Leydig cells are the main cells to express both growth factors and both receptors, whereas in the mouse, adult Sertoli cells can produce PDGF-A (235, 236, 238). Studies done in the human have confirmed that in the adult, it is the Sertoli cells and Leydig cells that mainly express PDGFs and their receptors, and not the germ cells (239). Both PDGFRα and β are present in the epididymis and thus, it is possible that PDGF is involved in epididymal maintenance (240).

Furthermore, not only is PDGF-BB involved in gonocyte proliferation (117), but studies have shown that it is also involved in the contractility process of the PMCs and in migration of the mesonephric cells, which is necessary for normal testicular development (241, 242). It is also believed that as testicular development progresses, there is a shift of PDGF and PDGFR production to the Leydig cells, which in turn may affect testosterone production (235). Both PDGF-C and D are found to be present in the testis, but their exact role remains to be determined (235). Studies have also shown that in PDGF-A deficient mice, there is a lack of Leydig cells and spermatogenic arrest occurs, indicating that PDGF-A is important for Leydig cell development (243). In addition, organ culture studies have shown that upon PDGFR inhibition, the seminiferous tubules become swollen (244). There is a significant decrease in the number of tubules per testis area and the diameter of each tubule increased due to tubule fusion (244).

To continue, PDGF-BB and PDGFRβ are known to be involved in both proliferation and migration during gonocyte development. Our group has previously described a variant form of PDGFRβ, termed V1-PDGFRβ (99). This variant form retains the tyrosine kinase domain but lacks the ligand binding domain and thus, can function independently of ligand binding (99). V1-PDGFRβ is only expressed at specific stages of testis development and the transcript contains sequences from intron 6 until exon 23. In our study with PDGF-BB, it is likely that the full length receptor is being activated due to the necessity of the ligand to induce proliferation (99). However, in the studies determining the involvement of PDGFRβ in gonocyte migration, it is not clear whether the full length or variant receptor form is involved (121). All in all, it is clear that PDGFs and PDGFRs are not only important during gonocyte development, but they also play key roles in overall testicular development and, as discussed later, in testicular tumors.

1.5 Testicular Cancer

It has been suggested that improper gonocyte development can lead to the formation of carcinoma-in-situ (CIS), the precursor pathology of testicular germ cell tumors (TGCTs) (134, 245). This renders gonocytes as an important cell type to study because the incidence of TGCTs has been steadily increasing for the past few decades for reasons that are still not completely known (246). CIS is believed to be at the origins of TGCTs due to their similarities in gene expression profiles and morphology within the seminiferous tubules (247, 248).

1.5.1 Types of Testicular Cancer

Testicular cancer (TC) is a relatively rare form of cancer but it represents the most commonly diagnosed malignant solid tumor in young men between the ages of 15-35 years old (249, 250). A large majority of TC cases are TGCTS, while only 5% of cases are non-germ cell tumors, including Leydig cell and Sertoli cell tumors (251). TGCTs in young adults can be further divided into two main categories: classic seminomas and non-seminomas, which include embryonal carcinomas, teratomas, yolk sac tumors, and choriocarcinomas (249, 252, 253). It is the classic seminoma tumors that are the most common types of testicular tumors and they are the ones likely to arise from improper gonocyte development. Although the initial malignant event by which tumors will be formed is likely occurring in utero in humans, the TGCTs themselves will not arise before puberty (247, 254). Thus, it is likely that there is a puberty-related trigger (i.e.: hormones) which promotes the initial transformation that occurs in utero to form a tumor later in life (254).

1.5.1.1 Seminoma-type Testicular Germ Cell Tumors

Seminomas and embryonal carcinomas share common gene profiles but are characteristically different regarding both morphology and a few specific markers. For example, seminoma tumors express SOX17 and do not express SOX2, while the reverse is true for embryonal carcinomas (255). While markers such as PLAP (placenta alkaline phosphatase) (256), NANOG (257), and OCT3/4 (258) are seen in a variety of TGCTs, seminomas alone have high expression levels of AP2γ (259) and c-KIT (252) alongside SOX17. These gene markers can be used to clinically diagnose seminoma tumors in patients (252). Furthermore, Palumbo C et al have shown that there is a 1.5kb variant form of the platelet-derived growth factor receptor

alpha (PDGFRα) that is only present in seminoma tumors (and not in the normal testis) and could potentially be used as a marker for diagnostic patient screening (260). The presence of a variant form of PDGFR in seminoma type tumors is interesting due to the importance of PDGF and its receptors in gonocyte development, as described above.

1.5.2 Testicular Dysgenesis Syndrome

TGCTs are part of a larger group of male reproductive pathologies known as testicular dysgenesis syndrome (TDS) (110). TDS related pathologies also include hypospadias (abnormal opening of the urethra), cryptorchidism (undescended testis), and decreased semen quality (255). The likely causes of TDS are genetic defects, but more importantly, the increased use of endocrine disruptors, including estrogenic compounds, in our environment (110, 261, 262). The likelihood that environmental factors are at the base of these male reproductive pathologies, especially TC, is strong due to the correlation of increased TC incidences in already developed nations, and the increased use of endocrine disruptor compounds in these countries (262).

Although there are genetic-related risks to developing TGCTs (brothers and sons of men with TGCTs have higher risks of developing this type of tumor), the environment is a key factor (263). Studies have shown that there are high rates of TGCT formation in Denmark and low levels in Finland (262). However, when men from each of these countries move to Sweden, they maintain risk levels of developing TGCT similar to their native countries, indicating a possible genetic component as well (264). However, when these men have children, the boys that are now the first generation born in Sweden, have TGCT developing risks similar to the men native to Sweden, probably due to the fact that they were exposed to the same environment starting at gestation (264). This shows that the environment plays an important role in TGCT development, as it possibly alters how the fetal or prepubertal testis develops.

1.5.3 Risk Factors for Developing Testicular Germ Cell Tumors

There are many risk factors that are involved in TGCT formation other than the environment and genetics. These factors include TDS, birth order, birth weight, maternal age, and ethnicity. Studies have shown that as TDS becomes more severe (from decreased semen quality to cryptorchidism), there is an increase in TGCT risk (110). Birth order is a known key risk as boys who are the first born child within a family have higher chances of developing TGCTs later in

life compared to boys born with older siblings (260). This is due to the higher estrogen levels found in women early during their first pregnancy compared to subsequent pregnancies (265). Boys who have low birth weights are also at a higher risk of developing all of the male reproductive pathologies that are characteristic of TDS (266). Especially for seminoma tumors, studies have shown that the older the maternal age at the birth of the child, the higher the risk of developing TGCTs (265). Finally, population studies have indicated that in general, Caucasian men are more susceptible to developing TGCTs compared to African-American and Asian men (267). Therefore, although the environment is playing a likely role in TGCT development, it is not the only factor involved.

1.5.4 Testicular Cancer Treatment, Survival, and Side-Effects

Unlike a large variety of other tumor types, TC has an effective cure and a survival rate after 5 years of treatment of over 90% (268). The current standard of treatment involves a unilateral orchidectomy (removal of the tumoral testis) followed by chemotherapy consisting of various combinations of bleomycin, etoposide, and cisplatin (BEP) depending on the severity of the disease (269, 270). However, although there is a high cure rate, these men experience reproductive difficulties after treatment (271). In brief, these difficulties include a reduced number of spermatozoa, sperm motility, and difficulty fathering children (272-275). Also, it is not clear whether this chemotherapy treatment will have negative effects on the future generations produced from men having undergone TGCT treatment (271).

Furthermore, like any chemotherapy regiment, there are many side effects including gastrointestinal, pulmonary, vascular, renal, neurological, and cardiovascular toxicity (276, 277). Although the data available for side effects and future fertility in humans are limited and not always clear, many studies have been done by treating rats with the BEP chemotherapeutic cocktail. In brief, studies have shown that in rats, BEP exposure leads to an oxidative stress response in round spermatids and induces pathways that can possibly result in the survival of damaged cells and production of abnormal sperm (271). To add, studies have shown that BEP exposure decreases litter size, alters gene expression profiles in the progeny, results in sperm chromatin remodeling, and alters sperm fertilizing capacity (278, 279).

All in all, there is growing evidence that the available treatments for TC, although effective, are likely having negative affects on the reproductive capacity of the afflicted men. Thus, it is important to better understand the underlying causes of TC development, by better understanding gonocyte development, in order to develop better, more targeted therapeutics.

1.5.5 Available Cell-line Model for Studying Human Testicular Seminoma Tumors

The main limitation in studying TGCT development is the lack of animal models for seminoma tumors and the lack of readily available tumor patient biopsies. Thus, the need for a cell line model was apparent until 1993, when Mizuno Y et al were able to propagate tumoral cells deriving from a 35-year old patient with a testicular seminoma (280). These cells were named TCam-2 cells and at the time, were thought to have similar gene expression profiles to seminoma tumors (280, 281). However, over time, studies have shown that TCam-2 cell gene expression profiles not only resemble those of seminomas but also those of embryonal carcinomas and thus, indicate the possibility of TCam-2 cells deriving from a patient tumor that was not completely 100% a seminoma but was a mixed-tumor instead (seen in ~15% cases in patients) (252, 282, 283). Although it is now accepted that TCam-2 cells do not represent entirely a seminoma, these cells are currently the cell-line model of choice when studying seminomas due to the lack of any other true seminoma cell line.

Although there is no cell line model available to properly study gonocyte development, using primary cultures of isolated gonocytes alongside the TCam-2 human testicular seminoma cell line, has allowed us to identify common genes and mechanisms involved in both gonocyte development, in particular differentiation, and seminoma cell functions. Despite being the result of fetal/perinatal initiation and subsequent peripubertal promotion and progression events of unknown origin, identifying genes or pathways common to gonocytes and TGCTs should provide mechanistic clues on the origins of these cancers.

Rationale for Thesis

The development of male germ cells is a complex process that requires many phases of proliferation, migration, differentiation, and apoptosis amongst other key regulatory events.

From the initial establishment of PGCs to the formation of mature spermatozoa ready for fertilization, there are many processes that govern the development of testicular germ cells to ensure the continuation of the species. The formation of a reservoir of SSCs is necessary to ensure the production of sperm throughout the lifetime of a male and this stem cell pool arises from the differentiation of its precursors cells, the gonocytes.

Arising from PGCs, gonocytes have been suggested to be at the origin of TGCT formation, as a result of improper development, in particular differentiation, preventing SSC formation and likely leading to the formation of carcinoma-in-situ, the precursor pathology to testicular germ cell tumors. Given the lack of information available on neonatal gonocyte differentiation and the steadily increasing incidence of testicular cancer, a better understanding of the molecular mechanisms and processes involved in gonocyte differentiation is needed. A more thorough understanding of gonocyte differentiation could in turn improve our comprehension of the events leading to testicular tumor formation.

The purpose of this thesis was to determine the molecular mechanisms and processes involved in gonocyte differentiation, a developmentally important process, that up until now, has not been well characterized.

To address this issue, the following questions were asked:

(1) What are the signaling pathways involved in gonocyte differentiation? (Chapter 2)
    a. Retinoic acid is known to induce gonocyte differentiation, but is activation of the RA receptor necessary for differentiation to occur, or does RA have its affects via a RAR/RXR independent signaling pathway?
    b. PDGFR pathway activation is known to be important in the differentiation of a variety of different cell types and has previously been shown to be involved in neonatal gonocyte proliferation. PDGFRα is also a known indirect target gene activated upon RAR activation. Does it also play a role in gonocyte differentiation?
    c. If so, what are the downstream pathways cross-talking with RA in gonocyte differentiation?

(2) What other biological processes are involved in gonocyte differentiation? (Chapter 3)

a. In order to properly develop, gonocytes must undergo a plethora of processes including proliferation, migration, differentiation, and apoptosis. Alongside these processes, there is much needed protein turnover and cellular reorganization that occurs in a timely and spatially regulated manner. Thus, we hypothesized that the ubiquitin proteasome system, the main system responsible for protein degradation, was involved in gonocyte differentiation and tested this hypothesis.
b. If so, what enzymes/proteins related to the ubiquitin proteasome system are likely to play a role in gonocyte differentiation?

(3) What mechanisms are involved in gonocyte differentiation that could possibly be involved in the formation of testicular germ cell tumors? (Chapter 4)
    a. Once we determine key molecules or processes important for gonocyte development, is it possible that the disruption of these same molecules or processes would play a role in testicular germ cell tumor formation?

To address these questions, we used isolated gonocytes in primary culture, our method of choice, to determine key factors necessary for gonocyte differentiation without the conflicting Sertoli cell culture alongside the gonocytes. We also performed comparative studies of the genes and protein profiles of gonocytes and spermatogonia, either freshly isolated or *in vivo*, to identify common *in vitro* and *in vivo* differentiation processes. Findings presented in this thesis have demonstrated the complexity by which gonocyte differentiation occurs and how, due to this complexity, there are multiple opportunities at which gonocyte development could possibly go off course and lead to eventual tumor formation.

Understanding the various processes at the base of gonocyte differentiation is a crucial step towards obtaining a more complete picture of the processes regulating SSC pool formation. Furthermore, finding how these processes could be dysregulated and possibly participate in tumor formation could, in the future, lead to the development of better targeted therapeutics with less reproduction-related side-effects.

Figures

Figure 1.1: Mammalian Testis Organization

(A) Schematic representation of a longitudinal cross-section through an adult testis, including the vas deferens and epididymis. (B) Schematic representation of a cross-section through seminiferous tubules, including the interstitial area. (C) Inside the seminiferous tubule, the developing germ cells are present at different stages that are closely located to the supporting Sertoli cells. The more immature germ cells are located close to the basement membrane whereas the more mature germ cells are located near the lumen. (D) Haploid spermatozoa are produced starting from diploid primordial germ cells. Adapted from Cooke and Saunders 2002 (284) and Krawetz, de Rooij, and Hedger 2009 (285).

Figure 1.1: <u>Mammalian Testis Organization</u>

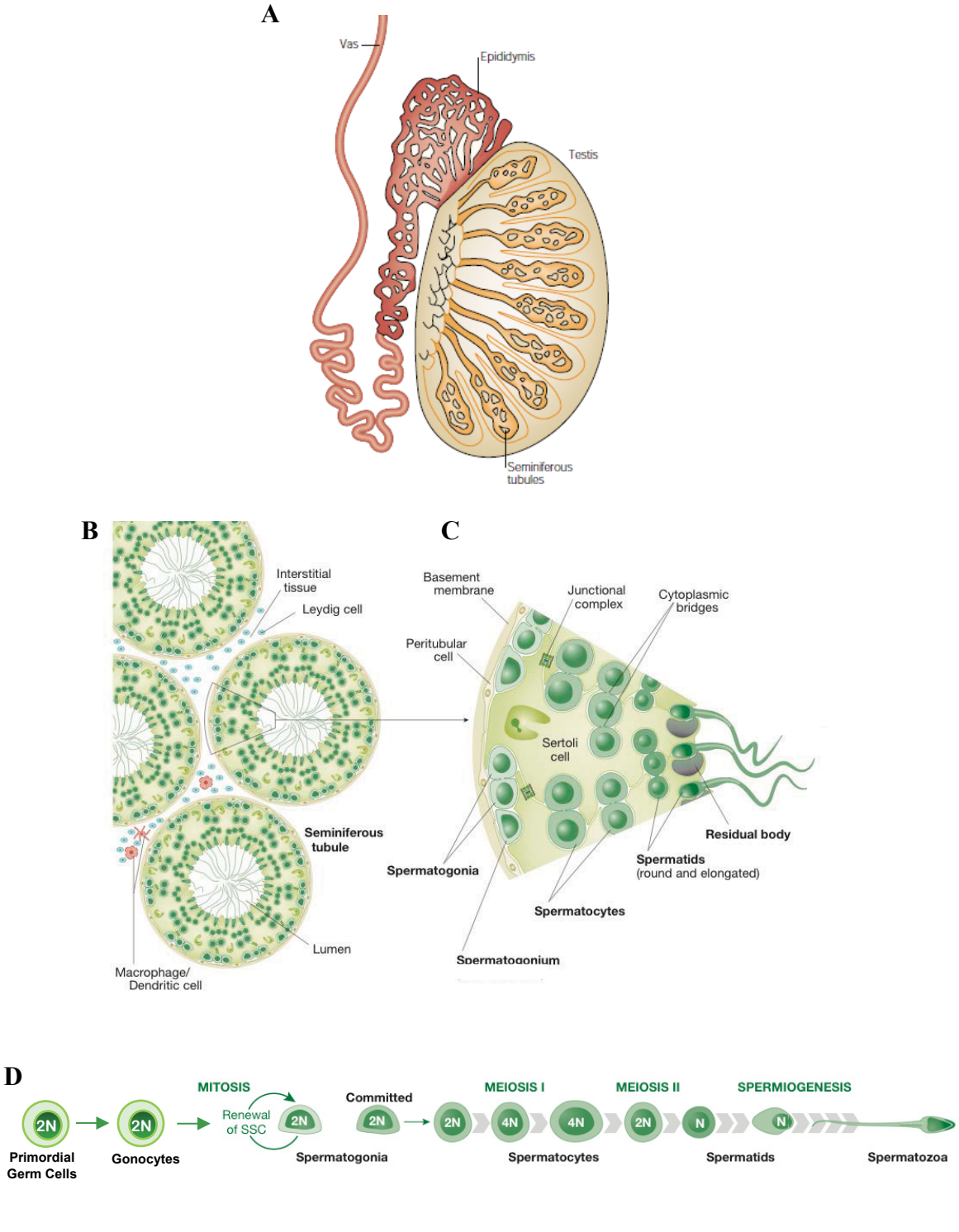


Figure 1.2: Timeline of Testicular Germ Cell Development

Schematic representation of the timeline of testicular germ cell development in (A) humans and in (B) rats as summarized from available literature. In brief, rat gonocytes, which are quiescent at birth, re-enter the mitotic phase at PND3. At this point, gonocytes undergo proliferation, migration toward the seminiferous cord basement membrane, and differentiation. Gonocytes that are unable to migrate will undergo apoptosis and be eliminated. It has been suggested that improper gonocyte development can lead to the formation of carcinoma-in-situ (CIS), the precursor pathology to testicular germ cell tumors. Adapted from Culty M 2009 (39) and Culty M 2013 (87).

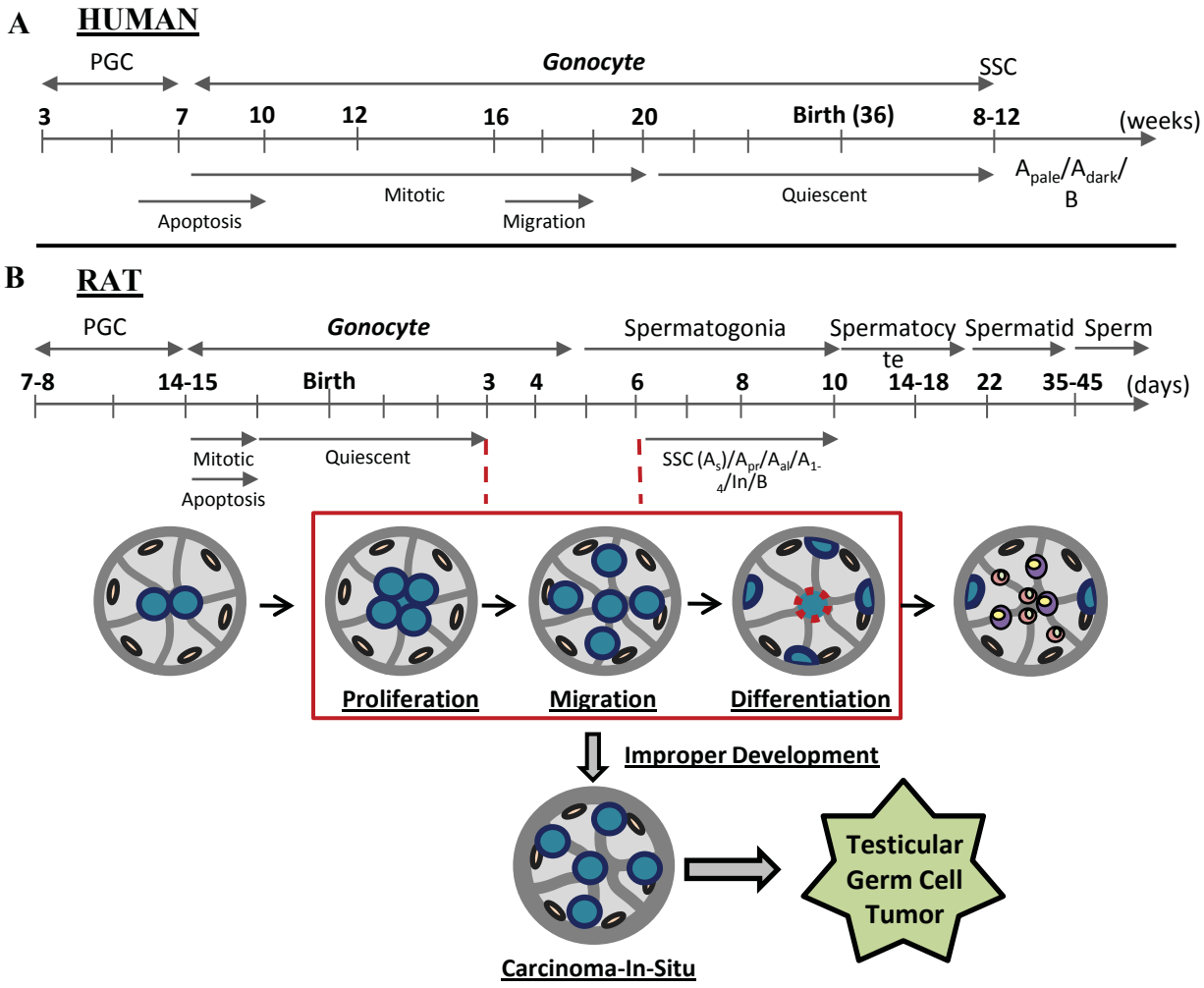


Figure 1.3: Vitamin A Metabolism in Germ Cells and Sertoli Cells

Schematic representation of how vitamin A metabolism has been hypothesized to occur in germ cells and Sertoli cells. In the postnatal testis, there are several possible ways in which RA is likely generated and delivered to the germ cell. Retinol (ROL) may be internalized (by binding to retinol binding protein (RBP)) of either Sertoli cells or germ cells and converted to retinaldehyde (RETALDH) and then retinoic acid (RA) by alcohol dehydrogenase (ADH) and retinaldehyde dehydrogenase (RALDH). RA can also be delivered to the germ cells from the supporting Sertoli cells or directly from the serum. RA will then interact with RA receptors (RAR) and stimulate gene transcription. Extra RA is metabolized by CYP26B1 into 4-OXO and 4-OH forms. These water soluble forms are then secreted from the cells and are eliminated. Adapted from Hogarth and Griswold 2010 (179) and Griswold, Hogarth, Bowles, and Koopman 2011 (286).

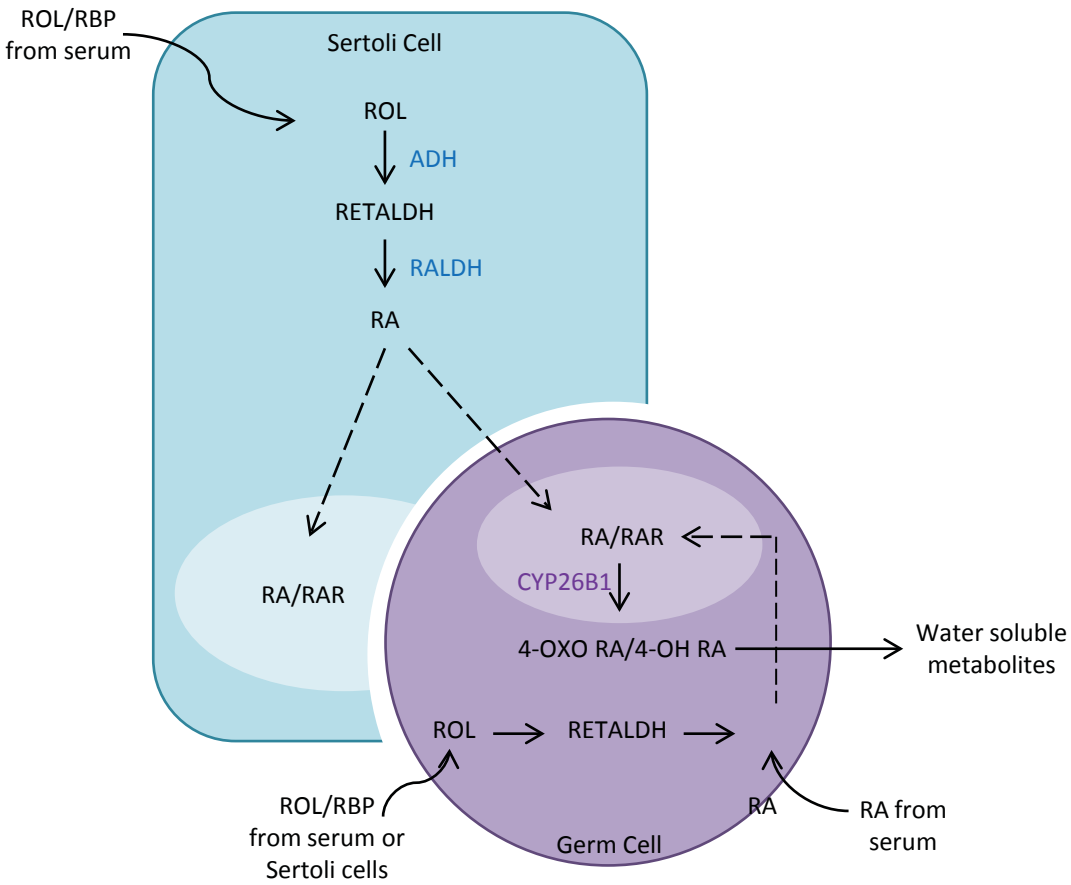


Figure 1.4: Gonocyte Isolation Protocol

In brief, PND3 rat pups are sacrificed and the testes are collected and decapsulated. The testes then undergo enzymatic digestion with collagenase and hyaluronidase, resulting in a mixture containing loose seminiferous tubules. This mixture is then further treated with trypsin and DNAse, and the resulting cells are filtered. These cells, now mainly Sertoli/myoid cells and gonocytes, are incubated overnight, allowing for Sertoli and myoid cells to adhere to the culture dishes, while the gonocytes remain floating, thus further separating the two cell populations. The next day, gonocytes and remaining Sertoli cells are further separated by sedimentation velocity at unit gravity on a modified small scale STAPUT gradient of 2-4% bovine serum albumin (BSA). An 80-90% pure gonocyte population is then collected and treated for further experiments.

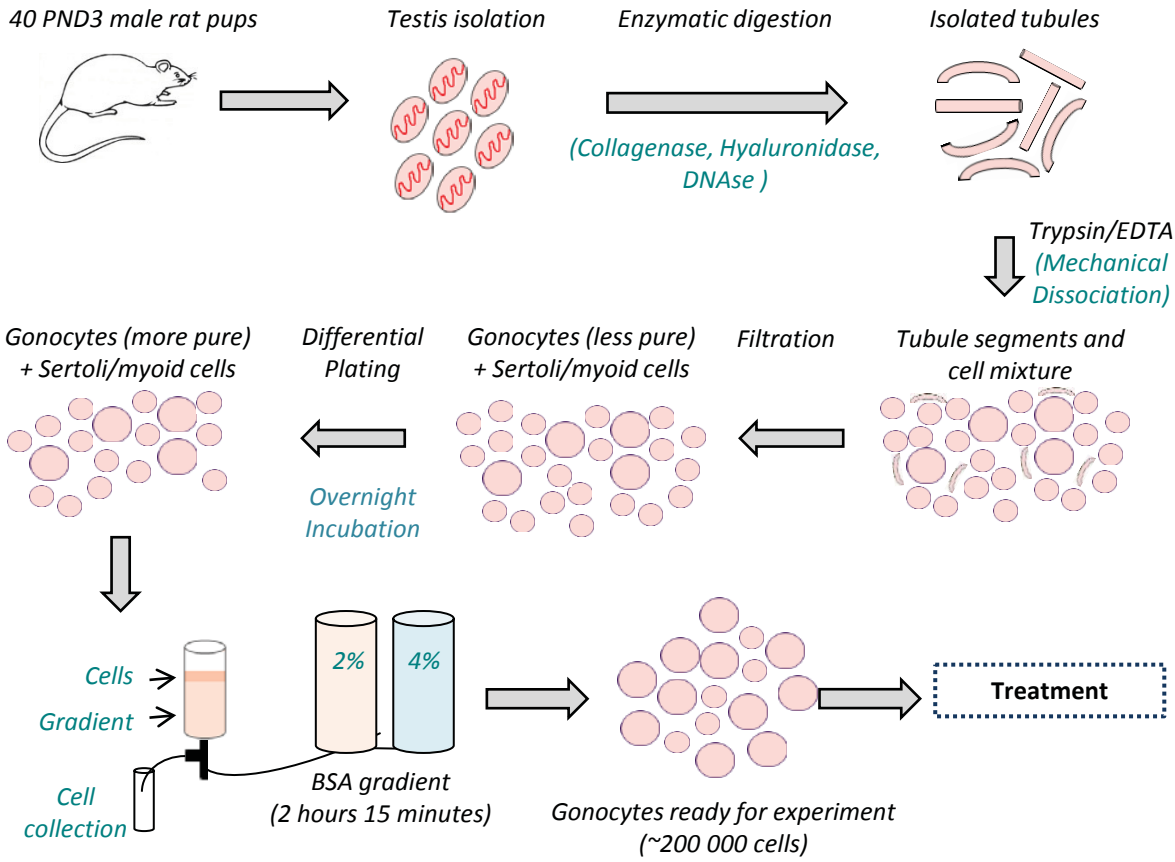
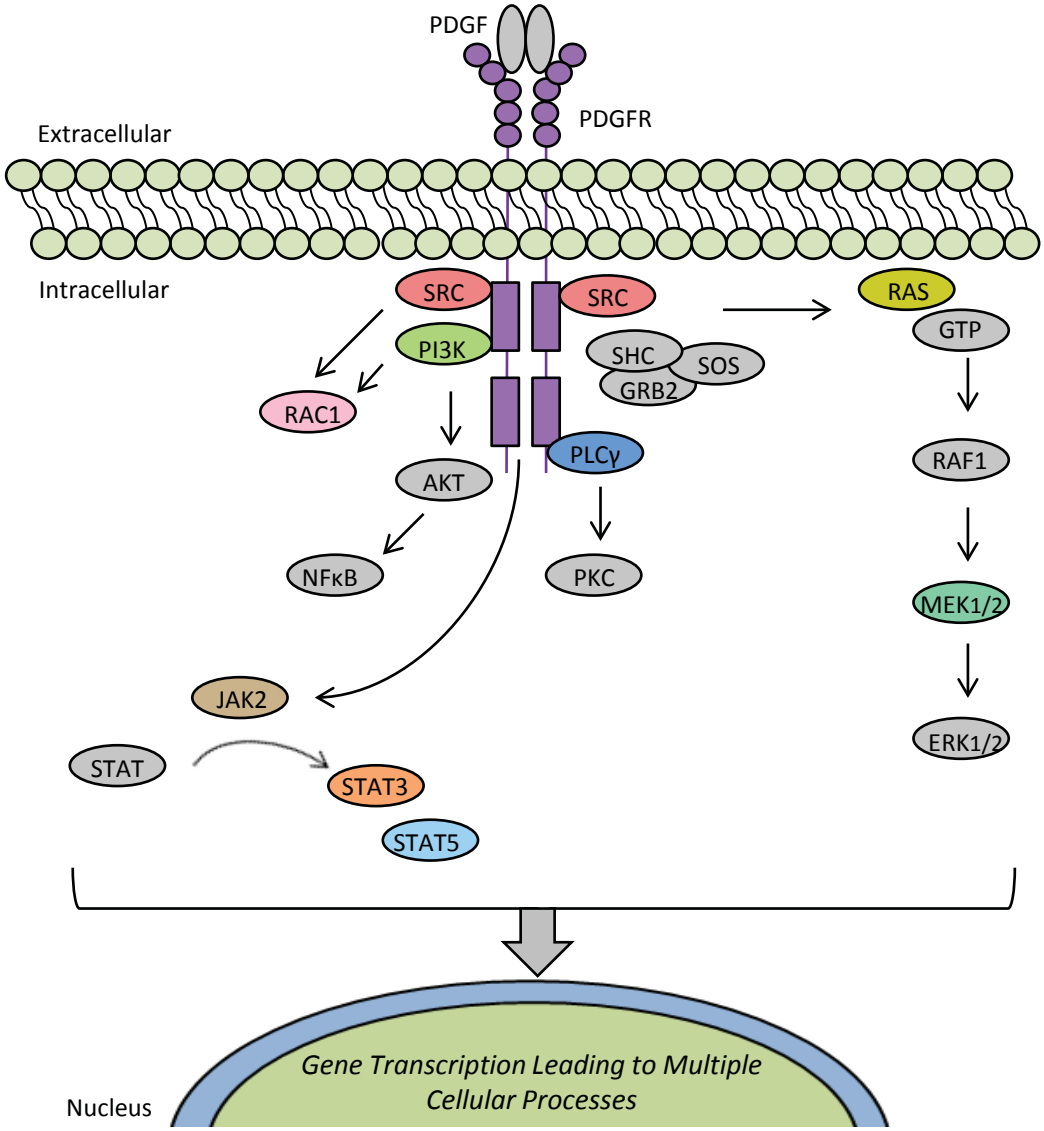


Figure 1.5: Platelet-Derived Growth Factor Receptor (PDGFR) Pathway

Schematic representation of the various downstream pathways of PDGFR activation. PDGF dimers activate PDGF receptors by binding to the extracellular domain and causing these receptors to dimerize. Upon dimerization, autophosphorylation of key tyrosine residues occurs by which downstream pathways can be further activated. Highlighted in color are the pathways known to be most commonly activated downstream of PDGFR activation. Summarized from the available literature.



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Chapter 2

Role of Retinoic Acid and Platelet-Derived Growth Factor Receptor crosstalk in the regulation of neonatal gonocyte and embryonal carcinoma cell differentiation

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Manuscript in Preparation

2.1 Abstract

Testicular neonatal gonocytes are direct precursors of spermatogonial stem cells (SSCs), the stem cell reservoir from which spermatozoa form. Although they are unipotent *in vivo*, gonocytes express many pluripotency genes common with embryonic stem cells. Gonocyte development includes phases of proliferation, migration, and differentiation and it has been suggested that improper gonocyte development could lead to formation of testicular germ cell tumors. Previously, we found that all-trans retinoic acid (RA) induced the expression of differentiation markers in isolated gonocytes. Similar effects occurred in F9 mouse embryonal carcinoma cells, an embryonic stem cell surrogate shown to differentiate into endoderm. In this study, we focused on better understanding molecular mechanisms and signaling pathways involved in RA-induced gonocyte and F9 cell differentiation, to determine if RA acted through the same mechanisms in gonocytes and early phases of embryonic development. In both cell types, RA induced increases in STRA8 expression in line with F9 cells corresponding to a stage prior to somatic-germline lineage specification. Northern blot analysis showed that neonatal testes express PDGFR α and β variants. Immunoblot analysis revealed PDGFRα variants in gonocytes and F9 cells, whose expressions were increased by RA. In both cell types, PDGFR tyrosine kinase activity inhibition reduced RA effects on the mRNA expression of Stra8, suggesting that one or more PDGFR forms are involved in differentiation. Inhibition of RARα activation in gonocytes also reduced RA effects on Stra8, indicating a potential crosstalk between RARα and PDGFR signaling pathways. MEK1/2 were activated during F9 cell differentiation towards the somatic lineage, whereas their inhibition promoted RA-induced Stra8 expression in F9 cells, suggesting that MEK1/2 acts as a switch for lineage specification in F9 cells. In contrast, RA-induced gonocyte differentiation was significantly reduced by inhibiting the SRC pathway, and JAK2 and STAT5 activities, indicating possible roles in gonocyte differentiation. These results suggest that neonatal gonocyte and F9 cell differentiation are regulated through crosstalks between RA and PDGFRs using different downstream pathways.

2.2 Introduction

Male germ cell development can be divided into two main phases: an earlier phase leading to the development of spermatogonial stem cells (SSCs), and a later phase ending with spermatozoa production (1). In the rat, gonocytes undergo two mitotic phases (1, 2), the first one taking place in utero, and the second, from post natal day (PND) 3 to PND5 (1-3). During this period, gonocytes migrate from the center of the seminiferous cords to their basement membrane and differentiate into spermatogonia, including SSCs and type A spermatogonia of the first wave of spermatogenesis (4-7). It is believed that alterations in gonocyte development may lead to the formation of carcinoma-in-situ (CIS), the precursor of testicular germ cell tumors (TGCTs) (1, 8). The incidence of TGCTs has been steadily increasing for the past few decades for unknown reasons, possibly related to increasing exposures to endocrine disruptors (9, 10). Thus, understanding the molecular mechanisms regulating gonocyte development could not only help better understand how SSCs are formed but could also provide insight into the origins of TGCTs.

Previously, we have shown that rat neonatal gonocytes differentiate upon retinoic acid (RA) stimulation, as reflected by increased expression of spermatogonial differentiation markers cKIT and STRA8 (stimulated by retinoic acid 8) in RA-treated gonocytes (11, 12). Similar results were also obtained in mice *in vitro* and *in vivo*, using vitamin A deficient mice (VAD) (13, 14). The importance of RA in spermatogenesis has been shown as extended vitamin A deficiency (VAD) in mice and rats results in spermatogenic arrest at undifferentiated spermatogonia phases. Upon vitamin A administration to these VAD animals, spermatogenesis is reinitiated (15, 16). The induction of STRA8 expression by RA also occurs in F9 mouse embryonal teratocarcinoma cells, a cell line commonly used as an embryonic stem cell surrogate for studying RA-induced cell differentiation (17). Although RA drives F9 cell differentiation along the somatic lineage, giving rise to cells with characteristics of the parietal or visceral endoderm, it also induces significant increases in STRA8 expression, which is in line with these cells corresponding to a stage prior to somatic-germline lineage specification (18, 19). Consequently, we were interested in comparing the signaling pathways associated with RA-induced differentiation in F9 cells and in gonocytes, using STRA8 as a common marker between the two cell types, as well as the somatic markers Collagen IV and Laminin B1 in F9 cells.

RA actions are mediated by the activation of nuclear retinoic acid receptors RAR (α, β, γ) and retinoid X receptors RXR (α, β, γ) (20). In classical pathway signaling, RARs and RXRs form homo- or hetero-dimers upon RA binding, which bind to RA response elements (RAREs) that then allow for a conformational change leading to transcriptional machinery recruitment and gene transcription regulation (21). Non-classically, RAR activation can affect many targets in an indirect manner, not involving RAREs directly but likely through a transcriptional intermediary (22). One such indirect target is platelet-derived growth factor receptor α (PDGFRα) (22).

The platelet-derived growth factor (PDGF) family consists of four members, PDGF-A, -B, -C, and -D which exert their action by binding to and dimerizing two receptor tyrosine kinases, the receptors PDGFRα and β (23). Activated PDGFRs and their downstream pathways play a critical role in various cellular processes (24). The importance of this signaling pathway is seen as both PDGFRα and β knockout mice are embryonic lethal (25). Human testicular seminoma tumors express a 1.5kb variant form of PDGFRα, not present in normal testicular tissue, representing a potential diagnostic marker for early tumor detection (26, 27). To add, transcripts for PDGF-A, PDGF-B, and the two receptors are found in 18 days post coitum (DPC) testicular tissue and reach the highest levels at PND5, at which point the expression begins to decline (25, 28). To add, we have shown that rat neonatal testicular gonocytes express both PDGFRα and β (29, 30) and proliferate in response to PDGF-bb and 17β-estradiol via activation of the MAPK pathway (29, 31). These data suggest that the PDGFR pathway may directly regulate germ cell development.

In the present study, we demonstrated that both gonocytes and F9 cells express a truncated form of PDGFRα (hereby termed T1-PDGFRα) that may be involved in RA-induced differentiation. Furthermore, using specific inhibitors, we showed that RA-induced differentiation for both F9 cells and gonocytes requires PDGFR pathway activation. For RA-induced gonocyte differentiation, the downstream pathways activated include the SRC family of kinases, JAK2, and STAT5. For F9 cells, it seems as though MEK1/2 acts as a switch, and depending on whether it is activated or inhibited, F9 cells differentiate towards the somatic cell lineage or express genes related to the germ-line lineage. Based on these results, we propose that PDGFRα plays a role in testicular germ cell development.

2.3 Materials and Methods

Animals

Newborn male Sprague Dawley rats were purchased from Charles Rivers Laboratories (Saint-Constant, QC, CA). GD18, PND3, PND8, and PND21 pups were euthanized and handled according to protocols reviewed and approved by the McGill University Health Centre Animal Care Committee and the Canadian Council on Animal Care.

Germ cell isolation

Gonocytes were isolated from PND3 rat testes using 30-40 pups per preparation, as previously described (13, 29, 30, 31, 32). In summary, testes were isolated, decapsulated, and gonocytes were isolated by sequential enzymatic tissue dissociation, filtration, and differential plating overnight in RPMI 1640 medium (Invitrogen, Burlington, ON, CA) with 5% fetal bovine serum (FBS) (Invitrogen), 2% penicillin/streptomycin (CellGro, Manassas, VA, USA), and 1% amphotericin B (CellGro). After overnight differential plating, the non-adherent cells, mainly gonocytes, were further enriched using a 2-4% bovine serum albumin (BSA) gradient (Roche Diagnostics, Indianapolis, IN, USA). Fractions that contained the most gonocytes, as judged by their morphology, were pooled, centrifuged, and collected with a final purity of at least 95% for gene array analysis and a purity of at least 85% for quantitative-PCR (qPCR) and immunoblot analysis. Spermatogonia were isolated from PND8 rat testes, using 10 pups per preparation. The method used to isolate spermatogonia was the same as described above (14, 30, 31, 32, 33). Experiments were performed using a minimum of three independent gonocyte or spermatogonia preparations. For pups at GD18 and PND21, the testes were extracted and frozen for RNA extraction and northern blot analysis.

Gonocyte culture

Once isolated, gonocyte cell populations of at least 85% purity were further treated for qPCR and immunoblot analysis as needed. Gonocytes (and spermatogonia) without any treatment were pelleted and frozen for later processing. For cell treatment, in brief, gonocytes were cultured in supplemented RPMI media at 37°C and 3.5% CO_2. Gonocytes were plated at a density of 8000-10000 cells/well in 24-well plates. The cells were treated with either control medium or all-trans retinoic acid (10^{-6}M) (Sigma, Oakville, ON, CA) for 24 hours (mRNA analysis) and 72 hours (protein analysis). Alongside the control and RA treatments, the cells

were also treated with different inhibitors at various concentrations: AG370 (50μM; Sigma), U73122 (1μM; Enzo Life Sciences, Brockville ON, CA), U0126 (10μM; Calbiochem, Billerica, MA, USA), Wortmannin (1μM; Sigma), SU6656 (0.5, 1μM; Sigma), AG490 (50, 100μM; Sigma), Stattic (1, 10μM; Sigma), Pimozide (1, 10μM; Tocris BioScience, Minneapolis, MN, USA), Farnesylthiosalicylic Acid (FTS) (50, 100μM; Cayman Chemical, Ann Arbor, MI, USA), NSC23766 (10, 25μM; Cayman Chemical), BMS195614 (10μM; Tocris Bioscience), and Cathepsin L inhibitor I (1μM; Calbiochem). Once the treatment periods were over, cells were collected for RNA extraction and protein analysis, as needed.

F9 cell culture

F9 mouse embryonal carcinoma cells were maintained in DMEM medium (Invitrogen) containing 10% fetal bovine serum (FBS) (Invitrogen) and 1% penicillin/streptomycin (CellGro) at 37°C and 5.0% CO_2. All culture flasks or dishes used for F9 cells were coated with 0.1% gelatin (Chemicon International, Billerica, MA, USA) before use. For F9 cell treatment, cells were plated on day 0 at a density of 30000 cells/well in 6-well plates. One day later, the cells were treated with either control medium or all-trans retinoic acid (10^{-7}M) (Sigma) (in DMEM media containing 10% FBS (Invitrogen) and 1% penicillin/streptomycin (CellGro)) for 72 hours. Alongside the control and RA treatments, the cells were also treated with different inhibitors at various concentrations as indicated above. For BrdU proliferation studies, F9 cells were treated with various concentrations of PDGF-AA (Sigma) and PDGF-BB (Sigma) for one day. Once the treatment periods were over, cells were collected for RNA extraction, ELISA analysis, and protein analysis, as needed.

RNA extraction and cDNA synthesis

As previously described for gene array and qPCR analysis, total RNA was extracted from cell pellets using the PicoPure RNA isolation kit (Arcturus, Mountain View, CA, USA) and digested with DNase I (Qiagen, Valencia, CA, USA) (33). For northern blot analysis, whole testes from GD18, PND3, and PND21 rats were first homogenized and RNA was then extracted using the PicoPure RNA isolation kit also. cDNA was then synthesized from the extracted RNA by using the single strand cDNA transcriptor synthesis kit (Roche Diagnostics) following the manufacturer's instructions.

Gene Array

Rat Illumina microarray analysis was performed by the McGill University's Genome Quebec facility with RNA samples extracted from isolated PND3 gonocytes, PND3 Sertoli/myoid cells, PND8 spermatogonia and PND8 Sertoli/myoid cells. As previously described, three independent RNA samples were analyzed for the germ cells and two independent samples for the somatic cells (33, 34). To have sufficient RNA concentrations (100ng/μl) per sample, each gonocyte sample preparation (at least 95% pure) included 60-90 PND3 pups, while each spermatogonia sample preparation required 10 PND8 pups. After RNA quality was verified at Genome Quebec, the samples were analyzed using the RatRef-12 Expression BeadChip for genome-wide expression analysis (Illumina, San Diego, CA, USA). This Illumina chip contains 22523 probes selected mainly from the NCBI RefSeq database. Data normalization and preliminary data analysis was performed by Dr. Jaroslav Novak (35). All data was normalized using the quantile normalization method, corrected for the background signal, and abbreviated by eliminating the low-expression-end of the data spectrum. Here, the gene array analysis was used to find overall expression levels of a variety of different genes in the samples.

Northern blot analysis

Northern blot analysis was performed as previously described (13, 36). In brief, RNA samples from GD18, PND3, and PND21 rat testes were prepared and run in an agarose gel. The gel was then transferred to a positively charged nylon membrane using capillary transfer. The membrane was hybridized with a ^{32}P-labeled PDGFRα cDNA probe generated from random priming (Promega, Madison WI, USA) that recognized the C-terminus of rat wild-type PDGFRα (accession number: NM_012801, region: bp 1907-2417). The blot was imaged using the LAS-4000 gel documentation system (Fujifilm, Mississauga, ON, CA). The same blot was then stripped and re-probed with a ^{32}P-labeled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe used as an internal control.

Quantitative real time PCR (qPCR)

QPCR analysis was done using a LightCycler 480 with a SYBR Green PCR Master Mix kit (Roche Diagnostics). Gene and species specific primers were designed using the Roche primer design software (Roche Diagnostics) and are listed in Table 1. QPCR cycling conditions used

were: an initial step at 95°C followed by 45 cycles at 95°C (10 sec), 61°C (10 sec), 72°C (10 sec), and then followed by melting and cooling cycles. Detection of PCR products occurred by measuring increase in fluorescence caused by SYBR Green dye binding to double-stranded DNA. The comparative threshold cycle (C_t) method was used to analyze the data. We have previously shown that, in these samples, the ideal housekeeping gene to use is 18S rRNA (35). The expression levels of the various genes were normalized to the reference gene (18S rRNA) by calculating the value of $2^{\Delta Ct}$, where ΔC_t is the difference between the C_t value of the gene of interest and that of the 18S rRNA. The final data were expressed in a relative unit representing the mRNA levels of the gene of interest present in the samples tested. Assays were performed in triplicate. All experiments were performed using a minimum of three independent sample preparations and the mean ± SEM are shown.

BrdU ELISA measurement

In order to determine the rates of proliferation in F9 cells upon PDGF-AA and PDGF-BB stimulation, BrdU ELISA proliferation assays were used. In brief, F9 cells were seeded in 6 well plates at a density of 5000 cells per well. After overnight incubation, the cells were treated with various concentrations of PDGF-AA (Sigma) and PDGF-BB (Sigma) for 24 hours. BrdU (Sigma) was added in the culture medium six hours before the ELISA was performed. The cells were then fixed to denature BrdU-labeled DNA (Invitrogen). The fixed cells were incubated with peroxidase-coupled anti-BrdU antibody (Invitrogen), followed by a colorimetric reaction with TMB (tetramethyl-benzidine) (Invitrogen) that measured the signal at 450 nm after the reaction was stopped by H_2SO_4. The data were expressed as differences between the absorbance at 450nm and the absorbance at the reference wavelength of 690nm for each sample. Three independent experiments were performed with each individual sample run in triplicates.

Immunoblot analysis

Samples for immunoblot analysis were solubilized in Laemmli buffer and protein concentrations were determined using the BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). The proteins were then separated on pre-casted 4-20% tris-glycine gels (Invitrogen). Once the run was complete, the gels were transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA). After blocking with 5% milk, membranes were probed with various antibodies to determine protein expression (Table 2). After overnight

incubation at 4°, bound antibodies were detected using anti-biotin and horseradish peroxidase (HRP) coupled goat anti-rabbit (or goat-anti mouse) secondary antibodies and ECL-enhanced chemiluminescence (GE Healthcare, Mississauga, ON, CA). Images of the immunoblots were taken using the LAS-4000 gel documentation system (Fujifilm). Membranes were stripped away of the probed antibodies using Restore Plus Western blot stripping buffer (Thermo-Scientific), blocked with 5% milk and then probed with a second round of antibodies to be studied. Tubulin or GAPDH were used as loading references. Protein expression levels were quantified using the MultiGauge V3.0 program (Fujifilm).

Immunohistochemistry

Formaldehyde fixed, paraffin-embedded sections of PND3 and PND8 testes were stained using previously described methods (37). In summary, slides were dewaxed and rehydrated using Citrosolv (Fisher Scientific, Toronto, ON, CA) and Trilogy solution (Cell Marque IVD, Rocklin, CA, USA). Following treatment with Dako Target Retrieval solution (DAKO, Burlington, ON, CA), the sections were incubated with PBS (Invitrogen) containing 10% goat serum (Vector Laboratories, Burlington, ON, CA), 1% BSA (Roche Diagnostics) and 0.02% Triton X100 (Promega) for 1 hour to block non-specific protein interactions. The sections were then incubated with primary antibodies diluted in PBS (Invitrogen) containing 1% BSA (Roche Diagnostics) and 0.02% Triton X100 (Promega) at various dilutions (Table 3) overnight at 4°C. The next day, sections were incubated with biotin-conjugated secondary antibodies (diluted in PBS (Invitrogen) containing 1% BSA (Roche Diagnostics)) for 1 hour at room temperature (Table 3). The slides were then treated with streptavidin-peroxidase (Invitrogen), AEC single use solution (Invitrogen), and counter stained with hematoxylin (Sigma Aldrich). The sections were coated with Crystal Mount (Electron Microscopy Sciences, Hatfield, PA, USA), dried, and cover-slipped (Fisher Scientific). Slides were examined under bright-field microscopy with a BX40 Olympus microscope (Olympus, Center Valley, PA, USA) coupled to a DP70 Olympus digital camera (Olympus). Negative controls were done by incubating some sections with Rabbit IgG (pre-immune rabbit serum) and not a primary antibody (Invitrogen).

Immunoprecipitation

F9 cells were plated at a density of 150000 cells/150mm 0.1% gelatin coated petri dish in multiple petri dishes at day 0. The next day, the cells were treated with or without RA and kept

in culture for 72 hours. After this incubation, the cells were collected and protein was extracted using a pre-made extraction buffer including NaCl, MgCl2, and DTT (Invitrogen Dynabeads© Co-Immunoprecipitation kit, Invitrogen). A Bradford assay (Bio-Rad) was then performed to determine the protein concentration of samples to be immunoprecipitated. The PDGFRα (Upstate/Millipore, catalog number 07-276 (lot number 30083)) antibody was coupled to the Dynabeads© by following the manufacturer's protocol and overnight incubation at 37°. Once the antibody was coupled to the Dynabeads and the cell proteins were extracted, 1.5mg of antibody coupled to Dynabeads© was mixed with the total cell lysates (equal protein amounts to be used for all samples) and incubated for 60 minutes at 4° while constantly rotating. After this incubation, the Dynabeads©/protein mixture (containing the protein of interest) was washed multiple times. Afterwards, the beads were separated from the protein complex using an elution buffer and magnetic separation. The resulting supernatant contained the purified protein complex. This complex sample was then analyzed on immunoblot to confirm PDGFRα expression. This approach allowed to concentrate PDGFRα within the samples before analyzing them by mass spectrometry.

Mass Spectrometry

F9 cell extracts were first separated using a pre-casted 10% tris-glycine gel (Invitrogen) and then silver stained to detect proteins after the electrophoretic separation using the SilverQuest Silver Staining kit (Invitrogen) following manufacturer's instructions. Selected protein spots were then cut and sent for mass spectrometry analysis (performed by Dr. Josip Blonder). In brief, protein spots of interest underwent an in-gel digestion procedure previously described (38). Afterwards, these digests were desalted using C18 Zip Tips (Millipore) and analyzed by nano-flow reversed phase liquid chromatography (nanoRPLC) using the Agilent 1100 LC system (Agilent Technologies Inc., Paolo Alto, CA, USA). This was coupled to a linear ion trap (LIT) mass spectrometer (LTQ, Thermo Scientific). Reversed-phase separations were performed using 75µm i.d.×360µm o.d.×10cm long slurry packed capillary columns (Polymicro Technologies Inc., Phoenix, AZ, USA). The column was then injected with 5µl of sample and washed for 20 minutes with 98% solvent A (0.1% formic acid in water). The peptides were eluted for 110 minutes at a constant flow rate of 250nl/min using a linear gradient of 2% solvent B (0.1% formic acid in 100% acetonitrile) to 85% solvent B. Furthermore, each full MS scan was followed by seven MS/MS scans where the most abundant peptide molecular ions were

dynamically selected for collision-induced dissociation (CID). The resulting spectra were searched against a non-redundant human protein database using SEQUEST (Thermo Scientific) and results were tabulated for each identified peptide/protein (39).

Statistical analysis

Statistical analysis was performed using an unpaired two-tail Student's t-test or one-way ANOVA with Dunnett's correction using statistical analysis functions in the GraphPad Prism 5.0 program (GraphPad Inc., San Diego, CA, USA). All experiments were performed with N equal to a minimum of three independent experiments. A p-value less than 0.05 was considered statistically significant.

2.4 Results

PDGF signaling pathway is involved in F9 proliferation and RA-induced differentiation

We have previously shown that there is a significant increase in V1-PDGFRβ (variant form of PDGFRβ) and PDGFRα expression in F9 cells treated with RA (11). Based on these observations, we postulated that the PDGF signaling pathway might be involved in RA-induced differentiation of F9 cells. To investigate, the specific inhibitor AG370 was used to block PDGFR tyrosine kinase activity (40). As shown in Figure 2.1A, after 72 hours of treatment with 0.1μM RA and 50μM AG370, the morphology of the F9 cells was less scattered and flattened in comparison with cells treated with RA alone, while some cells formed compact clusters similar to those observed in cells cultured without RA. Since RA-induced increases in collagen IV and laminin B1 mRNAs are indicative of F9 cell differentiation (11, 41, 42), we examined the effects of AG370 on the expression of these two genes by qPCR analysis in RA-stimulated cells. Interestingly, in the presence of AG370, collagen IV and laminin B1 mRNA expression was significantly decreased by 75% and 88% of their RA-induced levels, respectively (Figure 2.1B). To further confirm the involvement of PDGFRs in RA-induced differentiation of F9 cells, another PDGFR specific inhibitor, AG1295, was used to block the tyrosine kinase activity of PDGFRs (Figure 2.1C) (43). Because the proto-oncogene cMYC is activated by PDGFRs and it had previously been shown to decrease upon RA stimulation of F9 cells, we also examined its expression (44). Both RA stimulation and PDGFR inhibition by AG370 induced significant decreases in cMYC expression in F9 cells, and the combination of RA and AG370 showed additivity in their inhibitory effects, suggesting that the inhibitory effect of RA did not use the same mechanism than that resulting of PDGFR inhibition (Figure 2.1D). Since the full length PDGFRα, but not β, is expressed in F9 cells, we examined whether PDGF-AA or BB had an effect on F9 cell proliferation. F9 cell proliferation was significantly increased by PDGF-AA, whereas PDGF-BB had no effect on proliferation (Figure 2.1E). However, these experiments did not allow us to determine which form(s) of PDGFRs promotes the RA-induced differentiation of F9 cells, since AG370 and AG1295 may block the tyrosine kinase activity of both PDGFRα and V1-PDGFRβ. Furthermore, these findings suggest that PDGF-AA might regulate cMYC expression in proliferating F9 cells. By contrast, the RA inhibitory effect on cMYC expression could reflect an anti-proliferative effect of the differentiation factor (45).

The PDGF signaling pathway is associated with MEK/ERK activation but not with PLCγ or PI3K in RA-induced differentiation of F9 cells

PLCγ and PI3K signaling pathways are commonly activated downstream of PDGFR activation (46-48). To verify whether PDGF signaling pathway promotes differentiation in RA-treated F9 cells through PLCγ or PI3K, the PLC inhibitor U73122 (49), and the PI3K-specific inhibitor Wortmannin were used (50). Treatment with U73122 or Wortmannin had no effects on RA-induced morphological changes in F9 cells (Figure 2.2A), although the cell numbers were apparently decreased following treatment with either inhibitor alone or in combination with RA. Moreover, the presence of either inhibitor did not significantly change the RA-induced increases of collagen IV and laminin B1 transcripts (Figures 2.2B, 2.2C), in agreement with their lack of effect on F9 cell morphological changes. These results indicate that PLCγ and PI3K signaling pathways are not involved in RA-induced differentiation of F9 cells.

Another important signaling pathway regulated by PDGF is the Raf/MEK/ERK cascade. To determine whether MEK1/2 and ERK1/2 were involved in the RA-induced differentiation of F9 cells, the inhibitor U0126 (51), which specifically blocks MEK1/2 activation, was used. Since MEK1/2 are the only known kinases to activate ERK1/2 (52), blocking MEK1/2 activities will directly block the activation of ERK. As shown in Figure 2.2A, the presence of U0126 interfered with the changes in morphology normally induced by RA in F9 cells. qPCR analysis of collagen IV and laminin B1 transcripts revealed that U0126 decreased the levels of both mRNA by 57% and 56% of the RA-dependent inductions, respectively (Figure 2.2D).

As shown in Figure 2.3A, the analysis of laminin B1 protein expression confirmed the mRNA findings by showing that the RA-dependent increase in Laminin B1 was also significantly reduced at the protein level by U0126. The activation of MEK1/2 and ERK1/2 during RA-induced F9 differentiation were also confirmed by immunoblot analysis (Figure 2.3B and 2.3C). In order to clarify whether MEK/ERK1/2 activation by RA was associated with F9 cell differentiation toward primitive or parietal endoderm, we compared ERK1/2 activation status in the presence of RA alone or with 8-bromo-cAMP, a combination known to support parietal endoderm development (Figure 2.3C). RA significantly induced ERK2 phosphorylation, while ERK1 showed a trend toward increased phosphorylation. The addition of cAMP reduced RA-induced ERK2 phosphorylation, indicating that ERK2 activation is involved in primitive but

not parietal endoderm formation. As shown in Figure 3D, the RA-induced increase in phospho-ERK2 levels was suppressed both by AG370 (51% decrease) and U0126 (47% decrease). These results suggest that RA crosstalks with the PDGFR pathway via ERK2 activation in RA-induced F9 cell differentiation.

Taken together, the data showing that inhibitors of PDGFR kinase activity and MEK1/2 activation suppressed RA-induced increases in Collagen IV and Laminin B1 expression, as well as MEK1/2 and ERK2 phosphorylation, suggest that the differentiation of F9 cells towards the primitive endoderm is regulated by a crosstalk process between RA and the PDGFR pathway via ERK2 activation.

A PDGFRα variant protein is expressed in RA-induced differentiating F9 cells

In view of the findings that inhibiting PDGFR tyrosine kinase activity blocked the RA-induced F9 cell differentiation, we hypothesized that PDGFRα likely plays an important role in this process. To test this hypothesis, we analyzed PDGFRα protein expression by immunoblot analysis. As shown in Figure 2.4A and 2.4B, F9 cells not only express full length (120kDa) PDGFRα form, but also two variant forms of 60 and 45 kDa respectively. The expression levels of the 45kDa variant were significantly increased upon RA stimulation of F9 cells in a time-dependent manner, showing maximal expression after three days in culture. The RA-dependent formation of this 45kDa variant was significantly reduced by the PDGFR kinase inhibitor AG370 (Figure 2.4B). By contrast, the protein expression of the 120 and 60 kDa bands was decreased in F9 cells treated with RA, and the addition of AG370 had no effect on these two proteins in control or RA-treated cells. Given that the 120 and 60 kDa PDGFRα bands were decreased by RA simultaneously to the increases in the 45 kDa band, we examined whether the smaller size band could be a proteolytic product from one of the larger size bands. For this, F9 cells were treated with an inhibitor of Cathepsin L, a protease commonly activated by PDGFs (53, 54), alone or with RA. We found that inhibition of cathepsin L activity did not have any significant effect on the expression patterns of PDGFRα variant forms seen upon RA-stimulation in F9 cells (Figure 2.4C). These data suggested that the 45 kDa PDGFRα variant was not due to proteolytic degradation. Interestingly, the addition of the MEK inhibitor U0126 together with RA prevented the formation of the 45 kDa variant (Figure 2.4D), indicating that MEK1/2 activation

is required for the RA-induction of this variant PDGFRα in F9 cells, confirming the importance of the MEK1/2 pathway in RA-induced F9 cell differentiation.

In order to confirm the identity of the variant bands seen in RA-induced differentiating F9 cells as being PDGFRα, we performed mass spectrometry analysis of the bands of interest. We first co-immunoprecipitated the samples using a PDGFRα antibody before mass spectrometry analysis. The three expected PDGFRα bands were visible before (Figure 2.5A) and after (Figure 2.5B) immunoprecipitation in RA-treated samples. Mass spectrometry analysis identified the presence of different combinations of peptide fragments belonging to the PDGFRα sequence in each band (Figure 2.5C). The PDGFRα fragments from the 120 kDa band corresponded to sequences located throughout PDGFRα sequence, confirming that this band was indeed the full length receptor. By contrast, the 60 and 45 kDa bands contained PDGFRα sequences located toward the C terminal end of the receptor, but not in the N terminal region. These results, together with the fact that the antibody used to visualize the bands was raised against a peptide from the C terminal region of PDGFRα, suggest that the 45kDa and 60kDa variants are truncated forms of PDGFRα missing the N terminal region.

MEK/ERK1/2 inhibition promotes RA-induced expression of the premeiotic germline marker Stra8 in F9 cells.

Given that F9 mouse embryonal carcinoma cells correspond to a stage of embryonic stem cells prior to the somatic-germline lineage specification (19), and they are known to express the premeiotic germline marker Stra8 upon RA treatment (18), we examined the effects of several PDGFR pathway inhibitors on RA-induced Stra8 expression in these cells. Interestingly, the simultaneous addition of a PDGFR kinase inhibitor with RA prevented the increase of Stra8 expression normally induced by RA (Figure 2.6A), indicating the requirement of PDGFR activation in this process. Inhibitors of SRC, RAC1, JAK/STAT, PLCγ. and PI3K had no effect on Stra8 induction by RA (data not shown). By contrast, the addition of the MEK1/2 inhibitor U0126 to RA doubled the increase of Stra8 expression observed with RA alone (Figure 2.6B), while co-treatment with the RAS inhibitor FTS showed an increasing trend in Stra8 expression, suggesting that the inhibition of MEK/ERK1/2 promotes the expression of germline-specific genes in F9 cells.

Taken together with the data presented above showing the requirement of PDGFR (likely the 45kDa PDGFRα variant) and MEK/ERK1/2 activation for the differentiation of F9 cells towards the primordial endoderm (Figures 2.1-2.3), these results suggest that MEK/ERK1/2 activation participates to F9 cell somatic differentiation whereas its inhibition allows F9 cells to express germline-related genes in response to RA.

PND3 gonocytes express a variant form of PDGFRα that is increased upon RA stimulation

We examined whether neonatal gonocytes express variant forms of PDGFRα, as seen in RA-treated F9 cells. First, northern blot analysis revealed that PND3 testes express not only the full length PDGFRα transcript (6 kb), but also 3 shorter transcripts of 3.5, 2.5 and 1.5 kb, respectively. Of interest, PDGFRα transcripts were much more abundant at PND3 than in fetal (GD18) and prepubertal (PND21) testes, suggesting a preferential role for PDGFRα in the neonatal testis (Figure 2.7A). Looking more precisely at germ cells, PND3 gonocytes expressed much higher levels of PDGFRα mRNA than PND8 spermatogonia, a cell population including SSCs and various phases of undifferentiated spermatogonia (Figure 2.7B), in agreement with earlier studies showing the downregulation of the protein in spermatogonia (29). Moreover, PDGFRα mRNA was increased in RA-treated gonocytes (Figure 2.7C) simultaneously to Stra8 used as an indicator of differentiation (Figure 2.7D), as previously published (11). Similarly to the results obtained with F9 cells, PDGFR activation appeared to be required for RA-induced gonocyte differentiation, since the presence of PDGFR inhibitor AG370 significantly decreased the induction of Stra8 expression in response to RA treatment (Figure 2.7D). Taken together, these results suggest that RA crosstalks with PDGFR in order to induce gonocyte differentiation.

Next, we analyzed PDGFRα protein expression profiles in neonatal gonocytes by immunoblot analysis. There was very little to no full length PDGFRα (120kDa) expressed in gonocytes in function of the cell preparation (Figure 2.7E), in agreement with previous studies showing PDGFRβ as the major full length PDGFR form in gonocytes (30). However, gonocytes expressed a 65 kDa PDGFRα variant form that was significantly increased upon RA treatment (Figure 2.7E). The specificity of the 65 kDa band was verified by pre-incubating the PDGFRα antibody with the peptide it was raised against, confirming that the signal on the membrane was lost when the antibody was saturated with the peptide (data not shown). Moreover, the formation of this variant was not altered by treatment with an inhibitor of Cathepsin L (data not shown),

similarly to what was seen in F9 cells, suggesting that the variant form was not a proteolytic product.

To continue, we examined the differential PDGFRα exon profiles in control and RA-treated gonocytes with qPCR analysis, using primer sets of comparable efficiencies, to better understand the variant present in gonocytes. The fact that the antibody used here recognized the C terminal region of PDGFRα suggested that the mRNA sequence coding for the 65 kDa variant included the 3' coding sequence of the full length transcript. This was supported by the fact that an increase in PDGFRα expression in RA-treated gonocytes was seen when using qPCR primers targeting the 3' end of PDGFRα, but not with primer sets designed to amplify the 5' end (Figure 2.7F). To further characterize the PDGFRα variant, we undertook an extensive qPCR analysis where 41 specific overlapping primer sets covering the entire mRNA sequence were designed. We then plotted the data from all 41 primer sets for control and RA-treated gonocytes, showing differential profiles between the two conditions. Adjacent exons showing similar pattern were grouped together to simplify the analysis (Figure 2.7G). The study showed that there was no significant change in PDGFRα expression in the areas covering exons 1-6 between control and RA-treated gonocytes (Figure 7G, left panel). By contrast, exons 7 to 11 all showed significantly decreased expression in RA-treated cells in comparison to untreated cells (Figure 2.7G, middle panel). Finally, when grouped together, PDGFRα expression in exons 12-23 showed a significant increase in RA-treated gonocytes compared to control cells (Figure 2.7G, right panel). Taken together with the fact that there is a significant increase in PDGFRα variant protein expression in RA-induced differentiating gonocytes, these results suggested that the variant form of this receptor contains exons 12-23, and may be lacking an internal sequence containing exons 7-11. This will need to be confirmed by determining the exact sequence of this variant receptor form. Taken together, these results revealed the presence of a novel variant PDGFRα form in gonocytes, now termed T1- PDGFRα, also indicating that this variant form likely contains the 3' end of the full length PDGFRα sequence.

RA acts in part through RARα activation to induce gonocyte differentiation

It has previously been reported that PDGFRα is one target gene that can indirectly be activated upon RAR activation by RA (22). RAR and RXR expression in gonocytes has previously been reported, although there was conflicting data regarding which type of RAR

isoforms were expressed (55, 56). Our gene array analysis showed that it is RARα that is most abundantly expressed in PND3 gonocytes (Supplemental Figure 2.1A). However, because the gene arrays did not include all rat RAR and RXR isoforms, we performed qPCR analysis, comparing the RAR and RXR expression profiles of PND3 gonocytes and PND8 spermatogonia. RARα, RARγ1 and 2 were more highly expressed in PND3 gonocytes compared to other RAR and RXR isoforms. RXRα and RXRβ were also highly expressed in gonocytes (Supplemental Figure 2.1B). In order to determine whether activation of RARα played a role in RA-induced gonocyte differentiation, we treated isolated gonocytes with RA and BMS195614, a specific RARα antagonist (57), and analyzed Stra8 gene expression (Supplemental Figure 2.1C). As expected, there was a significant increase in Stra8 mRNA expression, indicating gonocyte differentiation. Furthermore, the presence of BMS195614 significantly decreased the induction of Stra8 expression compared to RA alone, indicating that RARα activation is involved in gonocyte differentiation. However, Stra8 expression was only partially reduced by the RARα antagonist, suggesting that RARγ, the other strongly expressed RAR in gonocytes, or another regulatory process, might also play a role in gonocyte differentiation.

RA-induced gonocyte differentiation requires the activation of SRC and JAK2/STAT5 pathways

In view of our finding that inhibiting PDGFR activation suppressed RA-induced Stra8 expression, we next examined which downstream signaling pathway could be involved in this crosstalk. We performed gene array, qPCR, and immunohistochemical analyses of isolated gonocytes and PND3 testes sections respectively, to verify which of the common PDGFR downstream signaling pathways were present in gonocytes (data not shown). Immunoblot analysis was also performed (Supplemental Figure 2.2). Here, we found that all the proteins were expressed at variable levels. When analyzing PDGFRs phosphorylation, phosphorylated receptors were detected at the plasma membranes of gonocytes with an anti-phospho-PDGFRα/β antibody detecting the phosphorylation of tyrosine 849 and tyrosine 857 in PDGFRα and β respectively. PDGFRβ was phosphorylated in gonocytes at tyrosine 751, whereas PDGFRα phosphorylation was visible using an antibody against phospho-tyrosine 754. However, there was no apparent phosphorylation of PDGFRα at phospho-tyrosine 1018 (data not shown). Overall, phosphorylated forms of PDGFRα and β were detected in PND3 gonocytes.

Since there was no true dominant pathway from protein and gene expression screening, we examined the effects of a large panel of inhibitors on RA-induced Stra8 expression used as a differentiation marker, similarly to what was done with F9 cells. As shown in Figure 8A, three inhibitors significantly reduced RA-induced Stra8 expression in gonocytes. First, the SRC family inhibitor SU6656 induced a concentration-dependent inhibition of RA induction, reaching 50% inhibition. The involvement of the SRC family of kinases was also confirmed using a second inhibitor, Dasatinib (58), which significantly inhibited RA-induced STRA8 expression by 56% (p-value: 0.0087; data not shown). The inhibitor of JAK2, AG490, and that of STAT5, Pimozide, inhibited by 68% and 82% RA-induced Stra8 expression, respectively. Altogether, these results indicate that PDGFR, a member of the SRC family and JAK2/STAT5 pathways all participate in gonocyte differentiation by crosstalking with RA.

In an effort to identify which of the SRC proteins is involved in this process, we examined the phosphorylation levels of several of them in gonocytes *in vivo*, and treated *in vitro* with RA. SRC and its various family members (BLK, FGR, FYN, HCK, LCK, LYN, YES (59)) and their phosphorylated forms were all present in gonocytes in various levels, located at the plasma membrane for all except FYN, which appeared to be nuclear (data not shown). In the PND3 testis, FYN and LCK were most highly phosphorylated (Figure 8B). While SRC was similarly phosphorylated in control and RA-treated gonocytes (data not shown), a larger number of RA-treated gonocytes appeared to express FYN and phospho-FYN (Figure 8C). The protein showing the most difference between control and RA-treated gonocytes was LCK, which showed a high proportion of cells positive for the total protein in both conditions, but more phospho-LCK positive cells in RA-treated samples (Figure 8C). These data suggest the three proteins are active in gonocytes, but that the LCK activation pattern better correlates with differentiation.

2.5 Discussion

The goal of the current study was to identify molecules and signaling pathways interacting with retinoic acid to regulate cell differentiation in F9 mouse embryonal carcinoma cells, a surrogate model for embryonic stem cells, and neonatal testicular rat gonocytes. Here, we showed that in both cell types, RA-driven induction of differentiation required the activation of PDGFRs, whereas different downstream signaling cascades were activated as part of their differentiation process. Furthermore, we identified novel variant forms of PDGFRα in both cell types, that were up-regulated during cell differentiation.

Given that there is no cell line model to study gonocytes, and considering that they express a number of pluripotency genes similar to embryonic stem cells, we included in the study the F9 mouse embryonal carcinoma cells, a well accepted model to study cell differentiation induced by RA (17). Developmentally, F9 cells have been shown to correspond to embryonic day 6.5, a period at which the somatic-germline lineage specification has not yet occurred (19). Although F9 cells will, by default, always differentiate towards the somatic cell lineage upon RA induction, they also express genes representative of the germline lineage, such as the premeiotic germ line gene Stra8 (11, 18, 41, 42), which can be induced by RA treatment in these cells (19). Thus, F9 cells have retained the theoretical capability of differentiating towards a cell type more representative of a germ cell. In our studies, the differentiation markers used to represent the somatic cell lineage included Collagen IV and Laminin B1 (12, 42, 43), whereas germline lineage differentiation was assessed by measuring the expression of Stra8 (18).

We first confirmed some characteristics of differentiating F9 cells induced by RA, which included a change in morphology towards a primitive endoderm-like morphology (where cells become more flattened and sparse) and the induction of Collagen IV and Laminin B1 (11). We also confirmed PDGFR participation by inhibiting PDGFR activity using the tyrphostin compounds AG370 and AG1295. The finding that both inhibitors significantly decreased RA-induced Collagen IV and Laminin B1 gene expression in F9 cells indicated that PDGFR activation is involved in this process. Another common regulatory process between F9 cells and neonatal testicular gonocytes is the fact that both cell types proliferate in response to PDGFs, although through different ligand and receptor types. Indeed neonatal gonocytes proliferate in response to PDGF-BB and estradiol via PDGFRβ (29, 31, 60), whereas in the present study, we

found that F9 cells, which express full length PDGFRα but not β, proliferate in response to PDGF-AA but not PDGF-BB in a concentration-dependent manner. Coinciding with the ability of F9 cells to respond to PDGF-AA, we observed that RA induced a decrease in cMYC expression in F9 cells, in agreement with another study in F9 cells (61). cMYC is an oncogene known to be involved in both cellular growth regulation and cellular metabolism, as well as tumor progression and cellular transformation (62). Our finding that the RA-induced down-regulation of cMYC expression was further exacerbated by inhibiting PDGFR with AG370, and the fact that PDGF is known to regulate cMYC expression (45), suggests that cMYC expression is regulated by PDGF in proliferating F9 cells, and that RA decreases cMYC in order to prevent proliferation during RA-induced differentiation, as both processes cannot occur simultaneously. To our knowledge, this is the first study to report the necessary activation of PDGFR in RA-induced differentiation of F9 cells.

The observation that inhibition of PDGFR was leading to a downregulation in differentiation led us to question which PDGF-related downstream signaling pathways were activated during the differentiation process. In order to better understand which signaling pathways were involved in RA-induced F9 cell differentiation towards the somatic lineage, we used specific inhibitors for PI3K (Wortmannin), PLC inhibition (U73122), and MEK1/2 inhibition (U0126). Based on our results, the participation of PLCγ and PI3K in RA-induced F9 cell differentiation was ruled out. It is well established that the PI3K signaling pathway mainly regulates cell survival and proliferation, while the PLCγ signaling pathway preferentially regulates cell proliferation and motility (48, 63). Our results showing that PI3K inhibition did not alter the RA-induced differentiation of F9 cells differ from those of Bastien J et al., who reported that RA induced both an early activation and a later inhibition of AKT activity and that this early activation was induced by increased activity of PI3K (64). This difference could be due to the fact that we studied the effects of 72 hour treatments, whereas their maximal effects were seen after 48 hours treatments, and using a different inhibitor. The present study showed that the inhibition of MEK1/2 interfered with the RA-induced changes in the expression of Collagen IV and Laminin B1. The concomitant phosphorylation of MEK1/2 and ERK2 upon RA-treatment of F9 cells, as well as their dependence on PDGFR activation, were further confirmed, establishing a link between PDGFR pathway and these downstream molecules. The involvement of PDGF signaling in cell differentiation through the MEK/ERK-dependent pathway has previously been reported in

the induction of neural stem cell (NSC) differentiation to oligodendrocyte precursors by neurotrophin 3 (NT3) (65). This study also reported that the expression of PDGFRα was dramatically increased during NT3-induced differentiation of NSCs, and this increase could be suppressed by MEK specific inhibitor U0126 together with the inhibition of differentiation (65). These observations further indicated that the activation of ERK can regulate the expression of PDGFRα. In view of our data, we propose that PDGFR might be a downstream regulator of the MEK/ERK pathway in RA-induced differentiation of F9 cells toward the somatic lineage.

We have previously shown that F9 cells express both V1-PDGFRβ, a truncated form of PDGFRβ, and full length PDGFRα, and that their mRNAs are both up-regulated upon RA treatment (11). PDGFRα upregulation by RA during cell differentiation has been observed in other cell types (65). In the present study, we report the existence of variant forms of PDGFRα in F9 cells treated with RA, identified by immunoblot analysis in RA-treated cells. The identity of the immunoreactive bands as forms of PDGFRα was confirmed by two methods. First, we used a blocking peptide raised against the antibody and saw that the blocking peptide was able to prevent the interaction of the antibody with the PDGFRα bands on the immunoblot membranes (data not shown). Secondly, the identity of the 3 bands as being PDGFRα was confirmed by mass spectrometric analysis. Interestingly, RA upregulated in a time-dependent fashion the expression of a 45 kDa form of PDGFRα, but not the full length receptor. Moreover, this effect was dependent of the activation of PDGFR and MEK/ERK1/2 pathway, suggesting that the activation of another PDGFR such as V1-PDGFRβ, might drive the formation of the 45 kDa variant, or that the 45 kDa variant could exert a positive feedback on its own expression. It was initially thought that this short variant may be produced by the degradation of the full length or the 60 kDa forms, upon the RA-dependent activation of a protease, as it has been described in other cell systems (66). In order to determine whether this was the case, we treated the cells with an inhibitor for Cathepsin L, a protease that can be activated by PDGF or RA (54, 66). Our results showed that the presence of a Cathepsin L inhibitor did not prevent the RA-dependent formation of 45 kDa PDGFRα, suggesting that this variant PDGFRα is not a proteolytic cleavage product, although one cannot exclude that it might be produced by the action of another protease.

Although the significant increase of a 45kDa variant form of PDGFRα in F9 cells upon RA treatment is a novel finding, the presence of PDGFRα in F9 cells has previously been reported.

Mercola M et al have previously shown that undifferentiated F9 cells do not express PDGFRs in the absence of RA, but upon RA stimulation, there is increased expression of both PDGFRα and PDGFRβ (67). In our studies, we cultured the cells under a constant basal level of RA present in the fetal bovine serum, and thus, even in control conditions, there was expression of a small amount of full length and 60 kDa PDGFRα. Studies have also shown that in F9 cells, RARβ-/- cells do not allow for growth arrest, even when the cells have been treated with RA. Furthermore, $RAR\beta_2$ regulates both cMYC and PDGFRα, amongst other target proteins activated by RA (61). Also, it has been previously shown that RA functions at the transcriptional level to promote PDGFRα gene expression in F9 cells (68). In this same study, a variant form of PDGFRα at approximately 60kDa was observed, that was also decreased upon stimulation with RA. However, these cells were not only treated with RA, but they were treated with RA alongside cAMP and theophylline (68). Here, we found that MEK1/2 inhibition prevented the RA-dependent expression of the somatic markers collagen IV and laminin B1. Taken together with studies reporting that Ras/ERK activation prevents parietal endoderm formation from primitive endoderm in differentiating F9 cells (69), these results suggest that the crosstalk observed between RA, PDGFR, and MEK/ERK activation might be related to the differentiation of F9 cells toward primitive endoderm rather than parietal endoderm.

Although F9 cells, by default, will differentiate towards the somatic cell lineage, they do have the capability to express genes that are representative of the germ line lineage as well. One such example is the commonly used marker for germ cell differentiation, STRA8. We found that, similarly to what is seen for Collagen IV and Laminin B1 gene expression, F9 cells treated with RA also present a significant increase in Stra8 gene expression. This response was also reduced in the presence of a PDGFR inhibitor. This suggests that, similar to differentiation towards the somatic lineage, the RA-driven expression of germline marker also involves PDGFR activation. However, it is possible that different PDGFRs are involved in these two processes, a question that cannot be answered at present. We then questioned which downstream pathway could be involved in the crosstalk between RA and PDGFR leading to Stra8 expression, using a panel of signaling inhibitors. SU6656 is a commonly used inhibitor for the SRC family of kinases (70). JAK2 is inhibited by AG490 (71) and Stattic can be used to inhibit STAT3 activity (72). STAT5 activation is inhibited by Pimozide (73) and RAS can be inhibited by Farnesylthiosalicyclic Acid

(FTS) (74). As previously described, MEK1/2 can be inhibited by U0126 (51), PLC activation is inhibited by U73122 (49), and PI3K can be inhibited by Wortmannin (50). Finally, in order to inhibit RAC1 activation, a compound known as NSC23766 was used (75). Interestingly, none of these inhibitors affected RA-induced Stra8 expression in F9 cells. However, our results showed opposite effects of MEK1/2 inhibition on the somatic markers and Stra8, with MEK1/2 inhibition significantly reducing RA-induced Collagen IV and Laminin B1 gene expression, while increasing the RA-induction of Stra8 expression. These results imply that MEK1/2 may act as a switch, and depending on whether it is activated or inhibited, there would be RA-induced differentiation towards the somatic- or germline-lineage. The fact that RA induces a significant increase in MEK1/2 and ERK2 phosphorylation upon RA stimulation in F9 cells is in agreement with these cells by default undergoing differentiation towards the somatic lineage.

Although our study is the first to report MEK1/2 as a potential switch in determining F9 cell differentiation lineage fate, MEK1/2 and its possible role in determining cell lineage and acting as a 'switch' has also been seen in the CD4/CD8 lineage commitment of thymocytes where, alongside calcineurin inhibition, MEK1/2 inhibition will help determine whether these thymocytes undergo CD4 lineage commitment or CD8 lineage commitment (76).

Furthermore, concurrent with the increase in STRA8 expression in RA-treated gonocytes, we observed a significant increase in PDGFRα expression in RA-treated gonocytes. Interestingly, targeting either the 5' or 3' ends of the PDGFRα sequence in qPCR analysis of control and RA-treated gonocytes revealed differences in how each domain of the sequence was affected by RA, with only the 3' of PDGFRα being significantly upregulated in gonocytes treated with RA (compared to untreated cells). Furthermore, immunoblot analysis revealed very little, if any, expression of the full length PDGFRα in PND3 gonocytes. It should be noted however, that we previously detected a faint band of the protein using a different antibody (29). Instead, gonocytes expressed a 65kDa variant form that was significantly upregulated upon RA treatment. Similarly to F9 cells, treating gonocytes with an inhibitor of cathepsin L, a protease produced by adjacent Sertoli cells (77), did not affect the formation of the variant protein in RA-treated gonocytes. The presence of a variant PDGFRα form in gonocytes was quite striking for two main reasons. First, it was not surprising because northern blot analysis of whole rat testes at PND3 had revealed the presence of three variant transcripts besides the full length PDGFRα.

Moreover, all forms were more abundantly expressed at PND3 compared to fetal and prepubertal ages, again confirming its likely important role in a time- and stage-specific manner. Regarding the full length PDGFRα, it is known that its main sources in the testis are the Leydig, myoid, and Sertoli cells (78). Also, the presence of a variant form of PDGFRα in gonocytes is of interest because a 1.5kb variant PDGFRα mRNA has been characterized in human seminoma patients (26, 27). Thus, if both testicular tumors and gonocytes, their supposed cell of origin, contain a variant PDGFRα, it is possible that the retention of such a variant form might be linked to the process of testicular tumor formation. Given that there was a significant increase in PDGFRα targeted to the 3' end of the sequence in RA-treated gonocytes, in order to better analyze the exact location of this variant, we performed a thorough quantitative analysis of PDGFRα exon profiles between control and RA-treated gonocytes. This approach allowed us to examine how PDGFRα expression was altered along the whole sequence, which should show differential profiles if a variant transcript was formed/increased in response to RA. We analyzed 41 overlapping, gene specific primer sets for qPCR analysis that all amplified products sizes no larger than 125bp. Here, we found that throughout the gene, there were areas that showed similar expression between untreated and RA-treated gonocytes. When grouped together, this analysis indicated that there was no significant change in the levels of PDGFRα sequences ranging from exons 1 to 6, and that the expression of PDGFRα in exons 7-11 was significantly decreased in gonocytes treated with RA, whereas the expression of the sequences comprised between exons 12-23 was significantly upregulated by RA. Taken together these data suggest that the variant PDGFRα form in gonocytes does not contain areas of the gene in exons 7-11 but does contain exons 12-23, thus confirming its presence at the 3' end of the sequence.

This study is the first to report the presence of a PDGFRα variant in PND3 rat gonocytes. However, the similarities between the 65kDa PDGFRα variant seen in PND3 gonocytes and the 45kDa and 60kDa variants seen in F9 cells still remains to be determined. Basciani S et al have previously performed northern blot analysis of PDGFRα gene expression in human fetal and adult testes where they did not detect any PDGFRα variant expression (79). This is not surprising, at least in the adult, as a PDGFRα variant seen in human seminoma tumors is specific only to tumors, making it a valid diagnostic tool for great clinical use (27). It is known that specific promoter haplotype combinations of PDGFRα can affect human neural tube defects (80). Furthermore, studies have shown that mutations in PDGFRα are commonly seen in

gastrointestinal stromal tumors (81, 82). Although variant forms of PDGFRα are not commonly seen, truncated forms of other tyrosine kinases, including KIT (tr-KIT) and SRC family member HCK, have been reported in the testis and are known to be involved in spermatogenesis (83).

Given that gonocyte differentiation is induced via all trans-RA, we wanted to determine the relative abundance of the various isoforms of the receptors in PND3 gonocytes. Using gene array and qPCR analysis, we found that both RARα and RARγ are abundantly expressed in PND3 gonocytes. RXRα and RXRβ were also expressed abundantly. However, since *in vitro* differentiation of gonocytes is induced by all-trans RA (11) which is only able to bind to RARs and not RXRs (85), RXRs are probably not directly involved in gonocyte differentiation. The finding that RARα and RARγ are most abundant in PND3 gonocytes adds to the literature of already conflicted findings on the topic. Boulogne B et al have reported that in germ cells at PND3, both RARα and RXRα are expressed (55), whereas Vernet N et al have reported the lack of RARα expression in gonocytes (56). One likely reason for this discrepancy is due to the different antibodies that were used. Here, we report that, at the mRNA level, both RARα and RARγ are expressed in PND3 gonocytes. To determine whether RARα was playing a role in RA-induced differentiation, we used BMS195614, a specific RARα inhibitor (57), and found that co-treatment with BMS195614 significantly reduced RA-induced STRA8 expression. This indicates that RA-induced gonocyte differentiation is in part due to RARα activation. Taken together with the results presented above, these findings suggests that both a variant form of PDGFRα and RARα likely play a role in gonocyte differentiation via a crosstalk that remains to be determined.

RA crosstalk with other signaling pathways is an idea worth considering. Studies originally done in F9 cells and embryonic stem (ES) cells using high-throughput methods have revealed that RA can activate over 300 genes that have, in some way, been correlated to differentiation (20). However, a large portion of these genes are actually targeted independently of RA-induced RARs binding to their response elements (i.e.: the classical pathway of RAR activation). Instead, RA can indirectly target other signaling pathways via crosstalk to induce a response. Such a process has been observed in the crosstalk between FGF and RA, where there is an initial rapid RA-induced induction of FGF8 (which contains RAREs), followed by a long term FGF4 repression, which results in ES cell neuronal differentiation (20, 85). Although FGF8 contains RAREs, FGF4 does not and thus, the repression seen in FGF4 is indirect and has been proposed

to likely be due to upstream repression of transcription factor OCT-4 (20, 86). Furthermore, although RA can also exert downstream effects without RAR activation (87), in RA-induced gonocyte differentiation, RARs are likely involved in view of our finding that inhibition of RARα resulted in a reduction of RA-induced differentiation, although the RAREs may not be involved since RA induces the indirect activation of PDGFRα.

To continue, at the protein level, we found that *in vivo*, a significant amount of PDGFR phosphorylation occurred at the gonocyte plasma membrane in PND3 testes, especially at tyrosines 849 and 754 for PDGFRα and tyrosines 857 and 751 for PDGFRβ. At tyrosine 754, it is SHP-2 that is interacting with PDGFRα whereas at tyrosine 1018, it is PLCγ (48). It is not surprising that tyrosine 1018 was not phosphorylated, as we have already shown that PLCγ is not involved in gonocyte proliferation (31, 48), and the present work showed that it is not involved in RA-induced gonocyte differentiation either. At tyrosine 751, it is PI3K that is binding to PDGFRβ (48). This phosphorylation status indicates that PI3K plays a role not yet determined in PND3 gonocytes. Our phosphorylation data also further stresses that neonatal gonocytes are not a synchronous population (1), since there were gonocytes within the same testis/cords that were differentially phosphorylated. Furthermore, the fact that some gonocytes presented phosphorylated PDGFRs at PND3 adds to the growing evidence that PDGFR activation is necessary, not only for gonocyte differentiation in particular, but for gonocyte development in general.

Moving forward, we found that gonocytes at PND3 strongly express SRC and its various family members, mainly at the plasma membrane. In our analysis, Fyn protein expression appeared to be nuclear. However, even with nuclear FYN expression, the phosphorylation of FYN expression was only seen at the plasma membrane, indicating that likely, only FYN located at the plasma membrane is phosphorylated, or, that once activated, FYN translocates to the plasma membrane. It has previously been shown that the various members of the SRC family of kinases are present in the adult mouse testis and that an increase in protein tyrosine phosphorylation is seen in sperm capacitation (88). Goupil S et al characterized the expression levels of various SRC family kinases using RT-PCR and found that these kinases, although members of the same family, have distinct patterns of expression, and thus, likely, each play a specific role in sperm integrity. In our analysis it was apparent that FYN and LCK were the most

abundantly and specifically expressed in gonocytes, thus likely playing a role in gonocyte development.

In order to determine which downstream pathways were activated during RA-induced gonocyte differentiation, we treated isolated gonocytes with RA alongside a variety of different inhibitors, similar to the approach used in F9 cells. In brief, we found that inhibition of the SRC family of kinases using SRC family inhibitors SU6656 and Dasatinib, JAK2 using AG490, and STAT5 using Pimozide significantly reduced RA-induced gonocyte differentiation. Dasatinib is a second generation receptor tyrosine kinase inhibitor that is used in the treatment of chronic myeloid leukemia (89). These two inhibitors are commonly used for SRC family inhibition, when there is a need to determine the role of SRC and its family members in PDGF-related signaling pathways. SU6656 is especially used for this type of signaling determination because other known inhibitors commonly inhibit tyrosine phosphorylation in both PDGFR and SRC kinases, whereas SU6656 has very little effect on PDGFR activation (71). Furthermore, AG490 is a commonly used inhibitor for JAK2 activity and prevents JAK2 phosphorylation. JAKs are known to function upstream of the STAT proteins. Here, we found that STAT5 was the likely candidate activated by JAK2. Pimozide is a known psychotropic drug that is commonly used for STAT5 phosphorylation inhibition as it does not inhibit other tyrosine kinases and is specific for STAT5 (74). Taken together, our data suggests that these three downstream pathways are likely involved in this differentiation process.

It is not surprising to find that there is more than one downstream pathway activated during RA-induced gonocyte differentiation, due to the common overlap seen amongst these pathways and the fact that the majority of the elements downstream of PDGFR can interact with each other. For example, although it is commonly known that JAK proteins activate STAT proteins, studies using megakaryocyte progenitors and stem cell factor have shown that both JAK2 and SRC can mediate phosphorylation of the STAT5 tyrosine and that synergistic activation of both JAK2 and SRC may be responsible for increased STAT5 signalling (90). The finding that SRC can also activate STAT5 was also reported in F-36P human leukemia cells where it was found that SRC contributed to growth hormone receptor-mediated signal transduction via STAT5 activation (91). Thus, both JAK2 and SRC have the capability of activating STAT5. However,

which of these factors, or if both, are responsible for STAT5 activation during RA-induced gonocyte differentiation remains to be determined.

Given the complexity by which cellular differentiation occurs, it is likely that the PDGFR pathway is not the only pathway activated during this process. However, the presence of many of its downstream elements in abundant amounts at both the mRNA and protein levels suggests that, even if it is not the only pathway involved in RA-induced gonocyte differentiation, it at least plays an important part in this process.

All in all, our study is the first to compare the signaling mechanisms involved in RA-induced differentiation of PND3 gonocytes and F9 mouse embryonal carcinoma cells. Here, we report that variant PDGFRα (T1-PDGFRα) are expressed in both F9 cells and PND3 gonocytes, and our data strongly suggests that these variants are involved in RA-induced differentiation. Finally, we found that while both cell types require PDGFR activation for RA-induced differentiation, F9 cells require the downstream activation of MEK1/2 for differentiation towards the somatic-lineage, whereas MEK1/2 inhibition promotes the expression of a germline-lineage marker. On the other hand, gonocyte differentiation requires further activation of the SRC family of kinases, JAK2, and STAT5 signaling pathways (Diagram 1).

Overall, a better understanding of the signaling mechanisms involved in gonocyte differentiation should help to provide a more in depth understanding of how SSCs are formed and how the disruption of gonocyte differentiation may lead to testicular tumor formation. Proteins such as the specific variant forms (V1-PDGFRβ and T1-PDGFRα) provide new potential targets that could be used for better targeted drug development to be used in the treatment of male reproductive pathologies, such as TGCTs. Moreover, the comparative study of RA effects on F9 embryonal carcinoma cells and neonatal gonocyte differentiation unveiled striking similarities and differences in their downstream pathways that might help better understand mechanisms that are critical for ES cell and gonocyte differentiation, respectively.

2.6 Acknowledgements

We would like to thank Dr. Jaroslav Novak for assistance with microarray data analysis and biostatistics.

2.7 Figures

Figure 2.1: PDGFR inhibitors block RA-induced differentiation of F9 cells

F9 cells were treated for 72 hours with 0.1μM RA, 50μM AG370, 10μM AG1295 or the different combinations indicated. (A) Morphological changes of F9 cells following the various treatments. (B, C) mRNA levels of two differentiation markers of somatic lineage differentiation (Collagen IV and Laminin B1) were quantified using qPCR analysis for F9 cells treated with or without RA, AG370, and AG1295. (D) mRNA levels of cMYC were quantified for F9 cells treated with or without RA and AG370. (E) Levels of F9 cell proliferation in response to varying concentrations of PDGF-AA and PDGF-BB were determined using a BrdU ELISA proliferation assay. Results shown represent the mean ± SEM of at least 3 independent experiments for each condition (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

Figure 2.1: PDGFR inhibitors block RA-induced differentiation of F9 cells

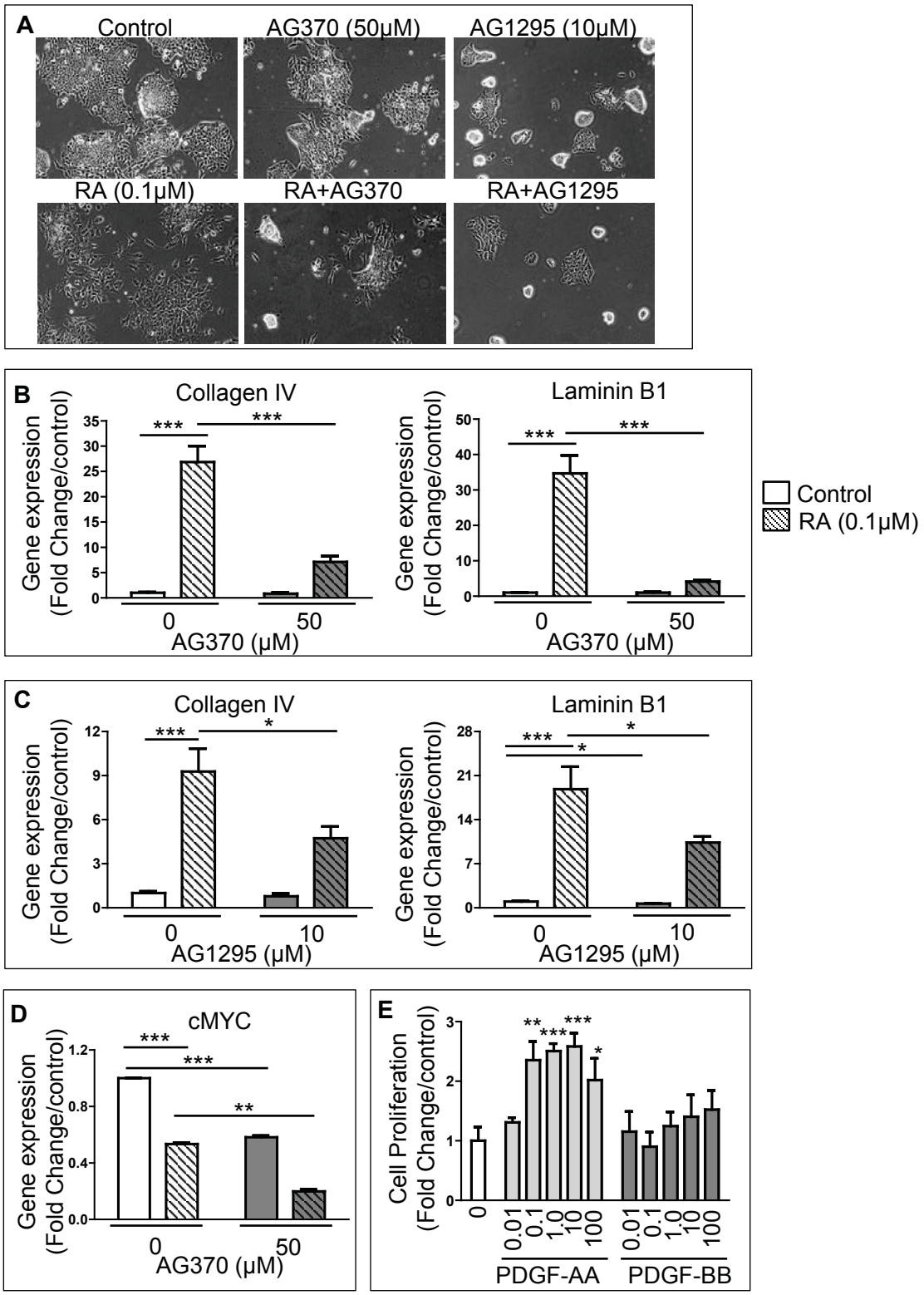


Figure 2.2: Effect of PLCγ inhibition (U73122), PI3K inhibition (Wortmannin), and MEK1/2 inhibition (U0126) on RA-induced differentiation of F9 cells

F9 cells were treated for 72 hours with 0.1μM RA, 1μM U73122, 1μM Wortmannin, 10μM U0126 or the different combinations indicated. (A) Morphological changes of F9 cells following the various treatments. (B, C, D) mRNA levels of two differentiation markers of somatic lineage differentiation (Collagen IV and Laminin B1) were quantified using qPCR analysis. Results shown represent the mean ± SEM of at least 3 independent experiments for each condition (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

Figure 2.2: <u>Effect of PLCγ inhibition (U73122), PI3K inhibition (Wortmannin), and MEK1/2 inhibition (U0126) on RA-induced differentiation of F9 cells.</u>

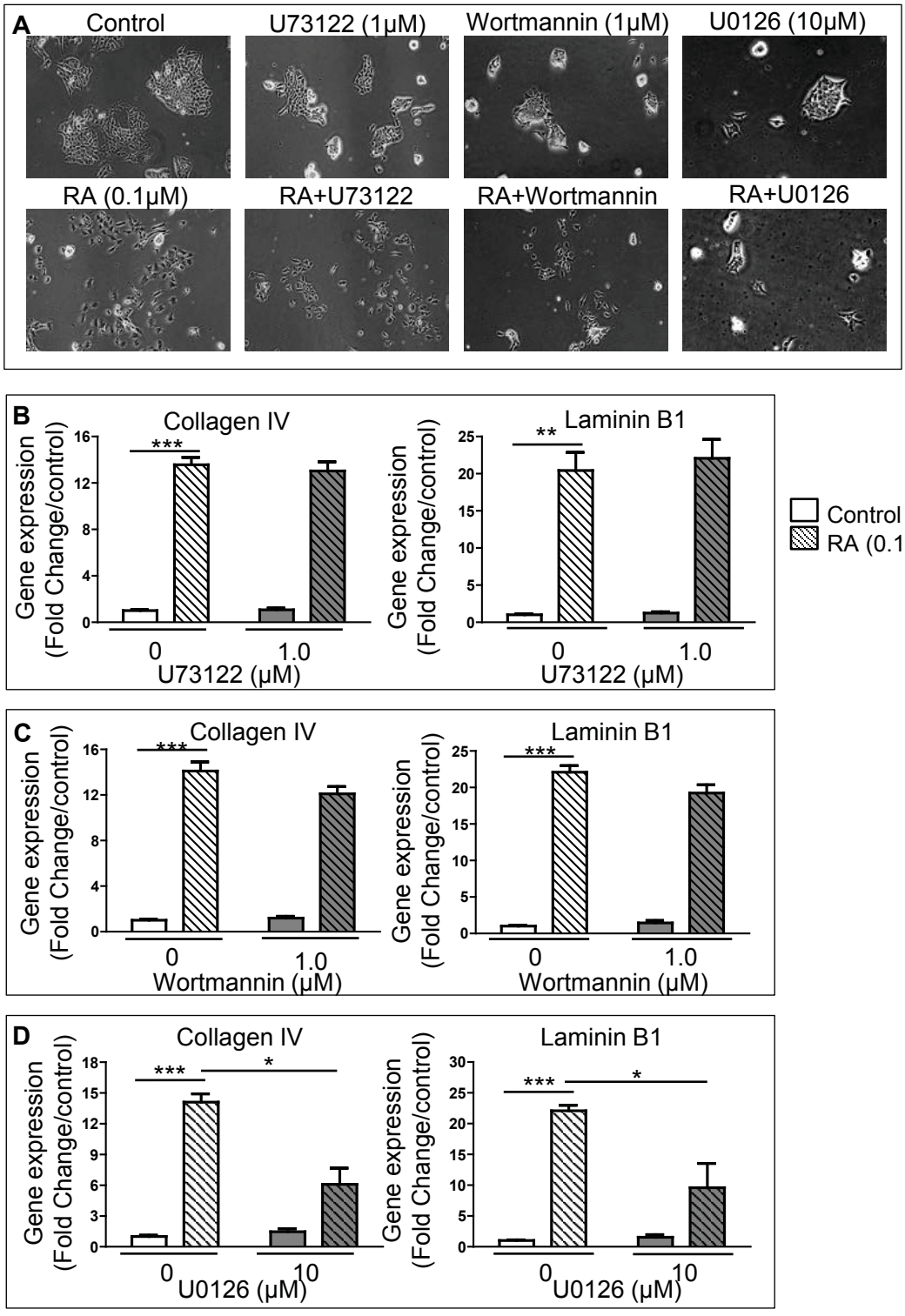


Figure 2.3: <u>RA-induced somatic differentiation in F9 cells requires MEK/ERK activation</u>

F9 cells were treated for 72 hours with 0.1μM RA, 50μM AG370, 10μM U0126, and 250μM cAMP alone or in the different combinations indicated. The proteins were examined by immunoblot analysis, and signal intensity analysis of immunoreactive bands was quantified by densitometry and the results were normalized against GAPDH or tubulin levels (loading control). (A) Protein level confirmation for results obtained at the mRNA level for F9 cells treated with or without RA and U0126 and their affect on Laminin B1 expression. (B) Immunoblot and quantitative analysis of MEK1/2 phosphorylation levels relative to total MEK1 expression in F9 cells treated with or without RA. (C) Immunoblot and quantitative analysis of ERK1/2 phosphorylation levels relative to total ERK1/2 expression in F9 cells treated with or without RA and cAMP. (D) Immunoblot and quantitative analysis of ERK1/2 phosphorylation levels relative to total ERK1/2 expression in F9 cells treated with or without RA, AG370, and U0126. The results shown represent the mean ± SEM of at least 3 independent experiments for each condition (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

Figure 2.3: <u>RA-induced somatic differentiation in F9 cells requires MEK/ERK activation</u>

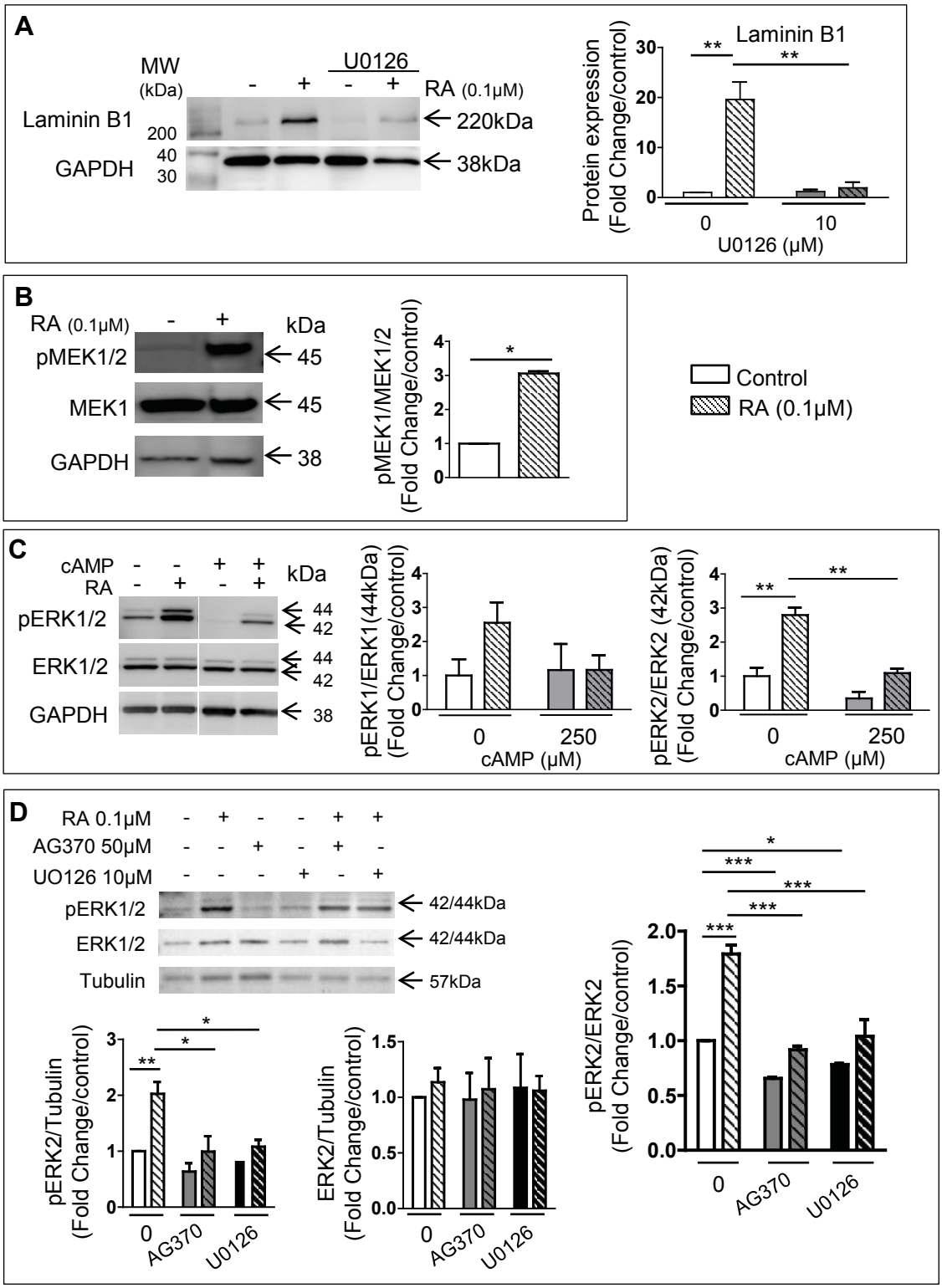


Figure 2.4: <u>RA induces the formation of truncated forms of PDGFRα in differentiating F9 cells which are dependent on PDGFR and MEK1/2 activation</u>

F9 cells were treated for 72 hours with or without RA and AG370. (A) Representative immunoblot shown of results obtained for PDGFRα expression in RA-treated F9 cells with or without AG370 treatment. (B) Signal density analysis of the immunoblot in (A) was performed. Results were normalized to GAPDH (loading control). (C) Immunoblot analysis of F9 cells treated for 72 hours with or without RA and Cathepsin L inhibitor. Representative blot shown. (D, E) Representative immunoblot shown alongside signal density analysis of the 45kDa variant PDGFRα expression in F9 cells treated with or without RA and the MEK1/2 inhibitor U0126. Results were normalized to GAPDH (loading control). Results shown represent the mean ± SEM of at least 3 independent experiments for each condition (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

Figure 2.4: <u>RA induces the formation of truncated forms of PDGFRα in differentiating F9 cell which are dependent on PDGFR and MEK1/2 activation</u>

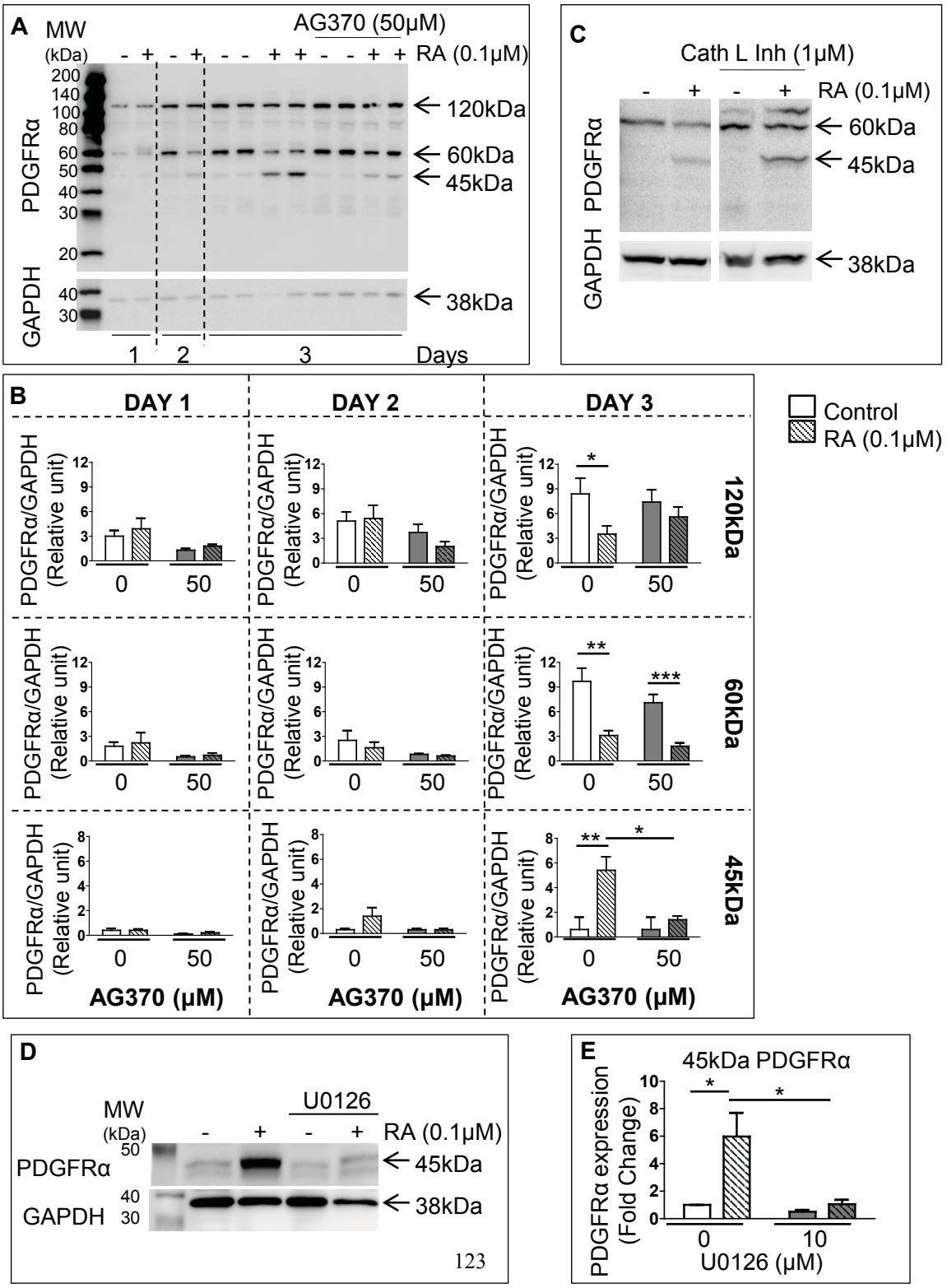
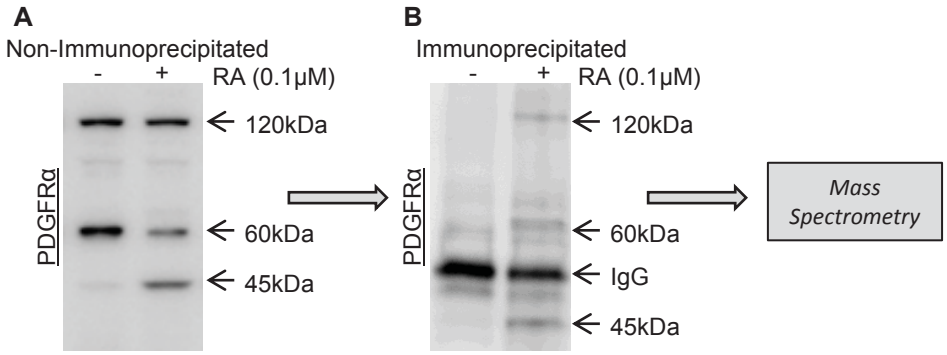


Figure 2.5: Mass spectrometry analysis of variant PDGFRα expression in RA-induced differentiation of F9 cells

F9 cells were treated for 72 hours with or without 0.1μM RA. (A) Immunoblot analysis of PDGFRα in F9 cells treated with or without RA, non-immunoprecipitated. (B) Immunoblot analysis of PDGFRα in F9 cells treated with or without RA, immunoprecipitated. (C) Mass spectrometry analysis of immunoprecipitated bands of interest and the position of the identified peptides in the overall PDGFRα sequence. Fragments highlighted in red were identified in the 120kDa band. Fragments enclosed by boxes were identified in the 45kDa band. Fragment highlighted in the solid black lined box was identified in the 60kDa band. (Trypsin cleavage sites indicated by amino acids in bold).



C Identification of various PDGFRα peptides

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1 MGTSHQVFLV LSCLLTGPGL ISCQLLLPSI LPNENEKIVQ LNSSFSLRCV GESEVSWQHP 60
  MSEEDDPNVE IRSEENNSGL FVTVLEVVNA SAAHTGWYTC YYNHTQTDES EIEGRHIYIY 120
  VPDPDMAFVP LGMTDSLVIV EEDDSAIIPC RTTDPETQVT LHNNGRLVPA SYDSRQGFNG 180
  TFSVGPYICE ATVKGRTFKT SEFNVYALKA TSELNLEMDA RQTVYKAGET IVVTCAVFNN 240
  EVVDLQWTYP GEVRNKGITM LEEIKLPSIK LVYTLTVPKA TVKDSGEYEC AARQATKEVK 300
  EMKRVTISVH EKGFVEIEPT FGQLEAVNLH EVREFVVEVQ AYPTPRISWL KDNLTLIENL 360
  TEITTDVQKS QETRYQSKLK LIRAKEEDSG HYTIIVQNED DVKSYTFELS TLVPASILDL 420
  VDDHHGSGGG QTVRCTAEGT PLPEIDWMIC KHIKKCNNDT SWTVLASNVS NIITELPRRG 480
  RSTVEGRVSF AKVEETIAVR CLAKNNLSVV ARELKLVAPT LRSELTVAAA VLVLLVIVIV 540
  SLIVLVVIWK QKPRYEIRWR VIESISPDGH EYIYVDPMQL PYDSRWEFPR DGLVLGRILG 600
  SGAFGKVVEG TAYGLSRSQP VMKVAVKMLK PTARSSEKQA LMSELKIMTH LGPHLNIVNL 660
  LGACTKSGPI YIITEYCFYG DLVNYLHKNR DSFMSQHPEK PKKDLDIFGL NPADESTRSY 720
  VILSFENNGD YMDMKQADTT QYVPMLERKE VSKYSDIQRS LYDRPASYKK RSMLDSEVKN 780
  LLSDDDSEGL TLLDLLSFTY QVARGMEFLA SKNCVHRDLA ARNVLLAQGK IVKICDFGLA 840
  RDIMHDSNYV SKGSTFLPVK WMAPESIFDN LYTTLSDVWS YGILLWEIFS LGGTPYPGMM 900
  VDSTFYNKIK SGYRMAKPDH ATSEVYEIMV QCWNSEPEKR PSFYHLSEIV ENLLPGQYKK 960
  SYEKIHLDFL KSDHPAVARM RVDSDNAYIG VTYKNEEDKL KDWEGGLDEQ RLSADSGYII 1020
  PLPDIDPVPE EEDLGKRNRH SSQTSEESAI ETGSSSSTFI KREDETIEDI DMMDDIGIDS 1080
  SDLVEDSFL
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Figure 2.6: <u>RA crosstalk with several PDGFR-related signaling pathways in differentiating F9 cells</u>

F9 cells were treated for 72 hours with or without 0.1μM RA and a variety of different inhibitors. (A) mRNA levels of STRA8 gene expression in F9 cells treated with or without RA and AG370. (B) mRNA levels of STRA8 gene expression in F9 cells treated with or without RA and MEK1/2 inhibitor U0126. (C) mRNA levels of STRA8 gene expression in F9 cells treated with or without RA and RAS inhibitor FTS. Results shown represent the mean ± SEM of at least 3 independent experiments for each condition (* $p<0.05$; ** $p<0.01$).

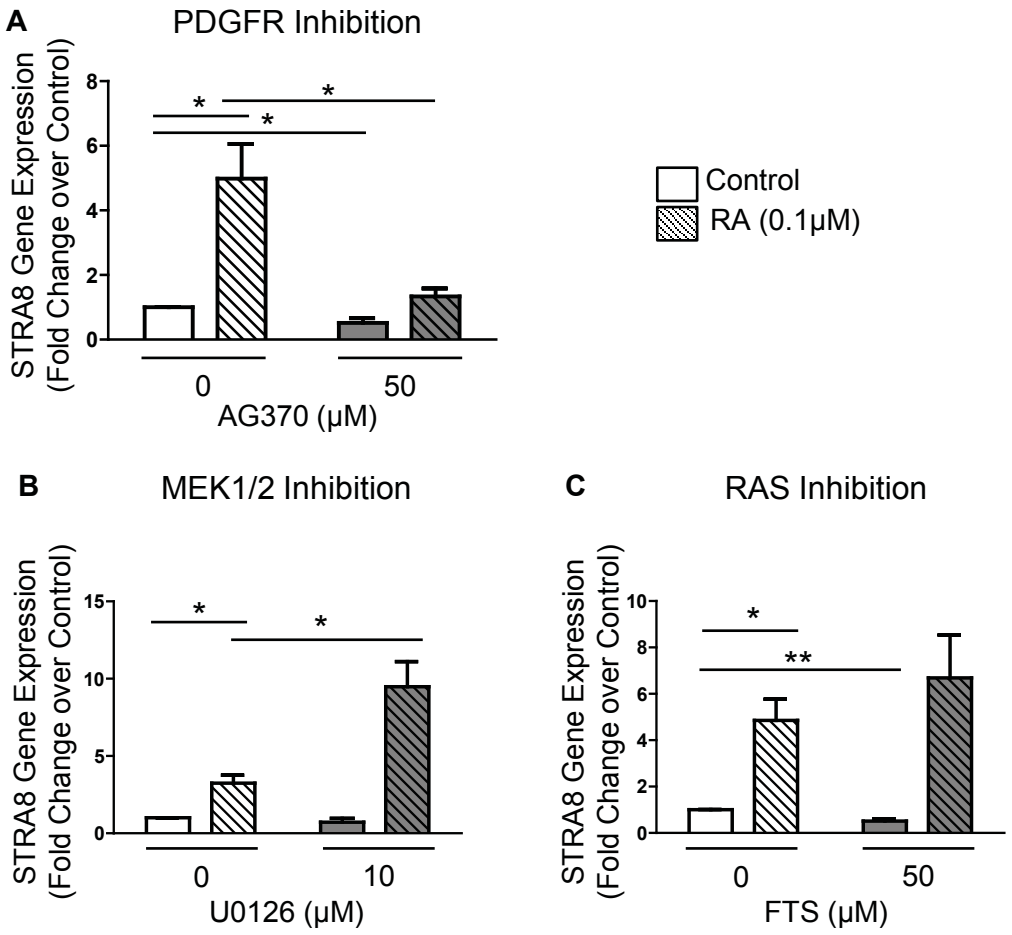


Figure 2.7: <u>PDGFRα expression in PND3 gonocytes</u>

(A) Northern blot analysis of PDGFRα expression in GD18, PND3, and PND21 whole rat testes. GAPDH used as loading control. (B) mRNA levels of PDGFRα in PND3 gonocytes (G3) compared to levels in PND8 spermatogonia (G8) using primers specific to the 3' end of the PDGFRα sequence. (C) mRNA levels of PDGFRα in gonocytes treated with RA in a concentration-dependent manner. (D) mRNA levels of STRA8 gene expression in gonocytes treated for 24 hours with or without RA and AG370. (E) Immunoblot and signal density analysis of PDGFRα expression of gonocytes treated with RA for 72 hours. (F) mRNA levels of PDGFRα in gonocytes treated with or without RA, specifically aimed at the 5' and 3' end of the PDGFRα sequence. (G) mRNA levels of PDGFRα in gonocytes treated with or without RA. Expression grouped by exon specific areas of the PDGFRα sequence. qPCR analysis was performed for each exon separately, and the data further pooled to cover 3 main areas, the 5' end, central exons, and 3' end of the sequence. Results shown represent the mean ± SEM of at least 3 independent experiments for each condition (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

Figure 2.7: PDGFRα expression in PND3 gonocytes

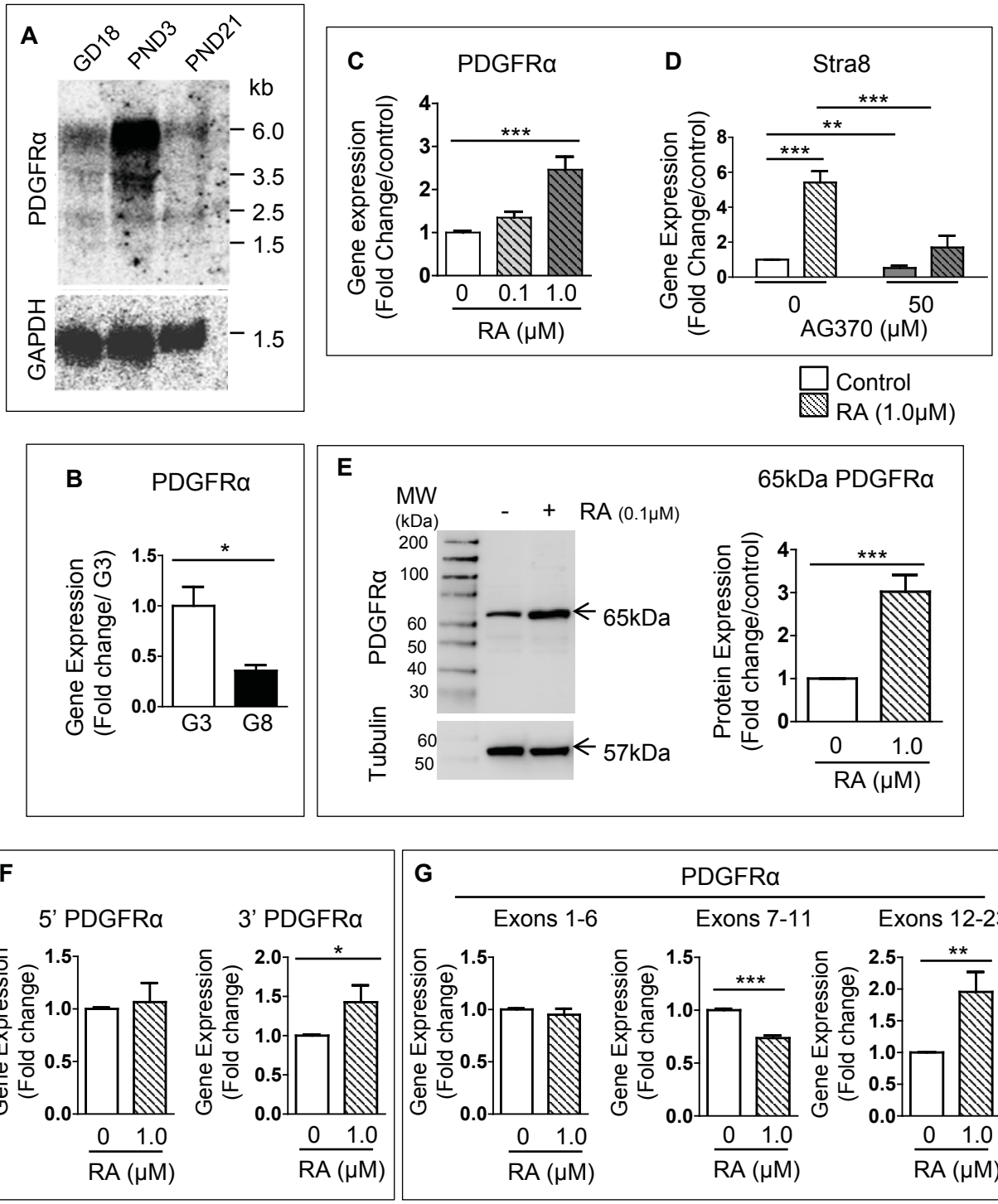


Figure 2.8: <u>Possible downstream pathways implicated in RA-induced gonocyte differentiation</u>

(A) Gonocytes were treated for 24 hours with or without 1.0μM RA and inhibitors SU6656 (SRC family of kinases), AG490 (JAK2), or Pimozide (STAT5). mRNA levels of STRA8 gene expression are shown. Results shown represent the mean ± SEM of at least 3 independent experiments for each condition (* $p<0.05$; ** $p<0.01$; *** $p<0.001$). (B-C) Immunohistochemical/immunocytochemical analysis of protein expression levels (native and phosphorylated forms) of key SRC kinase family members SRC, FYN, and LCK in (B) PND3 testes and (C) isolated PND3 gonocytes treated with or without RA. Representative images shown.

Figure 2.8 Possible downstream pathways implicated in RA-induced gonocyte differentiation

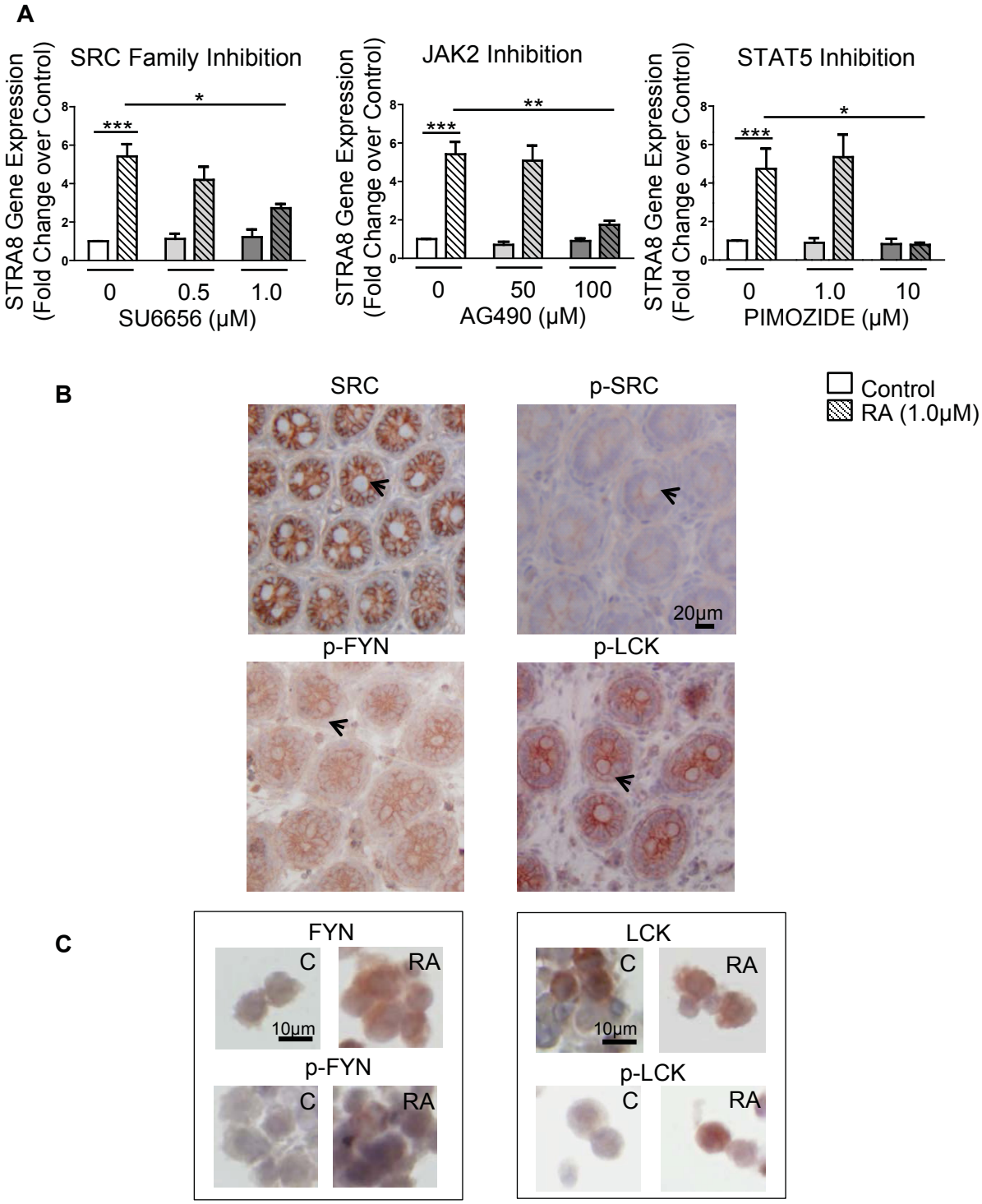
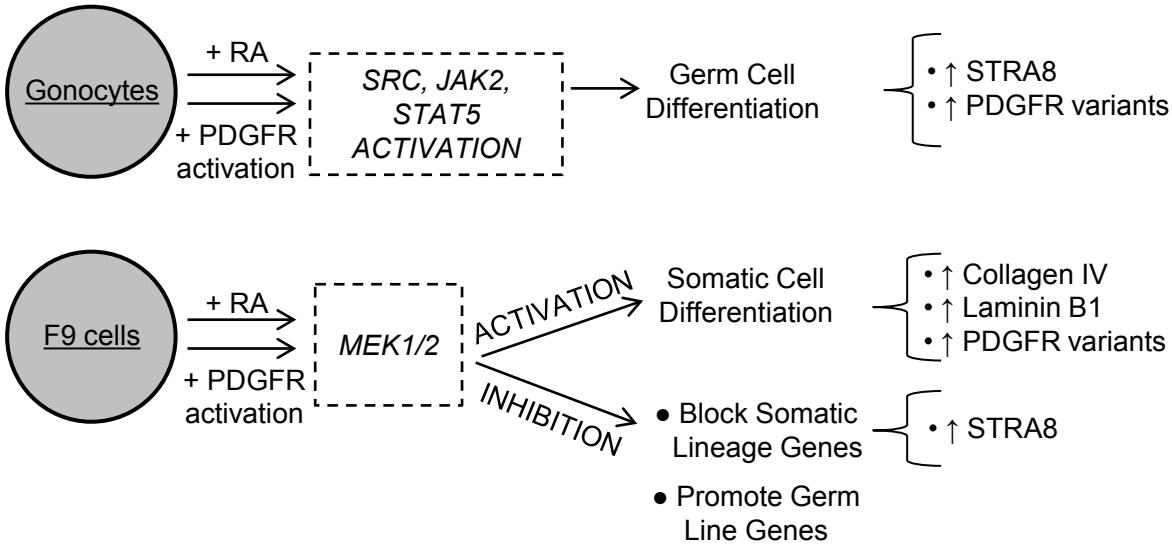


Diagram 2.1: <u>Summary diagram of the downstream pathways crosstalking with RA in the regulation of the differentiation of PND3 gonocytes and F9 embyonal carcinoma cells</u>



2.8 Tables

Table 2.1: (A) List of primers used for F9 mouse embryonal carcinoma cell qPCR analysis

Gene	Accession No.	Primer Sequence (F/R)	Start BP
cMYC	AH005318	CCTCAACGTGAACTTCACCAAC	2544
		ACGGAGTCGTAGTCGAGGTCAT	2594
COLLAGEN IV	NM_009931	ACTAACACACCCCGCTTCGT	5402
		CCTGTCCTTCTCCTTCAGCAAA	5452
LAMININ B1	NM_008482	CATGCTGATCGAGCGGTCT	712
		CTGTACACGCCCCAAGCCTTC	762
STRA8	NM_009292	cggcCCTCCTCCACTCTGTTGCCG	703
		TGCTGTTCATGGTCAGGAGGTC	792
18S	NR_003278.3	TCCCAGTAAGTGCGGGTCAT	1643
		CCAATCGGTAGTAGCGACGG	1723

(B) List of primers used for PND3 gonocytes and PND8 spermatogonia

Gene	Accession No.	Primer Sequence (F/R)	Start BP
PDGFRα	NM_012802	GAGATCGAAGGCAGGCACATTT	461
(5' end)		cggcGACTAAAGAATCCGTCATGCCG	544
PDGFRα	NM_012802	cgggtAGTGCTGGAACAGTGAACCCG	2922
(3' end)		CCAGGCAGCAGATTCTCCACT	2997
RARα	NM_031528	TTGGAATGGCTCAAACCAC	384
		AGGGCTGGGCACTATCTCTT	450
RARβ	NM_031529	GGGCAGATCCTGGATTTCTA	576
		CTGGTACTCTGTGTCTCGATGG	718
RARγ (V1)	NM_001135249	GCTATCTGCCTCATCTGTGGA	1486
		CAGCTTGTCCACCTTCTCG	1548
RARγ (V2)	NM_001135250	CCCTGACCTACCCAACTGTG	1633
		GCGGTATCTGGGAAATGGT	1696
RXRα	NM_012805	ACATGCAGATGGACAAGACG	1091
		GGGTTTGAGAGCCCCTTAGA	1172
RXRβ	NM_206849	GTTCTTCCATGGGGTCTCCT	365
		GGAGCGACACTGTGGAGTTAAT	460
RXRγ	NM_031765	GGGCATGAAGAGGGAAGC	836
		CTGGCACATTCTGCCTCAC	913
STRA8	XM_006236282	TGCTTTTGATGTGGCGAGCT	3305
		GCGCTGATGTTAGACAGACGCT	3367
18S	X01117	cgggTGCTCTTAGCTGAGTGTCCCG	768
		CTCGGGCCTGCTTTGAACAC	842

(C) List of primers used for Gonocyte 'walk-along' analysis

Set	Primer Sequence (F/R)	Start BP	Exon	Amplicon
1	TCCTCAGCTGTCTCCTCACA	159	2	98
	ACTCAGCGGCACAATCTTCT	256	3	
2	AGAAGATTGTGCCGCTGAGT	237	3	100
	ACGTTGGGGTCCTCTTCTTC	366	3	
3	CCCCATGTCTGAAGAAGAGG	307	3	84
	ACTTCCAGCACCGTGACAA	390	3	
4	GTGCTGGAAGTGGTCAACG	380	3	96
	CCTGCCTTCGATCTCACTCT	475	3	
5	CGGCATGACGGATTCTTTAG	523	4	80
	CAGTGTCTGGATCCGTGGT	602	4	
6	AGGGCTTCAACGGAACCTT	657	4	83
	AAACTCGCTGGTCTTGAACG	739	4	
7	ACGTTCAAGACCAGCGAGTT	719	4	106
	CGATCGTTTCTCCTGCCTTA	824	5	
8	AGAAACGATCGTGGTGACCT	814	5	99
	AGCATGGTGATGCCTTTGTT	912	6	
9	AACTCCCGTCCATCAAACTG	924	6	85
	CGGGCAGCACATTCATAATC	1008	6	
10	CGGTGAAGGACAGTGGAGAT	972	6	11
	TGATCTGGACGAAGCCTTTC	1082	7	
11	CCACGAAAGAGGTCAAGGAA	1014	6	106
	TGCAGGTTCACAGTTTCCAG	1119	7	
12	TTTGGCCATCTGGAAACTGT	1091	7	101
	AAGTTGTCCTTCAGCCAGGA	1191	7	
13	TCCTGGCTGAAGGACAACTT	1172	7	108
	CCGGATCAGCTTTAATTTGC	1279	8	
14	AGAGGAGCCAGGAGACGAG	1233	7	79
	ATGGTATAATGGCCGCTGTC	1311	8	
15	TCGAGTGGATGATCTGCAAG	1464	9	102
	GCTGGTGGAACTCCGTGATA	1565	10	
16	CAGCGAGGCAGGAGTACAGT	1562	10	97
	CAATGCCAAGGTCGTTCTTT	1658	10	
17	CAAAGAACGACCTTGGCATT	1638	10	106
	ACAATCACCAACAGCACCAG	1743	11	
18	CTGGTGCTGTTGGTGATTGT	1724	11	95
	TCAATGACCCTCCATCGAAT	1818	12	
19	AATTCGATGGAGGGTCATTG	1798	12	93
	TCCCATCTGGAGTCGTAAGG	1890	12	
20	AGCTGCCTTACGACTCCAGA	1866	12	101
	AGCTGTGCCTTCAACCACTT	1966	13	
21	TTTGGGAAAGTGGTTGAAGG	1940	13	103
	TCTCACTAGACCTGGCTGTGG	2042	14	
22	GTGGCTGTGAAGATGCTCAA	2000	13	119
	GCTCCCAGCAAATTCACAAT	2118	14	
23	ACCGCATCTGAACATTGTGA	2086	14	92
	CCAAATCCCCATAGAAGCAG	2177	15	
24	CTGCTTCTATGGGGATTTGG	2158	15	80
	TCTTTGGCTTCTCTGGGTGT	2237	15	
25	GACACCCAGAGAAGCCAAAG	2217	15	123
	CCTGCTTCATGTCCACGTAGT	2339	16	
26	GCTGGAAAGGAAGGAGGTTT	2365	16	125
	TCATCATCCGAAAGGAGGTT	2487	17	
27	AACCTCCTTTCGGATGATGA	2468	17	97
	TCGAAGCCAAAAATTCCATC	2564	17	
28	GGGATGGAATTTTTGGCTTC	2543	17	95
	TCACAGATCTTCACGATTTTCC	2637	18	
29	GCAGGGGAAAATCGTGAAG	2611	18	82
	GCTGCCCTTTGACACGTAG	2692	18	
30	GGCTGGCCAGAGACATCAT	2643	18	73
	ATCCACTTCACAGGCAGGAA	2715	19	
31	TTCCTGCCTGTGAAGTGGAT	2695	19	90

	GAGCAGAACGCCATAAGACC	2785	19	
32	GGTCTTATGGCGTTCTGCTC CCATCCTGTATCCGCTCTTG	2766 2876	19 20	111
33	CAAGAGCGGATACAGGATGG CTCGCTGAGGTGGTAGAAGG	2857 2974	20 21	118
34	GGAACAGTGAACCCGAAAAG TTGTACTGTCCAGGCAGCAG	2928 3006	21 21	79
35	GAATCTGCTGCCTGGACAGT TGTACGCGTTGTCAGAGTCC	2983 3095	21 22	113
36	GGACTCTGACAACGCGTACA TAGCCACTATCTGCGCTCAA	3076 3183	22 22	108
37	AGCTGAAGGAATGGGAAGGT CTCCTCTGGAACAGGGTCAA	3126 3223	22 22	98
38	TTGAGCGCAGATAGTGGCTA GTTCCTCTTGCCCAGGTCTT	3164 3244	22 22	81
39	AAGACCTGGGCAAGAGGAAC CATGTCGATGTCCTCAATGG	3225 3346	22 23	122
40	GACGGGTTCCAGTAGTTCCA TCCACCAGGTCTGAGGAATC	3283 3384	23 23	102
41	GAGGACGAGACCATTGAGGA GAGGCTCCCAGAGAAGATGA	3317 3441	23 23	125

Table 2.2: List of primary and secondary antibodies and dilutions used for immunoblot protein analysis

Antibody	Company	Catalog #	Species	WB
ERK1/2	Cell Signaling	4695	Rabbit	1:1000
GAPDH	Trevigen	2275-PC-100	Rabbit	1:3000
JAK2	Santa Cruz	sc-294	Rabbit	1:500
LAMININ B1	Santa Cruz	sc-5583	Rabbit	1:500
MEK1	Santa Cruz	sc-219	Rabbit	1:500
PDGFRα	Upstate	07-276	Rabbit	1:1000
pERK1/2	Cell Signaling	4370	Rabbit	1:1000
PI3K(III)	Cell Signaling	3358	Rabbit	1:1000
PLCγ1	Cell Signaling	2822	Rabbit	1:1000
pMEK1/2	Cell Signaling	9121	Rabbit	1:1000
RAC 1,2,3	Cell Signaling	2465	Rabbit	1:1000
RAS	Santa Cruz	sc-166691	Mouse	1:500
SRC	Cell Signaling	2108	Rabbit	1:1000
STAT3	Cell Signaling	9132	Rabbit	1:1000
STAT5	Cell Signaling	9363	Rabbit	1:1000
TUBULIN	Abcam	ab40742	HRP conjugated	1:2000
Anti-Biotin	Cell Signaling	7075	HRP conjugated	1:2500
Anti-Mouse IgG	Cell Signaling	7076	HRP conjugated	1:1000
Anti-Rabbit IgG	Cell Signaling	7074	HRP conjugated	1:1500

Table 2.3: List of primary and secondary antibodies used for immunohistochemistry protein analysis

Antibody	Company	Catalog #	Species	IHC	Phosphorylation
FYN	Santa Cruz	sc-28791	Rabbit	1:150	--
LCK	Santa Cruz	sc-13	Rabbit	1:150	--
pFYN	Santa Cruz	sc-377555	Mouse	1:100	Thr 12
pLCK	Cell Signaling	2751	Rabbit	1:100	Tyr 505
pSRC	Cell Signaling	2105	Rabbit	1:100	Tyr 527
SRC	Cell Signaling	2108	Rabbit	1:300	--
Anti-Mouse	BD Pharmingen	550337	Biotinylated	1:100	--
Anti-Rabbit	BD Pharmingen	550338	Biotinylated	1:100	--

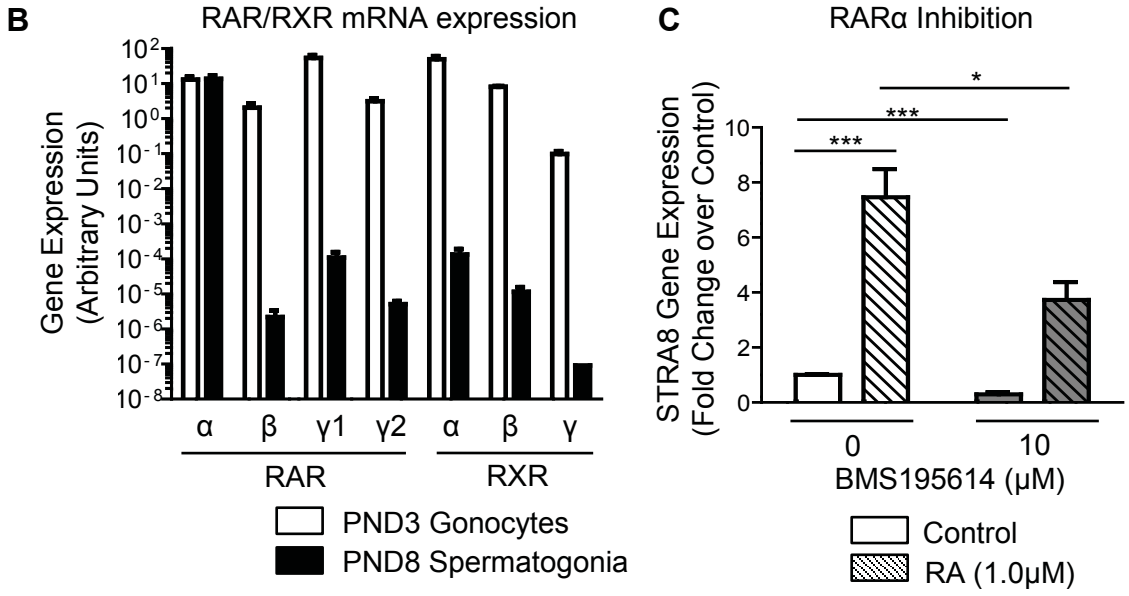
2.9 Appendix

Supplemental Figure 2.1: Importance of RARs in RA-induced gonocyte differentiation

(A) Expression values of RARs and RXRs (and their isoforms) present in the rat Illumina array. G3=PND3 gonocytes, G8=PND8 spermatogonia, S3=PND3 Sertoli/myoid cells, S8=PND8 Sertoli/myoid cells. (B) mRNA levels of RARs and RXRs (and their isoforms) in PND3 gonocytes and PND8 spermatogonia as analyzed by qPCR. Results shown are relative to RARα expression in PND3 gonocytes. (C) mRNA levels of STRA8 gene expression of gonocytes treated with or without RA and BMS195614 for 24 hours. Results shown represent the mean ± SEM of at least 3 independent experiments for each condition (*p<0.05; *** p<0.001).

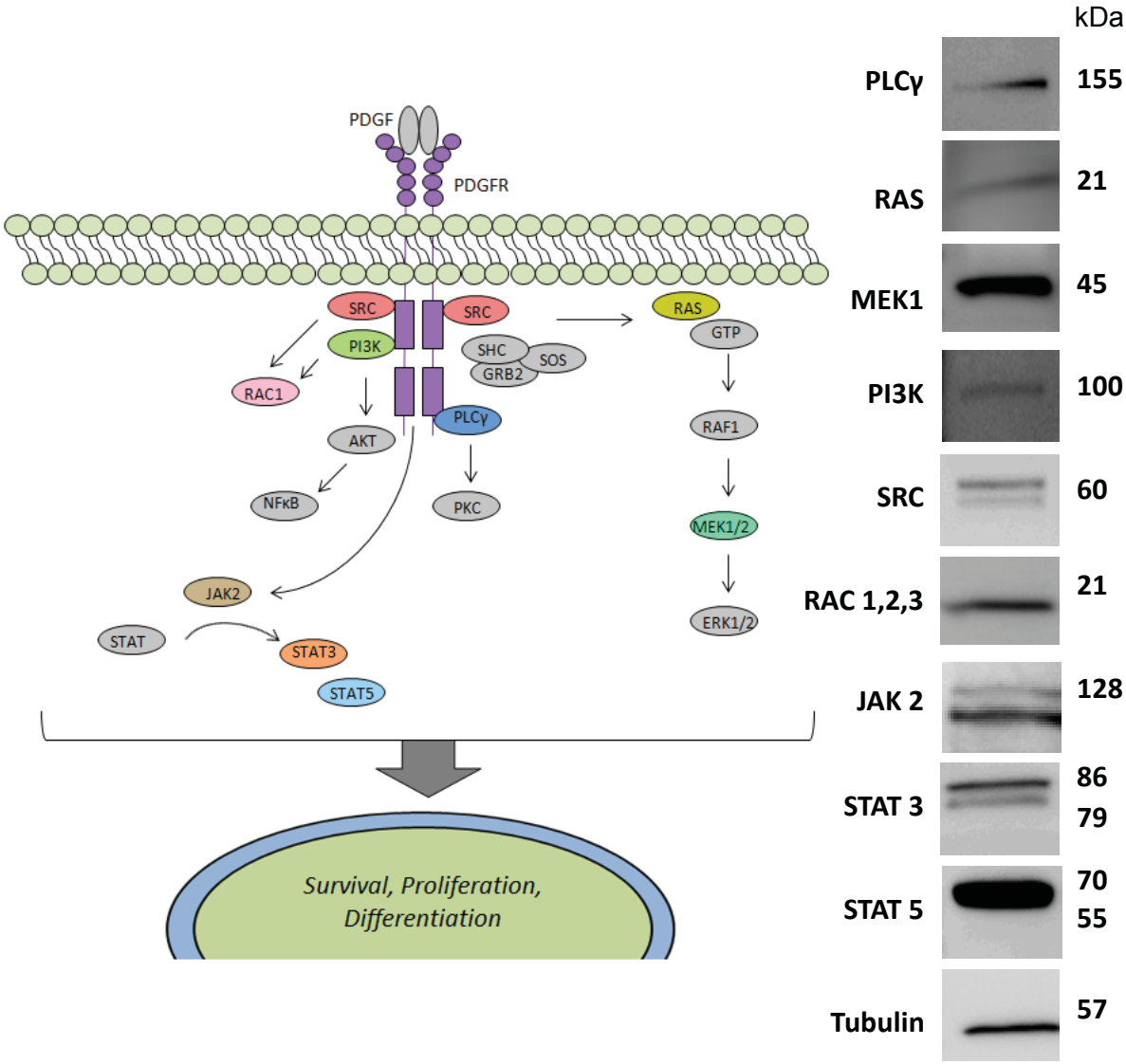
A

GENE	ACCESSION	G3 ± SEM	G8 ± SEM	S3 ± SEM	S8 ± SEM
RARα	NM_031528.1	40.4 ± 7.6	34.3 ± 4.9	60.4 ± 3.3	58.0 ± 2.9
RARβ	XM_223843.3	8.8 ± 0.6	6.3 ± 0.5	12.4 ± 3.8	11.6 ± 0.2
RXRα	NM_012805.1	14.5 ± 0.7	13.2 ± 0.9	11.8 ± 2.1	13.0 ± 0.8
RXRβ	XM_574720.1	17.9 ± 1.2	20.9 ± 0.3	21.7 ± 1.9	21.1 ± 0.4
RXRγ	XM_341151.2	16.3 ± 3.3	7.6 ± 0.3	6.6 ± 0.1	5.0 ± 0.4



Supplemental Figure 2.2: <u>Possible downstream pathways activated upon PDGFR activation</u>

Immunoblot analysis of PDGFRs and the various downstream pathways commonly activated upon PDGFR activation using isolated PND3 gonocytes. Representative immunoblots shown.



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Connecting Text: Chapter 2 and 3

The primary objective of chapter 2 was to determine the signaling mechanisms involved in gonocyte differentiation. As previously mentioned, gonocyte differentiation is induced by retinoic acid. In chapter 2, we demonstrated that RA treatment induced the expression of a PDGFRα variant form, and that the inhibition of PDGFR activation had a drastic inhibitory effect on RA-induced gonocyte differentiation, assessed by measuring changes in STRA8 mRNA expression. Furthermore, the analysis of possible PDGFR downstream pathways indicated that during gonocyte differentiation, the activation of a member of the SRC family of kinases and JAK2/STAT5 pathways were necessary. Similarly to gonocytes, the RA-induced differentiation of F9 mouse embryonal carcinoma cells, an embryonic stem cell model commonly used to study RA-induced differentiation, led to the formation of a PDGFRα variant form and was significantly downregulated upon PDGFR inhibition. However, in F9 cell differentiation, it was the activation or inhibition of the MEK1/2 pathway that pushed the F9 cells to differentiate towards the default somatic cell lineage or towards the expression of the germ cell marker Stra8, respectively. This indicated that F9 cells share some regulatory processes with gonocytes during differentiation, but that their downstream pathways are different. In chapter 3, we decided to examine the contribution of the ubiquitin proteasome system in gonocyte differentiation, because of the extensive remodeling and protein turnover required to support the complex processes that gonocytes undergo within a short period of time (proliferation, migration, apoptosis, and differentiation). Thus, in chapter 3, we examined the activation of the ubiquitin proteasome system and identified genes that may play a role in this process.

Chapter 3

Expression of the Ubiquitin Proteasome System in neonatal rat gonocytes and spermatogonia: Role in gonocyte differentiation

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3.1 Abstract

The Ubiquitin Proteasome System (UPS) consists of a cascade of enzymatic reactions leading to the ubiquitination of proteins, with consequent degradation or altered functions of the proteins. Alterations in UPS genes have been associated with male infertility, suggesting a role of UPS in spermatogenesis. In the present study, we questioned whether UPS is involved in extensive remodeling and functional changes occurring during the differentiation of neonatal testicular gonocytes to spermatogonia, a step critical for the establishment of the spermatogonial stem cell population. We found that the addition of the proteasome inhibitor lactacystin to isolated gonocytes inhibited their retinoic acid-induced differentiation in a dose-dependent manner, blocking the induction of the spermatogonial gene markers *Stra8* and *Dazl*. We then compared the UPS gene expression profiles of Postnatal Day (PND) 3 gonocytes and PND8 spermatogonia, using gene expression arrays and quantitative real time PCR analyses. We identified 205 UPS genes, including 91 genes expressed at relatively high levels. From those, 28 genes were differentially expressed between gonocytes and spermatogonia. While ubiquitin activating enzymes and ligases showed higher expression in gonocytes, most ubiquitin conjugating and deubiquitinating enzymes were expressed at higher levels in spermatogonia. Concomitant with the induction of spermatogonial gene markers, retinoic acid altered the expression of many UPS genes, suggesting that the UPS is remodeled during gonocyte differentiation. In conclusion, these studies identified novel ubiquitin-related genes in gonocytes and spermatogonia and revealed that proteasome function is involved in gonocyte differentiation. Considering the multiple roles of the UPS, it will be important to determine which UPS genes direct substrates to the proteasome and which are involved in proteasome-independent functions in gonocytes and to identify their target proteins.

3.2 Introduction

The ubiquitin proteasome system is the main pathway by which proteins are degraded in eukaryotes, occurring through the ligation of ubiquitin peptides to the target proteins (1-3). Ubiquitination has also been implicated in nonproteolytic functions such as the regulation of protein interactions, signal transduction, kinase activation, DNA repair, endocytosis, protein trafficking, cell cycle progression (1-4), intercellular communication (5), cell proliferation (6) and apoptosis (7). Consistent with its many functions, alterations of the UPS have been implicated in many pathological processes including neurological and immune disorders, cancer (1-4, 8), diabetes and obesity (9), and infertility (10-13). Multiple enzymatic reactions are involved in the attachment of a monoubiquitin or polyubiquitin chain to a substrate protein, including an E1 activating enzyme, an E2 conjugating enzyme, and an E3 ligase (3, 4). This is a complex process in which the E1 activating enzyme activates the ubiquitin molecule which is then transferred to a specific residue of the E2 conjugating enzyme and finally to an E3 ligase that will attach the ubiquitin tag onto its specific substrate. The 26S proteasome complex will then recognize and degrade the ubiquitinated substrate. The UPS also comprises deubiquitinating enzymes that can act antagonistically to the ubiquitination process (14-16).

Spermatogenesis relies on the existence of a pool of germline stem cells, the spermatogonial stem cells (SSCs), that will support the production of mature spermatozoa throughout the lifetime of a male (17, 18). Precursors of these SSCs are termed gonocytes (also called pre- or prospermatogonia) (19). Gonocyte development relies on the occurrence of timed events, including phases of quiescence, proliferation, migration within the seminiferous cords, differentiation and apoptosis, taking place between the fetal and neonatal periods (19). These processes are associated with major reorganization of the cell structure, changes in the responsiveness to Sertoli cell-secreted factors, and intracellular signaling pathways, some of which we unveiled in earlier studies (19-23). The plasticity of these cells is clearly illustrated by their ability to proliferate in response to a combination of PDGF and estradiol at Postnatal Day 3 (PND3) but not at PND2 (20) and the down-regulation of PDGF receptors and up-regulation of c-KIT expression as the gonocytes progress in their development (22-24). Similarly, gonocyte differentiation, which occurs in a precisely timed manner under the control of retinoic acid (22), also requires the cell to migrate and make contact with the basement membrane of the

seminiferous cord (19), implying major changes in the behavior and signaling profile of these cells. The disruption of gonocyte differentiation is believed to be at the origin of testicular cancer, the most common cancer in young men (25), and can also lead to infertility, underlining the importance of understanding these early phases of germ cell development. We have shown in previous studies that both ubiquitination and deubiquitination play a role in later phases of spermatogenesis (26-29). These studies were carried out in PND10-PND65 mice, ages that encompass all types of spermatogonia, from the undifferentiated type A single to the final differentiated type B spermatogonia (17). While knocking down the E2 conjugating enzyme UBC4 resulted in mice with delayed spermatogenic cycle (27), those lacking the deubiquitinating enzyme USP2 presented abnormal spermatozoa motility and a severe defect in fertilization (12). More recently, mice lacking the polyubiquitin B gene were found to be infertile and to present multiple testicular gene expression alterations, illustrating the complexity of UPS involvement in testicular germ cell development (30).

In view of the extended phenotypic and functional changes taking place during gonocyte differentiation, we hypothesized that UPS proteins might be involved in this process. To this end, we examined the effects of a proteasome inhibitor on retinoic acid-induced gonocyte differentiation. Because UPS genes had not been studied in gonocytes before, we also determined which UPS genes were expressed in PND3 rat gonocytes in comparison to PND8 spermatogonia, using gene expression array and quantitative real time PCR (qPCR). Further clarification of the role of these genes in gonocyte differentiation and the identification of the signaling pathways regulated by UPS should bring new light to the mechanisms underlying SSC formation and provide novel candidate molecules with which to study testicular cancer and infertility.

3.3 Materials and Methods

Animals

Newborn male Sprague Dawley rats were purchased from Charles Rivers Laboratories (Saint-Constant, QC, Canada). PND3 and PND8 pups were euthanized and handled according to protocols approved by the McGill University Health Centre Animal Care Committee and the Canadian Council on Animal Care.

Cell Isolation

Gonocytes were isolated from PND3 rat testes using 30–40 pups per preparation as previously described (20-23, 31). In summary, decapsulated testes were submitted to sequential enzymatic tissue dissociation, filtration, and differential plating overnight in 5% fetal bovine serum (FBS) (Invitrogen, Burlington, ON, CA) to allow for somatic cell adhesion. The next morning, nonadherent cells were further separated on a 2%–4% bovine serum albumin (BSA) (Roche Diagnostics, Indianapolis, IN) gradient in the absence of serum. Fractions that contained the most gonocytes (as judged by their morphology and large size) were chosen and pooled to obtain final cell suspensions presenting 80-90% purity or above 95% purity for preparations used in gene array analysis. After centrifugation, the cells were either directly frozen for later RNA or protein extraction, or collected on microscopic slides by cytospin centrifugation, or kept for 1 day in culture. Spermatogonia were isolated from PND8 rat testes using 10-20 pups per preparation. The method used for spermatogonia cell isolation was exactly that used for PND3 gonocytes described above (22). Enriched spermatogonia were either stored at -80°C until further processing or collected by cytospin centrifugation. All experiments were performed on three to four independent gonocyte or spermatogonia preparations. Enriched Sertoli cell preparations that contained ~20% myoid cells were also collected during the process of cell isolation at PND3 or PND8. These cells were used as a comparison for gene and protein analysis.

Gonocyte Cultures

Isolated gonocytes were cultured for 1 day to examine the effects of all-trans retinoic acid (RA) (Sigma-Aldrich, Oakville, ON, Canada) as previously described (22), or the proteasome inhibitor lactacystin (Sigma-Aldrich). Gonocytes from the final BSA pool (with a purity of ~80-90%) were resuspended in RPMI 1640 medium (Invitrogen) supplemented with 2.5% FBS (Invitrogen), 2% penicillin/streptomycin (CellGro; Mediatech, Manassas, VA), and 1%

amphotericin B (CellGro). Gonocytes were then plated in 24-well plates at a density of 10 000 cells in a 500μL final volume containing medium alone or RA (1μM; stock solution in ethanol) and/or the proteasome inhibitor lactacystin (stock solution in ethanol; Sigma-Aldrich) at 0.1, 0.5 or 1.0μM. In some experiments, we also tested the effects of the proteasome inhibitor bortezomib (Velcade; product no. PS-341; stock solution in dimethyl sulfoxide [DMSO]; Selleck Chemicals, Houston, TX) at 5, 10, 25, 50nM. Some experiments were performed to determine whether the solvents used to prepare the stock solutions of RA and the two proteasome inhibitors affected the transcripts levels in control cells by measuring the mRNA expression levels of representative genes, including *Stra8*, *Dazl*, *Tubulin*, *Gapdh,* and *Huwe1*. Combinations of 0.1-1 % ethanol with or without 0.0025-0.5 % DMSO were used to reproduce the final solvent levels present in treated samples and were compared to samples with medium alone. These experiments showed that the presence of the two solvents, alone or in combination, did not affect the basal mRNA levels of any of the genes examined (data not shown). Thus, using these samples or medium alone as a control to calculate the fold changes in treated samples did not make a difference in the results. Because of the limited numbers of gonocytes per experiment and the lack of effect of the solvents as compared to medium alone, control samples consisting of medium alone were used in subsequent experiments. The cells were incubated for 1 day in a 3.5% CO_2 incubator at 37°C. At the end of the incubation period, the cells were pelleted, and their RNA was extracted for qPCR analysis. Each condition was tested in duplicate wells, and at least three independent experiments were performed for each condition examined.

RNA Extraction and RT-PCR Analysis

Total RNA was extracted from cell pellets using the PicoPure RNA isolation kit (Arcturus, Mountain View, CA) and digested with DNase I (Qiagen, Santa Clarita, CA). cDNA was synthesized from the isolated RNA by using the single-strand cDNA transcriptor synthesis kit (Roche Diagnostics). Expression of various ubiquitin-related genes in various cell types was examined by reverse transcriptase (RT)-PCR. The primer sets used are listed in Table 1. The reactions were carried out using Platinum *Taq* DNA polymerase and amplified using the iCycler thermal cycler (Bio-Rad; Hercules, CA). PCR cycle conditions were 94°C for 3 min; then 40 cycles of 94°C for 30 sec, at 50°-60°C (depending on primer set) for 30 sec, and 72°C for 30 sec; followed by a 5 min extension at 72°C. PCR products were then separated on a 2% agarose gel. Simultaneous runs of the samples were performed using 18S primers as a housekeeping gene.

Gene Expression Array Analysis

Illumina microarray analysis was done by the McGill University's Genome Quebec facilities on RNA samples extracted from isolated PND3 gonocytes and PND8 spermatogonia preparations, as well as their corresponding Sertoli/myoid cell fractions. Three independent RNA extracts of PND3 gonocytes and PND8 spermatogonia and two independent preparations of corresponding Sertoli/myoid cells were processed for gene array analysis. In order to get sufficient amounts (100ng/µl) of RNA per sample, each gonocyte RNA sample was prepared from a total of 60-90 pups, while each spermatogonial RNA was isolated from 10 PND8 pups. Moreover, only the highest purity fractions from the BSA gradients were used in these studies to ensure more than 95% purity in the gonocytes and spermatogonia cell preparations. After we validated the RNA quality and purity, the samples were analyzed using the RatRef-12 Expression BeadChip for genome-wide expression analysis, which contains 22523 probes selected primarily from the NCBI RefSeq database. Data analysis was performed by Dr. Jaroslav Novak (32) and contained quality control analysis, normalization, abbreviation, and dispersion analysis, differential analysis of gene expression, and gene set enrichment analysis. Data were normalized using quantile normalization, corrected for the background signal, and abbreviated by eliminating the low-expression-end of the spectrum. Abridgement of data reduced the tables to 19100 from the original 22523 genes and expressed sequences. The comparisons between PND3 gonocytes and PND8 spermatogonia were done using the Bayesian approach, which emphasizes the significance of variation between replicates of the same sample rather than excluding genes according to a "fold-change" approach. However, a cut-off was applied in the selection of UPS genes for which the comparisons were made. In order to determine an appropriate cut-off value to use, we determined the proportion of genes represented in various ranges of signal intensities on the arrays. We found that the majority (65%) of all genes on the arrays had a relative signal intensity ≤ 20, while 23% of the genes had an intensity ≥ 50, 10% $\geq$ 300, and only 1% ≥ 2000. Thus, a cut-off of 50 appeared reasonable and was used to select the genes to study further.

Real-Time Quantitative PCR (qPCR)

qPCR was performed with a LightCycler 480 using a SYBR Green PCR Master Mix kit (Roche Diagnostics) and primers specific for the genes of interest, designed either with the DLUX program from Invitrogen or the Roche primer design software (Table 2). The cycling

conditions consisted of an initial step at 95°C, followed by 45 cycles at 95°C for 10 sec, 61°C for 10 sec, and 72°C for 10 sec. This was followed by melting curves and cooling cycles. The comparative threshold cycle (Ct) method was used to analyze the data. The amounts of the various genes were normalized to the endogenous reference (18S rRNA) by calculating the value of $2^{\Delta Ct}$, with ΔCt being the difference between the threshold cycle point (Ct/Cp) of the gene of interest and that of 18S rRNA. Data obtained for 18S rRNA showed that it presented no or minimal changes in Cp values between samples. Because stimulatory agents and culture conditions can alter the expression of genes otherwise considered "housekeeping", we initially determined the Cp values of four potential housekeeping genes, *Gapdh*, *Tubulin*, β-*Actin,* and 18S rRNA, as well as the Cp values for two UPS genes, *Ubc2* and *Ubc4*, in cDNA samples from isolated gonocytes cultured for 1 day with medium with or without RA. We compared the data obtained for *Ubc2* and *Ubc4* by using housekeeping genes to the values obtained using cDNA standard curves established for each primer set, in which the template was a mixture of the cDNAs analyzed. This approach showed that 18S rRNA was very constant, with less than half a cycle variation between control and RA-treated samples, and that using it for normalization provided *Ubc2* and *Ubc4* data consistent with those obtained using the standard curves. Interestingly, RA treatment consistently decreased the expression of *Tubulin* by 35% (n=9; $p<0.01$), *Gapdh* by 37% (n=9; $p<0.01$) and β-*Actin* by 67% (n=3; $p<0.001$) over several experiments, whereas other agents added to gonocyte cultures did not have such effects (data not shown). Thus, 18S rRNA was used for all experiments as the reference for data normalization. We also determined the primer efficiency for 8 of the primer sets used in qPCR analyses, including the 18S reference and genes that showed significant differences in expression between conditions as well as genes for which qPCR and gene array data either fitted well or diverged. For each primer set, qPCR reactions were carried out on serial dilutions of rat genomic testis DNA used as template. The resulting cycle numbers were plotted in log scale against DNA amounts, and the slope values were used to calculate primer efficiency (optimal efficiency = 2; ideal slope = -3.32), using the formula: primer efficiency = $10^{\wedge(-1/slope)}$. These experiments confirmed that 6 primer sets, including the 18S reference, had close to 100% efficiency, validating the qPCR results and the comparison of the relative abundances of these genes. The 2 primer sets that had low efficiency were those of genes presenting lower transcript levels in qPCR than expected from the gene array data, providing an explanation to the observed

discrepancies. The final data were expressed in arbitrary units representing the mRNA levels of the genes of interest present in the samples tested. Assays were performed in triplicate. For each treatment condition or cell type studied, the mRNA levels were determined in samples from three to four independent cell preparations. Results were expressed as means ± SEM of the fold changes in relative expression levels normalized to 18S rRNA between either gonocytes and spermatogonia or control and RA-treated gonocytes in culture.

Immunoblot Analysis

Aliquots of cell preparations were solubilized in Laemmli buffer, and proteins were separated on 4-20% Tris-glycine gels (Invitrogen). The gels were then transferred to a polyvinylidene fluoride membrane (Bio-Rad). After blocking with 5% milk in 1X Tween 20-Tris-buffered saline (TTBS), the membranes were incubated with specific primary antibodies diluted in TTBS overnight at 4°C (see Table 3), followed by incubation with a horseradish peroxidise-coupled secondary antibody (goat anti-rabbit, 1:1500 dilution; Cell Signaling) for 1 hour and then with ECL-enhanced chemiluminescence (GE Healthcare). Images were captured using the LAS-4000 gel documentation system (Fujifilm). Some membranes were first treated with rabbit immunoglobulin G (IgG) instead of a primary antibody to determine nonspecific bands and showed no signal. Each membrane was repeatedly processed with primary antibodies and stripped 3-4 times, using Restore Plus Western blot stripping buffer (Thermo Scientific). Tubulin was used as loading reference.

Immunocytochemistry

Protein expression of seven ubiquitin-related genes was examined by immunocytochemistry, following a previously described protocol (21, 31). Briefly, gonocytes (or spermatogonia) were collected right after BSA gradient separation, washed with PBS, fixed with 4% paraformaldehyde in PBS for 5 min, centrifuged, washed, and then collected onto microscopic slides by cytospin centrifugation. The slides were dried and treated with a mixture of acetone/methanol (60/40), followed by DakoCytomation target retrieval solution (Dako North America Inc., Carpinteria, CA). The slides were incubated overnight at 4°C with various primary antibodies (Table 3). The next day, the slides were treated for colorimetric staining using biotin-coupled secondary antibodies (BD Pharmingen, Mississauga, ON, CA) and streptavidin-coupled horseradish-peroxidase (HRP)/colorimetric HRP chromogen (AEC; Invitrogen), further

counterstained with hematoxylin (Invitrogen) and cover-slipped. Negative controls were produced by incubating some samples with nonspecific rabbit IgG (Invitrogen). Representative results are shown.

Immunohistochemistry

Testes were fixed in 3.5% buffered formaldehyde, embedded in paraffin, and treated for immunohistochemical analysis as previously described (33). Briefly, 5μm sections were dewaxed, rehydrated, and treated for antigen retrieval with DAKO and processed for immunostaining using primary antibody incubation overnight at 4^{o}C, followed by secondary antibodies, HRP/colorimetric HRP chromogen (AEC) and hematoxylin counterstaining as described above. Nonspecific IgG was used as negative control. Representative results are shown.

Statistical analysis

Statistical analysis was performed by unpaired two-tail *t*-test with statistical analysis functions in GraphPad Prism version 5.0 software (GraphPad Inc., San Diego, CA) or one-way ANOVA with a Bonferroni correction. All experiments were performed three to four times independently, and *P* values less than 0.05 were considered statistically significant.

3.4 Results

Proteasome Activity is Required for Gonocyte Differentiation

We have previously shown that the differentiation of neonatal gonocytes can be studied *in vitro* by measuring increases in the expression of genes characteristic of differentiating spermatogonia in gonocytes incubated with RA (22). As shown in Figure 3.1A, the addition of 1μM RA to gonocytes for 24 hours induced a nearly 6-fold increase in the mRNA expression of Stimulated by retinoic acid 8 (*Stra8*), a gene marker of differentiating spermatogonia involved in cell progression toward meiosis (34-36). Similarly, the mRNA expression of Deleted in Azoospermia-like (*Dazl*), an RNA binding protein found to increase in differentiating spermatogonia (37), increased by 50% upon RA treatment (Fig. 3.1B). However, addition of the non-reversible natural proteasome inhibitor lactacystin (38, 39) significantly inhibited in a dose-dependent manner the effects of RA on both genes, with greater than 80% suppression at the highest concentrations. While lactacystin alone had no effect on the basal expression of *Stra8* (Fig. 3.1) and several genes, including UPS and housekeeping genes (see Supplemental Figure 3.1), it induced a significant decrease in the basal levels of *Dazl* transcripts. We also examined the effects of bortezomib, another proteasome inhibitor (39), and found that it had inhibitory effects on *Stra8* and *Dazl* induction by RA similar to those of lactacystin, except that the inhibition was less pronounced on *Stra8* (data not shown). These results showed that gonocyte differentiation requires an active proteasome.

Protein Expression Profiles in PND3 Gonocytes and PND8 Spermatogonia of UPS Genes Previously Identified in Juvenile and Adult Testicular Germ Cells

In search of genes potentially involved in gonocyte proteasome activity, we examined whether UPS genes previously found in spermatogonia to spermatids were already expressed in germ cells prior to spermatogonia formation. The mRNA and protein expression levels of seven UPS genes were measured in PND3 gonocytes and the corresponding Sertoli/myoid and interstitial cells. They included the E2 Ubiquitin conjugating enzymes UBC2 (UBC2E; E217kB) and UBC4, the ubiquitin ligases UBR5 (EDD) and HUWE1 (LASU1, $E3^{histone}$, MULE, ARFBP1, HectH9), ubiquitin and the deubiquitinating enzymes USP19 and USP2 (UBP-testis/UBP41) which include two variants, the USP2a/UBP69 long isoform (Ubp-t2.Ubpa;

69kDa), and the short isoform Usp2b/UBP45 (Ubp-t1, Ubpb; 45kDa). As shown in Figure 3.2A, mRNAs for all seven genes were present in testicular germ and somatic cells at PND3.

Immunoblot analysis showed that the corresponding proteins were all expressed in gonocytes (Fig. 3.2B). The immunoreactive bands were positioned at the expected size and presented signals indicative of high levels for UBC2, HUWE1, UBR5 and ubiquitin mono-, di- and trimers, as a diffuse band for USP19, and at low levels for UBC4 and the 69kDa form of USP2. The two UBR5 and USP19 proteins were present in gonocytes but not detected in Sertoli/myoid cells, while gonocytes did not seem to express the short 45kDa USP2 isoform, which was visible in somatic cells (Fig. 3.2B).

Protein levels were also examined by immunocytochemical analysis of paraformaldehyde-fixed isolated PND3 gonocytes and PND8 spermatogonia (Fig. 3.2C). UBC2, UBC4, HUWE1 and ubiquitin were strongly expressed in the cytoplasm of gonocytes. USP2 immunoreactivity was weak but consistently found in the nucleus of gonocytes, while it localized to the cytoplasm in spermatogonia. In these experiments, one could not detect UBR5 immunoreactivity in gonocytes, although a positive signal was found in isolated spermatogonia. USP19 expression was very low at both ages, below detection levels in gonocytes and as a faint cytoplasmic staining in spermatogonia. In contrast, the other UPS proteins showed a strong immunoreactivity in spermatogonia, with UBC4, HUWE1, and ubiquitin present mainly in nuclei (Fig. 3.2C).

Immunohistological analysis of the proteins in tissue sections confirmed that PND3 and PND8 germ cells expressed the seven UPS proteins *in vivo*, which appeared more abundant in germ cells than in the surrounding somatic cells (Fig. 3.2D). Indeed, USP19 and UBR5 protein expression were detected only in gonocytes, in agreement with the immunoblot data, and despite the presence of transcripts in the somatic cells (Fig. 3.2A and 3.2D). Both proteins also appeared preferentially expressed in germ cells at PND8. Interestingly, UBR5, HUWE1, USP2, and ubiquitin were strongly expressed in the cytoplasmic areas where germ cells had established contact with the basement membrane of the seminiferous tubules (Fig. 3.2, arrows). Noticeably, at PND8, many germ cells had reached the basement membrane but presented an intermediate elongated phenotype, indicating that they had not yet established full alignment with the basement membrane nor achieved full differentiation to spermatogonia.

Gene Expression Array Analysis of UPS Genes in Gonocytes, Spermatogonia and Sertoli/Myoid cells

To determine thoroughly the UPS gene profile of gonocytes, we performed gene expression array analysis of several PND3 gonocyte preparations and compared them to those of PND8 spermatogonial preparations, using a rat Illumina gene expression platform. Gene expression arrays were also performed on PND3 and PND8 Sertoli and myoid cell mixtures isolated during the cell isolation procedure, as a way to better identify potential germ cell-specific UPS genes. A global comparison of the gene expression profiles obtained for germ cells and Sertoli/myoid cells by using principal component analysis showed very different profiles for the somatic and germ cells, confirming the efficient enrichment of the germ cells (see Supplemental Fig. 3.2). Out of the 19100 genes and expressed sequences validated on the arrays, we identified 205 that were related to the ubiquitin system. Among those, 191 were UPS enzymes and 14 were either donors of ubiquitin moieties, proteins interacting with ubiquitin enzymes, or scaffolding proteins (see Supplemental Table 3.1). Most of the UPS genes expressed in germ cells were E3 ubiquitin ligases, followed by deubiquitinating enzymes. However, approximately half of these genes were expressed at low levels, presenting signal intensities below 50. Applying a cut-off intensity of 50 led to the selection of 91 UPS genes for which the fold changes in transcript levels between gonocytes, spermatogonia, and Sertoli/myoid cells were calculated (Table 3.4). Among these highly expressed transcripts, the most abundant UPS transcripts in gonocytes and spermatogonia were those of E3 ubiquitin ligases, representing 55% of the genes, followed by the deubiquitinating enzymes (DUBs) (21%), E2 ubiquitin conjugating enzymes (13%), other UPS-related genes (9%) and only 2% were E1 activating enzymes (Fig. 3.3A).

The polyubiquitin gene *Ubb,* ubiquitin A-52 residue ribosomal protein fusion product 1 (*Uba52*), ubiquitin-like protein neural precursor cell expressed developmentally downregulated 8 (*Nedd8*), and ubiquilin 1 (*Ubqln1*) genes were highly expressed (signals above 700) and had the most abundant UPS-related mRNAs both in gonocytes and spermatogonia. Amongst E3 ubiquitin ligases, the most abundant transcripts (≥ 700) were those of the Cullin 1 (*Cul1*), the tripartide motif family members *Trim28* (*TIF1*β; transcriptional intermediary factor 1), the F-Box (FBX) *Fbx15*, *Mdm2* and the Ring-type E3 ligase *Rnf19*. *Trim47* (*RNF100*) transcripts were nearly 2-fold higher in gonocytes than in spermatogonia and Sertoli/myoid cells at both ages

(Table 3.4). Several E3 messengers were more prominent in germ cells than in somatic cells (~2-fold), including *Mdm2*, *Fbxo7*, *Fbxo33, Rnf134, Rnf149, Rnf139, Rnf138, Rnf125* and *Rnf17*. Regarding E2 conjugating enzymes, only a few presented high transcript levels, including *Ube2e3*, *Ube2r2* and *Arih1*, the latter being 1.8-fold more prominent in germ cells. *Ube2l6* transcript levels were 1.7-fold higher in gonocytes than spermatogonia and 1.5-fold higher in germ cells than somatic cells. Among DUB enzymes, the most abundant mRNA was that of the ubiquitin specific protease (Usp) *Usp14*, present at similar levels in all cell types, followed by *Usp3,* which was the highest in gonocytes. *Usp3, Uchl1* (ubiquitin carboxy-terminal hydrolase L1), *Usp2, Usp28* and *Usp24,* were significantly higher by 1.7 to 4-fold in germ cells. There were only two E1 enzymes with expression signals above 50, *Ube1dc1*, more abundant in germ cells than Sertoli/myoid cells, and *Ube1c*. It should be noted that the most common E1 activating enzymes, *UBA1* and *UBA6*, were not represented on the rat Illumina arrays, similar to several of the genes examined in previous studies, such as *Ubc2, Ubc4* and *Ubr5*. However, the array included several *Ube2* genes that were described as homologs of *Ubc4/5*. Some of the genes presented in Table 4 were further validated by qPCR analysis.

Functional annotation clustering using the David Bioinformatics resource version 6.7 software (National Institutes of Health, Bethesda, MD) (40, 41), revealed that the majority of the 91 genes selected were functionally related to ubiquitin mediated proteolysis (Table 3.5, clusters 1, 5 and 10). A number of genes belonged to more than one functional cluster, suggesting that they might be involved in several functions, while 14 of the UPS genes did not belong to any of the identified functional clusters. Six genes were related to histone and chromatin structure and modifications (cluster 12, 17); 9 were related to cell cycle and mitosis (cluster 10); 29 were related to negative or positive regulation of transcription and nucleoside/nucleotide binding (clusters 12, 13, 16, 18); 24 were related to protein biogenesis, assembly and modifications (clusters 3, 19); 10 were related to apoptosis (clusters 15, 22); and 7 were related to cell morphogenesis and fractions (clusters 20, 21), suggesting that the UPS genes expressed in germ cells are involved in a variety of functions besides regulated proteolysis (Fig. 3.3B, Table 3.5).

Differential Expression of UPS Genes in PND3 Gonocytes and PND8 Spermatogonia

The goal of this analysis was to identify UPS genes that would be preferentially expressed in gonocytes and might be characteristic of the neonatal germ cell and its functions, as well as UPS genes differentially expressed in gonocytes and spermatogonia that would be indicative of differences in the ubiquitin systems of the two developmental phases. This comparative analysis showed that 28 genes presented statistically significant differences in their transcripts levels between gonocytes and spermatogonia, while 38 genes showed significantly higher mRNA levels in germ cells than somatic cells (Table 3.4). The transcript levels of some of these genes were further quantified by qPCR analysis. In addition to these genes, we also quantified the mRNA levels of genes previously reported to be expressed in spermatogonia, as well as the two main E1 ubiquitin activating enzymes *Uba1* and *Uba6* (ubiquitin-like modifier activating enzyme 6; *Ube1l2*) that were not represented on the Illumina arrays. The primer efficiency was successfully validated for 6 primer sets, including the reference *18S* rRNA, *Ubb*, *Ube2i*, *Huwe1*, *Ubr5* and *RNF149* genes. However, the qPCR primers of 2 of the genes presenting discrepancies between arrays and qPCR data were found to be sub-optimal, explaining the discrepancy found between the two methods. Thus, these genes were not included in the final qPCR results. There was a good agreement between the differential expressions observed in the arrays and by qPCR analysis for most genes.

The expression levels of UPS genes in gonocytes were compared to those in spermatogonia. As shown in Figure 3.4, the transcript levels of E1 activating enzymes *Uba1* and *Uba6* in spermatogonia were only 10% of their values in gonocytes. Similarly, the transcript levels of the E3 ligases *Huwe1*, *Trim47* and *Rnf149* were 2-fold higher in gonocytes, in agreement with the gene array data. However, the transcript level of *Rnf134* was 2-fold higher and that of *Rnf138* was 7-fold higher in spermatogonia. In contrast, most E2 conjugating enzymes were expressed at levels in spermatogonia that were higher than in gonocytes, the largest differences being in *Ube2e3*, *Ube2g1*, *Ubc4* and *Ube2c*, which were, respectively, 10-fold, 6-fold, 4-fold and 3.5-fold higher in spermatogonia than in gonocytes. Among the DUBs, *Usp24* was 3-fold higher and *Usp25* 1.8-fold higher in spermatogonia than in gonocytes, presenting the same trend as on the gene array, while *Uchl1* showed higher levels in spermatogonia by qPCR, but not on the gene array. The other DUBs measured by qPCR showed expression levels comparable between the two cell types, while some of these genes appeared differentially expressed on the array. It should be noted that we measured separately the two transcripts of *Usp2*, *Usp2a* corresponding

to the mRNA of the long 69kDa variant, and *Usp2b* corresponding to the messenger of the short 45kDa isoform. The data showed that the expression of the short *Usp2* transcript was higher than that of the long transcript in spermatogonia. *Ubb and Uba52* were more than 4-fold higher in spermatogonia than gonocytes. It should be noted that not all changes observed by qPCR were in agreement with the data obtained by gene array, probably due to the different approaches and sequences targeted in these methods. Overall, these data highlighted the existence of large differences between the UPS mRNA profiles of gonocytes and spermatogonia.

Retinoic Acid Affects Expression of Spermatogonial Markers and UPS Genes Differently in Gonocytes

In view of the differences in UPS gene expression profiles between gonocytes and spermatogonia and our finding that the proteasome activity is required for RA-induced gonocyte differentiation, we further examined whether UPS genes were altered by RA treatment as a way to determine whether UPS remodeling occurs during gonocyte differentiation and to identify genes more specific for gonocytes than later phases of development. As shown in Figure 3.5, RA treatment significantly increased the expression of the spermatogonial markers *Stra8* by 6-fold and *Dazl* by 1.7-fold, as well as that of the germ cell markers *Vasa* and *Mili* by 1.6 and 1.4-folds respectively. Simultaneously, the transcript levels of the E1 activating enzymes and half of the E3 ligases examined were not changed, while the E3 ligases *Ubr5*, *Fbxo7*, *Rnf139*, *Rnf149* and *Rnf10* were significantly decreased upon RA-induced differentiation (Fig. 3.5). Among those, *Rnf149* was also found to be less abundant in spermatogonia than in gonocytes, suggesting that this UPS gene is decreased during the process of differentiation and remains low thereafter. Most DUBs were decreased by RA treatment, as were all but one E2 conjugating enzymes (Fig. 3.5). Taken together with the fact that these enzymes were either unchanged or higher in spermatogonia than gonocytes, these results suggest that RA altered their expression in a transient manner in differentiating gonocytes. Thus, these results indicated that some components of the proteasome system were altered during gonocyte differentiation, whereas others remained constant, and pinpointed *Rnf149* as being preferentially expressed in gonocytes.

3.5 Discussion

This report shows for the first time that the regulated degradation of proteins via the ubiquitin proteasome system is likely central to gonocyte differentiation into spermatogonia and provides an extensive profiling of the UPS genes expressed during this critical period of germ cell development. The formation of spermatogonial stem cells from neonatal gonocytes provides the foundation from which spermatogenesis will proceed and is essential for male fertility. We hypothesized that the ubiquitin proteasome system participates to the extensive remodeling that takes place in differentiating gonocytes. This hypothesis was validated by our finding that the proteasome inhibitor lactacystin blocked the ability of RA to increase the expression of the differentiating spermatogonia markers *Stra8* and *Dazl* in gonocytes. *Stra8* was chosen as an indicator of gonocyte differentiation because of previous studies, including ours, in which RA was found to increase the mRNA expression of *Stra8* in isolated gonocytes (22, 35), and to increase STRA8 protein expression in PND2 gonocytes in organ culture (36). STRA8 protein was also detected in PND5 mouse gonocytes *in vivo* (36), an age at which germ cells are still gonocytes, most of them in the process of migrating toward the basement membrane of the seminiferous cords where they will finalize their differentiation. In search of other germ cell-specific genes potentially altered during gonocyte differentiation, we found that the mRNA expression of *Dazl*, a gene known to be expressed in differentiating spermatogonia (37), was increased in RA-treated gonocytes, simultaneously with *Stra8*, suggesting that *Dazl* could be used as a marker of gonocyte differentiation. Lactacystin, a nonreversible proteasome inhibitor synthesized by members of the *Streptomyces* genus, binding the β-1 subunit of the 26S proteasome catalytic core (38, 39), blocked the ability of RA to induce gonocyte differentiation *in vitro*, as reflected by its inhibitory effects on *Stra8* and *Dazl* mRNA expression. Similar effects were obtained with bortezomib, a dipeptide boronic acid acting on the same site of the proteasome as lactacystin, but in a reversible manner (39), confirming the fact that the effects observed were indicative of proteasome inhibition. The involvement of proteasomal activity in cell differentiation is not unique to gonocytes, as proteosomal degradation was also shown to be required for the differentiation of preadipocytes into adipocytes (42). Lactacystin was also found to block RA-induced G_1 arrest and differentiation of RA-sensitive breast cancer cells by preventing the RA-induced degradation of a critical scaffolding protein mediating insulin-like growth factor type-I receptor signaling in these cells (43). In contrast, blocking proteasomal

degradation in osteoblasts *in vitro* and *in vivo* had the opposite effect on differentiation by preventing the degradation of phosphorylated Smad members (44). The existence of biological systems in which proteasome activity can either block or support cell differentiation highlights the complexity of the ubiquitin system and the multiplicity of its target proteins (1-4). The observation that the mRNA expression of *Dazl* was reduced by proteasome inhibitors below its basal level suggests that proteasomal degradation is involved either in the regulation of *Dazl* transcription or the stability of its messenger, and the maintenance of Dazl homeostasis in gonocytes. However, this does not appear to be a common mechanism in gonocytes, since proteasome inhibitors did not have an effect on the basal transcript levels of other genes, including *Stra8* and several UPS and housekeeping genes in gonocytes.

Gene expression profiling identified a large number of UPS transcripts in gonocytes and spermatogonia. Functional annotation of these genes indicated that, besides belonging to gene clusters linked to proteasomal degradation, a number of UPS genes were included in categories related to processes such as transcriptional regulation (negative or positive), protein biogenesis, cell cycle, and histone and chromatin modifications. This suggests that UPS proteins might participate in the regulation of proliferation and gene transcription, directly or via chromatin modifications, in early phases of germ cell development. Indeed, some of the genes highly expressed in gonocytes are well known for their involvement in histone and chromatin remodeling. Among those, the E2 enzyme UBC2 is believed to regulate transcriptional activation by catalyzing the mono-ubiquitination of histones and to be a RA-responsive gene during rat embryo development (45), two important processes in gonocyte development. The E3 ligase Trim28 (TIF1β), abundant in germ and somatic cells, was also shown to affect chromatin structure (46), and to be a transcriptional corepressor required for proper spermatogenesis (47). Similarly, HUWE1 (*ARF-BP1; LASU1*), corresponding to the most abundant E3 ligase transcript in gonocytes, is highly expressed in the nuclei of spermatogonia and spermatocytes and was shown to poly-ubiquitinate histones *in vitro* (28) and proposed to play a role in histone removal during chromatin condensation in elongating spermatids (48). However, the localization of HUWE1 in gonocyte cytoplasm and in cytoplasmic extensions of germ cells making contact with the basement membrane of the seminiferous cords suggest that Huwe1 has a distinct role unrelated to chromatin remodeling at this stage of germ cell development. Histone deubiquitination via the action of USP3, an abundantly expressed DUB in gonocytes and

spermatogonia, may also play a role in chromatin remodeling and genome stability (49) in these cells. The strong expression of these genes in gonocytes is in agreement with the high levels of DNA methylation and chromatin remodeling that take place in neonatal gonocytes as part of the paternal imprinting process (50).

Among the UPS genes potentially involved in gonocyte and spermatogonia cell cycle is Ubr5 (*EDD)*, the ubiquitin protein ligase E3 component n-recognin 5, previously found to play a role in cell cycle progression and G_2/M DNA damage checkpoints (51, 52). It has become evident that some ubiquitin ligases stabilize proteins rather than mark them for degradation. This is the case of Ubr5, which was reported to stabilize β-catenin via its ubiquitination, resulting in the upregulation of the Wnt signaling pathway (53). Interestingly, both Huwe1 and Ubr5 are HECT-type E3 ligases recently shown to play a role in cell cycle progression by interacting with the tumor suppressor p53 (54-56). Moreover, gonocytes and spermatogonia express high levels of *Mdm2* transcript, an oncogenic ring finger E3 enzyme that targets p53 for proteosomal degradation (57). Gene array analysis showed that p53 transcript was present in gonocytes and spermatogonia and their aged-matched Sertoli/myoid cells (data not shown). Thus, neonatal gonocytes and PND8 spermatogonia appear to be equipped with several alternative ways to modulate p53 levels. This might be important for the regulation of the cell cycle and maintenance of germ cell genomic integrity. Our immunohistological studies showed that Ubr5 accumulated at the same subcellular location as HUWE1, inside cytoplasmic extension contacting the basement membrane, together with ubiquitin and the DUB USP2. The colocalization of several members of the UPS cascade at these sites suggests that UPS is involved in the contact formation between germ cells and the basement membrane and the extensive remodeling occurring at this location. These findings could be important clues toward understanding the events that take place upon anchorage of the germ cell to the basement membrane, a requirement for the cell to progress in its differentiation program.

Among the genes identified in the study, only a few were preferentially expressed in germ cells, positioning them as good candidates for being involved in germ cell function at these ages. Despite not being very abundant in terms of mRNA levels, *Rnf17* was the only gene showing much higher mRNA levels in germ cells than in somatic cells, while it was expressed at similar levels between gonocytes and spermatogonia. *Rnf17* has been shown to be essential for

spermatocyte and spermatid differentiation in studies using a RNF17 knockout mouse model, which presented male sterility and an arrest in round spermatids (58). The present findings suggest that Rnf17 also might play a role in earlier phases of germ cell development. *Cul1*, the most abundant E3 ligase in gonocytes, was also significantly more highly expressed in germ cells than in somatic cells. The covalent binding of Nedd8 onto Cul1 has been shown to increase the ubiquitination activity of the ligase (59). The finding that Nedd8 is among the highest expressed UPS gene in gonocytes and spermatogonia suggests that neddylation might be involved in the regulation of the ubiquitination process in these cells.

A large number, but still a select minority of genes, showed significant regulation upon transitioning from gonocytes to spermatogonia: E1 enzymes were markedly down regulated; and E2 enzymes were mainly upregulated, while E3 ligases showed both up- and down- regulation, indicating that these critical substrate-recognizing proteins must be precisely regulated. A few DUBs were also specifically upregulated, as were ubiquitin-encoding genes, presumably to ensure adequate supply of ubiquitin for spermatogonial proliferation and differentiation. The transcripts of E1 enzymes *Uba1* (*Uae*) and *Uba6* were strikingly expressed at higher levels in gonocytes than in spermatogonia. UBA1 was shown to be expressed in adult germ cells and is required for sperm capacitation and fertilization (60), while UBA6 was reported to play a role in the transition from mitosis to meiosis (61, 62). Considering that the two enzymes mediate different types of functions despite being coexpressed in some cell types (4, 63, 64), our findings further support the idea that UPS acts at multiple levels in gonocytes.

Amid the E2 enzymes expressed at high levels in gonocytes and spermatogonia, *Ube2e3* appears to be an interesting gene to study further because its silencing in epithelial cells was shown to block cell proliferation, while cell differentiation decreased its expression, suggesting that it plays a role in the balance between proliferation and differentiation (65). Two other potentially important E2 enzymes are UBC2 and UBC4, both of which were expressed in gonocyte cytosol but appeared to be either cytosolic or nuclear in spermatogonia. In earlier studies, we had found that both enzymes were present in rodent testis, in which UBC4 was restricted to spermatids, and that UBC4 deletion led to a delay in spermatogenesis (27, 66). The present findings showing that UBC2 and UBC4 proteins are also present in gonocyte and spermatogonial suggest that they might also participate to early phases of germ cell development.

Another level of regulation of the ubiquitin system is provided by the existence of deubiquitinating enzymes. *USP2* was one of the few DUBs expressed at higher level in gonocytes than in spermatogonia and in germ cell than in somatic cells. We previously showed that the *USP2* gene generates two isoforms in testis by using different promoters, resulting in proteins with different N termini and substrate specificities (29). Interestingly, gonocytes expressed both transcripts at similar levels but a higher level of USP2a protein, whereas spermatogonia presented higher levels of *USP2b* transcripts, suggesting that the two isoforms were differentially regulated during gonocyte and spermatogonial differentiation. While USP2 protein signal was localized in the cytoplasm of spermatogonia both in situ and *in vitro*, USP2 appeared to shift from the cytoplasm to the nucleus between gonocytes observed in situ and those isolated from the Sertoli cells for few hours. This change, which was seen only for USP2, suggests that the subcellular localization of USP2 in gonocytes is regulated by the Sertoli cells. We recently reported that $USP2^{-/-}$ mice present defects in spermatid development, as well as fertilization-deficient spermatozoa (15). Together with the present data, this suggests that USP2 might play a role at different periods of germ cell development. Regarding USP19, the germ cell-restricted expression of the protein places it as an interesting candidate gene to study further. Although nothing is yet known of its potential role in testis, USP19 has been shown to regulate the stability of a cyclin-dependent kinase inhibitor involved in the control of G_1 to S phase progression (6) and to play a role in the turnover of endoplasmic-reticulum-associated degradation proteins (67).

In the present study, we found that the responses to RA in term of UPS gene expression differed from the profiling data between gonocytes and spermatogonia, suggesting that the dynamics of regulation are highly stage-dependent between gonocytes and spermatogonia. RA treatment of gonocytes increased the mRNA expression of *Stra8* as expected, as well as the transcript levels of the germ cell markers *Dazl, Vasa* and *Mili*. Vasa is a germ cell-specific RNA helicase that is believed to act as a post-transcriptional regulator (68), whereas Mili is a piwi-interacting RNA binding protein expressed from gonocytes to early spermatocytes that was shown to be essential for spermatogonial stem cell self renewal and differentiation (69). These data demonstrate that isolated gonocytes can recapitulate several aspects of their differentiation process *in vitro*, further validating the use of this model to study RA-induced gonocyte differentiation. In contrast to its effects on spermatogonial markers, RA decreased the expression

of most of UPS genes examined. Our results suggest that most UPS genes are transiently down-regulated during gonocyte differentiation, returning to higher expression levels at later stages of development. The only gene that appeared to be altered in a similar manner during *in vitro* and *in vivo* gonocyte differentiation was *Rnf149* (also called DNA polymerase-transactivated protein 2; DNAPTP2). RNF149 belongs to the Ring-type E3 ligases, a family of E3 ligases characterized by a common RING finger motif composed of a linear series of cysteine and histidine residues, which can bind two zinc atoms (70). However, nothing is yet known of the role of this protein. Taken together, the decreased expression of *Rnf149* in differentiating gonocytes *in vitro* and its lower level of expression in spermatogonia in comparison to gonocytes, suggest that the downregulation of this gene might be part of the differentiation process.

The importance of the UPS system in spermatogenesis and male fertility is supported by the existence of knockout mouse models in which the absence of a specific UPS gene resulted in the disruption of spermatogenesis. Indeed, several of the genes identified in gonocytes and spermatogonia have been reported to play a role in spermatogenesis. This is the case of *Ubb*, a polyubiquitin gene producing 3 to 4 ubiquitin repeats, for which gene disruption was shown to prevent spermatogenesis beyond the pachytene stage and to lead to infertility (10). Another example is *Usp14*, which was shown to be required for spermatogenesis and male fertility in studies using the homozygous ataxia mouse model in which the reduction of USP14 was found to disrupt spermiogenesis (11). Our finding that *Usp14* is the most abundant DUB in gonocytes and spermatogonia suggests that this gene is also important in early germ cell development. Another DUB that stands out from our data is *Uch-l1*, which appeared upregulated in spermatogonia, but transiently decreased during gonocyte differentiation. UCH-L1 was originally proposed to play a role in the early apoptotic wave responsible of removing abnormal germ cells during spermatogenesis (71). However, it was recently shown to be differentially expressed in spermatogonia and proposed to play a role in the maintenance and asymmetrical division of spermatogonial stem cells undergoing differentiation and self-renewal processes (72). Our results are in agreement with these data, showing a major role of UCH-L1 after the first postnatal week, coinciding with the period at which germ cells that failed to migrate to the basement membrane are eliminated by apoptosis.

In conclusion, this study revealed that the proteasome participates in the process of gonocyte differentiation, suggesting that specific proteins need to be degraded, most likely proteins involved as functional brakes or inhibitors preventing the gonocyte-spermatogonia transition. We also identified novel UPS genes in gonocytes and spermatogonia, many of them never described in testis or germ cells before (Fig. 6). While some UPS genes were expressed at high levels in both germ cell types and others overexpressed in spermatogonia, several genes showed a noticeably higher expression in gonocytes, suggesting a specific role in gonocyte functions. The exact function of UPS genes in germ cells and the identity of their substrates and interacting proteins remain to be determined. Noticeably, several of the UPS genes identified in gonocytes have been previously associated with functions that are critical for gonocyte development, such as cell cycle progression during mitosis, gap junction remodeling, likely to take place during germ cell migration, endocytosis required for the turnover of cell surface receptors, and DNA remodeling during paternal imprinting and cell differentiation. Thus, the identification of the UPS genes present in gonocytes is only the first step in the understanding of the network of interactions taking place between UPS proteins and their substrates during gonocyte differentiation. The present study will provide a framework on which to design targeted studies that will elucidate the role of UPS and the mechanisms regulating gonocyte development.

3.6 Acknowledgements

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3.7 Figures

Figure 3.1: <u>Effect of the proteasome inhibitor lactacystin on in vitro gonocyte differentiation</u>

Isolated PND3 gonocytes were induced to differentiate *in vitro* by the addition of 1μM RA to the medium containing 2.5% FBS for 1 day. Gonocyte differentiation was determined by measuring the mRNA expression of *Stra8* and *Dazl*, two genes markers of spermatogonial differentiation, by qPCR analysis. The role of the proteasome in this process was evaluated by adding the proteasome inhibitor lactacystin at the concentrations of 0.1μM, 0.5μM, and 1μM, alone or with RA. The addition of lactacystin inhibited in a dose-dependent manner the effects of RA on gonocyte differentiation. The results represent the means ± SEM of 3 independent experiments per treatment, with each sample performed in duplicate wells.

* $p<0.05$; ** $p<0.01$; *** $p<0.001$

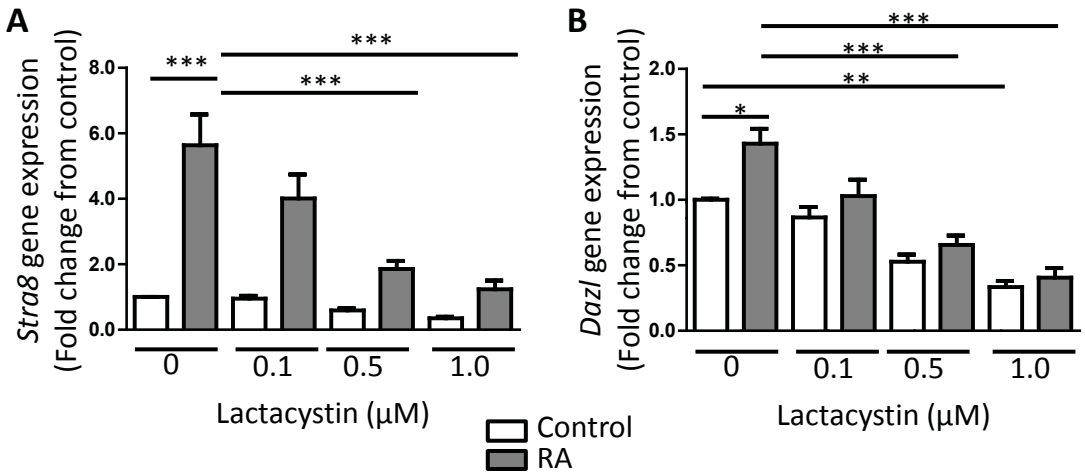


Figure 3.2: Messenger RNA and protein expression in PND3 gonocytes and PND8 spermatogonia of UPS genes previously identified in juvenile and adult testes

The mRNA (A) and protein (B-D) expression of seven UPS genes, including two E2 ubiquitin conjugating enzymes (UBC2, UBC4), two E3 ubiquitin ligases (UBR5, HUWE1), two deubiquitinating enzymes (USP2, USP19) and ubiquitin, were examined in isolated cells (A-C) and testes sections (D). (A) RT-PCR analysis of the mRNA expression of UPS genes in PND3 gonocytes (G3) compared to their expression in PND3 Sertoli/myoid cells (S/M3) showed that the 7 genes were expressed in germ and somatic cells at PND3. (B) Protein expression was evaluated by immunoblot analysis and showed the presence of bands of the expected sizes in gonocytes. Lad: ladder; arrows indicate expected size of proteins; Tub: Tubulin (loading control). (C) Freshly isolated gonocytes and spermatogonia were collected on microscopic slides by cytospin centrifugation and immunostained for the indicated proteins. Bar=20μm. Representative pictures are shown. (D) Protein expression was evaluated by immunohistochemistry on sections from PND3 and PND8 testes. Background signal was determined by using rabbit IgG instead of primary antibody on some slides. Arrows indicate areas of the germ cells in contact with the tubules' basement membrane.

Figure 3.2: <u>Messenger RNA and protein expression in PND3 gonocytes and PND8 spermatogonia of UPS genes previously identified in juvenile and adult testes</u>.

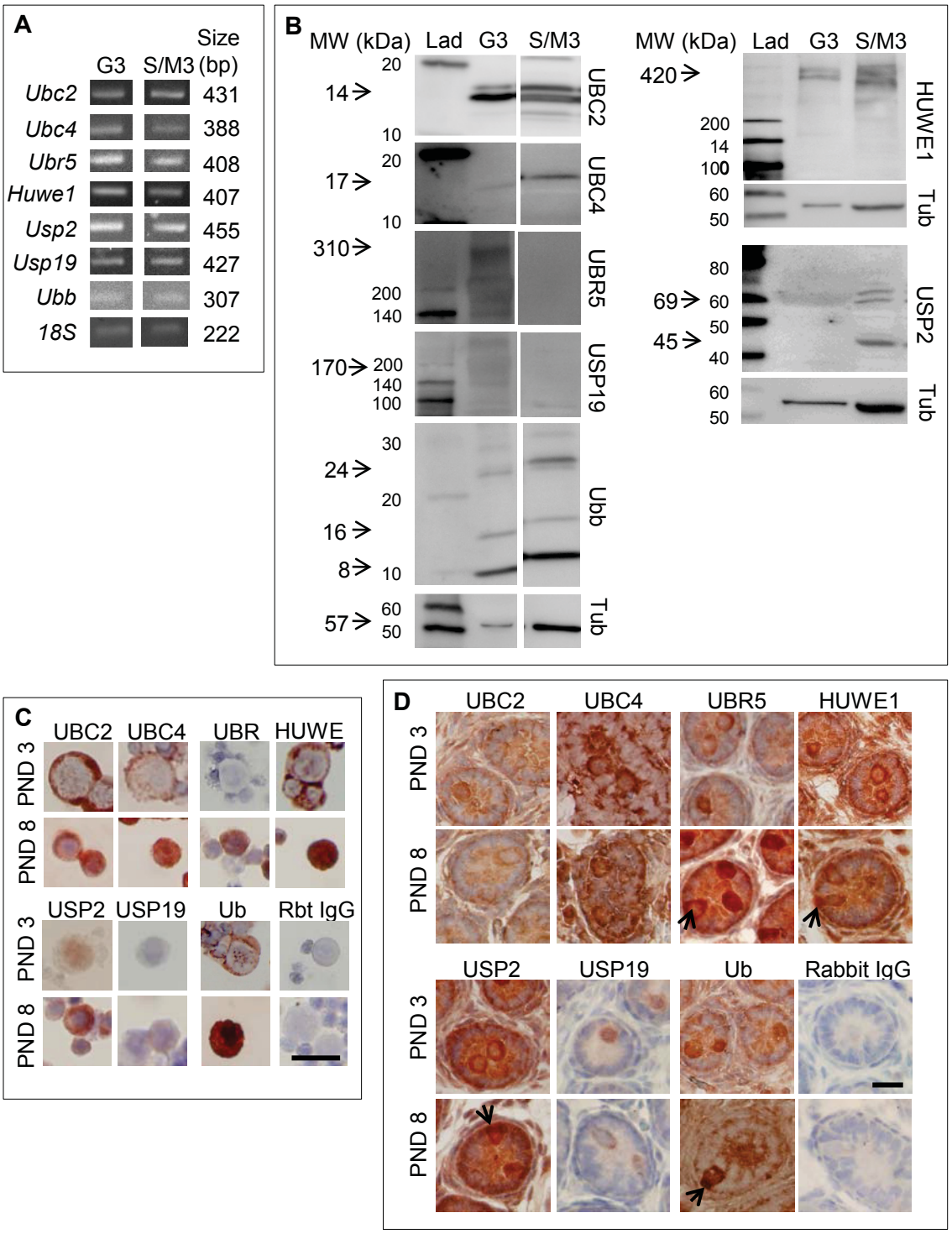


Figure 3.3: Relative proportion of each type of UPS genes expressed in gonocytes and main functional clusters

UPS genes expressed in gonocytes and spermatogonia were identified on gene arrays. Genes expressed at a signal intensity equal to or above 50 were selected. (A) The proportions represented by each type of enzyme (E1, E2, E3, DUB) and other UPS-related genes were evaluated, showing that more than 50% of the genes expressed in germ cells were E3 ligases. (B) Functional annotation clustering of the UPS genes, using David Bioinformatics Resources version 6.7 software (NIH). Although proteasomal degradation was the most common function involving UPS genes in germ cells, other functions were also well represented among the genes expressed in germ cells.

Figure 3.3: Relative proportion of each type of UPS genes exprressed in gonocytes and main functional clusters

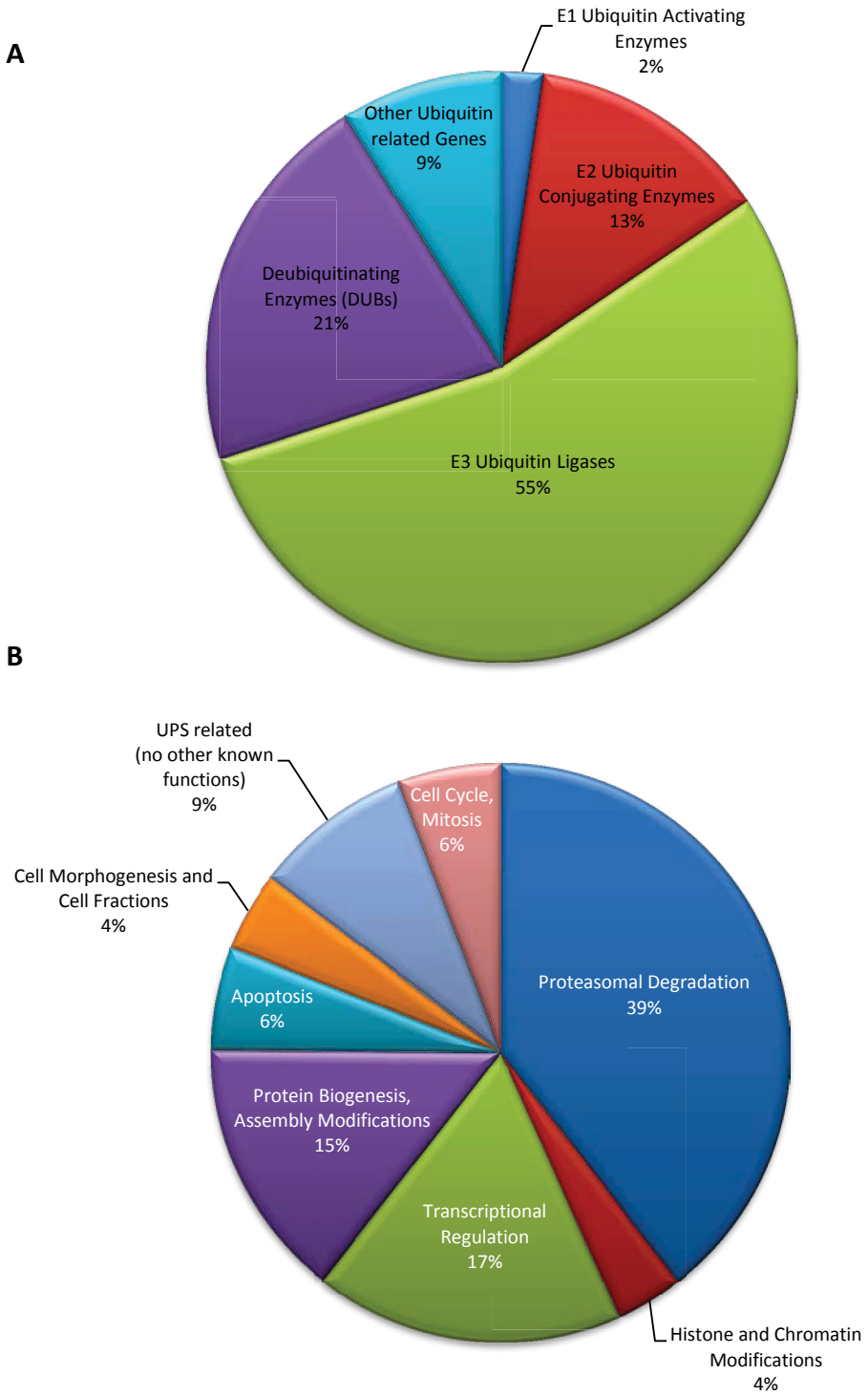


Figure 3.4: <u>Differential expression of UPS genes between gonocytes and spermatogonia</u>

Germ cells were isolated from PND3 and PND8 rat testes, their RNA extracted and processed for mRNA quantification by qPCR. The transcript levels of 30 UPS genes were determined. Results are expressed as fold change values over expression levels in gonocytes, and are means ± SEM of data obtained from 3 to 4 independent preparations, with each sample performed in triplicate. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Figure 3.4: <u>Differential expression of UPS genes between gonocytes and spermatogonia</u>

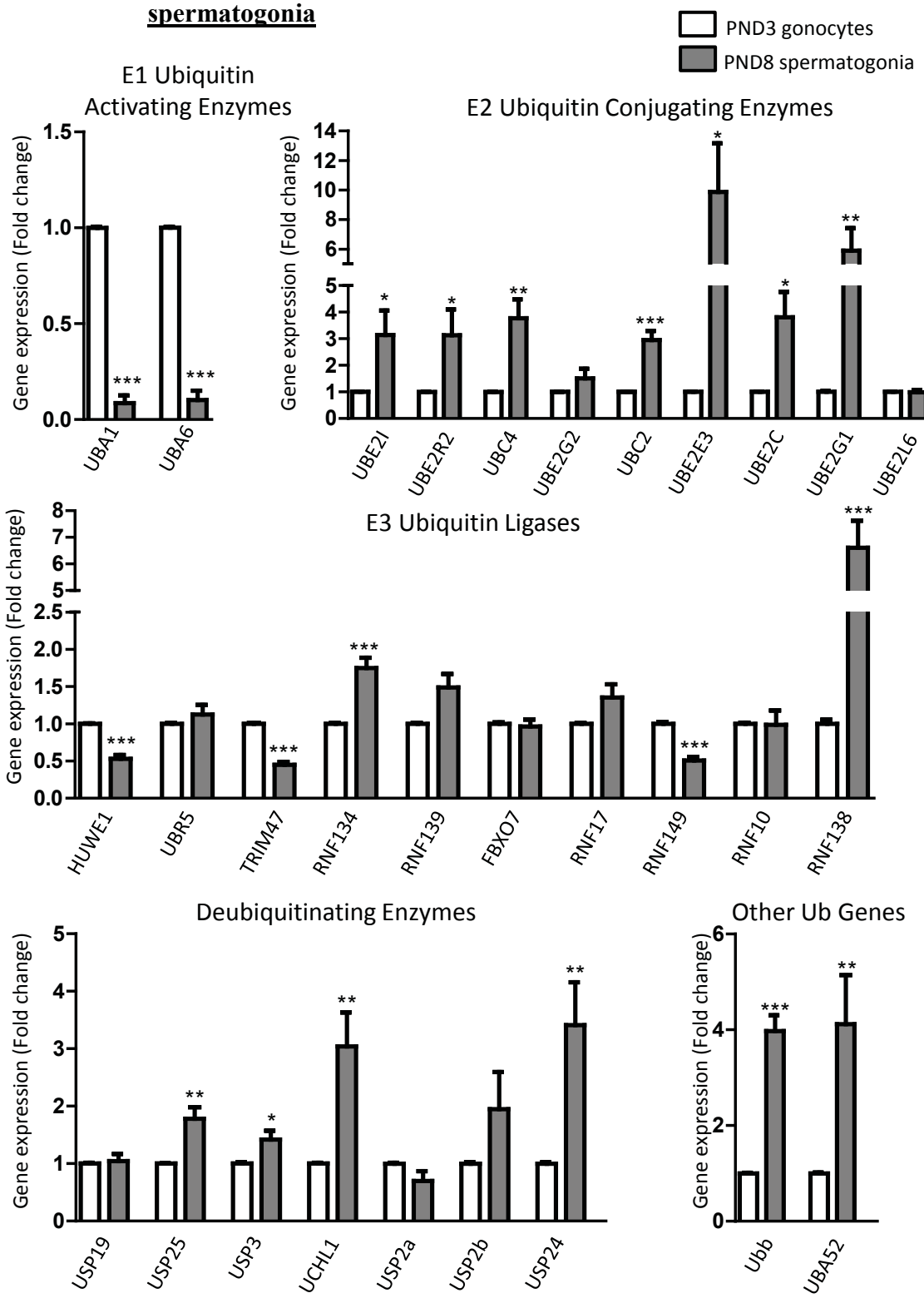


Figure 3.5: Effects of RA on the mRNA expression of differentiation markers and on UPS gene expression levels in isolated gonocytes

Gonocytes were isolated from PND3 rat testes and incubated for 1 day with medium alone or supplemented with 1μM all-trans RA and further processed for mRNA analysis as described in the legend to Figure 1. Results are fold change values over expression levels in control gonocytes and are expressed as means ± SEM of data obtained from 3 independent preparations, with each sample performed in triplicate. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Figure 3.5: Effects of RA on the mRNA expression of differentiation markers and on UPS gene expression levels in isolated gonocytes

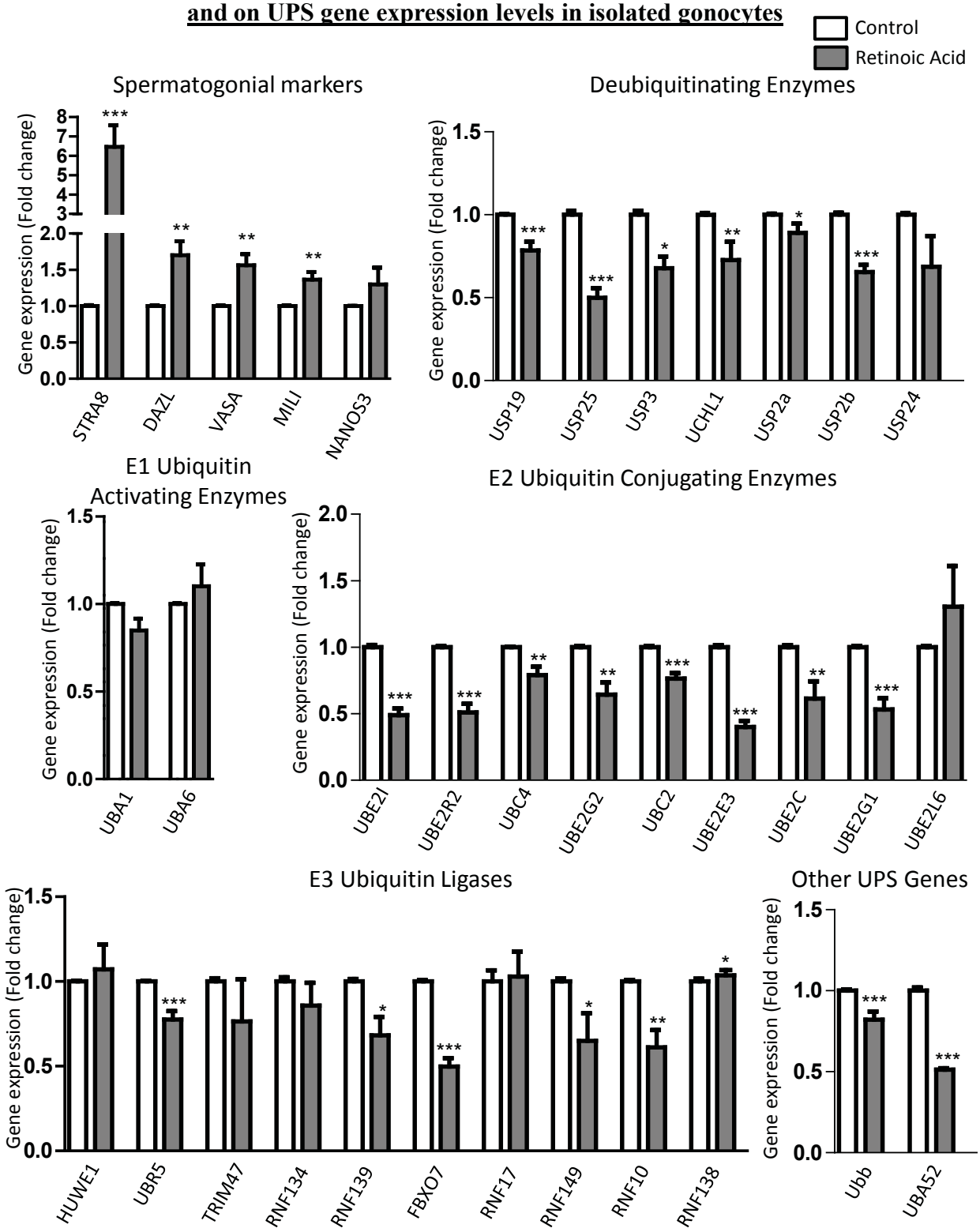
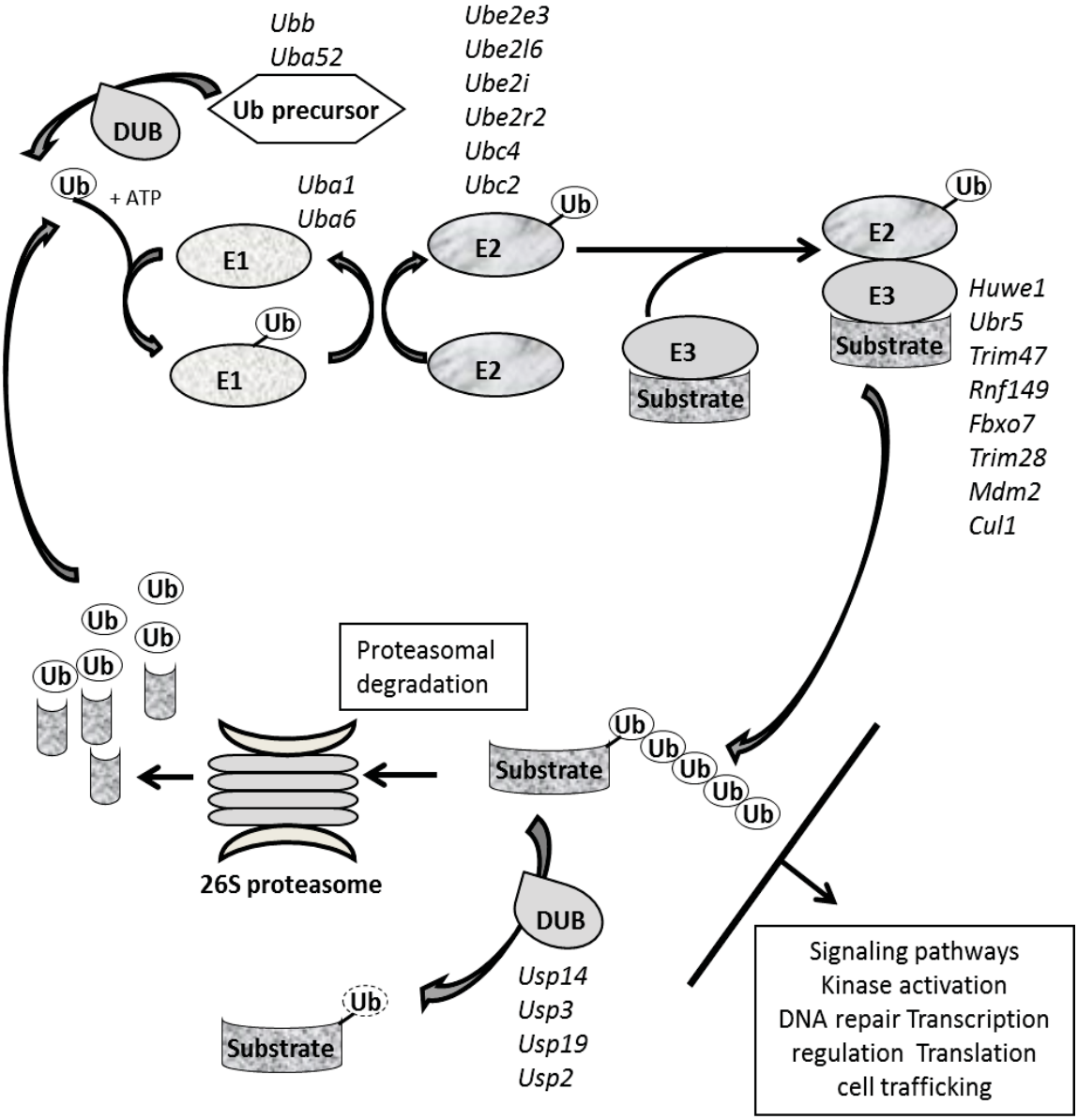


Figure 3.6: Schematic representation of the ubiquitin proteasome system in gonocytes

Ubiquitin is provided from processing of products of polyubiquitin or Uba52 ubiquitin fusion genes, or from recycling from ubiquitinated cellular proteins. Ubiquitin is activated by an E1 ubiquitin-activating enzyme in an ATP-dependent manner. Ubiquitin is then transferred to an E2 ubiquitin conjugating enzyme. The E2 enzyme interacts with an E3 ubiquitin ligase carrying the protein substrate to be ubiquitinated, resulting in the transfer of activated ubiquitin to the substrate. Successive conjugation of ubiquitin molecules onto the substrate will lead to the formation of a polyubiquitin chain, acting as a recognition signal for the 26S proteasome that will then degrade the substrate in small peptides and release reusable ubiquitin molecules. Deubiquitinating enzymes (DUBs) can modulate the E1-E3 pathway or stabilize protein substrates by removing ubiquitin moieties from a tagged substrate. As indicated, the UPS can also participate in the regulation of functions not related to proteasomal degradation. Genes highly or preferentially expressed in gonocytes are indicated in italics.

Figure 3.6: <u>Schematic representation of the ubiquitin proteasome system in gonocytes</u>



3.8 Tables

Table 3.1: List of primers used for RT-PCR analysis

Species	Name	GenBank Accession no.	Primers (5'-3')	F/R†	Start Site (bp)	Tm (°C)‡	Amplicon (bp)
Mouse	HUWE1*	AY929611	AAACTTTCAACTGGGCTCTATCC	F	3503	53	406
			TTCTTCTTCTCCTCGAGATCCTT	R	3909		
Rat	UBB	NM_138895	GGAAGATGGCCGCACCCTCTC	F	(213, 441, 669, 897)	56	762, 534
			GCCACCCCTCAGGCGGA	R	975		306, 78
Rat	UBC2	U13176	AGAATCCACAAGGAATTGAATGA	F	87	50	430
			TACATGGCATACTTCTGAGTCCA	R	517		
Rat	UBC4	U13177	TGACAGCCCATATCAAGGTG	F	326	52	307
			TCTGAGTCCATTCCCGAGAT	R	633		
Rat	UBR5	XM_001061308	CTGCTGTAAGTGGCCGGGCC	F	3308	60	388
			CAGGCGAGCCGAGCTAGACG	R	3696		
Rat	USP2a	NM_053774	CCAGAGATATGCACCAC	F	1195	52	454
			GGAACCTCTGGACAGAGA	R	1649		
Rat	USP19	AY605065	CACCTGCTTCATGAATAGTGTCA	F	1629	52	426
			AAACAGGTCCACAATGAATGAGT	R	2055		
Rat	18S	X01117	TCCCAGTAAGTGCGGGTCAT	F	1648	53	222
			GATCCTTCCGCAGGTTCAC	R	1870		

*HUWE1 (LASU1) primers were determined from the mouse sequence as there is no available sequence in rat.
†F: forward; R: reverse
‡Tm: annealing temperature

Table 3.2: List of primers used for quantitative-real time PCR analysis

Name	GenBank Accession no.	Primers (5'-3')†	F/R‡	Start Site
DAZL	NM_001109414	cggttGATGTTAGGATGGATGAAACCG	F	340
		AGCCCTTCGACACACCAGTTC	R	442
FBXO7	XM_576203.1	CCAGGCTAACATCCCAGATGAA	F	367
		cggacCATATTGGGTGACCTGTCCG	R	428
HUWE1*	AY929611	GATCCCTCCTTTCTGGCTGCT	F	8905
		cgggCTGGTTCTGTAGGACTTCCCG	R	8964
MILI	NM_001107276.1	GGGTCTCTTCTTGCTCGCTGA	F	1293
		cggaaGACATCCAGTACAGAGTCATTCCG	R	1356
NANOS 3	NM_001105945.1	cagcatAAGGCAAAGACACAGAATGCTG	F	430
		TGGAACCCGCATAGACACCTG	R	499
RNF10	NM_001011904	TTCAGACCCCCTCTCTGACA	F	2767
		CAGCAGCTTCTGCTTCTGC	R	2826
RNF17	XM_224231.3	GAGGTTGTGGGAGGCACCAT	F	3298
		cggagGCACTGTGGGATCTTCTCCG	R	3366
RNF134	NM_001013154	GCCCCAAATGCAACATAGTC	F	563
		TGCCGGTCCAACCTTATATT	R	627
RNF138	NM_053588.2	TCTCTTCTGTCATTCCAAATGTTAAG	F	391
		CAGATGCAGATGTTTCACTCCT	R	468
RNF139	XM_235338.3	GAGGACACATGGAAGCGGATT	F	550
		cgggAATCCTTGTTAGCCAGAAGACCCG	R	613
RNF149	XM_343561	TGCACCTTCAAGGACAAGGT	F	403
		GCGCTCCTGGTTGTAGACC	R	477
STRA8	XM_575429.2	TGCTTTTGATGTGGCGAGCT	F	861
		GCGCTGATGTTAGACAGACGCT	R	923
TRIM47	NM_001109585	CTCAAGCCTGCCTTCTTCC	F	1855
		TTCTTGAGGGGTCCAATCTG	R	1916
UBA1	NM_001014080.1	GGAGCAGCCACTCAGTGCTATG	F	814
		cggtgCAAGGCAGGTAACCACACCG	R	883
UBA6	NM_001107213	cggtaATCTTGAAGCAGCAGATACCG	F	1311
		GGAGAAACTCTTCATGCCCAAG	R	1370
UBA52	NM_031687	GATCTGCCGCAAGTGCTACG	F	331
		cgccTTTGACCTTCTTCTTGGGGCG	R	433
UBB	NM_138895	AAGAGTCAACCCTGCACCTG	F	251
		TTCACGTTCTCGATGGTGTC	R	371
UBC2	U13176	cgagcGGAATTGAATGACCTGGCTCG	F	98
		TTGTAGCCTGCCAATGAAACAT	R	184
UBC4	U13177	cacggAACTTAGTGATTTGGCCCGTG	F	229
		CCCATAATTGTGGCTTGCCAAT	R	320
UBE2C	NM_001106542	cggaaTCTGGTGACAAAGGAATTTCCG	F	186
		TGGTTCCTACCCATTTGAACAGG	R	249
UBE2E3	NM_001047857	cgggaTGAAAGACAACTGGAGTCCCG	F	826
		TCGTGTTCTGCTCTGTTGGTCA	R	965
UBE2G1	NM_022690	cggagCCCAAAAGATTATCCCCTCCG	F	487
		TTGGGTGCCAAATCTCTGTAATG	R	548
UBE2G2	NM_001106380	cgggTTGAGTTTGGTGTTTTCCCCG	F	218
		TCGGAGGGCTCAAAGGGTAGT	R	280
UBE2I	NM_013050	cggagCTGGAAAGAAGGGGACTCCG	F	218
		GGTGGTGAGGACGGATAGTCA	R	300
UBE2L6	NM_001024755	CTAGCGATGATGCCAATGTG	F	543
		TTGAGGCGATAGGGAAGTTG	R	612
UBE2R2	NM_026275	cacattCCTTTTCTCCAGCCAATGTG	F	456
		CTTTGCTGTCCCTCCATTTCCT	R	516
UBQLN1	NM_053747	cgggCCAACAAATGCAGAACCCCG	F	1474
		CAACGTCTGCAAGCCCTGCT	R	1567
UBE2M	NM_001108471	GTCGCCCAAGTCAGGGTCTTTA	F	696
		cgggCTTCCACTAAGCATTTCCCG	R	760
UBR5	XM_001061308	ATTCAGGCAGCCCATGACT	F	6155
		GAAATTCACTTTGTCGTCTACCC	R	6214
UCHL1	NM_017237	cggcCTCTACCAAGCAACCAGCCG	F	468

		CCCTTGCGGCCATAGTTCTC	R	533
USP2a	NM_053774	GGCGACAGATGTGGAGGAAGTA	F	719
		cggatACGAAGAGATCCCCAATCCG	R	783
USP2b	AF106658	cggcTATGCAGTTCACGAAGGCCG	F	780
		CCCAACGGTCTCCTCATCAGT	R	842
USP3	XM_343415.2	cggcATTCGTATGAGAAGGGGCCG	F	569
		AGAGGTATCCCTGCGGCTCTC	R	604
USP19	AY605065	CCACTTTAAAAACAGAATGAAAGTGT	F	999
		TCCCTCTCCAAACACCTCTG	R	1030
USP24	XM_001064937	cggttCAGGGAAACTGGCATAACCG	F	552
		ATGCTGGCTTCAAGAACTCTGCT	R	613
USP25	NM_001107114	cgaatCAGTTTGGGCACTCAATTCG	F	542
		TCAGCCTCCCTGGAGTAGCAC	R	560
VASA	NM_001077647	cgaatCAGTTTGGGCACTCAATTCG	F	1430
		CTCGGGCCTGCTTTGAACAC	R	1500
18S	X01117.1	cggttGATGTTAGGATGGATGAAACCG	R	768
		AGCCCTTCGACACACCAGTTC	F	842

*HUWE1 (LASU1) primers were determined from the mouse sequence as there is no available sequence in rat.

†Lowercase letters indicate nucleotides added by the primer design program that are not part of the gene sequences.

‡F: forward; R: reverse

Table 3.3: List of primary antibodies and dilutions used for protein analyses

Antibody	Species	Dilutions for ICC and IHC*	Dilution for immunoblots
UBC 2	Rabbit	1:100	1:1000
UBC 4	Rabbit	1:100	1:1000
USP 2	Rabbit	1:100	1:300
USP 19	Rabbit	1:100	1:100
HUWE1	Rabbit	1:100	1:100
EDD	Rabbit	1:100	1:100
Ubiquitin	Rabbit	1:100	1:50
Tubulin	Rabbit	NA	1:2000

*ICC: immunocytochemistry; IHC: immunohistochemistry; NA: not available

Table 3.4: Comparative expression of UPS genes in PND3 and PND8 germ and Sertoli/myoid cells

Symbol	Accession no.	G3	G8	Fold G3/G8	P value G3/G8	S3	Fold G3/S3	P value G3/S3	S8	Fold G/S	P value G/S
E1 ubiquitin-activating enzymes											
Ube1dc1	NM_001009669.1	625 ± 40	668 ± 53	0.9		442 ± 8	1.4	0.020	405 ± 49	1.5	0.009
Ube1c	NM_057205.1	122 ± 15	133 ± 3	0.9		136 ± 28	0.9		192 ± 47	0.8	
E2 ubiquitin-conjugating enzymes											
Ube2e3_predicted	XM_215754.3	731 ± 32	808 ± 70	0.9		709 ± 18	1.0		725 ± 61	1.1	
Ube2r2_predicted	XM_216864.3	585 ± 67	540 ± 39	1.1		648 ± 11	0.9		705 ± 1	0.8	
Arih1_predicted	XM_217157.3	328 ± 50	382 ± 15	0.9		171 ± 4	1.9	0.047	222 ± 22	1.8	0.002
Ube2g1	NM_022690.2	266 ± 16	290 ± 23	0.9		297 ± 48	0.9		354 ± 30	0.9	
Ube2g2_predicted	XM_215371.3	162 ± 5	210 ± 9	0.8	0.004	125 ± 17	1.3	0.039	133 ± 2	1.4	0.017
Ube2l6_predicted	XM_215762.2	159 ± 28	94 ± 7	1.7	0.041	90 ± 2	1.8		76 ± 3	1.5	0.010
Ube4a	NM_207610.1	121 ± 20	151 ± 19	0.8		133 ± 14	0.9		145 ± 2	1.0	
Ube2m_predicted	XM_341790.2	91 ± 17	78 ± 9	1.2		142 ± 3	0.6	0.051	132 ± 12	0.6	
Ube2d1_predicted	XM_342125.2	73 ± 6	68 ± 8	1.1		107 ± 3	0.7	0.014	112 ± 4	0.6	
Ube2c_predicted	XM_215924.2	71 ± 8	102 ± 8	0.7	0.024	130 ± 0	0.5	0.005	78 ± 11	0.8	
Ube2q_predicted	XM_215612.3	71 ± 7	58 ± 3	1.2		94 ± 0	0.8	0.036	85 ± 0	0.7	
Ube2i	NM_013050.1	40 ± 2	61 ± 4	0.7	0.006	59 ± 6	0.7	0.018	65 ± 1	0.8	
E3 ubiquitin ligases											
Cul1_predicted	XM_342679.2	1310 ± 144	1289 ± 61	1.0		984 ± 78	1.3		852 ± 45	1.4	0.013
Trim28	XM_344861.2	989 ± 75	969 ± 17	1.0		963 ± 20	1.0		896 ± 77	1.1	
Fbxl5_predicted	XM_223508.3	869 ± 49	933 ± 26	0.9		896 ± 30	1.0		702 ± 9	1.1	
Mdm2_predicted	XM_235169.3	754 ± 25	917 ± 52	0.8	0.024	269 ± 56	2.8	0.001	392 ± 135	2.5	0.0004
Rnf19_predicted	XM_343228.2	654 ± 154	657 ± 26	1.0		575 ± 47	1.1		511 ± 13	1.2	0.038
Rnf10_predicted	XM_573410.1	644 ± 17	606 ± 45	1.1		547 ± 16	1.2	0.015	582 ± 15	1.1	
Rnf11_predicted	XM_237327.3	364 ± 77	327 ± 3	1.1		475 ± 43	0.8		545 ± 0	0.7	
Trim8_predicted	XM_217908.3	363 ± 68	262 ± 25	1.4		464 ± 5	0.8		383 ± 9	0.7	
Fbxo7_predicted	XM_576203.1	348 ± 34	296 ± 3	1.2		172 ± 21	2.0	0.016	148 ± 11	2.0	0.001
Fbxo18_predicted	XM_214505.3	321 ± 9	298 ± 6	1.1	0.040	342 ± 50	0.9		318 ± 0	0.9	
Rnf111_predicted	XM_236380.3	299 ± 23	341 ± 14	0.9		251 ± 19	1.2		340 ± 57	1.1	
Rnf134_predicted	XM_219963.3	298 ± 80	251 ± 31	1.2		118 ± 3	2.5		133 ± 17	2.2	0.001
Rnf149_predicted	XM_343561.2	292 ± 16	231 ± 33	1.3		178 ± 10	1.6	0.007	178 ± 8	1.5	0.011
Fbxo22_predicted	XM_217146.3	281 ± 11	312 ± 5	0.9	0.029	285 ± 8	1.0		276 ± 23	1.1	
Fbxw4_predicted	XM_219944.3	259 ± 29	240 ± 16	1.1		334 ± 60	0.8		294 ± 25	0.8	
Fbxl11_predicted	XM_341983.2	242 ± 28	248 ± 13	1.0		167 ± 10	1.5		162 ± 20	1.5	0.008
Ubadc1	NM_001007742.1	208 ± 39	255 ± 24	0.8		157 ± 23	1.3		180 ± 21	1.4	0.017
Trim27_predicted	XM_214485.3	182 ± 13	272 ± 23	0.7	0.014	249 ± 20	0.7	0.030	262 ± 7	0.9	
Nedd4a	XM_343427.2	177 ± 39	128 ± 21	1.4		285 ± 24	0.6		230 ± 8	0.6	
Fbxo30	NM_001007690.1	163 ±10	169 ± 16	1.0		107 ± 9	1.5	0.015	96 ± 1	1.6	0.005
Fbxo11	NM_181631.2	159 ± 16	166 ± 11	1.0		154 ± 12	1.0		150 ± 2	1.1	
Fbxl20	NM_022272.1	155 ± 28	145 ± 11	1.1		81 ± 12	1.9		76 ± 0	1.9	0.001
Fbxl6	NM_001005563.1	152 ± 25	198 ± 22	0.8		144 ± 0	1.1		135 ± 7	1.3	0.039

Rnf25_predicted	XM_576586.1	142 ± 25	162 ± 11	0.9		140 ± 21	1.0		120 ± 9	1.2	0.059
Fbxo6b	NM_138917.1	142 ± 19	112 ± 5	1.3		162 ± 6	0.9		146 ± 5	0.8	
Fbxo33_predicted	XM_234205.3	138 ± 12	147 ± 15	0.9		91 ± 2	1.5	0.027	68 ± 9	1.8	0.003
Rnf2_predicted	XM_222726.3	131 ± 14	149 ± 29	0.9		113 ± 10	1.2		117 ± 9	1.2	0.048
Rnf20_predicted	XM_232995.3	127 ± 11	166 ± 5	0.8	0.015	104 ± 11	1.2		132 ± 22	1.2	0.039
Rnf3_predicted	XM_223729.3	118 ± 1	106 ± 3	1.1	0.014	94 ± 13	1.2	0.050	102 ± 7	1.1	
Trim41_predicted	XM_220357.3	116 ± 5	119 ± 10	1.0		97 ± 11	1.2		96 ± 12	1.2	0.043
Rnf166	XM_579073.1	115 ± 18	137 ± 16	0.8		102 ± 11	1.1		89 ± 10	1.3	0.028
Cul3_predicted	XM_217454.3	110 ± 28	178 ± 19	0.6	0.058	173 ± 19	0.6		178 ± 1	0.8	
Rnf34	NM_001004075.1	105 ± 8	131 ± 16	0.8		140 ± 7	0.8	0.028	163 ± 7	0.8	
Rnf7_predicted	XM_217235.3	104 ± 13	138 ± 3	0.7	0.027	115 ± 7	0.9		144 ± 30	0.9	
Trim47_predicted	XM_221124.3	100 ± 20	56 ± 8	1.8	0.056	66 ± 19	1.5		57 ± 1	1.3	0.041
Cul2_predicted	XM_341542.2	97 ± 17	97 ± 11	1.0		91 ± 14	1.1		89 ± 1	1.1	
Fbxl3a_predicted	XM_224478.3	97 ± 5	112 ± 3	0.9	0.035	74 ± 7	1.3	0.042	76 ± 8	1.4	0.017
Wwp1_predicted	XM_216361.3	93 ± 14	86 ± 3	1.1		49 ± 3	1.9	0.050	63 ± 11	1.6	0.004
Rnf139_predicted	XM_235338.3	88 ± 5	111 ± 8	0.8	0.043	44 ± 2	2.0	0.005	55 ± 12	2.0	0.002
Fbxo9_predicted	XM_576433.1	83 ± 2	89 ± 5	0.9		76 ± 4	1.1		77 ± 1	1.1	
Rnf31_predicted	XM_344409.2	62 ± 7	81 ± 1	0.8	0.033	95 ± 11	0.7	0.039	96 ± 5	0.8	
Rnf17_predicted	XM_224231.3	79 ± 29	91 ± 19	0.9		5 ± 0	16.3		8 ± 1	13.6	0.0001
Fbxo8_predicted	XM_573904.1	77 ± 13	82 ± 5	0.9		76 ± 3	1.0		73 ± 1	1.1	
Rnf138	NM_053588.2	75 ± 12	96 ± 4	0.8		45 ± 6	1.7		53 ± 3	1.7	0.003
Trim21_predicted	XM_219011.3	74 ± 8	68 ± 2	1.1		59 ± 9	1.2		51 ± 1	1.3	0.027
Rnf125_predicted	XM_341581.2	68 ± 16	38 ± 4	1.8		25 ± 9	2.7		12 ± 2	2.9	0.001
Hace1_predicted	XM_342160.2	61 ± 4	58 ± 3	1.0		80 ± 5	0.8	0.024	67 ± 0	0.8	
Fbxw9_predicted	XM_213838.2	56 ± 8	79 ± 3	0.7	0.025	46 ± 3	1.2		39 ± 4	1.6	0.009
Cul5	NM_022683.1	55 ± 3	66 ± 6	0.8		52 ± 3	1.0		79 ± 25	0.9	
Fbxo8_predicted	XM_573904.1	49 ± 11	65 ± 2	0.8		73 ± 11	0.7		86 ± 6	0.7	
Deubiquitinating enzymes											
Usp14	NM_001008301.1	995 ± 14	1175 ± 71	0.8	0.034	906 ± 72	1.1		977 ± 62	1.2	
Usp3_predicted	XM_343415.2	519 ± 38	383 ± 35	1.4	0.028	307 ± 8	1.7	0.011	222 ± 14	1.7	0.005
Usp7_predicted	XM_340747.2	280 ±20	288 ± 19	1.0		273 ± 8	1.0		380 ± 127	0.9	
Usp16_predicted	XM_213676.3	272 ± 10	265 ± 21	1.0		280 ± 32	1.0		293 ± 30	0.9	
Usp47_predicted	XM_218997.3	198 ± 20	235 ± 14	0.8		245 ± 17	0.8		221 ± 4	0.9	
Usp48	NM_198785.1	188 ± 26	181 ± 7	1.0		170 ± 9	1.1		172 ± 7	1.1	
Uchl1	NM_017237.1	185 ± 55	190 ± 37	1.0		49 ± 0	3.8		42 ± 0	4.1	0.0001
Usp5_predicted	XM_238380.3	172 ± 16	146 ± 11	1.2		171 ± 5	1.0		169 ± 7	0.9	
Usp15	NM_145184.1	149 ± 4	168 ± 3	0.9	0.009	110 ± 3	1.4	0.002	104 ± 4	1.5	0.012
Usp19	XM_573962.1	148 ± 1	146 ± 8	1.0		148 ± 6	1.0		148 ± 10	1.0	
Uchl5_predicted	XM_573467.1	147 ± 23	165 ± 18	0.9		147 ± 28	1.0		181 ± 12	1.0	
Usp2	NM_053774.2	108 ± 11	75 ± 8	1.4	0.039	42 ± 3	2.6	0.011	31 ± 5	2.5	0.001
Usp30_predicted	XM_222274.3	98 ± 8	114 ± 2	0.9		71 ± 9	1.4	0.059	73 ± 6	1.5	0.011
Usp39_predicted	XM_216173.3	87 ± 3	100 ± 5	0.9	0.043	90 ± 6	1.0		81 ± 1	1.1	
Usp1_predicted	XM_233217.3	70 ± 5	91 ± 12	0.8		90 ± 17	0.8		91 ± 17	0.9	
Usp45_predicted	XM_232828.3	69 ± 9	73 ± 5	1.0		98 ± 3	0.7	0.048	108 ± 7	0.7	
Usp28_predicted	XM_236240.3	54 ± 17	52 ± 9	1.0		27 ± 1	2.0		25 ± 4	2.0	0.002

Usp25_predicted	XM_221722.3	45 ± 2	105 ± 6	0.4	0.0003	64 ± 5	0.7	0.013	79 ± 6	1.1	
Usp24_predicted	XM_233260.3	41 ± 12	59 ± 0	0.7		24 ± 2	1.7		26 ± 0	2.0	0.002
Other ubiquitin-related genes											
Ubb	XM_579661.1	4742 ± 141	4766 ± 312	1.0		3815 ± 29	1.2	0.007	3448 ± 44	1.3	0.027
Uba52	NM_031687.2	2472 ± 109	2842 ± 29	0.9	0.015	2579 ± 8	1.0		2596 ± 267	1.0	
Ubqln1	NM_053747.2	896 ± 26	1017 ± 15	0.9	0.007	1043 ± 1	0.9	0.010	1120 ± 12	0.9	
Nedd8	NM_138878.1	822 ± 85	1039 ± 36	0.8	0.039	784 ± 15	1.0		932 ± 14	1.1	
Ube4b_predicted	XM_233679.3	213 ± 12	172 ± 10	1.2	0.027	224 ± 10	0.9		202 ± 17	0.9	
Ubap2_predicted	XM_232901.3	171 ± 29	179 ± 11	1.0		152 ± 17	1.1		157 ± 4	1.1	0.059
Ubtd1_predicted	XM_219869.3	104 ± 2	69 ± 5	1.5	0.001	116 ± 2	0.9	0.011	116 ± 4	0.7	
Ubl3_predicted	XM_344075.2	86 ± 16	101 ± 11	0.9		75 ± 14	1.1		94 ± 14	1.1	

*Genes that were expressed in gonocytes (G3) above a cut-off point set at 50 are shown and compared to their expression levels in spermatogonia (G8) and somatic cells (S3, S8).

†Values are means ± SEM

‡$P<0.06$ are shown, and differences with a P value ≤ 0.05 were considered significant

Table 3.5: Functional annotation clustering of the UPS genes expressed in gonocytes and spermatogonia

Gene	Cluster number																					
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Ube1dc1	+						+									+						
Ube1c	+	+	+			+				+		+	+			+		+				
Ube2e3_predicted	+	+				+																
Ube2r2_predicted	+	+				+																
Ube2g1	+	+	+			+										+						
Arih1_predicted	+						+															
Ube2g2_predicted	+	+				+				+												
Ube4a	+	+	+																			
Ube2l6_predicted	+	+				+										+						
Ube2m_predicted		+	+							+												+
Ube2c_predicted	+	+	+							+		+			+			+				
Ube2d1_predicted	+	+	+							+		+			+			+				
Ube2q_predicted		+				+																
Ube2i	+	+	+			+		+	+	+	+	+	+		+	+		+				
Cul1_predicted	+	+							+	+					+			+				
Trim28							+			+		+	+					+		+		
Fbxl5_predicted																						
Mdm2_predicted	+	+	+				+		+	+		+	+					+	+		+	
Rnf19_predicted																						
Rnf10_predicted							+			+		+	+					+				
Rnf11_predicted	+	+	+				+															
Trim8_predicted							+			+												
Fbxo18_predicted																+						
Rnf111_predicted																						
Fbxo22_predicted				+																		
Fbxw4_predicted																						
Fbxo7_predicted	+			+																		
Trim27_predicted							+			+		+							+			
Rnf149_predicted							+															
Rnf134_predicted							+			+		+	+					+				
Fbxl11_predicted																						
Nedd4a	+	+	+			+			+	+		+		+		+		+			+	
Fbxl6				+																		
Fbxo11	+	+		+			+															
Cul3_predicted	+	+							+	+										+		
Rnf25_predicted							+															
Fbxo30	+			+			+															
Fbxo6b	+	+		+					+	+												
Rnf20_predicted	+	+	+						+	+		+	+				+	+				
Rnf34	+	+				+	+			+	+		+		+							
Rnf2_predicted	+	+	+			+	+			+		+	+				+	+				
Rnf7_predicted		+	+							+					+							+
Fbxl20	+			+																		
Fbxo33_predicted				+																		
Rnf166							+						+									
Trim41_predicted							+															
Rnf3_predicted							+															
Cul2_predicted	+	+																				
Fbxl3a_predicted																						
Fbxo9_predicted	+			+																		
Rnf31_predicted							+			+												
Rnf139_predicted							+			+												
Fbxo8_predicted	+			+																		
Wwp1_predicted	+	+				+				+		+										
Trim47_predicted																						

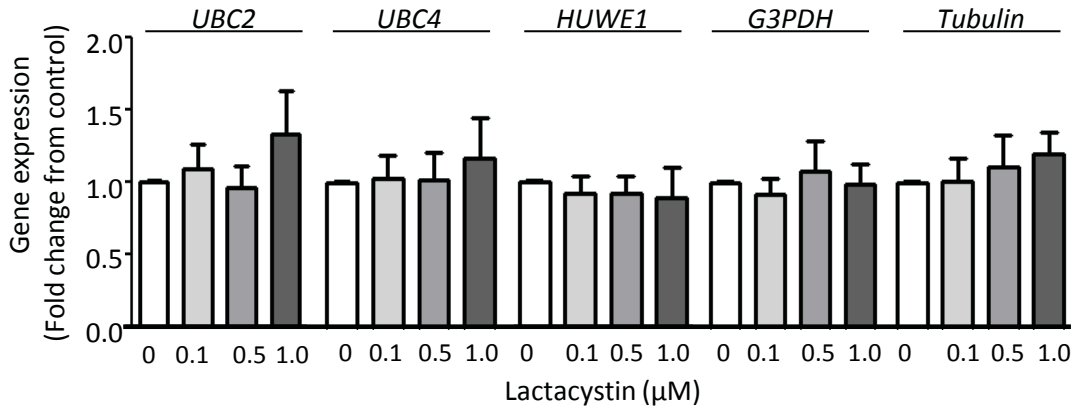
Rnf138	+					+	+		+		+		+									
Fbxo8_predicted	+			+																		
Hace1_predicted																						
Trim21_predicted																		+				
Cul5	+	+	+					+	+	+	+			+							+	
Fbxw9_predicted				+																		
Rnf17_predicted																						
Rnf125_predicted							+															
Usp14	+				+			+													+	
Usp3_predicted	+				+		+															
Usp7_predicted	+		+		+						+											
Usp16_predicted	+		+		+		+			+		+	+				+	+	+			
Usp47_predicted	+				+																	
Usp48	+				+			+														
Usp5_predicted																						
Uchl5_predicted	+				+																	
Usp19	+				+		+			+												
Usp15	+				+																	
Uchl1	+		+		+	+				+										+		
Usp30_predicted																						
Usp39_predicted	+				+		+															
Usp1_predicted	+		+		+					+	+			+								
Usp45_predicted																						
Usp25_predicted	+				+							+										
Usp2	+				+							+										
Usp28_predicted																						
Usp24_predicted	+				+																	
Ubb	+	+	+					+		+	+	+	+		+			+				+
Uba52								+		+	+	+										
Nedd8	+	+	+					+			+	+										
Ubadc1	+							+														
Ube4b_predicted	+	+	+											+	+							
Ubap2_predicted																						
Ubtd1_predicted								+														
Ubl3_predicted								+														
Ubqln1								+		+				+								

* The presence of genes in clusters is indicated by a +. DAVID Bioinformatics resources version 6.7 software was used to analyze the UPS genes identified on gene expression arrays. Cluster summaries: 1: proteolysis, catabolic processes; 2: ligase activity; 3: protein modifications by small protein conjugation; 4: Fbox proteins; 5: ubiquitin thiolesterase, hydrolase, protease, peptidase activity; 6: ubiquitin conjugating enzymes and ligases; 7: Zinc finger, ion binding; 8: ubiquitin proteins; 9: Cullin proteins; 10: regulation, proteasomal ubiquitin-dependent catabolic process, organelle components, mitotic cell cycle, protein modification; 11: ubiquitin conjugation, isopeptide bonds; 12: negative regulation of gene expression, transcription repressor, chromatin, chromosome, regulation of RNA metabolic process; 13: transcription regulation, repressor, Zinc finger; 14: response to UV, to light stimulus, to radiation; 15: apoptosis, programmed cell death; 16: ATP binding, nucleoside/nucleotide binding; 17: histone and chromatin modifications; 18: positive regulation of transcription, transcription cofactor, positive regulation biosynthetic process; 19: protein biogenesis, protein assembly, oligomerization; 20: cell morphogenesis involved in differentiation, cellular component morphogenesis; 21: cell fraction, insoluble fraction, membrane fraction; 22: regulation of apoptosis.

3.9 Appendix

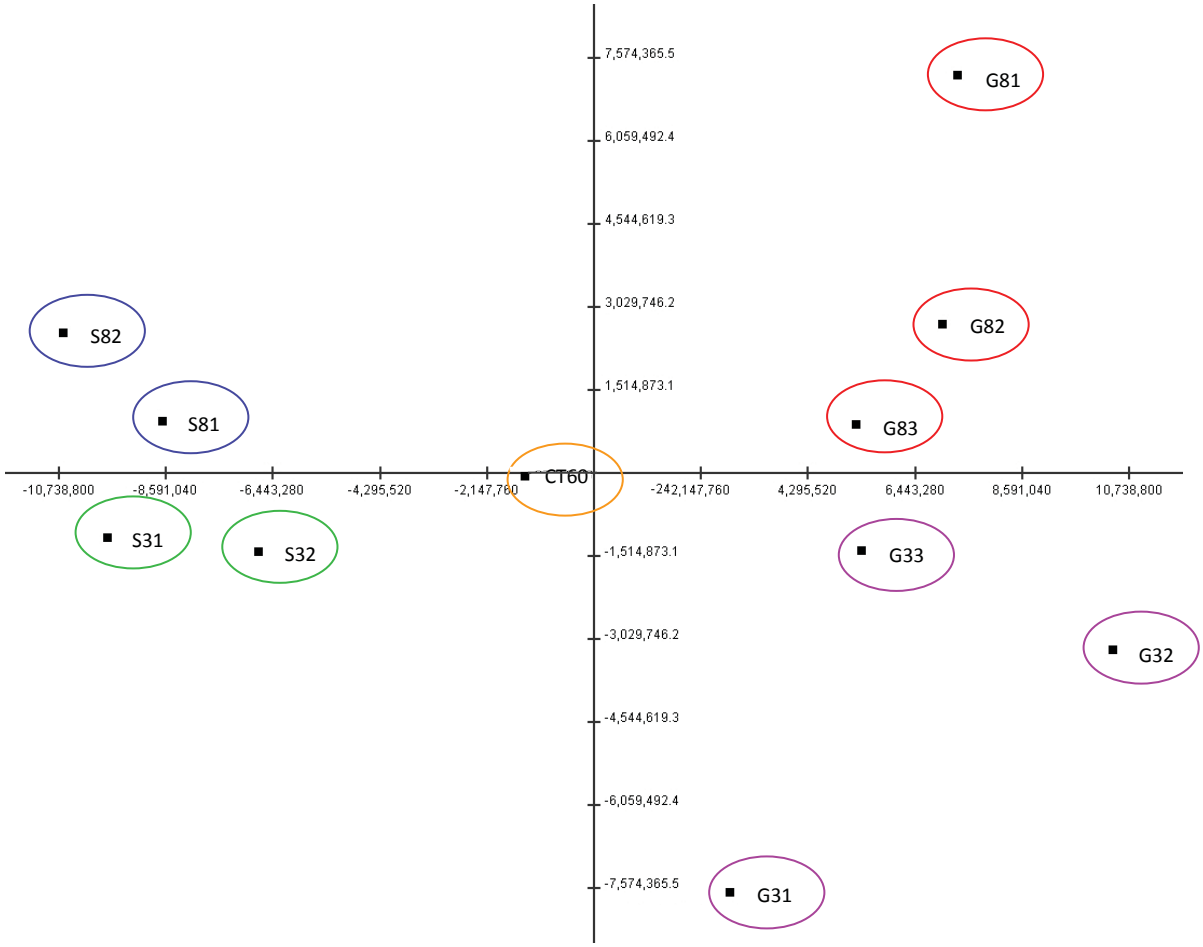
Supplemental Figure 3.1: Effect of the proteasome inhibitor lactacystin on the mRNA expression of UPS and housekeeping genes

The experiments were performed as indicated in Figure 1, with various concentrations of lactacystin, and transcripts levels were determined by qPCR. The presence of the inhibitor did not affect the basal transcripts levels of any of the genes.



Supplemental Figure 3.2: <u>Principal component analysis of gene expression arrays</u>

The PCA graph of quantile normalized data indicates that PND3 gonocytes (G31-33), PND8 spermatogonia (G81-83), and Sertoli/myoid cells from PND3 (S31, 32) and 8 (S81, 82) present very distinct gene expression profiles, validating the efficient enrichment of each cell type. The profile on an adult testis RNA extract is also shown (CT60), illustrating the great difference in gene expression profiles between early postnatal ages and adulthood.



Supplemental Table 3.1: List of UPS-related genes identified by Illumina gene expression arrays

Individual samples and averages (Av) of each cell type are shown. G3: PND3 gonocytes; G8: PND8 spermatogonia; S: Sertoli/myoid cells at PND3 (S3) and 8 (S8).

Symbol	Accession no.	G3-1	G3-2	G3-3	Av G3	G8-1	G8-2	G8-3	Av G8	S3-1	S3-2	Av S3	S8-1	S8-2	Av S8
E1 Ubiquitin Activating Enzymes															
Ube1dc1	NM_001009669.1	563.1	700.7	612.3	625.4	575.6	759.7	668.3	667.9	433.9	449.9	441.9	356.2	453.4	404.8
Ube1c	NM_057205.1	133.9	91.5	141.1	122.2	127.7	131.1	139.4	132.7	163.9	108.3	136.1	145.0	238.9	191.9
Uble1b_predicted	XM_218502.3	8.0	15.3	13.3	12.2	20.0	23.1	20.2	21.1	6.3	7.7	7.0	10.4	8.3	9.3
Ube1l_predicted	XM_217252.3	2.7	2.9	3.3	2.9	1.9	3.7	4.2	3.3	2.3	3.7	3.0	3.8	4.8	4.3
E2 Ubiquitin Conjugating Enzymes															
Ube2e3_predicted	XM_215754.3	708.5	793.9	690.9	731.1	944.1	766.8	713.9	808.3	726.7	690.9	708.8	664.8	786.0	725.4
Ube2r2_predicted	XM_216864.3	684.2	457.6	611.8	584.5	492.1	510.6	618.2	540.3	659.0	636.5	647.7	705.5	703.8	704.6
Arih1_predicted	XM_217157.3	229.6	392.9	362.7	328.4	410.7	375.4	360.9	382.3	174.8	167.3	171.0	199.9	243.1	221.5
Ube2g1	NM_022690.2	248.4	297.4	251.0	265.6	335.3	275.3	258.0	289.5	344.8	249.4	297.1	324.8	384.0	354.4
Ube2g2_predicted	XM_215371.3	164.9	167.8	152.1	161.6	224.6	210.6	194.1	209.8	107.6	141.6	124.6	134.9	130.4	132.7
Ube2l6_predicted	XM_215762.2	118.2	147.9	211.8	159.3	107.9	87.3	85.9	93.7	88.1	91.4	89.8	79.1	72.5	75.8
Ube4a	NM_207610.1	159.8	104.3	98.5	120.9	114.2	164.4	174.8	151.2	147.3	118.4	132.9	147.1	143.1	145.1
Ube2m_predicted	XM_341790.2	113.7	58.7	101.3	91.3	65.8	73.9	95.7	78.5	144.6	139.2	141.9	143.5	120.2	131.9
Ube2d1_predicted	XM_342125.2	79.3	60.8	79.8	73.3	80.8	54.8	67.5	67.7	109.8	104.2	107.0	108.0	115.1	111.6
Ube2c_predicted	XM_215924.2	80.3	56.2	77.6	71.4	116.8	94.2	94.0	101.7	129.8	129.8	129.8	89.0	66.1	77.6
Ube2q_predicted	XM_215612.3	73.6	81.1	58.9	71.2	54.6	57.3	63.2	58.4	93.9	94.4	94.2	85.2	85.0	85.1
Ube2i	NM_013050.1	42.3	35.4	42.0	39.9	57.9	68.8	55.4	60.7	64.9	53.0	59.0	64.1	65.2	64.7
Ube2d3	NM_031237.1	29.6	24.9	24.7	26.4	31.7	32.7	42.5	35.7	30.0	29.6	29.8	33.3	34.7	34.0
Ube2l3_predicted	XM_344046.2	28.2	16.5	20.4	21.7	23.6	19.8	26.8	23.4	46.3	42.4	44.4	40.1	35.2	37.6
Ube2n	NM_053928.1	10.5	9.4	11.6	10.5	10.6	10.0	11.4	10.7	14.4	14.0	14.2	15.8	21.8	18.8
Ube2j1_predicted	XM_216362.3	13.2	6.8	9.2	9.7	11.5	9.8	15.8	12.4	19.8	19.7	19.8	22.7	27.1	24.9
Ube2v1_predicted	XM_215948.3	10.6	7.0	10.1	9.3	11.7	6.9	8.6	9.1	12.0	12.3	12.1	11.9	15.1	13.5
Ube2e2_predicted	XM_341288.2	7.7	6.9	9.3	8.0	5.7	8.6	11.9	8.7	7.0	10.1	8.5	8.2	8.3	8.3
Ube2v2	NM_183052.1	7.7	5.4	7.3	6.8	7.3	9.8	10.4	9.2	19.4	6.8	13.1	16.1	19.6	17.8
E3 Ubiquitin Ligases															
Cul1_predicted	XM_342679.2	1126.1	1594.1	1209.5	1309.9	1411.1	1227.6	1227.6	1288.8	1062.3	906.5	984.4	897.2	807.0	852.1
Trim28	XM_344861.2	1033.6	1090.1	842.1	988.6	989.9	935.8	980.0	968.6	942.5	983.1	962.8	818.8	972.8	895.8
Fbxl5_predicted	XM_223508.3	859.7	958.5	788.3	868.8	980.0	929.7	890.4	933.4	926.0	865.4	895.7	711.8	693.1	702.5
Mdm2_predicted	XM_235169.3	703.8	782.1	775.7	753.9	816.1	989.9	946.0	917.4	212.9	324.8	268.9	257.2	525.9	391.5
Rnf19_predicted	XM_343228.2	962.0	483.9	516.5	654.1	614.9	649.1	705.5	656.5	621.1	528.2	574.6	497.7	523.4	510.6
Rnf10_predicted	XM_573410.1	673.1	643.4	614.9	643.8	519.2	672.3	625.5	605.7	531.1	562.6	546.8	567.4	597.0	582.2
Rnf11_predicted	XM_237327.3	481.5	219.0	391.8	364.1	323.6	325.3	332.6	327.2	517.3	431.8	474.5	545.4	544.5	545.0
Trim8_predicted	XM_217908.3	483.9	248.6	355.2	362.6	231.7	241.7	312.6	262.0	468.8	459.0	463.9	374.0	392.4	383.2
Fbxo18_predicted	XM_214505.3	327.5	331.9	304.4	321.3	307.6	296.7	288.4	297.6	291.7	391.4	341.6	318.7	318.2	318.4

Rnf111_predicted	XM_236380.3	336.4	304.1	257.4	299.3	316.4	341.5	364.9	340.9	232.4	270.4	251.4	283.5	397.2	340.4
Fbxo22_predicted	XM_217146.3	281.9	299.4	262.0	281.1	303.0	320.3	314.1	312.5	293.6	276.7	285.1	252.9	299.1	276.0
Fbxw4_predicted	XM_219944.3	312.9	212.6	252.4	259.3	208.6	254.0	258.7	240.4	274.4	393.1	333.7	318.5	268.8	293.7
Fbxo7_predicted	XM_576203.1	294.5	411.1	339.5	348.3	293.1	302.6	291.6	295.7	151.2	192.9	172.0	158.7	136.9	147.8
Trim27_predicted	XM_214485.3	207.3	174.3	163.1	181.5	318.8	248.4	249.5	272.2	228.9	269.5	249.2	269.1	254.9	262.0
Rnf149_predicted	XM_343561.2	323.0	271.7	280.5	291.7	165.8	257.0	270.3	231.0	187.9	167.5	177.7	170.2	186.7	178.5
Rnf134_predicted	XM_219963.3	173.0	445.5	275.9	298.2	312.0	229.8	209.8	250.5	114.3	121.1	117.7	116.5	149.8	133.1
Fbxl11_predicted	XM_341983.2	223.1	296.5	205.8	241.8	273.3	235.6	234.3	247.7	176.4	156.8	166.6	142.1	181.1	161.6
Nedd4a	XM_343427.2	253.5	122.6	154.8	176.9	91.6	127.7	165.8	128.3	309.4	261.5	285.5	238.6	221.9	230.2
Fbxl6	NM_001005563.1	119.1	200.5	137.3	152.3	241.0	184.0	169.6	198.2	144.2	144.6	144.4	128.3	141.8	135.0
Fbxo11	NM_181631.2	151.4	190.0	136.1	159.2	171.1	146.2	181.6	166.3	166.0	141.3	153.6	151.8	148.6	150.2
Cul3_predicted	XM_217454.3	158.4	60.4	110.4	109.7	142.1	185.5	206.3	178.0	192.1	153.4	172.7	179.2	177.7	178.4
Rnf25_predicted	XM_576586.1	179.2	94.9	151.7	141.9	142.2	178.0	166.9	162.4	119.5	160.9	140.2	128.6	110.7	119.6
Fbxo30	NM_001007690.1	166.8	178.0	144.7	163.2	200.8	153.5	152.3	168.8	97.5	116.0	106.7	97.4	95.5	96.5
Fbxo6b	NM_138917.1	175.3	110.3	140.9	142.2	104.1	111.5	121.6	112.4	156.4	168.1	162.3	150.9	140.2	145.5
Rnf20_predicted	XM_232995.3	106.2	143.9	130.4	126.8	156.6	171.6	170.2	166.1	114.9	94.0	104.5	110.2	153.4	131.8
Rnf34	NM_001004075.1	91.0	118.5	105.9	105.1	163.6	111.8	117.5	131.0	146.5	133.0	139.8	156.7	170.0	163.4
Rnf2_predicted	XM_222726.3	120.8	157.7	114.2	130.9	207.7	123.8	115.5	149.0	123.2	102.9	113.1	107.8	126.1	117.0
Rnf7_predicted	XM_217235.3	123.0	80.1	107.6	103.6	132.3	140.7	142.4	138.5	121.3	108.3	114.8	114.3	174.0	144.1
Fbxl20	NM_022272.1	98.6	184.7	181.1	154.8	134.8	165.7	133.3	144.6	69.6	93.2	81.4	75.9	76.5	76.2
Fbxo33_predicted	XM_234205.3	132.0	160.0	121.2	137.7	176.4	132.5	130.9	146.6	93.1	89.9	91.5	77.2	59.0	68.1
Rnf166	XM_579073.1	94.0	149.4	100.6	114.7	169.3	123.1	119.2	137.2	113.4	90.5	101.9	78.5	99.1	88.8
Trim41_predicted	XM_220357.3	120.9	122.2	105.6	116.2	138.7	107.2	110.3	118.7	85.5	108.1	96.8	107.3	84.1	95.7
Rnf3_predicted	XM_223729.3	119.0	115.9	118.1	117.6	112.0	105.7	100.8	106.1	81.1	107.5	94.3	95.5	108.8	102.2
Cul2_predicted	XM_341542.2	121.1	64.9	104.2	96.7	76.3	101.4	112.4	96.7	104.4	76.9	90.6	90.5	88.0	89.2
Fbxl3a_predicted	XM_224478.3	86.1	103.7	100.3	96.7	118.2	110.5	107.5	112.1	67.3	81.6	74.4	68.1	83.1	75.6
Fbxo9_predicted	XM_576433.1	79.3	86.4	82.4	82.7	97.7	88.5	81.2	89.2	71.7	80.6	76.2	75.4	78.2	76.8
Rnf31_predicted	XM_344409.2	76.5	51.8	59.0	62.5	80.7	79.9	81.9	80.8	84.3	105.5	94.9	90.8	100.6	95.7
Rnf139_predicted	XM_235338.3	97.5	87.2	78.8	87.8	97.5	108.1	126.2	110.6	46.7	42.2	44.4	43.3	66.7	55.0
Fbxo8_predicted	XM_573904.1	98.4	55.0	79.0	77.5	73.8	80.6	91.2	81.9	79.1	73.6	76.3	71.8	74.5	73.1
Wwp1_predicted	XM_216361.3	65.4	113.5	99.6	92.8	79.9	87.2	89.5	85.6	51.8	46.5	49.1	51.6	74.0	62.8
Trim47_predicted	XM_221124.3	134.0	63.4	103.4	100.3	39.8	60.6	66.5	55.7	46.1	84.9	65.5	57.0	58.0	57.5
Rnf138	NM_053588.2	99.6	63.5	63.3	75.5	102.5	89.9	96.6	96.3	51.8	39.2	45.5	56.5	50.2	53.4
Fbxo8_predicted	XM_573904.1	63.7	27.6	56.7	49.3	62.5	69.1	63.4	65.0	83.3	61.8	72.6	79.6	92.5	86.0
Hace1_predicted	XM_342160.2	59.3	67.4	55.4	60.7	60.4	62.4	51.8	58.2	85.2	74.7	80.0	67.3	67.4	67.4
Trim21_predicted	XM_219011.3	61.6	89.3	71.1	74.0	72.2	66.2	66.5	68.3	50.1	68.3	59.2	52.0	50.7	51.4
Cul5	NM_022683.1	48.2	59.4	56.1	54.6	66.3	56.3	75.9	66.2	54.9	49.9	52.4	54.4	103.9	79.1
Fbxw9_predicted	XM_213838.2	49.3	71.9	47.6	56.3	79.5	83.8	74.2	79.2	42.5	49.1	45.8	35.8	43.0	39.4
Rnf17_predicted	XM_224231.3	38.0	135.4	64.1	79.2	128.5	72.4	71.6	90.8	5.2	4.5	4.9	8.3	6.9	7.6
Trim39	NM_213562.1	41.8	45.6	40.1	42.5	56.0	45.2	47.7	49.7	53.9	66.3	60.1	62.3	66.9	64.6
Rnf110_predicted	XM_213447.3	40.0	32.5	34.3	35.6	34.7	43.1	48.2	42.0	36.6	51.0	43.8	46.5	63.9	55.2
Rnf38	NM_134467.1	38.5	42.2	48.4	43.0	37.9	49.4	47.3	44.8	23.0	36.2	29.6	32.8	49.2	41.0
Rnf125_predicted	XM_341581.2	99.0	50.9	53.9	68.0	46.5	31.3	36.5	38.1	33.9	15.9	24.9	14.0	9.4	11.7
Trim3	NM_031786.1	40.9	15.7	27.3	28.0	21.3	25.8	32.9	26.7	51.8	68.2	60.0	57.7	45.2	51.5
Rnf103	XM_579558.1	45.0	27.2	34.7	35.6	38.8	42.3	42.2	41.1	39.2	31.7	35.5	35.9	34.2	35.0

Fbxo5_predicted	XM_214756.3	33.0	31.7	30.7	31.8	47.1	38.4	39.2	41.6	42.4	37.1	39.8	33.9	31.1	32.5
Trim25_mapped	NM_001009536.1	28.0	31.5	29.9	29.8	30.3	23.1	28.4	27.3	36.1	45.9	41.0	35.5	47.3	41.4
Rnf121_predicted	XM_238909.3	36.0	26.3	21.9	28.1	21.8	29.4	37.5	29.5	30.6	32.5	31.5	38.5	35.7	37.1
Trim23	XM_342183.2	21.1	30.9	20.1	24.0	33.4	33.0	29.2	31.9	33.9	29.7	31.8	28.9	35.2	32.1
Rnf8_predicted	XM_342108.2	23.9	20.9	20.1	21.6	33.1	26.3	22.4	27.3	34.5	35.3	34.9	31.5	35.9	33.7
Fbxo10_predicted	XM_342829.2	19.9	38.1	21.0	26.4	62.9	38.6	32.6	44.7	17.6	18.2	17.9	13.3	17.3	15.3
Fbxo34_predicted	XM_223938.2	29.3	25.3	25.6	26.7	35.8	33.8	35.1	34.9	29.2	22.3	25.8	22.6	20.5	21.5
Fbxo4_predicted	XM_227090.3	24.4	10.7	20.5	18.5	20.4	23.8	25.3	23.2	41.6	33.4	37.5	39.2	38.0	38.6
Trim33_predicted	XM_345266.2	20.6	29.6	20.0	23.4	46.4	40.3	31.8	39.5	25.6	20.0	22.8	20.4	22.3	21.3
Trim37_predicted	XM_340872.2	16.1	23.9	18.0	19.3	56.0	35.4	30.9	40.8	22.3	21.5	21.9	17.5	17.8	17.6
Trim36_predicted	XM_225947.3	28.5	25.1	27.7	27.1	32.2	33.2	37.7	34.4	18.8	15.7	17.2	18.5	17.7	18.1
Trim45_predicted	XM_215666.3	15.4	34.9	24.1	24.8	51.1	28.6	24.2	34.6	17.8	18.6	18.2	18.7	14.9	16.8
Fbxw5_predicted	XM_342382.2	30.4	14.0	20.6	21.7	18.4	18.3	19.4	18.7	23.6	29.4	26.5	28.3	27.8	28.0
Rnf41_predicted	XM_576140.1	21.2	16.2	16.7	18.0	25.8	21.0	22.6	23.2	17.9	32.4	25.1	27.6	26.7	27.1
Trim2_predicted	XM_342268.2	24.0	26.5	18.4	23.0	23.1	23.3	21.6	22.6	18.9	21.9	20.4	24.9	19.5	22.2
Rnf4	NM_019182.1	21.8	20.4	16.2	19.5	28.8	23.3	21.5	24.5	21.8	21.7	21.7	22.9	20.6	21.8
Fbxo28_predicted	XM_223125.3	20.4	17.9	18.7	19.0	25.1	28.2	27.2	26.8	17.4	21.3	19.3	18.1	19.5	18.8
Rnf141	XM_579065.1	12.0	17.1	19.2	16.1	26.4	26.2	19.2	23.9	17.4	19.2	18.3	22.3	24.2	23.3
Rnf151_predicted	XM_220225.3	18.6	31.0	30.1	26.6	15.2	18.2	16.2	16.5	18.6	14.7	16.7	13.9	14.9	14.4
Rnf146_predicted	XM_574289.1	20.7	12.9	20.3	18.0	23.7	20.4	19.8	21.3	20.7	18.0	19.4	18.1	15.5	16.8
Rnf153_predicted	XM_215286.3	16.3	7.6	8.9	10.9	7.5	11.6	14.1	11.1	40.6	27.1	33.8	18.0	28.7	23.3
Trim34_predicted	XM_219045.3	11.4	11.9	11.4	11.6	16.4	17.7	18.7	17.6	19.0	19.2	19.1	19.4	19.8	19.6
Herc6	XM_342700.2	22.9	22.6	20.3	21.9	13.3	16.9	14.5	14.9	12.3	14.2	13.3	12.4	12.9	12.6
Ube3a_predicted	XM_341867.2	11.7	9.3	9.7	10.2	10.8	14.2	20.3	15.1	16.4	18.4	17.4	22.0	19.9	21.0
Fbxo15_predicted	XM_341633.2	15.2	22.2	17.3	18.2	17.0	15.0	13.4	15.1	13.4	11.1	12.2	15.3	11.9	13.6
Fbxw2_predicted	XM_231162.3	14.8	15.2	13.0	14.3	14.3	14.4	17.5	15.4	15.6	16.7	16.1	14.3	13.2	13.8
Fbxl12_predicted	XM_233735.1	19.5	9.1	10.1	12.9	12.0	12.4	13.3	12.5	18.8	15.4	17.1	11.9	13.0	12.4
Itch	NM_001005887.1	8.6	12.3	12.2	11.0	14.9	11.9	13.6	13.5	12.0	9.2	10.6	13.4	15.7	14.5
Fbxw8_predicted	XM_222223.3	10.9	8.4	9.3	9.5	12.8	7.1	10.3	10.0	15.9	14.4	15.1	14.8	15.7	15.3
Fbxo46_predicted	XM_218414.2	10.3	13.0	9.1	10.8	15.4	8.4	9.4	11.1	9.0	13.7	11.4	13.4	15.6	14.5
Trim13_predicted	XM_573800.1	10.0	17.7	12.4	13.4	13.5	8.5	8.3	10.1	10.9	13.4	12.2	13.1	8.1	10.6
Rnf39	NM_134374.1	13.5	10.6	11.7	11.9	10.2	17.0	15.1	14.1	10.6	9.4	10.0	7.9	7.1	7.5
Rnf26_predicted	XM_236181.2	8.5	9.0	6.6	8.0	12.9	9.8	8.0	10.2	18.7	11.1	14.9	13.8	10.1	12.0
Rnf6_predicted	XM_221888.3	14.7	8.2	8.7	10.5	10.4	9.3	10.1	9.9	11.5	12.0	11.8	13.2	10.4	11.8
Fbxw11_predicted	XM_220281.3	12.8	11.1	11.2	11.7	9.5	11.3	11.2	10.7	9.7	10.9	10.3	10.7	9.8	10.2
Trim14_predicted	XM_232992.3	11.4	10.1	8.0	9.8	14.4	10.7	11.4	12.2	9.7	9.0	9.3	10.8	11.8	11.3
Trim26	XM_574717.1	12.6	6.2	11.0	9.9	7.0	8.5	9.7	8.4	10.9	8.4	9.6	13.7	12.3	13.0
Rnf40	NM_153471.1	9.2	7.0	8.3	8.2	8.1	8.2	9.9	8.7	12.0	12.7	12.4	9.2	14.3	11.7
Trim29_predicted	XM_236207.3	7.5	11.7	8.6	9.2	20.4	14.3	11.8	15.5	7.5	5.1	6.3	5.7	4.9	5.3
Fbxl19_predicted	XM_219356.3	8.0	9.4	7.7	8.4	12.2	6.7	7.5	8.8	11.9	8.9	10.4	10.4	13.5	11.9
Fbxo16_predicted	XM_224297.3	10.5	13.5	10.9	11.7	19.1	10.3	6.5	12.0	7.0	3.6	5.3	5.0	6.0	5.5
Fbxl4_predicted	XM_232833.3	5.9	6.2	7.6	6.6	5.1	5.4	5.8	5.4	13.8	11.0	12.4	12.5	11.2	11.8
Fbxo39_predicted	XM_220647.3	7.2	6.8	7.4	7.1	10.0	6.5	6.9	7.8	9.1	7.8	8.4	7.7	10.0	8.8
Trim17	NM_022798.1	6.4	7.5	7.5	7.1	7.9	6.7	6.7	7.1	8.1	9.3	8.7	6.9	8.6	7.7
Trim16_predicted	XM_220552.3	8.6	9.6	7.6	8.6	7.3	6.7	8.2	7.4	6.8	7.8	7.3	6.1	5.4	5.8

Fbxo42_predicted	XM_342963.2	7.7	5.1	6.8	6.5	7.2	4.1	6.0	5.8	8.3	7.3	7.8	8.9	9.3	9.1
Fbxo43_predicted	XM_576249.1	8.7	10.2	10.0	9.6	8.0	8.2	8.4	8.2	4.5	4.7	4.6	4.1	3.7	3.9
Trim46_predicted	XM_227414.3	5.1	8.7	6.5	6.7	4.4	7.2	5.6	5.7	8.6	6.7	7.7	7.1	9.1	8.1
Rnf135_predicted	XM_573156.1	4.7	5.0	5.8	5.2	6.5	5.6	6.7	6.3	9.3	10.4	9.9	6.5	7.2	6.8
Rnf41_predicted	XM_576140.1	6.0	5.1	7.4	6.1	4.1	5.5	7.1	5.6	8.4	6.9	7.6	8.1	9.2	8.6
Park2	NM_020093.1	6.3	4.9	6.2	5.8	5.9	5.7	6.7	6.1	8.9	6.4	7.7	8.3	7.9	8.1
Fbxo9_predicted	XM_576433.1	5.3	6.8	6.9	6.3	6.5	5.8	4.7	5.7	5.9	9.7	7.8	6.5	8.3	7.4
Fbxo32	NM_133521.1	8.1	5.9	4.4	6.1	4.9	2.9	5.7	4.5	8.6	5.8	7.2	9.7	7.9	8.8
Rnf30_predicted	XM_343026.2	7.9	14.1	14.0	12.0	5.1	4.2	4.1	4.4	3.5	2.5	3.0	3.2	3.3	3.2
Fbxl17_predicted	XM_237458.3	4.3	5.5	5.6	5.2	5.8	6.0	4.9	5.6	8.7	5.5	7.1	7.3	7.1	7.2
Trim31_predicted	XM_227942.2	5.2	6.6	6.2	6.0	6.4	5.8	6.6	6.3	5.8	6.3	6.1	5.2	5.2	5.2
Rnf32_predicted	XM_575314.1	5.5	5.8	7.0	6.1	6.4	5.2	4.9	5.5	5.4	6.6	6.0	4.9	6.4	5.6
Rnf44_predicted	XM_341494.2	6.3	5.2	5.5	5.7	7.1	5.9	5.8	6.3	5.1	3.5	4.3	8.0	5.1	6.5
Trim63	NM_080903.1	5.2	8.0	7.9	7.0	5.1	5.7	4.1	5.0	5.0	5.5	5.2	5.1	5.7	5.4
Fbxo2	NM_053511.1	5.9	6.8	5.8	6.2	7.6	6.8	7.6	7.3	2.9	5.5	4.2	5.0	3.3	4.1
Fbxo24_predicted	XM_222016.3	5.2	5.2	6.9	5.8	4.9	5.2	5.3	5.2	5.0	6.6	5.8	5.5	7.1	6.3
Trim10	XM_579280.1	5.7	5.2	4.8	5.3	4.8	6.4	5.2	5.5	5.7	5.0	5.3	6.8	7.2	7.0
Trim13_predicted	XM_573800.1	4.5	5.3	3.7	4.5	4.8	8.2	6.3	6.5	4.5	6.0	5.3	8.9	4.3	6.6
Fbxo36_predicted	XM_343610.2	6.0	8.2	5.8	6.6	6.2	5.1	4.6	5.3	4.5	3.6	4.0	4.2	5.5	4.8
Trim50	NM_181080.1	6.3	6.9	4.5	5.9	5.3	5.3	4.6	5.1	5.7	4.3	5.0	3.8	6.4	5.1
Fbxl14_predicted	XM_232330.3	5.5	4.1	4.1	4.6	8.7	6.0	5.9	6.9	3.5	5.0	4.2	6.2	3.7	5.0
Fbxl7_predicted	XM_342204.2	4.1	2.5	4.0	3.5	2.8	4.2	4.1	3.7	5.6	4.3	4.9	7.4	11.1	9.2
Rnf24_predicted	XM_342522.2	5.1	3.3	4.4	4.3	5.7	4.6	6.0	5.4	2.7	4.8	3.8	6.2	5.0	5.6
Trim11_predicted	XM_340806.2	4.2	3.5	3.6	3.7	3.2	6.8	4.5	4.8	5.0	4.5	4.8	4.6	6.3	5.4
Rnf36_predicted	XM_230524.2	3.3	3.4	2.0	2.9	3.7	5.4	4.1	4.4	4.2	3.1	3.6	4.7	6.0	5.4
Fbxo40_predicted	XM_344023.2	2.7	5.0	3.9	3.9	2.2	3.6	4.5	3.4	4.4	2.8	3.6	3.2	6.6	4.9
Rnf29_predicted	XM_574905.1	3.0	4.7	4.4	4.0	2.0	2.7	4.7	3.2	4.8	2.8	3.8	3.4	2.6	3.0
Deubiquitinating Enzymes															
Usp14	NM_001008301.1	969.6	998.8	1016.2	994.9	1043.4	1193.9	1286.7	1174.7	978.0	834.6	906.3	914.5	1039.1	976.8
Usp3_predicted	XM_343415.2	453.7	583.5	519.7	519.0	452.1	347.6	348.5	382.7	299.1	314.1	306.6	236.5	208.0	222.2
Usp7_predicted	XM_340747.2	296.8	303.2	240.1	280.0	251.7	297.8	314.8	288.1	280.7	264.7	272.7	254.1	506.9	380.5
Usp16_predicted	XM_213676.3	290.4	256.3	269.7	272.1	227.2	269.0	298.3	264.8	312.9	248.1	280.5	263.0	322.1	292.5
Usp47_predicted	XM_218997.3	187.2	236.1	169.3	197.5	207.3	249.2	248.8	235.1	262.5	228.1	245.3	225.0	217.5	221.2
Usp48	NM_198785.1	150.0	238.4	175.2	187.9	172.2	193.8	177.3	181.1	179.2	160.7	170.0	164.5	178.6	171.5
Uchl1	NM_017237.1	83.6	274.2	198.4	185.4	252.9	192.4	123.3	189.5	49.2	48.3	48.8	41.9	42.2	42.0
Usp5_predicted	XM_238380.3	201.0	145.8	167.9	171.5	123.8	156.2	156.8	145.6	176.8	166.1	171.5	161.6	175.7	168.7
Usp15	NM_145184.1	142.8	155.5	149.8	149.3	173.1	167.0	162.8	167.7	107.1	112.3	109.7	100.7	107.7	104.2
Usp19	XM_573962.1	148.6	145.3	148.8	147.6	144.1	160.4	133.5	146.0	153.9	141.8	147.8	138.4	157.7	148.0
Uchl5_predicted	XM_573467.1	119.5	129.9	192.9	147.4	201.5	142.8	151.1	165.1	175.4	118.9	147.1	169.0	193.8	181.4
Usp2	NM_053774.2	93.8	130.3	100.0	108.0	90.8	62.2	71.7	74.9	38.9	45.7	42.3	26.1	36.4	31.3
Usp30_predicted	XM_222274.3	101.9	109.4	82.0	97.8	110.5	117.3	113.8	113.8	62.6	79.6	71.1	67.2	79.2	73.2
Usp39_predicted	XM_216173.3	82.8	91.9	87.2	87.3	109.3	94.7	95.1	99.7	95.6	84.1	89.9	80.4	81.9	81.2
Usp1_predicted	XM_233217.3	74.6	74.7	60.6	70.0	113.0	74.9	83.7	90.5	106.7	72.5	89.6	74.4	107.6	91.0
Usp45_predicted	XM_232828.3	87.8	60.1	59.6	69.1	63.3	76.5	78.4	72.7	101.5	95.5	98.5	100.3	115.3	107.8
Usp28_predicted	XM_236240.3	27.0	84.2	52.1	54.4	68.2	50.8	36.7	51.9	26.6	28.1	27.3	29.7	20.9	25.3

Usp25_predicted	XM_221722.3	48.2	44.8	43.0	45.3	95.2	115.8	103.4	104.8	68.9	58.3	63.6	72.2	85.1	78.6
Usp52	XM_579254.1	33.4	58.8	41.8	44.7	56.4	52.1	40.9	49.8	26.4	25.1	25.7	20.7	18.6	19.6
Usp24_predicted	XM_233260.3	23.5	64.2	35.2	41.0	59.0	59.1	57.7	58.6	21.7	25.7	23.7	25.9	25.6	25.8
Usp12_predicted	XM_341033.2	31.9	40.5	49.3	40.6	45.9	42.3	41.4	43.2	33.3	34.6	33.9	43.4	59.8	51.6
Usp13_predicted	XM_227025.3	47.1	22.8	34.0	34.6	26.5	44.2	43.7	38.1	53.6	44.5	49.0	59.2	52.3	55.7
Usp11	XM_579253.1	34.2	35.9	32.8	34.3	29.9	28.8	25.7	28.1	42.2	41.2	41.7	33.5	33.3	33.4
Usp8_predicted	XM_215821.3	36.0	26.9	32.2	31.7	44.3	38.2	34.0	38.8	48.3	39.1	43.7	43.8	46.7	45.2
Usp53_predicted	XM_215700.3	23.3	31.1	23.0	25.8	29.1	26.0	25.0	26.7	13.8	15.7	14.7	11.6	14.1	12.8
Usp19	XM_573962.1	21.2	23.5	26.7	23.8	33.1	38.3	38.3	36.6	10.5	14.1	12.3	14.3	12.5	13.4
Usp42_predicted	XM_237865.3	19.8	28.0	18.0	21.9	26.6	17.7	16.8	20.4	20.8	20.6	20.7	24.7	23.1	23.9
Uchl3	XM_575294.1	21.9	12.1	27.6	20.5	34.7	25.8	21.4	27.3	24.7	25.3	25.0	31.7	27.4	29.5
Usp36_predicted	XM_221143.3	21.1	14.0	22.4	19.2	20.3	23.0	27.1	23.5	14.8	16.0	15.4	16.5	20.4	18.5
Usp49_predicted	XM_236917.3	14.9	8.8	12.2	12.0	15.7	11.6	14.5	13.9	9.9	10.1	10.0	13.8	11.8	12.8
Usp33_predicted	XM_227811.3	14.0	10.7	11.0	11.9	18.3	18.3	19.0	18.5	20.2	16.5	18.3	21.2	30.5	25.8
Usp43_predicted	XM_220582.3	7.7	14.5	8.5	10.2	6.9	7.7	6.2	7.0	7.1	4.5	5.8	8.4	5.4	6.9
Usp44_predicted	XM_235070.3	8.6	12.6	7.6	9.6	15.3	12.4	12.7	13.4	6.2	3.2	4.7	5.5	7.1	6.3
Usp40_predicted	XM_237371.3	9.2	11.2	7.9	9.4	17.7	18.2	12.2	16.0	22.7	20.4	21.6	16.6	15.2	15.9
Usp38_predicted	XM_226362.3	9.3	6.9	8.2	8.2	11.0	8.2	11.7	10.3	10.7	9.8	10.3	10.2	10.9	10.5
Usp6nl_predicted	XM_214508.3	9.5	6.5	8.7	8.2	9.1	5.7	6.5	7.1	8.9	8.5	8.7	6.2	8.4	7.3
Otub2_predicted	XM_234481.3	7.8	7.6	7.7	7.7	6.8	8.1	8.9	7.9	8.2	9.9	9.1	5.8	8.5	7.2
Usp54	XM_579255.1	7.8	7.1	6.6	7.2	9.3	7.1	10.5	9.0	9.6	10.6	10.1	9.5	8.5	9.0
Usp20_predicted	XM_231148.3	6.9	7.2	5.5	6.5	5.7	6.6	6.5	6.3	7.6	7.7	7.7	8.3	8.7	8.5
Usp32_predicted	XM_220798.3	6.8	7.1	5.0	6.3	16.0	14.4	10.0	13.5	12.7	8.7	10.7	11.5	14.2	12.8
Atxn3	NM_021702.1	6.4	5.9	5.2	5.8	8.6	8.8	9.7	9.0	8.8	7.0	7.9	9.0	6.6	7.8
Usp31_predicted	XM_219292.3	5.4	4.9	4.5	5.0	5.7	4.6	6.6	5.6	4.5	4.7	4.6	7.5	3.6	5.5
Other Ubiquitin-related Genes															
Ubb	XM_579661.1	4469.4	4820.2	4937.6	4742.4	5382.7	4378.3	4538.5	4766.5	3843.7	3786.4	3815.0	3492.0	3403.9	3448.0
Uba52	NM_031687.2	2557.9	2256.2	2601.3	2471.8	2799.9	2898.3	2827.7	2842.0	2587.3	2570.6	2578.9	2862.1	2329.7	2595.9
Ubqln1	NM_053747.2	857.9	884.8	944.1	895.6	992.3	1015.3	1043.4	1017.0	1041.8	1043.4	1042.6	1105.0	1133.9	1119.5
Nedd8	NM_138878.1	793.9	690.9	980.0	821.6	1100.5	1041.8	975.5	1039.3	799.1	769.1	784.1	918.6	946.0	932.3
Ube4b_predicted	XM_233679.3	235.6	199.4	202.4	212.5	154.1	174.6	186.9	171.9	233.7	214.2	224.0	219.7	185.1	202.4
Ubap2_predicted	XM_232901.3	126.6	224.7	162.6	171.3	199.7	175.6	160.3	178.5	169.3	135.6	152.4	153.0	161.6	157.3
Ubtd1_predicted	XM_219869.3	104.0	106.7	100.6	103.8	59.9	73.1	74.1	69.0	118.6	113.9	116.3	120.4	112.3	116.4
Ubl3_predicted	XM_344075.2	114.7	59.6	83.2	85.8	96.9	84.4	120.5	100.6	89.6	61.1	75.3	80.1	107.3	93.7
Ubap1_predicted	XM_575802.1	51.8	23.1	50.2	41.7	40.7	41.1	43.9	41.9	25.9	29.6	27.8	30.4	22.4	26.4
Ubd	NM_053299.1	34.6	11.8	19.6	22.0	6.1	7.8	7.7	7.2	7.6	32.9	20.3	6.6	6.0	6.3
Uhrf2_predicted	XM_219801	3.1	2.2	6.4	3.9	4.8	3.4	3.1	3.7	4.0	4.1	4.0	4.9	4.4	4.6

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Connecting Text: Chapter 3 and 4

The primary objective of chapter 3 was to determine other molecular processes involved in gonocyte differentiation. Given the large number of changes that gonocytes must undergo within a short period of time in order to develop properly, we hypothesized that the ubiquitin proteasome system was activated during RA-induced differentiation. Using specific, commonly used inhibitors to block the proteasome (Lactacystin and Bortezomib), we found that inhibiting proteasome activity prevented the RA-induced upregulation of both STRA8 and DAZL mRNAs, indicating that when the proteasome was inhibited, so was gonocyte differentiation. Furthermore, gene array analysis found a large number of UPS related genes expressed in varying amounts between PND3 gonocytes and PND8 spermatogonia, demonstrating how the UPS itself was remodeled during differentiation. In depth analysis of the gene expression profiles of UPS-related genes and enzymes showed that an E3 ubiquitin ligase, RNF149, was not only downregulated in RA-treated gonocytes but also, in PND8 spermatogonia. Thus, RNF149 either plays a preferential role during gonocyte development and is downregulated thereafter, or it is actively downregulated in order for gonocyte differentiation to occur. As seen in both chapters 2 and 3, there are many processes that govern gonocyte differentiation. Given that improper gonocyte development has been suggested as the likely cause for testicular germ cell tumor formation, the results from the previous chapters showed that there are many processes that could be perturbed, preventing regular gonocyte development, including PDGFR signaling and UPS activation. Thus, the goal of chapter 4 was to determine what genes/processes are not only involved in gonocyte differentiation, but may also play a role in testicular germ cell tumor formation. For this, we performed gene array analyses and explored a variety of genes, their expression patterns, and functional networks in gonocyte differentiation and in testicular tumors. All in all, findings from chapter 4 provide a better insight into how improper gonocyte development could lead to testicular tumor formation.

Chapter 4

Identification of genes overexpressed in testicular seminoma tumors and downregulated during gonocyte differentiation as potential players in the origins of testicular cancer

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Manuscript in Preparation

4.1 Abstract

Testicular germ cell tumors (TGCTs) are the most common type of cancer in young men today and their incidence has been increasing steadily for the past decades. Little is known about the underlying causes of TGCT and its precursor stage, Carcinoma In-Situ (CIS). It has been suggested that CIS and TGCT arise from the deficient differentiation of primordial germ cells or gonocytes, the precursors of spermatogonial stem cells. In this present study, our goal was to uncover possible molecular mechanisms involved in gonocyte development and retained in testicular tumors to better understand tumor formation. To identify common gene signatures between gonocytes and seminomas, we performed comparative gene expression analyses of postnatal day (PND)3 gonocytes versus PND8 spermatogonia, and human normal testicular versus TGCT specimen, including the human TCam-2 seminoma cell line. We found that TCam-2 cells and seminoma biopsies expressed a 1.5kb PDGFRα variant previously identified in TGCTs, which was not seen in normal testicular tissue. Western blot analysis of PND3 gonocytes also showed presence of a PDGFRα variant. Moreover, PDGFRα protein expression paralleled that of OCT4, a transcription factor overexpressed in seminomas. Gene expression arrays and pathway analysis identified cell adhesion molecules as a functional category of genes that was highly upregulated in both PND3 gonocytes and seminoma tumors. Quantitative PCR analysis of these molecules confirmed that various members of the claudin family were significantly upregulated in both cell types. Claudins are normally involved in tight junctions and both under- and over-expression of these proteins has been reported in various tumor types. The high expression of claudin 6 and 7 in seminoma tumors suggests that these genes failed to be downregulated during the transition from gonocyte to spermatogonia, providing a potential link between their deregulation and tumor formation. Our findings not only support the idea that TGCTs arise from a disruption of early male germ cell development, but also provide potential new gene targets, such as the claudin family, to study further in relation to TGCT formation.

4.2 Introduction

There are three main types of testicular tumors in humans based on a patients age. Teratomas and yolk sac tumors are found in newborns and infants, seminomas and non-seminomas are found in adolescents and young adults, and spermatocytic seminomas are found in the older population (1). Testicular germ cell tumors (TGCTs) are the most common solid malignant tumors in young men aged between 17-45 years old and make up approximately 98% of all testicular cancers (2). TGCTs in young adults can be divided into two main categories: classic seminomas (55%) and non-seminomas (44%) including embryonal carcinomas (ECs), teratomas, yolk sac tumors, and choriocarcinomas (2, 3, 4). The incidence of TGCTs has shown much variation among different countries and different ethnic backgrounds (5) and, in general, the rates of this type of tumor have been increasing for the past several decades (6). Although the exact reasons remain to be elucidated, the rapidity of this increase suggests it may be due to environmental factors; more specifically, the exposure of endocrine disruptors during embryogenesis (7). Despite environmental factors being considered as the main culprit, it is likely that genetic factors also play an important role in TGCT development (8, 9). Although many factors may play a role, the exact etiology of the disease still remains unknown.

Pure seminoma tumors are weakly differentiated and are quite similar to primordial germ cells/gonocytes (10). TGCTs have a number of markers that provide better means of characterization of these tumors. Where markers such as PLAP (placenta alkaline phosphatase) (11), NANOG (12), and OCT3/4 (13) are seen in a variety of TGCTs, seminomas alone have high expression levels of AP2γ (14), c-KIT (3), and SOX17 (15). Therefore, it is a combination of these genes that is currently used as clinical markers to diagnose seminoma tumors in patients (3). Furthermore, Palumbo C et al have shown that there is a 1.5kb variant form of the platelet-derived growth factor receptor alpha (PDGFRα) that is only present in seminoma tumors (and not in the normal testis) and can potentially be used as a marker for diagnostic patient screening (16).

One major problem in studying TGCTs is the lack of available animal and cell line models. Although some cell lines, such as JKT-1, have been established (17), further characterization has revealed that these cells are more similar to non-seminoma tumors and do not accurately portray seminoma tumors (18). The lack of a proper seminoma cell line model was apparent until 1993,

when in Japan, Mizuno Y et al established the TCam-2 human testicular seminoma cell line, originating from a 35 year old patient (19, 20). Not unlike other cell lines, the TCam-2 cell line may eventually behave differently in vitro than the original in vivo tumor, further transforming with successive passages in culture conditions that do not mimic its native physiological environment (10). However, as of yet, this is the only cell-line model available to study seminoma tumors.

Although the exact origin of TGCTs is not known, it has been suggested that seminoma tumors arise from a failure of gonocytes (the fetal germ cells) to develop properly. This failure could lead to the formation of carcinoma in-situ (CIS), the precursor lesion from which TGCTs arise (21, 22). Gonocytes, also known as pre- or pro-spermatogonia in their later phase of development, are the precursor cells to spermatogonial stem cells (SSCs) (23). Spermatogenesis is a complex process that relies on the existence of a pool of SSCs that will support the production of spermatozoa (24, 25). Proper gonocyte development requires multiple timed events, including phases of quiescence, proliferation, migration, differentiation, and apoptosis (23). Previously, we have shown that gonocytes proliferate in response to PDGF and 17β-estradiol, via activation of the estrogen receptor (ER) and PDGFR pathways at post natal day (PND) 3 (26, 27). Interestingly, PDGFR is not only important in proliferation, but we have recently shown that this pathway is also activated during retinoic acid (RA)-induced gonocyte differentiation (28). Gonocyte differentiation also requires the cell to migrate and make contact with the basement membrane of the seminiferous cord (23), implying that there are major changes in the behavior and signaling profile of these cells. Given the importance of PDGFR pathway activation during both gonocyte proliferation and differentiation and presence of a 1.5kb variant form of PDGFRα in human testicular seminoma tumors (16), it is possible that the PDGFR pathway is among the mechanisms deregulated during gonocyte development that plays a role in TGCT formation.

In view of the potential relationship between disrupted gonocyte development and TGCT formation, this study focused on comparing gene and protein expression between PND3 gonocytes, the TCam-2 cell line and human specimen of normal testes and testicular seminoma. From this analysis, several gene candidates emerged that might bring new light to the mechanisms involved in TGCT formation.

4.3 Materials and Methods

Animals

Newborn male Sprague Dawley rats were purchased from Charles Rivers Laboratories (Saint-Constant, QC, CA). PND3 and PND8 pups were euthanized and handled according to protocols approved by the McGill University Health Centre Animal Care Committee and the Canadian Council on Animal Care.

Germ Cell Isolation

Using 30-40 pups per preparation, gonocytes were isolated from PND3 rat testes as previously described (26, 27, 29-31). Briefly, testes were first decapsulated and then isolated by sequential enzymatic tissue dissociation, filtration, and differential plating overnight in RPMI 1640 medium (Invitrogen, Burlington, ON, CA) with 5% fetal bovine serum (FBS) (Invitrogen), 2% penicillin/streptomycin (CellGro, Manassas, VA, USA), and 1% amphotericin B (CellGro). The next morning, non-adherent cells were further separated using a 2-4% bovine serum albumin (BSA) (Roche Diagnostics, Indianapolis, IN, USA) gradient. Gonocytes were judged by their morphology and larger size compared to Sertoli/myoid cells. Fractions that contained the most gonocytes were pooled, centrifuged, and collected with a final purity of at least 95% for the gene array analysis and a purity of at least 85% for the reverse transcriptase polymerase chain reaction (RT-PCR), quantitative-PCR (qPCR), and immunoblot analysis. After collection, the cells were directly frozen for later RNA extraction. Using 10 pups per preparation, spermatogonia were isolated from PND8 rat testes. The method used to isolate spermatogonia was the same that was used for gonocyte isolation described above (26, 27, 29-31). All experiments were performed using a minimum of three independent gonocyte or spermatogonia preparations. Enriched Sertoli cell populations (that also contained ~20% myoid cells) were collected at both PND3 and PND8 ages and used as comparison samples in the gene array analysis.

TCam-2 Cell Culture

TCam-2 human testicular seminoma tumor cells (kindly provided by Dr. Sohei Kitazawa, Kobe University, Kobe, Japan) were maintained in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 2% penicillin/streptomycin (CellGro), and 1% amphotericin B (CellGro) at 37 °C and 5.0% CO_2. Cells were collected once they were ~80% confluent and cell pellets were frozen for later RNA or protein extraction. For

immunocytochemical analysis of non-treated cells, TCam-2 cells were grown directly in 8-chamber microscopic slides (BD Falcon, Mississauga, ON, CA). Once cells were ~80% confluent, chambers were washed with phosphate buffered saline (PBS) (Invitrogen), fixed with 3.7% paraformaldehyde (Invitrogen) in PBS for 7 minutes, washed using PBS (Invitrogen) for 5 minutes, and then treated with 100% ethanol (Commercial Alcohols Inc., Brampton, ON, CA) for 5 minutes. To examine TCam-2 cell responsiveness to retinoic acid (RA), cells were plated on day 0 at a density of 10000 cells/well in 8-well chamber slides. One day later, the cells were treated with either control media or all-trans retinoic acid (10^{-7}M) (Sigma Aldrich, Oakville, ON, CA) for 3 days. The cells were then fixed using protocol described above. Chamber slides were stored at 4° with PBS until ready for analysis. All experiments were performed using a minimum of three independent TCam-2 cell passages from different initial batches of cells.

Human Testicular Tissue

Human testicular tissues (both normal and tumoral specimen) for mRNA analysis were provided by Dr. Peter Chan (Department of Surgery, Division of Urology, McGill University, Montreal, QC, CA) or extracted RNA was purchased from Oncomatrix (San Marcos, CA, USA). Microscopic slides of normal and tumoral testicular tissues were provided by Dr. Fadi Brimo (Department of Pathology, McGill University, Montreal, QC, CA). All samples were obtained under supervision of the institutional ethics review board and informed consent was obtained from all subjects who provided the biopsy samples.

RNA extraction and cDNA synthesis

Total RNA was extracted from cell pellets using the PicoPure RNA isolation kit (Arcturus, Mountain View, CA, USA) and digested with DNase I (Qiagen, Valencia, CA, USA). Total RNA was extracted from human testicular tissue (normal and tumoral biopsies) using a RNeasy Protect Mini Kit (Qiagen). For RT-PCR and qPCR analysis, cDNA was synthesized from the extracted RNA by using the single strand cDNA transcriptor synthesis kit (Roche Diagnostics).

Reverse transcriptase (RT)-PCR analysis

Expression of various genes in rat and human samples was examined by RT-PCR analysis. The primer sets used were designed using Primer3 software available online (32, 33) (Table 4.1). Reactions were carried out using Platinum Taq DNA polymerase (Invitrogen) and amplified using the Bio-Rad iCycler thermal cycler (Bio-Rad, Hercules, CA, USA). PCR cycle conditions

were: 94°C for 3 min; 40 cycles of 94°C for 30 sec, 50°-60°C (depending on primer set) for 30 sec, and 72°C for 30 sec; followed by a 5 min extension at 72°C. Resulting PCR products were separated on a 2% agarose gel (Agarose: Bioline, Taunton, MA, USA; TAE buffer: Bio-Rad) and samples were made visible using an EZ-Vision One DNA loading dye (Amresco, Solon OH, USA). Gels were then viewed using the LAS-4000 gel documentation system (Fujifilm, Mississauga, ON, CA). For all samples, 18S primer was used as loading control.

Gene Array

Rat Illumina microarray analysis was performed by the McGill University's Genome Quebec facility. As previously described, three independent RNA extracts were analyzed for rat germ cells and two independent extracts were processed for somatic cells (34). To have sufficient RNA concentrations (100ng/µl) per sample, each gonocyte sample preparation included 60-90 PND3 pups, while each spermatogonia sample preparation used 10 PND8 pups. Each sample was prepared from germ cell populations at a purity of at least 95%. After RNA quality was verified at Genome Quebec, the samples were analyzed using the RatRef-12 Expression BeadChip for genome-wide expression analysis (Illumina, San Diego, CA, USA). This chip contains 22523 probes selected primarily from the NCBI RefSeq database. Human Affymetrix microarray analysis was also performed by the McGill University's Genome Quebec facility. Here, the RNA analyzed was either isolated from human testicular normal or tumoral tissues. RNA extracted from two independent passages of TCam-2 cells was also analyzed. All human samples were analyzed using the HuGene-1_0-st-V1 array chip (Affymetrix, Santa Clara, CA, USA). This chip contains 33297 probes selected mainly from the NCBI RefSeq database. Data normalization and preliminary data analysis for both series of arrays, including quality control analysis, dispersion analysis, differential analysis of gene expression, and gene set enrichment analysis, was performed by Dr. Jaroslav Novak (35). All data were normalized using the quantile normalization method, corrected for the background signal, and abbreviated by eliminating the low-expression-end of the data spectrum. The various comparisons made between samples were done using the Bayesian approach, which emphasizes the significance of variation between the different replicates of the same sample and does not take into consideration the commonly used "fold-change" method. However, using the Bayesian approach, no comparison remaining in either the rat or human gene array had a fold change smaller than 2.0 (rat array) or 3.6 (human array) once calculated. This indicates that for our data, the Bayesian

approach was a well suited method for analysis. For both arrays, a cut off of signal intensity was applied, in which genes that had an average value of signal intensity at less than 50 in both samples in each comparison were eliminated. Then, genes that were significantly changed within each comparison (0.9 confidence interval, p-value $\leq$0.05) were analyzed using the DAVID Bioinformatics Resources version 6.7 software (NIH, Bethesda, MD, USA) and its built-in KEGG (Kyoko Encyclopedia of Genes and Genomes) pathway analysis to determine functional pathway clustering. The gene array data was also analyzed using the Ingenuity Pathway Analysis (IPA; Qiagen) for another source of pathway and cluster analysis. Finally, potential functional partners of candidate genes were obtained using 'Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) 9.05 (36) where functional partner predictions were based on available experimental data, data bases, textmining, and homology.

Quantitative PCR (qPCR)

qPCR was performed using a LightCycler 480 with a SYBR Green PCR Master Mix kit (Roche Diagnostics). Gene and species specific primers were designed using the Roche primer design software (Roche Diagnostics) (Table 4.2). The qPCR cycle conditions were as follows: an initial step at 95°C followed by 45 cycles at 95°C for 10 sec, 61°C for 10 sec, and 72°C for 10 sec. This was followed by both melting curves and cooling cycles. Direct detection of PCR products was monitored by measuring the increase in fluorescence caused by the binding of SYBR Green dye to double-stranded DNA, and the comparative threshold cycle (C_t) method was used to analyze the data. Analysis of both 18S rRNA and GAPDH as housekeeping genes showed that it was 18S rRNA expression that varied the least between samples, thus making it the ideal choice for a housekeeping gene. The final data were expressed in a relative unit representing the mRNA levels of the gene of interest present in the samples tested. Assays were performed in triplicate. All experiments were performed using a minimum of three independent sample preparations and the mean ± SEM are shown.

Immunoblot Analysis

PND3 gonocytes and TCam-2 cells were solubilized in Laemmli buffer and protein concentrations were determined using the BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). The proteins were then separated on pre-casted 4-20% tris-glycine gels (Invitrogen). Once the run was complete, the gels were transferred to a polyvinylidene fluoride (PVDF)

membrane (Bio-Rad). After blocking with 5% milk, membranes were probed with a PDGFRα antibody (Upstate/Millipore, catalog number 07-276 (lot number 30083), Billerica, MA, USA) to determine protein expression. After an overnight incubation at 4°, bound antibodies were detected using anti-biotin and horseradish peroxidase (HRP) coupled goat anti-rabbit secondary antibodies (Cell Signaling, Danvers, MA, USA) and ECL-enhanced chemiluminescence (GE Healthcare, Mississauga, ON, CA). Images were taken using the LAS-4000 gel documentation system (Fujifilm). After PDGFRα detection, the antibody was stripped away with Restore Plus Western blot stripping buffer (Thermo-Scientific) and the membrane was further probed with the loading reference Tubulin (Abcam, catalog number ab40742, Toronto, ON, CA).

Immunohistochemistry

All slides were stained using previously described methods (37). In brief, slides were first dewaxed and rehydrated using Citrosolv (Fisher Scientific, Toronto, ON, CA) and Trilogy solution (Cell Marque IVD, Rocklin, CA, USA). Following treatment with Dako Target Retrieval solution (DAKO, Burlington, ON, CA), the sections were incubated with PBS (Invitrogen) that contained 10% goat serum (Vector Laboratories, Burlington, ON, CA), 1% BSA (Roche Diagnostics) and 0.02% Triton X100 (Promega, Madison, WI, USA) for 60 minutes to block non-specific protein interactions. The sections were then incubated with primary antibodies diluted in PBS (Invitrogen) containing 1% BSA (Roche Diagnostics) and 0.02% Triton X100 (Promega) at various dilutions (Table 4.3) overnight at 4°C. Once the overnight incubation was complete, the sections were incubated with biotin-conjugated secondary antibodies (diluted in PBS (Invitrogen) containing 1% BSA (Roche Diagnostics)) for 60 minutes at room temperature (Table 4.3). Immunoreactivity was detected using a combination of streptavidin-peroxidase (Invitrogen) and AEC single use solution (Invitrogen). The sections were then counter-stained with hematoxylin (Sigma Aldrich), coated with Crystal Mount (Electron Microscopy Sciences, Hatfield, PA, USA) and dried, and then cover-slipped. Slides were examined with a BX40 Olympus microscope (Olympus, Center Valley, PA, USA) coupled to a DP70 Olympus digital camera (Olympus). Negative controls were done by incubating some sections with Rabbit IgG (pre-immune rabbit serum) (Invitrogen) instead of primary antibody.

Immunocytochemistry

For immunocytochemical analysis, TCam-2 cells were grown directly in 8-chamber microscopic slides (BD Falcon), fixed when ~80% confluent and stored at 4° with PBS (Invitrogen) until ready to stain. The protocol was as previously described (26, 30). In brief, cells were treated with Dako Target Retrieval solution (DAKO) and then blocked with PBS (Invitrogen) that contained 10% goat serum (Vector Laboratories), 1% BSA (Roche Diagnostics) and 0.02% Triton X100 (Promega) for 60 minutes to block non-specific protein interactions. The cells were then incubated with primary antibodies diluted in PBS (Invitrogen) containing 1% BSA (Roche Diagnostics) and 0.02% Triton X100 (Promega) at various dilutions (Table 4.4) overnight at 4°C. Once the overnight incubation was complete, the cells were incubated with a biotin-conjugated secondary antibody (diluted in PBS (Invitrogen) containing 1% BSA (Roche Diagnostics)) for 60 minutes at room temperature (Table 4.4). Immunoreactivity was detected using a combination of streptavidin-peroxidase (Invitrogen) and AEC single use solution (Invitrogen). The cells were counter-stained with hematoxylin (Sigma Aldrich) and coated with Crystal Mount (Electron Microscopy Sciences). TCam-2 immunocytochemistry results were viewed with a BX40 Olympus microscope (Olympus) coupled to a DP70 Olympus digital camera (Olympus). Pre-immune rabbit serum (Invitrogen) was used as a negative control.

Statistical analysis

For the gene array analysis and qPCR results, statistical analysis was performed using an unpaired two-tail Student's t-test using statistical analysis functions in the GraphPad Prism 5.0 program (GraphPad Inc., San Diego, CA, USA). All experiments were performed where N was equal to a minimum of three independent experiments (cell preparations). A P-value less than 0.05 was considered statistically significant.

4.4 Results

Presence of platelet-derived growth factor receptor α in human seminomas

It has previously been shown that seminoma and embryonal carcinoma (EC) tumors express a 1.5kb variant PDGFRα form that is not present in normal adult testicular tissues (16). We analyzed a variety of human testicular samples (normal (N=12) and seminoma tumors (N=10)) and found that all samples tested expressed positive mRNA expression for both OCT-4 (transcription factor known to be present in TGCTs) and the full length 6.0kb PDGFRα (Figure 4.1A). When analyzing the 1.5kb variant form, 8/10 seminoma tumors tested expressed this variant, whereas it was not expressed in any normal testicular tissues analyzed. Thus, this PDGFRα variant has a potential use as a diagnostic marker for seminoma tumors specifically.

We also analyzed biopsy samples of over 20 different patients and found that CIS cells within seminomas presented similar expression patterns for both PDGFRα and OCT-4 cells along the thickened basement membrane of the tubules (Figure 4.1B). While PDGFRα expression is seen at the plasma membrane and cytosol, OCT-4 expression is nuclear. Overall, these results indicated that PDGFRα and OCT-4 proteins were co-expressed in seminoma tumors.

TCam-2 seminoma cells and neonatal gonocytes have common gene/protein expression profiles and functional responses

To determine whether TCam-2 cells presented expression levels of pluripotency and SSC genes reminiscent of gonocytes, we measured gene expression in PND3 gonocytes compared to PND8 spermatogonia, as well as in TCam-2 cells compared to non-tumoral human testicular samples. In Figure 4.2A, the data showed that gonocytes expressed higher levels of pluripotency genes than spermatogonia, with Nanog and Thy1 being significantly higher in gonocytes; Oct4, Sox2 and Stella also showed higher expression, although not statistically significant due to large variations within each group (Figure 4.2A). Similarly, TCam-2 cells expressed higher levels of Nanog, Oct4, and Thy1 than normal human testis. By contrast, Sox2 was conspicuously absent in TCam-2 cells, which was expected because seminoma tumors are known to be Sox2 negative (15). Stella was expressed at low levels both in normal human specimen and TCam2 cells. While Ap2γ and PLZF (ZBTB16) showed similar expression levels between gonocytes and

spermatogonia, Ap2γ expression was 200-fold higher in TCam-2 cells than normal testis. Another interesting gene is CD44, comprising a short standard/hematopoietic form and several longer variants, encoding for cell adhesion molecules expressed in cancer stem cells and during organogenesis (38, 39, 40). Standard CD44 was stronger in gonocytes than spermatogonia. All CD44 forms were expressed in varying amounts in TCam-2 cells (Figure 4.2A). Miwi2, a piwi protein previously shown to be expressed at higher levels in gonocytes than spermatogonia (41), showed higher expression in gonocytes and TCam-2 cells, but no statistical difference.

In Figure 4.2B, we found that the expression of GFRα1, usually associated with SSC self-renewal and survival, was significantly higher in spermatogonia compared to gonocytes, while its co-receptor Ret did not show difference in expression. Integrin α6, also considered as a SSC marker, showed higher expression, although not significant, in spermatogonia, and it was strongly expressed in TCam-2 cells in comparison to normal tissues. Integrin β3 showed a very different pattern of expression, being significantly higher in gonocytes than spermatogonia, but lower in TCam-2 cells than normal testes. Integrin β1 was similarly expressed in gonocytes and spermatogonia, but was upregulated in TCam-2 cells. Nanos2 and Nanos3 expression was not significantly different between the two phases of germ cell development, similarly to the germ cell markers Vasa and Mili. Nanos2 did not appear to be expressed in TCam-2 cells, but Nanos3 showed stronger signal in these cells than in normal testes. We also measured the gene expression of the spermatogonial differentiation markers Stra8, cKit, and Dazl, known to also increase during gonocyte differentiation (29). Stra8 mRNA expression was significantly higher in spermatogonia compared to gonocytes, while Dazl showed the same trend, but the levels of cKit transcripts were similar at both ages. The mRNA of ERα, ERβ and androgen receptor (AR) were also examined, showing that ERβ was generally the most expressed in developing germ cells, whereas both transcripts were present in TCam-2 cells. Surprisingly, low mRNA levels of AR were detected in gonocytes and spermatogonia, as well as in TCam-2 cells.

Considering the hypothesis that TGCTs originate from gonocytes, we then determined whether the TCam-2 cell protein profile was comparable to that of neonatal gonocytes reported in previous studies (26, 27, 29, 30, 42). The comparison of the protein expression patterns of PDGFRα in TCam-2 human seminoma cells and PND3 gonocytes by immunoblot analysis showed that TCam-2 cells expressed not only a full length receptor form but also a ~65kDa

variant form (Figure 4.2C), whereas only the variant form was visible in gonocytes. The presence of a variant form of PDGFRα in TCam-2 cells was in keeping with the expression of a PDGFRα truncated transcript in seminoma tumors. Immunocytochemical analysis revealed that the majority of PDGFRα appeared to be nuclear in TCam-2 cells (Figure 4.2C). In contrast to PND3 gonocytes, TCam-2 cells expressed minimal levels of PDGFRβ. TCam-2 cells also expressed PDGF-AA (Figure 4.2C), while only minimal signal for PDGF-AB and PDGF-BB was detected (data not shown). As shown in Figure 4.2D, TCam-2 cells showed strong OCT4 nuclear signal, similar to seminoma biopsies, although a minority of cells were negative. Their high proliferative activity was shown by a strong nuclear signal for PCNA (proliferating cell nuclear antigen), a protein expressed during DNA replication (43), in a majority of cells. TCam-2 cells expressed ERβ and Hsp90, similarly to gonocytes (42), but did not express ERα. Surprisingly, there was a positive signal for the androgen receptor (AR) in TCam-2 cells. Most TCam-2 cells expressed low levels of ERK1/2, but only 10% stained positive for phospho-ERK1/2, suggesting a limited role of ERK1/2 in these cells, likely not related to proliferation, unlike gonocytes. In our analysis, TCam-2 cells grown with FBS only did not express STRA8 protein. However, treatment with RA for 3 days induced Stra8 expression in the cells (Figure 4.2E), indicating that these cells respond to RA in a manner similar to gonocytes (29). Overall, these comparative studies highlighted similarities between gonocytes and TCam-2 cells in regards to both gene expression and ability to respond to RA.

Comparative analysis of pluripotency markers and germ cell related genes expression in testicular cancers and matching non-tumor specimen

We evaluated mRNA expression of the same panel of genes as above in human testicular specimen from patients with seminoma, EC, and other types of testicular cancers and matched non-tumor testicular tissues from the same patients (Figure 4.3). Genes that were generally highly expressed in patients with seminoma tumors were also highly expressed in patients with EC tumors and a variety of other testicular tumors, including Leydig cell tumors, yolk sac tumors, and teratomas. These genes included PLZF, full length CD44, ERα, and Integrin α6. Generally, most samples showed some level of gene expression for the majority of genes tested, with the exception of Nanos2, detected only in teratoma, and the variant 8 of CD44, weakly expressed in some of the tumor samples. Of particular note, ERα was strongly expressed in most

seminomas, whereas ERβ showed an opposite expression profile, being either low or non-detected in the same samples. Integrin β1 showed differential expression between seminomas (high expression in 40% samples) and their corresponding normal tissues (high expression in 80% samples), while Integrin α6 gave a strong signal both in seminomas and normal tissues. Noticeably, STRA8 was expressed in half of the ECs examined, 2/10 seminomas, and only in normal tissues dissected from testes presenting EC lesions. The standard form of CD44 was highly expressed in the majority of samples tested, regardless of their pathology. RT-PCR analysis indicated that CD44 variants 1 and 2 seemed to be more typical of seminomas. Moreover, variants 2, 5 and 7 showed preferential expression in seminomas in comparison to their matched normal tissues. There were only a few genes in this pre-selected list that were differentially expressed in seminomas, including up-regulated CD44 variants and downregulated ERβ and Integrin β1, in comparison to normal tissues and other tumor types.

Identification of genes preferentially expressed in gonocytes and seminomas by gene expression array analysis

In order to better identify genes that could have been dysregulated during gonocyte differentiation in relation to tumor formation, we performed parallel gene array analyses in rat germ cells and human testicular cancer specimens and their matching non-tumoral tissues (overview in Schematic 4.1). RNAs from PND3 rat gonocytes and PND8 rat spermatogonia were analyzed to identify genes altered between the 2 developmental phases. For the human arrays, we compared the gene expression profiles of seminoma tumors (including TCam-2 cells) and EC tumors to those of normal testicular tissue samples. Our goal was to compare gene profiles obtained in each series of arrays and to perform a functional analysis of the genes significantly altered in both rat and human sets, in order to detect genes and pathways regulated during gonocyte differentiation and presenting differential expression between testicular tumors and normal testes, thus providing a link between this pathology and its supposed cell of origin.

As previously shown, in the rat gene array analysis, effective germ cell enrichment was confirmed using principle component analysis, in which somatic cell populations clustered very differently from the germ cells (34). The efficient enrichment of the two cellular populations (germ and somatic) at each age (PND3 and PND8) was also confirmed by the fact that germ cell and somatic cell populations only had 65 genes commonly expressed (data not shown). A

dispersion plot analysis of genes expressed in gonocytes versus spermatogonia (0.9 confidence interval; p-value <0.05; fold change of >2.0) indicated that 102 genes were preferentially expressed in gonocytes and down-regulated in spermatogonia, whereas only 24 genes were identified as highly expressed in spermatogonia (Figure 4.4A). KEGG (Kyoto Encyclopedia of Genes and Genomes) analysis on the upregulated genes indicated that these genes belonged to cell adhesion molecules/tight junctions, focal adhesion molecules, genes related to leukocyte transendothelial migration, ECM-receptor interactions, and dilated cardiomyopathy (Figure 4.4B). KEGG pathway analysis of downregulated genes did not result in any pathways due to the small number of genes (Figure 4.4C). Thus, these upregulated genes likely play a role in gonocyte development and are largely cell adhesion molecules.

A similar approach was used to analyse the results of the human gene arrays. 2-D Principle component analysis (Figure 4.5A) indicated that the normal tissues and tumoral tissues were distinctly separate in terms of gene expression, whereas the seminoma and EC samples themselves clustered together, underlining the common CIS origin of these tumors (7). Interestingly, a testicular biopsy described by the pathologist as including areas of inflammation but no tumor was found in the 2-D principle component analysis to locate in a distinct position, indicating a gene expression profile different from those of normal and TGCT specimens. There were 494 genes commonly expressed between seminomas, TCam-2 cells, and EC tumors. Of these 494 genes, 468 were significantly downregulated in the tumors (compared to normal testicular tissue) and only 26 were significantly upregulated. Of note, TCam-2 cells, which are a model for seminoma tumors, and actual biopsies of seminomas had only 87 genes commonly expressed, while TCam-2 cells and EC tumors had 54 genes commonly expressed. In the human arrays, significantly altered genes were those with a confidence interval of 0.9, a p-value > 0.05 and a fold change of at least 3.6. There were 266 genes that were unique to the seminomas, of which 203 were significantly downregulated, while 63 genes were upregulated when compared to normal testicular tissues. For EC tumors, 113 genes were significantly altered, and of those, 69 genes were upregulated and 44 genes were downregulated. TCam-2 cells had the most genes significantly altered exclusively in these cells (465 genes). Of those, 287 genes were upregulated and 178 genes were downregulated when compared to normal testicular tissue. These data illustrated the differences in the transcriptomes of these types of tumors, while also stressing the shared expression of a smaller number of genes.

The dispersion plot of TCam-2 cells versus normal testis identified 351 genes significantly upregulated in TCam-2 cells, and 749 genes downregulated (Figure 4.5B(i)). KEGG pathway analysis of the upregulated genes indicated that the top 5 functional pathways of these genes were pathways involved in cancer, cell adhesion molecules and tight junctions, metabolism, cardiomyopathy, and the insulin signaling pathway (Figure 4.5B(ii)). When categorizing the downregulated genes, the pathways included signaling pathways, genes related to germ cell maturation, metabolism, cell adhesion molecules, and cytotoxicity (Figure 4.5B(iii)).

The dispersion plot analysis of seminomas versus normal testis showed that 149 genes were significantly upregulated in seminomas and 821 were downregulated (Figure 4.5C(i)). Functional pathway analysis revealed that the upregulated genes fell into categories including cell adhesion molecules, genes related to systemic lupus erythematous, hematopoietic cell lineage, prion disease, and the immune response. On the other hand, genes that were downregulated included genes related to metabolism, cell adhesion molecules, ErbB signaling pathways and epithelial cell signaling (Figure 4.5C(ii) and 4.5C(iii)). Interestingly, the dispersion plots of TCam-2 cells versus seminoma biopsies (Figure 4.5C(iv)) highlighted many differences between the two sample types, besides a large fraction of genes commonly expressed (shown in blue).

The dispersion plots of EC tumors indicated that 155 genes were significantly upregulated, while 629 genes were downregulated when compared to normal testicular tissue (Figure 4.5D(i)). KEGG functional analysis concluded that the genes that were upregulated and downregulated in ECs were related to a variety of different functions (Figure 4.5D(ii) and 4.5D(iii)). Furthermore, when comparing ECs with seminomas (Figure 4.5D(iv)), there were very few genes that were upregulated in seminomas compared to EC, the majority being downregulated in seminoma.

Ingenuity Pathway Analysis (IPA) is another method used to better analyze and identify relevant changes in gene array lists. Supplemental Table 4.1 summarizes the top diseases/disorders and molecular/cellular functions that were most closely related to reproduction. When analyzing genes upregulated in gonocytes (compared to spermatogonia), cancer and developmental disorders were most commonly seen. Genes upregulated in seminomas related to cancer and reproductive system diseases (including germ cell tumors) alongside cell adhesion molecules and SSC renewal. Similar results were seen with TCam-2 cells (data not shown). Interestingly, genes associated with asthenozoospermia, gametogenesis,

spermatogenesis, and spermiogenesis were also downregulated in seminoma, as in TCam-2 cells. Downregulated genes related to gametogenesis, spermatogenesis, and spermiogenesis most likely reflected the disappearance of normal spermatogenic cycles and cells in the diseased tissues, hence decreased expression of genes characteristic of spermatogenesis and spermiogenesis, rather than decreased expression related to TGCT functions.

STRING analysis is a method commonly used to determine how genes in a given list can possibly interact with each other. Supplemental Figure 4.1A represents a network of genes that were significantly upregulated in PND3 gonocytes (when compared to PND8 spermatogonia). While some genes were able to group with each other and form clusters, there were many genes that stood alone and were not predicted to interact functionally with any other gene present. The main networks that were formed centered around Mmp9 (matrix metallopeptidase 9), Selp (selectin-P), Npy (neuropeptide Y), and Spp1 (secreted phosphoprotein 1).

The network clustering of genes upregulated in TCam-2 cells was more complex (Supplemental Figure 4.1B) and showed two dominant clusters. In TCam-2 cells, the top most complex networks centered around CDH1 (cadherin 1), DNMT3B/3L (DNA (cytosine-5-)-methyltransferase 3β/3-like), and CCNA2 (cyclin A2). Overall, genes related to cell adhesion molecules, chromatin remodelling and DNA repair emerged as key pathways in TCam-2 cells.

Upregulated genes in seminoma tumors (Supplemental Figure 4.1C) clustered around NANOG, CXCR4 (chemokine (C-X-C motif) receptor 4), and PTPRC (protein tyrosine phosphatase, receptor type, C). This analysis highlighted high expression of pluripotency genes, as well as cell surface receptors and immune cell/inflammation-related genes in seminomas.

Overall, STRING analysis highlighted genes commonly found in TCam-2 cells and seminomas, including pluripotency genes such as Dppa4, Lin28b, Nanog, and Oct4 (Pou5f1), but also pointed at new genes of potential functional relevance in germ cells and testicular tumors.

Emergence of claudins as common genes highly expressed in gonocytes and seminomas

Given that KEGG analysis had indicated cell adhesion molecules as particularly upregulated in PND3 gonocytes and in seminomas, we further examined in more detail this gene category. Table 4.5 summarizes all cell adhesion molecules both upregulated and downregulated

throughout the gene array data (as indicated by KEGG pathway analysis). Mainly, members of the claudin family were significantly altered within this analysis. Claudins 3, 4, and 5 were significantly upregulated in PND3 gonocytes whereas claudins 6 and 7 were significantly upregulated in TCam-2 cells and claudin 6 was significantly upregulated in both seminoma and EC tumors. Claudin 11 was seen to be downregulated in both TCam-2 cells and seminoma tumors. Other cell adhesion molecules that were significantly upregulated in the tumor samples included cdh3 (cadherin 3), PTPRs (protein tyrosine phosphatases), Sell (selectin-L), F11R (also known as JAM1 (junctional adhesion molecule 1), TJP3 (tight junction protein 3) and OCLN (occludin). From these gene lists, the members of the claudin family represented key genes upregulated in both PND3 gonocytes and seminomas (including TCam-2 cells) and thus, presented ideal candidate genes to study further.

Claudin expression in rat testicular gonocytes

Various members of the claudin family were significantly up- and down-regulated in both PND3 gonocytes and seminomas. Although claudins were also seen to be significantly altered in EC tumors, our focus was better understanding seminomas, the most common of TGCTs. Thus, we analyzed the expression levels of various claudins showing altered expression in either the rat or human gene arrays, including claudins 3, 4, 5, 6, 7 and 11. qPCR analysis confirmed the significantly higher expression of claudin 3, 5, 6, and 7 in PND3 gonocytes, compared to PND8 spermatogonia (Figure 4.6A). Immunohistochemistry analysis revealed that claudin 3 expression seemed similar at both ages. Claudin 4 was more highly expressed at PND8 compared to PND3. The same was seen for claudin 5. However, claudin 6, 7, and 11 expression was downregulated in germ cells at PND8 in comparison to PND3 (Figure 4.6B). Thus, higher expression of claudin 6 and claudin 7 in gonocytes was confirmed at the mRNA and protein levels, pointing at these as preferentially altered during gonocyte development and thus, ideal candidates to study further.

Claudin expression in human normal testicular and seminoma tumor samples

The mRNA expression of the same six claudins was measured by qPCR in normal testicular tissue and seminomas (Figure 4.7A). This analysis showed that claudin 6 and 7 were significantly upregulated in seminomas, confirming the results found in the gene arrays. We then performed protein analysis of over 20 different patient specimen, including normal testicular

tissue, seminomas, and a variety of mixed tumor samples (representative expression patterns shown in Figure 4.7B). As shown in Figure 7B, there was minimal protein expression of claudin 6 at the basement membrane area of human adult seminiferous tubules presenting normal spermatogenesis (Figure 4.7B (i)). By contrast, claudin 6 was strongly expressed in seminomas, localizing at the plasma membrane, as shown in 3 representative samples (Figure 4.7B (ii-iv)), and in CIS cells (Figure 4.7B (v, vi)). However, the protein was only weakly expressed in EC (Figure 4.7B (vii)) and in yolk sac tumor samples (Figure 4.7B (viii)), further stressing its preferential expression in seminomas. Regarding Claudin 7, it showed a stronger expression than claudin 6 in normal seminiferous tubules (Figure 4.7C (i)), at the basement membrane of the tubules but also between Sertoli cells, possibly involving claudin 7 in the blood testis barrier. Claudin 7 was clearly upregulated in seminomas (Figure 4.7C (ii-iv)) and CIS cells (Figure 4.7C (v, vi)). Interestingly, it appears to have two different types of subcellular localization in seminomas and CIS cells, with some protein expressed at the cell-cell junctions (black arrows) and some found in the nucleus (white triangle arrows). Similarly to claudin 6, it was only weakly expressed in EC and yolk sac tumors (Figure 4.7C (vii and viii)). Protein analysis confirmed the transcript analysis and further underlined the high expression levels of claudin 6 and 7 in seminomas. Thus, claudin 6 and claudin 7 are two proteins that may provide a missing link between improper gonocyte development and seminoma formation.

Other genes of interest found by gene array analysis

We further examined the gene array data to find other key genes that may be of interest. The top 100 genes besides claudins have been listed in Table 4.6 alongside their fold change in each gene array. Key gene families included ADAM (ADAM metallopeptidase domain), CCND (cyclin D), CD molecules, the DAZ (deleted in azoospermia) , FBXO (F-box) proteins, GDFs and FGFs (growth differentiation and fibroblast growth factors), MMPs (matrix metalloproteinase), RNF (ring finger) proteins, SPATA (spermatogenesis associated) genes, TEX (testis expressed) genes, TRIM (tripartite motif containing) genes, and Ubiquitin specific genes. All in all, there was a large variety of genes that were commonly highly expressed between PND3 gonocytes and TGCTs other than claudins, that could potentially fail to be downregulated during dysfunctional gonocyte differentiation and participate to the emergence of a CIS phenotype, later resulting in seminoma.

4.5 Discussion

This report shows for the first time that members of the claudin family of proteins are expressed in neonatal testicular germ cells, and that their expression is disregulated in testicular germ cell tumors. The incidence of TGCTs has been steadily increasing in the past few decades for reasons that are not known and as a result, TGCTs have become one of the most common types of cancers occurring in young men today (44). Although chemotherapeutic drugs have increased the overall 5 year survival of testicular cancer patients to over 90% (45), these treatments not only decrease patient fertility but they also have grave side effects on patient health, including renal, pulmonary, gastrointestinal, and cardiovascular toxicity (46). Thus, despite being highly curable, testicular cancer is associated with serious health issues in young men, and better understanding its origins and the mechanisms regulating TGCTs formation could potentially help in decreasing its incidence and design better targeted treatments. It has been suggested that improper development of primordial germ cells or gonocytes can lead to formation of the TGCT precursor pathology, carcinoma-in-situ (CIS) (21). In general, gonocytes have not been that well studied and so, the goal of this study was to better understand molecular mechanisms involved in gonocyte development that could also be involved in tumor formation, providing us with a potential link between the two processes.

In order to unveil possible conserved genes and pathways between gonocyte and TGCTs, we performed both gene-targeted and gene array studies on developing rat neonatal germ cells, and human testicular normal and tumoral samples including the human seminoma cell line, TCam-2. Given that there is no proper animal model to study seminomas, and that there is limited access to human testicular normal and pathological specimen, using human cell line models represents a good alternative. Here, we used the TCam-2 seminoma cell line, originally propagated by Mizuno Y et al. from a primary lesion in a testicular seminoma originating from a young adult patient, presenting the histology of a typical pure seminoma, which was named TCam-2 cells (19). Although TCam-2 cells were shown to have similar gene expression profiles as seminomas, recent studies proposed that their expression profile also resembles those of ECs, suggesting that TCam-2 cells were derived from a tumor that was not uniquely seminoma but rather was a mixed tumor, as seen in ~15% of TGCT cases (3, 47, 48). Interestingly, Nettersheim D et al showed that TCam-2 cells were very plastic and could give rise to either seminoma/CIS when

transplanted into seminiferous tubules, or ECs when transplanted into the brain, further illustrating the importance of the stromal microenvironment in tumor fate (49). However, in the present study, TCam-2 cells did not show Sox2 expression, but presented high levels of SOX17, similar to the profiles found in seminomas, suggesting that in the conditions used, the cells had seminoma-like characteristics rather than ECs (15). Despite its possible mixed tumor origins, TCam-2 cells are currently the cell-line model of choice when studying seminomas.

The first genes examined among a list of genes selected for their relevance to pluripotency, germ cell development, and gonocyte or spermatogonia identity or function, were the PDGFRs. PDGFRs are tyrosine kinase receptors involved in cellular proliferation, differentiation, survival, and a multitude of other processes, which are expressed in both pre- and post-natal rat testis, with maximal expression occurring in the early post-natal periods (27, 50, 51, 52, 53). Interestingly, Palumbo C et al had previously described the expression of a 1.5kb alternative transcript of PDGFRα present only in seminoma and embryonal carcinoma tumors, along with OCT4 (16, 54). In the present study, we confirmed the presence of this variant mRNA in seminomas, and its absence in normal testicular tissue. PDGFRα expression has previously been detected in pulmonary metastases arising from a variety of different tumors, including renal cell carcinomas, sarcomas, colorectal carcinomas, and otolaryngologic carcinomas (55). Furthermore, PDGFRα mutants that retained tyrosine kinase activity have been shown to be present in gastrointestinal stromal tumors (56). The finding of a TGCT specific 1.5kb PDGFRα form remains one of the few studies implicating PDGFRs in testicular cancer, alongside the finding that patients with germ cell tumors have higher levels of serum PDGF compared to healthy patients (57). Finding characteristic hallmarks for any type of tumor, as this 1.5kb PDGFRα variant appears to be for seminoma tumors, is not always easy, but provides for a potentially useful diagnostic tool in patients, and an insight to understanding molecular mechanisms leading to tumor formation.

Furthermore, although the antibody used did not discriminate between full length and truncated proteins, immunoblot analysis confirmed the expression of the variant form at the protein level in TCam-2 cells, similarly to the variant protein expressed in neonatal gonocytes. These data support the current belief that CIS is the intermediary step between the failure of gonocytes to differentiate and the formation of TGCTs (21). To our knowledge, this is the first report of the presence of a variant PDGFRα in testicular neonatal/transitional gonocytes. Given

that seminoma tumors also express a variant form, this aberrant retention in gonocytes could be an active factor in the prevention of adequate cell differentiation leading to tumor formation, or it could reflect the retention of an undifferentiated neonatal germ cell phenotype in tumor cells.

PDGFRs are activated by PDGF ligand dimerization, leading to the activation of a downstream signaling cascade (58). We have previously shown that gonocyte proliferation requires the combined action of PDGF-BB and 17β-estradiol (26, 27). Interestingly, TCam-2 cells expressed PDGF-AA, PDGF-AB, and PDGF-BB mRNAs, but only PDGF-AA was noticeably expressed at the protein level in TCam-2 cells. An attempt to determine which growth factor(s) regulated TCam-2 cell proliferation showed that there was not a single factor, but rather multiple factors are able to increase proliferation in synchronized TCam-2 cells cultured in the presence of BSA and a minimal level of FBS or serum replacement (data not shown). PCNA (proliferating cell nuclear antigen) is a commonly used marker for cell proliferation expressed in the nuclei of cells during DNA replication (43). Not surprisingly, TCam-2 cells had strong positive expression for PCNA. Taken together with the ability of the cells to produce factors such as PDGF, this suggests an autocrine control of TCam-2 cell proliferation, a property more representative of tumor cells, even though TCam-2 cells have a long doubling time of 58 hours (3). Because gonocyte proliferation requires the activation of the ER pathway together with that of PDGFR (27), we examined which ER were expressed in TCam-2 cells and found that TCam-2 cells had higher expression of ERβ than ERα at the protein level, similarly to gonocytes (42). However, the analysis of ERα and ERβ mRNA expression profiles in a panel of testicular cancer specimen showed that ERα expression was increased from normal testicular tissue to seminomas, whereas ERβ appeared to be downregulated in seminoma tumors, in agreement with a study reporting decreased ERβ levels in seminomas and ECs (59). This was different from the results obtained with TCam-2 cells, where both mRNA species were relatively strong, but only ERβ protein was expressed. Despite the expression of ERβ in most germ cell stages in rodent and humans, including the presence of the ERβ2 variant in human fetal gonocytes (60, 61, 62), its role in normal and pathological germ cells remains so far a mystery, in view of the absence of a reproductive phenotype in male knockout mice (63).

Once PDGFRs are activated, there is a large number of potential downstream pathways that can be activated (50). The commonly activated MEK/ERK pathway (known to be activated

during gonocyte proliferation (27)), was also positively expressed in TCam-2 cells. However, only a minority of cells presented phosphorylated ERK1/2 at any given time. This low level of activation contrasted with the high proportion of cells positive for PCNA, suggesting that ERK1/2 activation is not a major player in TCam-2 cell proliferation. Another critical function of gonocytes is their RA-driven differentiation into spermatogonia. We have previously shown that aspects of RA-induced gonocyte differentiation could be replicated in vitro by treating isolated rat gonocytes with RA, leading to an increase in STRA8 (stimulated by retinoic acid 8) mRNA and protein expression (28, 29, 64). TCam-2 cells grown with serum but no additional RA (mainly undifferentiated cells) did not express the STRA8 protein. However, when the TCam-2 cells were treated with RA, there was an increase in STRA8 protein expression within 3 days of treatment, indicating the ability of TCam-2 cells to respond to RA. Such increase in STRA8 expression upon RA treatment of TCam-2 cells has not yet been reported. However, it is reminiscent of the increase in c-KIT transcript levels found in TCam-2 cells treated with Activin A, BMP4, and RA (65). Although it is known that ECs have the ability to differentiate (66), such ability has not been fully studied in seminoma. Previously, Nettersheim D et al stimulated TCam-2 cells in order to get better insight into the potential use of all-trans RA as a therapeutic agent for seminoma tumors. However, they concluded that TCam-2 cells were not able to undergo differentiation in response to RA, based on the lack of morphological or growth behaviour changes, as well as unaltered gene expression in markers of pluripotency, germ or somatic cells (66, 67). Where some groups believe seminoma tumors cannot differentiate in vivo (68, 69), others have shown that this is possible (70). Young JC et al showed that TCam-2 cell treatment with RA not only increases expression of various activin receptors but also enhances cell survival, proliferation, and self renewal alongside BMP4 (similar to fetal germ cell response to RA) (69). Considering the recently evidenced plasticity of TCam-2 cells, it is likely that these differences in responses to RA reflect different proportions of seminoma versus EC sub-populations in TCam-2 cells between different laboratories. These results suggest that TCam-2 cells have retained the ability to respond to RA, similarly to gonocytes and spermatogonia.

Surprisingly, TCam-2 cells showed positive expression for androgen receptor (AR) at the mRNA and protein level. Generally, AR is not considered to be expressed in germ cells, and only one group has reported its transient expression in late fetal gonocytes (71, 72). The expression of AR in TCam-2 cells could thus be related to re-acquisition of fetal or embryonic

gene expression characteristics that often occur in tumors. This finding is in line with recent reports that breast cancer cells, expected to express ERs and aromatase, can also express AR in relation to tumorigenesis and cancer progression (73). It is also interesting because it has been suggested that the steadily increasing incidence in testicular cancer may be due to the prevalence of endocrine disruptors in the environment, including some acting on androgen-estrogen responses and/or homeostasis (7). As a result, the acquisition of AR, alongside changes in the ratios of ERα and β, could indicate a susceptibility of TCam-2 cells (and seminoma tumors) to endocrine disruptors, potentially giving them the ability to respond to these types of chemicals.

A number of genes originally characterized as pluripotency markers were found to be expressed in SSCs and undifferentiated spermatogonia in variable levels. NANOG is a homeobox gene that is a key regulator in the self-renewal and pluripotency of embryonic stem cells (12). It has also been described as a marker for testicular CIS and germ cell tumors, and given the timing of its downregulation in fetal gonocytes, it is likely that NANOG acts upstream of OCT4 (12). NANOG mRNA was present in both gonocytes and TCam-2 cells and downregulated in PND8 spermatogonia, similarly to its fate in human germ cells (23). NANOG mRNA and protein expression have previously been shown in TCam-2 cells, in agreement with our data (3). GFRα1 was expressed clearly at much higher levels in spermatogonia than in gonocytes, but was minimally expressed in TCam-2 cells. NANOS2 and 3 (known to play important roles in the survival of embryonic germ cells and SSC maintenance after birth (74, 75)) were not significantly altered in either rat or human analysis. CD44 represents another interesting gene because it is considered as a stem cancer cell marker, several of its isoforms have been shown to be overexpressed in a variety of cancers (76), and to associate with Oct4 and Sox2 (42). Although there is only one standard form and one variant (V6) form of CD44 in the rat (77), in humans, there is a standard (hematopoietic) form as well as several variants (41, 78). CD44 is a cell surface receptor that is involved in a variety of processes, including extracellular matrix adhesion. The CD44 standard form was much stronger in gonocytes than in spermatogonia, suggesting a preferential role in gonocytes. In TCam-2 cells and human biopsies, the standard form and varying amounts of the eight variant forms were expressed, with variants 5 and 8 being the most highly expressed in TCam-2 cells, but variants 2, 5 and 7 showing higher expression levels in seminoma biopsies. To the best of our knowledge, this is the first time that the expression of CD44 isoforms is reported in gonocytes and seminomas. Overall, these data

confirmed that both PND3 gonocytes and TCam-2 cells share gene expression profiles of major pluripotency genes, further suggesting that the TCam-2 cell phenotype is closer to that of gonocytes than spermatogonia. However, there were also discrepancy in the expression patterns of several genes, not surprisingly, as seminomas are not simply cells frozen in an undifferentiated state, but they have likely evolved in response to successive cues from the surrounding stroma and the dynamic hormonal changes taking place during puberty.

We then analyzed the expression profiles of common germ cells and differentiating spermatogonia markers, in hopes to see differential expression in some of these genes. VASA (DDX4) and MILI are RNA binding proteins expressed in fetal to adult germ cells (79, 80). MILI, as other piwi protein family members, bind small piRNAs and has been shown to be critical for SSC self-renewal and differentiation (81). Both gonocytes and TCam-2 cells expressed the germ cell markers MILI and VASA at low levels. Weak VASA protein expression has previously been seen in seminoma tumors (81) and in TCam-2 cells (18). MILI expression had not been reported in TCam-2 cells. Another piwi gene usually associated with fetal to neonatal gonocytes is Miwi2 (82). The presence of these genes at low levels in TCam-2 cells further confirmed their germ cell origin, but did not provide new differentially expressed candidate genes. Gonocytes had relatively high expression of cKIT and much less of STRA8 and Dazl. The low expression of DAZL transcripts in seminomas had previously been shown (3, 83). The fact that cKIT was more strongly expressed in TCam-2 cells was expected from published studies on TGCTs (3, 20). Although cKIT is commonly used as a marker of spermatogonial differentiation (47), it is also a gene typical of embryonic stem cells (ESCs) and primordial germ cells, in which it is required for PGC migration to the genital ridge (84). Thus, positive expression in seminomas could indicate a process of re-acquisition of ESCs marks or alternatively, the retention of PGC markers.

In view of the small number of genes showing differential expression among the pre-selected list of genes, we next performed comparative inter-species gene array analyses, with the goal to identify molecular or functional processes that may be involved in gonocyte development and upregulated in testicular tumors. Although studies have been done comparing later stages of spermatogenesis with tumor development, our study was one of the first to use an approach focusing on neonatal germ cells, closer to the point at which germ cell development is believed

to become disrupted. Gene expression arrays of PND3 gonocytes and PND8 spermatogonia identified 102 genes significantly upregulated in gonocytes and 24 genes downregulated compared to spermatogonia. KEGG analysis of upregulated genes in gonocytes showed that they belonged to the main category of cell adhesion molecules and tight junctions. STRING analysis found that these upregulated genes clustered around Selp, MMP9, NPY, and SPP1. SELP (Selectin-P) is a cell adhesion molecule that is expressed at the surface of activated cells, known to be involved in tumor metastasis (85). NPY (neuropeptide Y) is one of the most abundant neuropeptides in human and is known to regulate various functions including food intake, circadian rhythm, blood pressure, reproduction, and to be involved in a variety of disease states including hypertension and anxiety (86). NPY has been found to be expressed in the human testis and in one study, both Y1 and Y2 NPY receptor were found to be present in 33% of the germ cell tumors tested (87, 88). However, in our analysis, NPY was not significantly altered in any of the tumor samples. Our study is the first to report that these genes were significantly upregulated in gonocytes when compared to spermatogonia, indicating a possible role for these genes in early germ cell development.

STRING analysis of TCam-2 cells pointed out to several noteworthy, upregulated genes. F11R is also known as JAM1 (junctional adhesion molecule 1). JAM proteins are tight junctional proteins in epithelial and endothelial cells (89). JAM1 expression has been found in various tumors including breast cancer and lung carcinomas (90, 91). It has also been suggested that JAM1 can be used as a novel oncology target as it can arrest tumor cell proliferation (92). Thus, genes upregulated in TCam-2 cells represent interesting genes that could be further studied.

STRING analysis of upregulated genes in seminoma biopsies also revealed important genes. For example, CXCR4 is a receptor involved in migration, proliferation and survival of primordial germ cells, gonocytes, and spermatogonial stem cells and has previously been shown to be highly expressed in seminoma tumors (93). Although the known ligand of CXCR4 is CXCL12, we found that CXCL12 was not significantly upregulated in seminomas, but instead it was CXCL10 that was upregulated in both seminoma and embryonal carcinoma tumors. Interestingly, although not significant, CXCL10 was more highly expressed in gonocytes than in spermatogonia, whereas CXCL12 expression was not significantly altered in any of the arrays.

Furthermore, we examined the expression of cell adhesion molecules, in particular claudin proteins, in both PND3 gonocytes and seminoma tumors in more detail. Claudins are tetraspan membrane proteins that represent the main proteins in tight junctions and regulate paracellular permeability (94). Claudins are 21-28kDa proteins that contain an N-terminal intracellular part, four transmembrane domains, two extracellular loops, an intracellular loop and an intracellular C-terminal end (94, 95). As of now, 27 different claudins have been identified and only 24 of them are present in humans, which do not have claudin 13, 26, and 27) (96). Here, we found that claudins 3, 4, 5, 6, and 7 (also known as the "classic claudins" based on sequence similarity) were found to be upregulated in rat gonocytes and human TGCTs, whereas claudin 11 (considered a "non-classic claudin") was downregulated in tumoral tissue (97). Of these, higher expression of claudin 6 and claudin 7 in gonocytes was confirmed at the protein level in PND3 and PND8 rat testes sections. This suggested that Claudin 6 and 7 are likely playing a role in gonocyte development and represent good candidate genes to study further. To our knowledge, this is the first report indicating the expression of claudin 3, 5, 6, 7, and 11 both at the mRNA and protein levels in testicular PND3 gonocytes.

Claudin 3 is known to be a part of the blood testis barrier (BTB) formed by tight junctions between adjacent Sertoli cells, and was recently found to help regulate the progression of meiosis in early stage spermatocytes (97). Concerning claudin 5, it is expressed not only in Sertoli cells, spermatogonia, and preleptotene spermatocytes, but it also plays a role in the BTB formation, as shown by increased expression of claudin 5 in mice at the time of BTB formation (98). Up until now, claudin 6 expression had not been reported in rat testes. There had been only one study showing weak expression of claudin 6 in adult seminiferous epithelium (99). The same study showed that claudin 7 was weakly expressed in the seminiferous tubules, whereas the epithelium of the rete testis was strongly positive for this claudin (99). Furthermore, in our analysis, the expression of claudin 5 in PND3 gonocytes appears to be nuclear. Claudins are cell adhesion molecules involved in tight junction formation and thus, it would seem out of the ordinary to find these molecules in nuclei. However, several studies have described the nuclear localization of cell adhesion molecules. For example, claudin 1 has a nuclear localization in human primary colon carcinoma and metastasis and in cell lines that are derived from both primary and metastatic tumor (100). Claudin 1 was also reported to be nuclear in nasopharyngeal carcinoma cells (101). Other cell junction proteins, such as zonula occludens (ZO-1, ZO-2) have been

shown to have nuclear localization as well (102, 103). In these studies, it was suggested that the nuclear localization of cell junction proteins correlated with oncogenic transformation, signal transduction, and cellular proliferation (102, 103). Furthermore, these studies proposed that cell junction protein localization would depend on cell density where, in conditions of sparse cells, junctional proteins would travel to the nucleus (in a NLS independent manner if necessary) to activate cellular proliferation (102, 103). However, this is not the case for gonocytes, which are surrounded and in direct contact with Sertoli cells, to which they are bound by gap junctions (104). Thus, although nuclear claudins might play a role in cell proliferation in gonocytes, this does not seem to be related to cell density.

The study of claudins in human normal testicular tissue and seminoma biopsies indicated that the expressions of claudins 5 and 11 were significantly decreased in seminoma tumors. Similarly to claudin 5, claudin 11 is involved in BTB formation (105). It is important to note that the BTB is not formed yet at PND3 and 6, the ages when claudin 5 and 11 expressions were observed in gonocytes and spermatogonia. Indeed, the decreased expression of claudins 5 and 11 in seminomas likely reflects the disappearance of tight junctions between the remaining Sertoli cells and the destruction of the BTB in human TGCTs. This is different from the situation in neonatal testis, where there seems to be a shift in the cell types expressing the two claudins, with claudins 5 and 11 being expressed in gonocytes at PND3, but at the basal surface of Sertoli cells at PND8. The findings that claudin 5 and claudin 11 are expressed in neonatal germ cells and downregulated in seminomas are novel. In contrast to claudins 5 and 11, the expressions of claudin 6 and 7 were significantly upregulated in seminomas as compared to normal testicular tissues, both at the mRNA and protein levels, findings that were confirmed on multiple biopsies. Interestingly, in seminomas and CIS, claudin 7 protein was expressed in nuclei as well as at the plasma membrane, suggesting that a fraction of the protein had retained the canonical function of cell adhesion molecules, while another fraction was exerting a different function unrelated to cell-cell interactions. This contrasted with claudin 6, which was also upregulated, but was present at the plasma membrane, indicating a potential role of junction proteins between tumor cells. The finding that claudin 6 and claudin 7 expressions are elevated in gonocytes and in seminomas suggests that these molecules might have failed to downregulate as part of the disruption in gonocyte differentiation. Although the role of claudins in testicular tumors is not understood, the importance of claudins in other types of tumors has been known for some time.

Interestingly, both upregulation and downregulation of various claudin proteins can be associated with tumorigenesis (106). Thus, finding the role of claudins in testicular tumors would add to the expanding knowledge about claudins and their role in tumorigenesis.

Interestingly, one of the CD44 isoforms (CD44-V6) has been shown to form a complex with Claudin 7, EpCam, and tetraspanins and to promote cancer progression (107). Although we have not determined the expression levels of CD44-V6 in gonocytes, the existence of interactions between claudin 7 and CD44, and complexes made of CD44, Oct4 and Sox2 in other cell types, suggest that such a network could exist in gonocytes and in TGCTs. The understanding of claudin functions has expanded over the past years, and it is clear that their roles in the regulation of the intercellular exchange of ions and small molecules might be as important as their role in preventing the passage of other molecules and their barrier function (108, 109). Knowing the partners of these claudins in gonocytes and TGCTs could provide functional information.

Finally, gene array analyses identified other genes that could potentially be important in the physiological development of gonocytes, and in the dysregulation leading to CIS. Of those, the top 100 genes (other than claudins) included several metalloproteases families, pluripotency genes, genes related to DNA methylation, chromatin remodeling and cell cycle. CCND1 (cyclin D1) is upregulated in gonocytes whereas cyclin D2 is upregulated in tumors. Although the upregulated of cyclin D2 has been previously shown in tumors (110), the upregulated expression of CCND1 in rat gonocytes is opposite to what was found in newborn mice, in which CCND1 expression was lower at PND1 than in PND7-9 Swiss mice (111). Such a difference observed between PND1 mice and PND3 rat in CCND1 expression could be important since most gonocytes are quiescent on the day after birth but mitotic at PND3, despite some species differences in the development timings. Together with our results, this suggests that CCND1 expression increases as gonocytes become mitotic. DNMT3L (DNA (cytosine-5-)-methyltransferase 3-like) was among the genes highly upregulated in both gonocytes and in TCam-2 cells. Although DNMT3L has no methyltransferase activity, it plays an important role in the de novo DNA methylation during gametogenesis alongside DNTM3a, which takes place mainly during the fetal to neonatal gonocytes phases, and is important in normal spermatogenesis (112, 113, 114). Thus, its high expression in rat gonocytes was in agreement with studies in mice

(115, 116). Interestingly, while it was not significantly upregulated in the seminoma and embryonal carcinoma specimens analyzed in the present study, DNMT3L had been found upregulated in the same types of pathologies before (116), and a recent study reported its upregulation during the acquisition of an embryonal carcinoma phenotype in TCam-2 (117). Thus, DNMT3L overexpression appears to be variable in function of the seminoma and embryonal carcinoma samples examined.

MMPs are a family of zinc-dependent endopeptidases important in extracellular matrix remodeling (118, 119). In our study, we found that MMP3, 9, and 12 were significantly upregulated in gonocytes, while MMP9 was upregulated in embryonal carcinomas. The balance between MMP2, MMP9 expression with that of TIMP1 and TIMP2 (TIMP metallopeptidase inhibitor 1 and 2) can determine germ cell tumor invasiveness (120). MMP2 and MMP9 have also been shown to be involved in PGC migration (121), but have not yet been reported to be expressed in PND3 gonocytes. Interestingly, it has been shown that claudin 1 overexpression in colon cancer cells significantly increases MMP2 and MMP9 expression (100) and that in ovarian epithelial cells, claudin 3 and claudin 4 increases lead to increases in MMP2 also (122). Our findings that gonocytes express high levels of claudin 3, 4, and 5 alongside highly expressed MMP3, 9, and 12 (gene array data), suggest the possibility that claudins and MMPs could interact in these cells as well as in TGCTs.

Many of the genes that were downregulated in TGCTs were not informative because they simply reflected the loss of normal spermatogenesis in the pathological tissues. Such genes included RNF genes (RING finger proteins), TRIM genes (tripartite motif containing proteins) and various ubiquitin conjugating enzymes. These genes are commonly related to the ubiquitin proteasome system, a system we have previously shown to be involved in gonocyte differentiation (34). Thus, the decreases in ubiquitin-related genes observed in TGCTs implies a disruption of the UPS, potentially linked to perturbations in gonocyte differentiation.

In summary, our study indicated that claudin 6 and claudin 7 were highly expressed in PND3 gonocytes, were downregulated during normal spermatogenesis, and were upregulated in seminomas. This suggests either an active participation to the process of transformation, or a passive expression due to the failure of differentiation and retention of genes normally found in fetal to neonatal gonocytes. We hypothesize that Claudins 6 and 7 are involved in junctions

between gonocytes and Sertoli cells, and that their lack of downregulation would prevent gonocyte movement and efficient attachment to the basement membrane, disrupting normal spermatogenesis and leading to arrested development and potential tumor formation. This hypothesis would fit for claudins expressed at the plasma membrane of tumor cells, but not to the nuclear fraction of claudin 7, which would likely serve another purpose. All in all, this study has unveiled a number of genes and proteins presenting high expression in neonatal gonocytes as well as TGCTs, further stressing their potential relationship and identifying new genes that could play a role in the formation of CIS. Deciphering their roles in gonocyte development and in seminomas should advance our understanding of a critical phase of germ cell development leading to stem cell formations and on TGCTs.

4.6 Acknowledgements

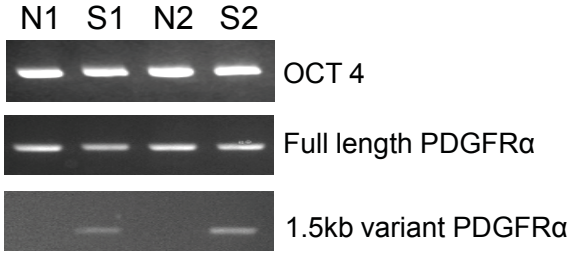
We would like to thank Dr. Jaroslav Novak for assistance with microarray data analysis and biostatistics, Dr. Daniel B. Martinez-Arguelles for assistance with microarray data and pathway analysis, and Annie Boisvert for outstanding technical assistance.

4.7 Figures

Figure 4.1: Presence of PDGFRα and OCT4 in human testicular normal tissue and seminoma tumors

(A) RT-PCR analysis of OCT4, full length PDGFRα, and 1.5kb variant PDGFRα in human testicular normal tissue and seminoma tumors (normal (N) human testicular samples (N=12) and seminoma-type tumor (S) samples (N=10)). (B) Immunohistochemistry analysis of OCT4 and PDGFRα protein expression in carcinoma in-situ samples.

A



Sample Type	OCT 4	Full length PDGFRα	Variant PDGFRα
Normal testes (N=12)	12	12	0
Seminoma tumors (N=10)	10	10	8

B

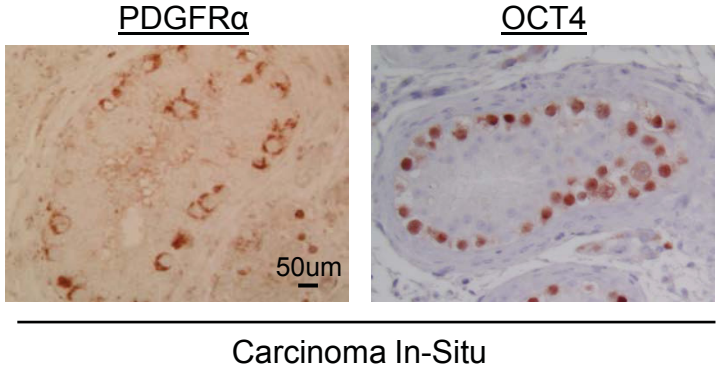


Figure 4.2: <u>Comparative mRNA and protein analysis of various genes in rat and human testicular tissue</u>

(A) RT-PCR and qPCR analysis of various genes, including markers of pluripotency. (B) RT-PCR and qPCR analysis of various genes, including markers of germ cells, differentiating spermatogonia, spermatogonial stem cells, and other genes. PND3 gonocytes=G3, PND8 spermatogonia=G8, N=normal testicular tissue, T=TCam-2 human seminoma cells. Representative RT-PCR bands shown. *$p<0.05$, **$p<0.01$, ***$p<0.001$. (C) (i) Immunoblot analysis of PDGFRα expression in PND3 gonocytes (G3) and TCam-2 cells. (ii) Immunocytochemistry analysis of PDGFRα, PDGFRβ, and PDGF-AA protein in TCam-2 cells. (D) Immunocytochemistry analysis of various proteins in TCam-2 cells. (E) STRA8 protein expression in TCam-2 cells treated with or without retinoic acid (RA). Representative images are shown.

Figure 4.2: <u>Comparative mRNA and protein analysis of various genes in rat and human testicular tissue</u>

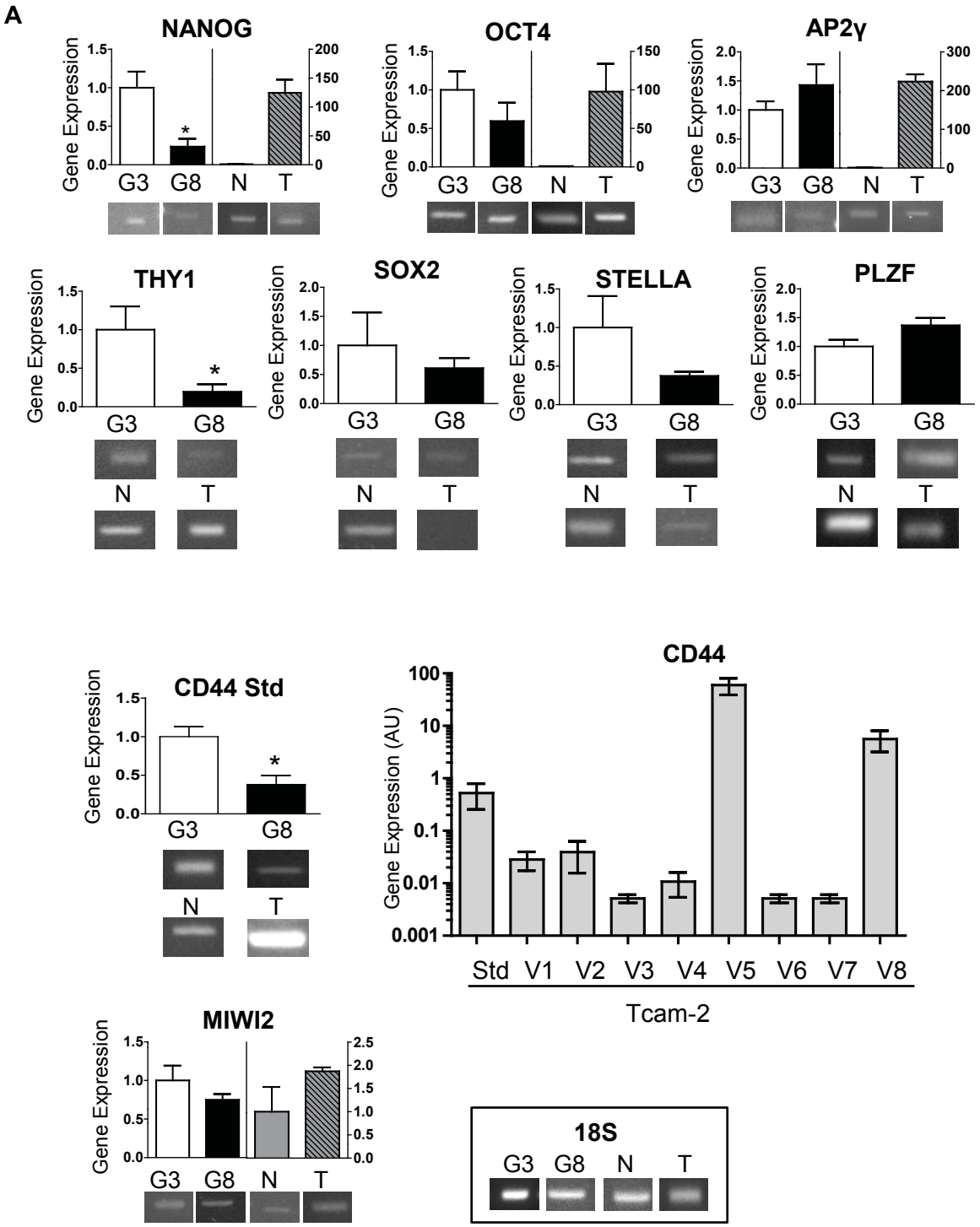


Figure 4.2: <u>Comparative mRNA and protein analysis of various genes in rat and human testicular tissue</u>

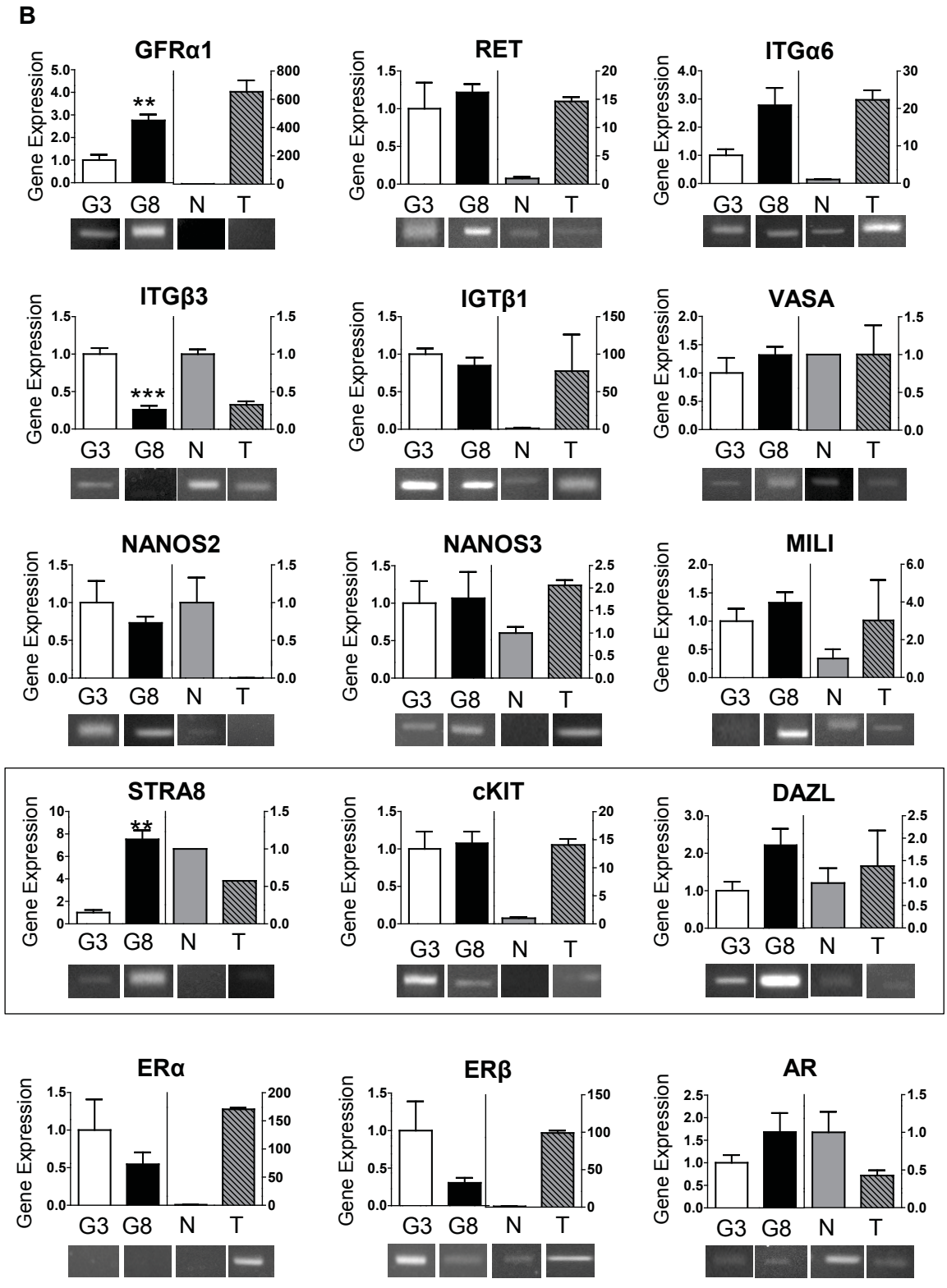


Figure 4.2: <u>Comparative mRNA and protein analysis of various genes in rat and human testicular tissue</u>

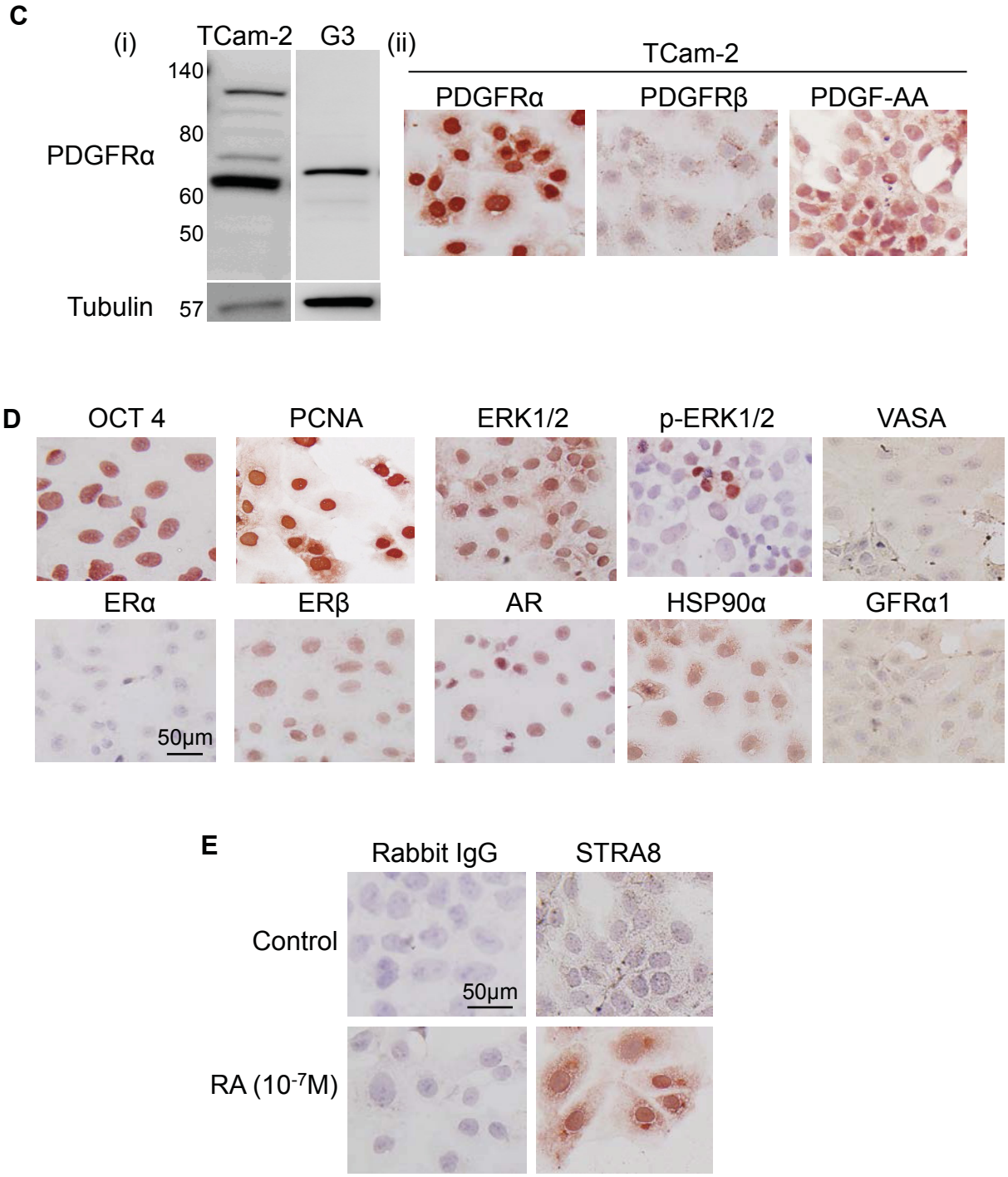
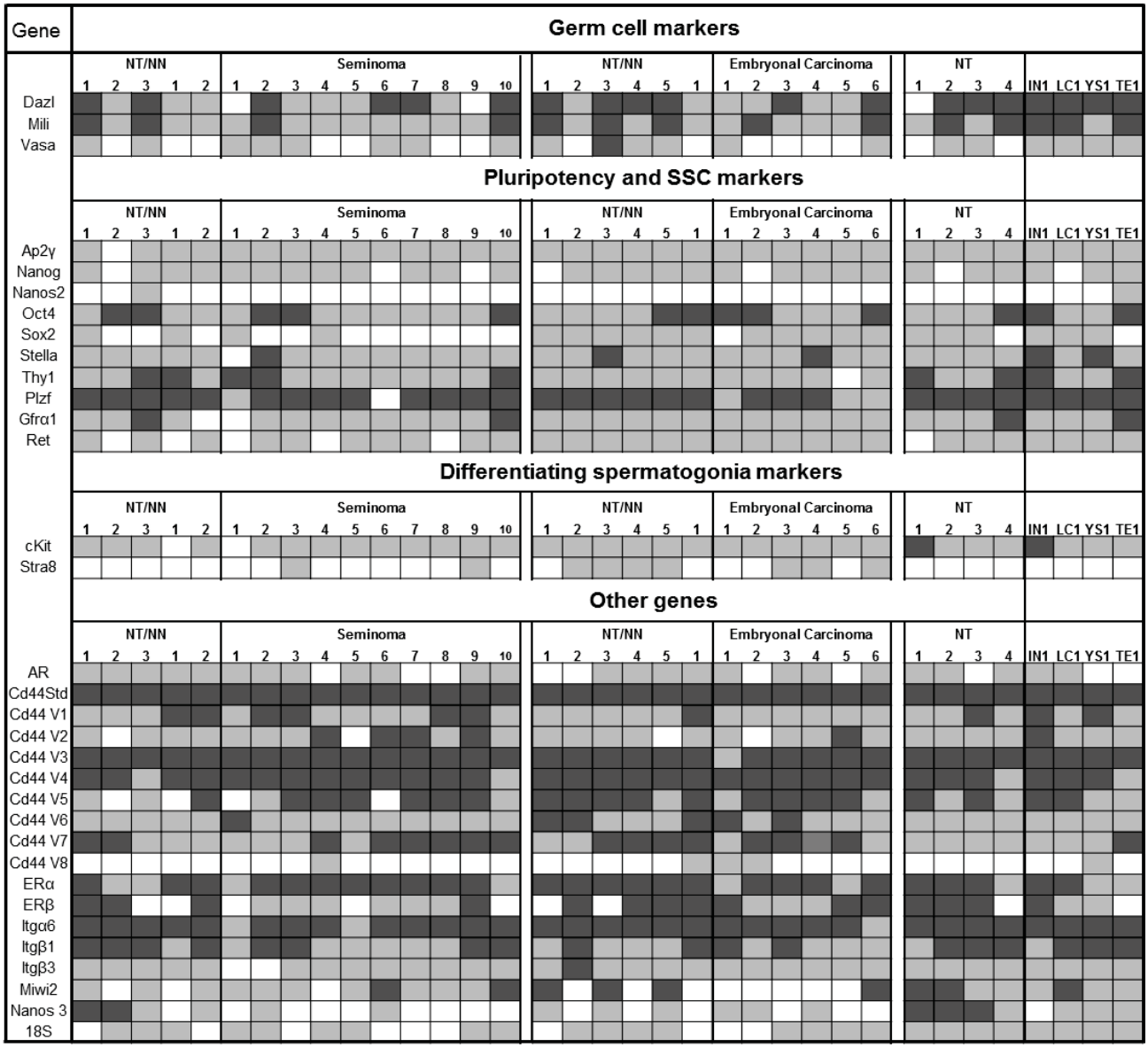


Figure 4.3: Differentiation gene expression in human testicular normal tissue and seminoma tumors

Gene specific primers were used and RT-PCR was run using an amplification step of 40 cycles and primer dependent T_m. Strong expression is indicated by a dark gray box, medium expression is indicated by a light gray box, and a white box indicates that, using our gene specific primers, the mRNA levels of that particular gene were undetectable but still may be present in extremely low quantities. Both GAPDH and 18S rRNA were used as loading controls. NT: normal testis, NN: non-testicular normal (spermatic cord), SE: seminoma tumor; EC: embryonal carcinoma tumor; IN: inflammation; LC: Leydig cell tumor; YS: yolk sac tumor; TE: teratoma tumor.

Figure 4.3: <u>Differential gene expression in human testicular normal tissue and seminoma tumors</u>



Schematic 4.1: Overview of gene array analysis

Illumina rat gene array analysis was conducted using samples of isolated PND3 gonocytes and PND8 spermatogonia. Corresponding-age Sertoli/myoid cells were also analyzed as controls for sample purity. Germ cell samples were analyzed in triplicate whereas somatic cell samples were processed in duplicate. Affymetrix human gene array analysis was conducted using testicular biopsy samples. This array included normal testicular tissue (N=3), seminoma tumor samples (N=3), TCam-2 cells (N=2), and embryonal carcinoma tumor samples (N=2).

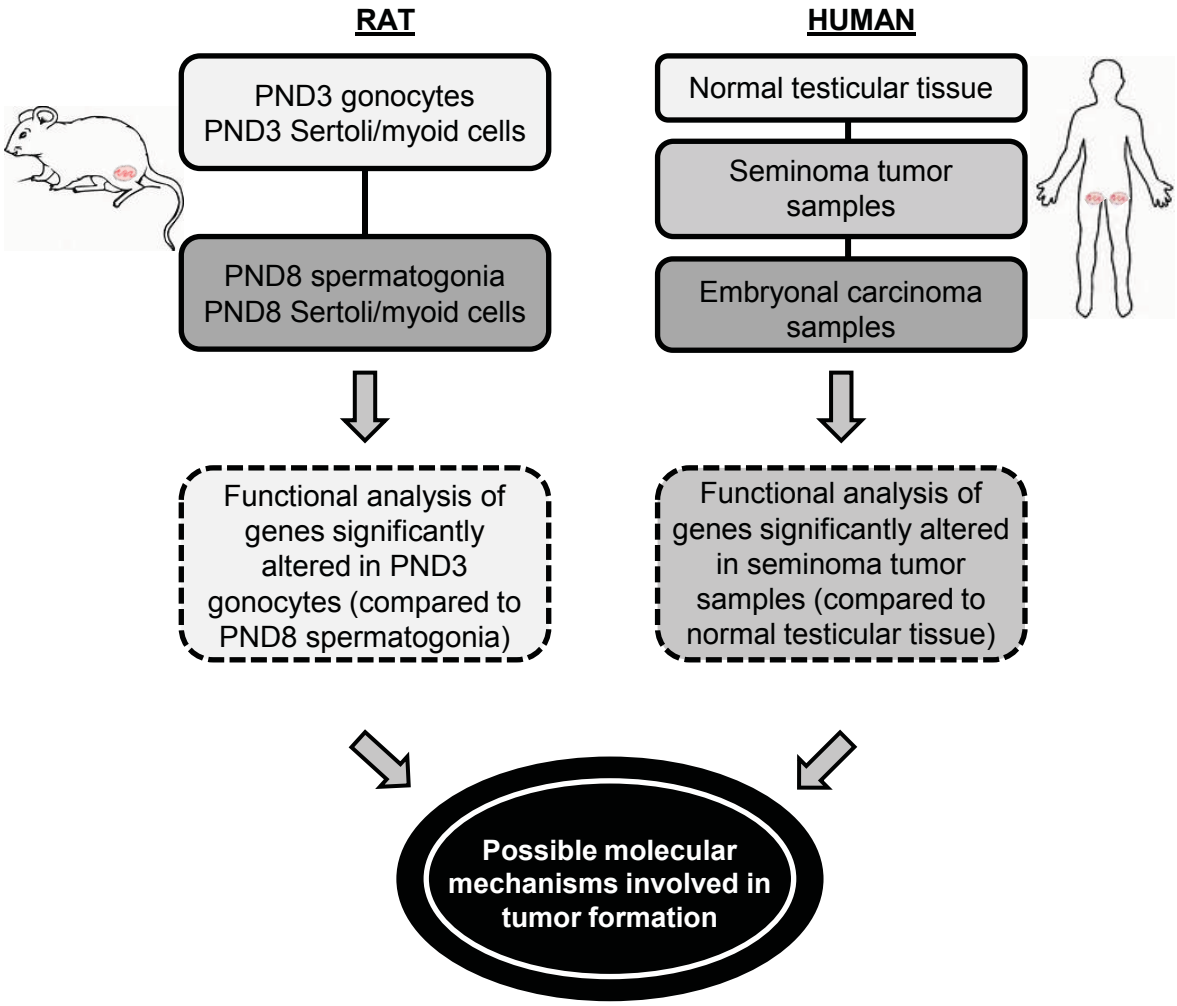
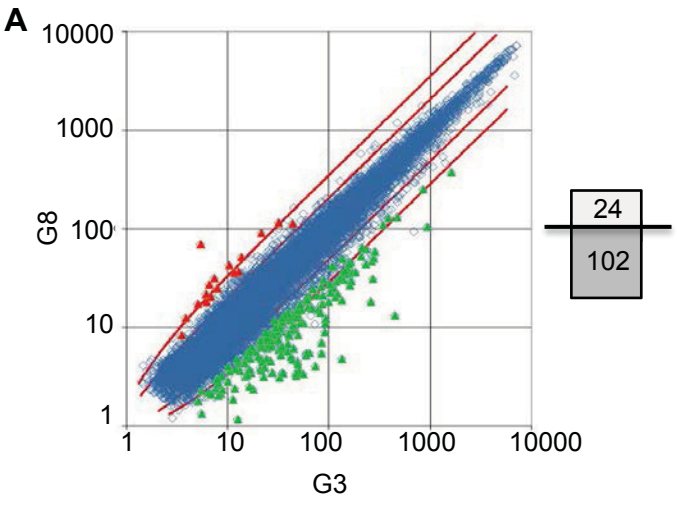


Figure 4.4: Gene array analysis: PND3 gonocytes compared to PND8 spermatogonia

Gene array analysis: PND3 gonocytes compared to PND8 spermatogonia. (A) 2-D Principle component analysis of samples tested G3: PND3 gonocytes, G8: PND8 spermatogonia, S3: PND3 Sertoli/myoid cells, S8: PND8 Sertoli/myoid cells. (B) Venn diagram representation of genes both commonly and uniquely present in the germ cell and somatic cell populations analyzed at both ages. (C) Gene expression dispersion plot of PND3 gonocytes and PND8 spermatogonia comparison. Red lines: boundaries of probability intervals 0.95 and 0.99; green triangles; candidate genes upregulated in PND3 gonocytes (compared to PND8 spermatogonia); red triangles: genes downregulated in PND3 gonocytes. (D) KEGG pathway analysis of genes upregulated in PND3 gonocytes. (E) KEGG pathway analysis of genes downregulated in PND3 gonocytes.



B Top 5 KEGG Functional Pathways

Genes Upregulated in PND3 Gonocytes
Cell adhesion molecules/Tight junctions
Focal adhesions
Leukocyte transendothelial migration
ECM-receptor interaction
Dilated cardiomyopathy

C Top 5 KEGG Functional Pathways

Genes Downregulated in PND3 Gonocytes
-NA-

Figure 4.5: <u>Gene array analysis: Normal testicular tissue compared to testicular tumor samples</u>

(A) (i) 2-D Principle component analysis of samples tested. NT: normal testis, SE: seminoma tumor samples, EC: embryonal carcinoma tumor samples; TC: TCam-2 human seminoma cell-line model; IN: testicular inflammation sample. (ii) Venn diagram analysis of the genes expressed in all tumor types. (B) TCam-2 human seminoma cell line analysis. (i) Gene expression dispersion plot of normal testicular tissue and TCam-2 cell comparison. Red lines: boundaries of probability intervals 0.95 and 0.99; red triangles: candidate genes upregulated in TCam-2 cells; green triangles: genes downregulated in TCam-2 cells compared to normal testicular tissue. (ii-iii) KEGG pathway analysis of genes upregulated and downregulated in TCam-2 cells (compared to normal testicular tissue). (C) Seminoma tumor gene array analysis. (i) Gene expression dispersion plot of normal testicular tissue and seminoma tumor comparison. Red lines: boundaries of probability intervals 0.95 and 0.99; red triangles: candidate genes upregulated in seminoma tumors; green triangles: genes downregulated in seminoma tumors compared to normal testicular tissue. (ii-iii) KEGG pathway analysis of genes upregulated and downregulated in seminoma tumors (compared to normal testicular tissue). (iv) Gene expression dispersion plot comparing TCam-2 cells and seminoma tumors. (D) Embryonal carcinoma tumor gene array analysis (i) Gene expression dispersion plot of normal testicular tissue and embryonal carcinoma tumor comparison. Red lines: boundaries of probability intervals 0.95 and 0.99; red triangles: candidate genes upregulated in embryonal carcinoma tumors while green triangles represent genes downregulated in EC tumors. (ii-iii) KEGG pathway analysis of genes upregulated and downregulated in EC tumors (compared to normal testicular tissue). (iv) Dispersion plot of gene expression differences while comparing seminoma tumors and EC tumor samples.

Figure 4.5: Gene array analysis: Normal testicular tissue compared to testicular tumor samples

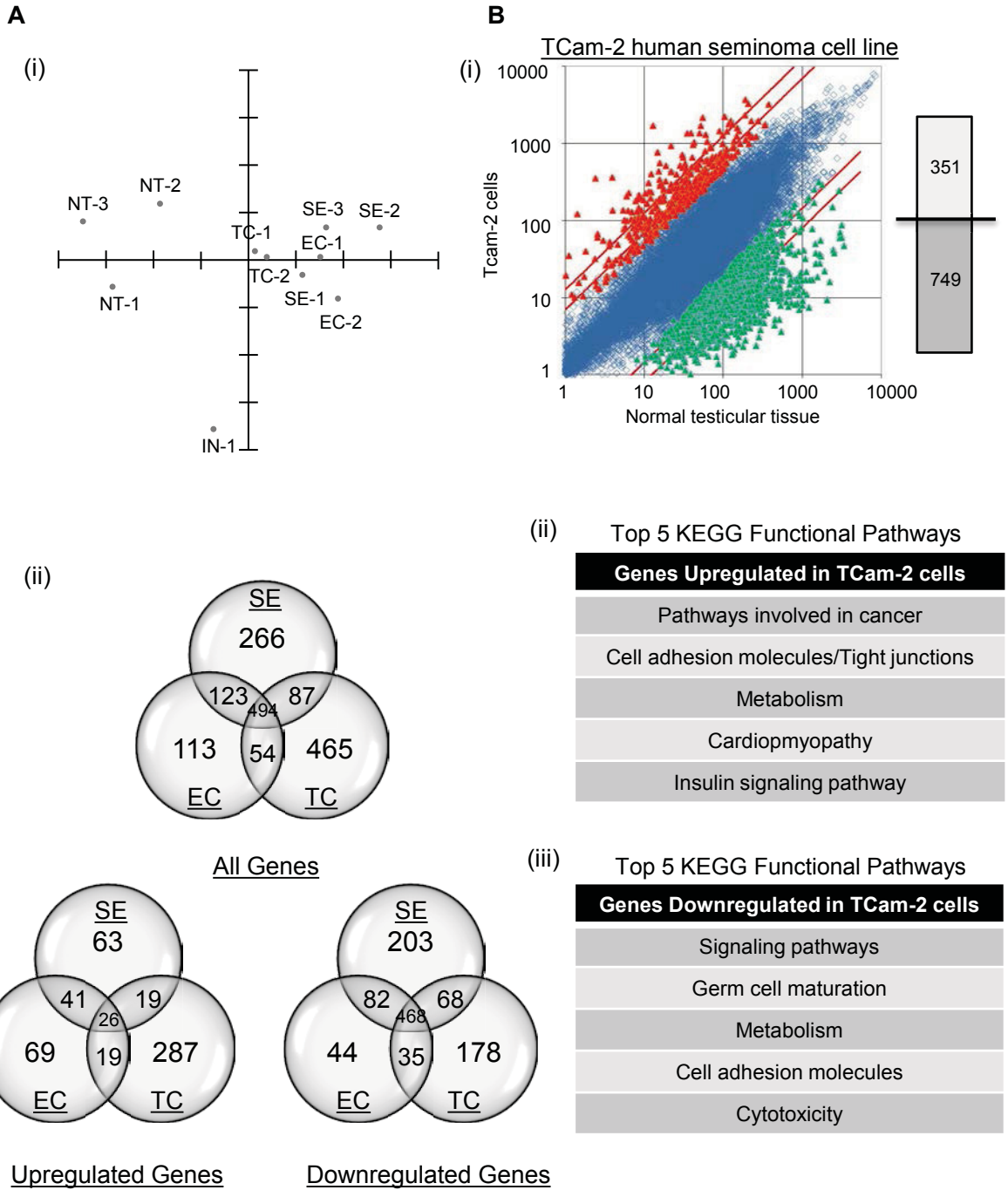
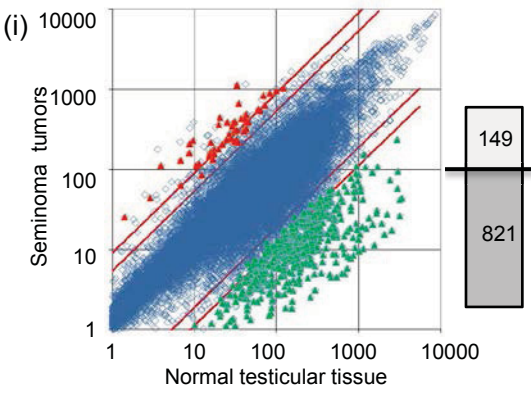


Figure 4.5: Gene array analysis: Normal testicular tissue compared to testicular tumor samples

C Seminoma tumors

(i)



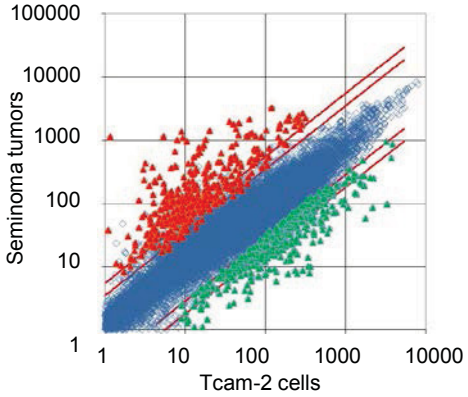
(ii) Top 5 KEGG Functional Pathways

Genes Upregulated in SE tumors
Cell adhesion molecules
Systemic lupus erythematosus
Hematopoietic cell lineage
Prion disease
Immune response

(iii) Top 5 KEGG Functional Pathways

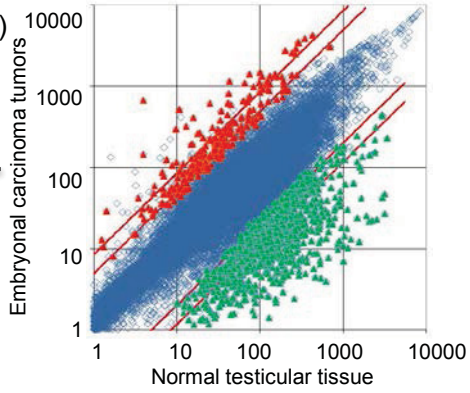
Genes Downregulated in SE tumors
Metabolism
Cell adhesion molecules
ErbB signaling pathway
Epithelial cell signaling
--

(iv)



D Embryonal Carcinoma tumors

(i)



(ii) Top 5 KEGG Functional Pathways

Genes Upregulated in EC tumors
Receptor interaction
Signaling pathways
Cell adhesion molecules
Immune response
Systemic lupus erythematosus

(iii) Top 5 KEGG Functional Pathways

Genes Downregulated in EC tumors
Meiosis
Dorso-ventral axis formation
Glycolysis/Gluconeogenesis
Pyruvate metabolism
--

(iv)

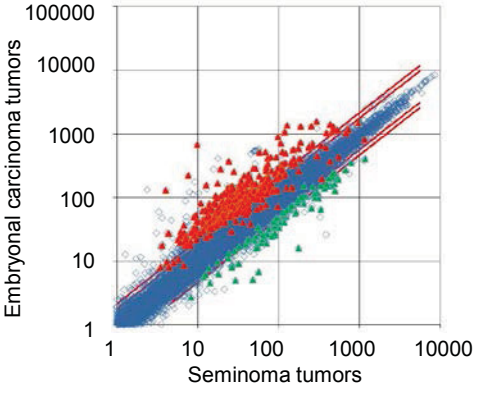


Figure 4.6: <u>Claudin expression in PND3 gonocytes and PND8 spermatogonia</u>

(A) qPCR analysis of claudin 3, 4, 5, 6, 7, and 11 using isolated PND 3 gonocytes and PND8 spermatogonia shown. Results are fold change values over expression levels in PND3 gonocytes and are expressed as means ± SEM of data obtained from 3-7 independent germ cell preparations, with each sample performed in triplicate. *$P<0.05$. (B) Protein expression of claudin 3, 4, 5, 6, 7, and 11 in PND3 and PND8 rat testes was evaluated by immunohistochemical analysis. Background signal was determined using rabbit IgG instead of primary antibody on some slides. Representative images are shown.

Figure 4.6: Claudin expression in PND3 gonocytes and PND8 spermatogonia

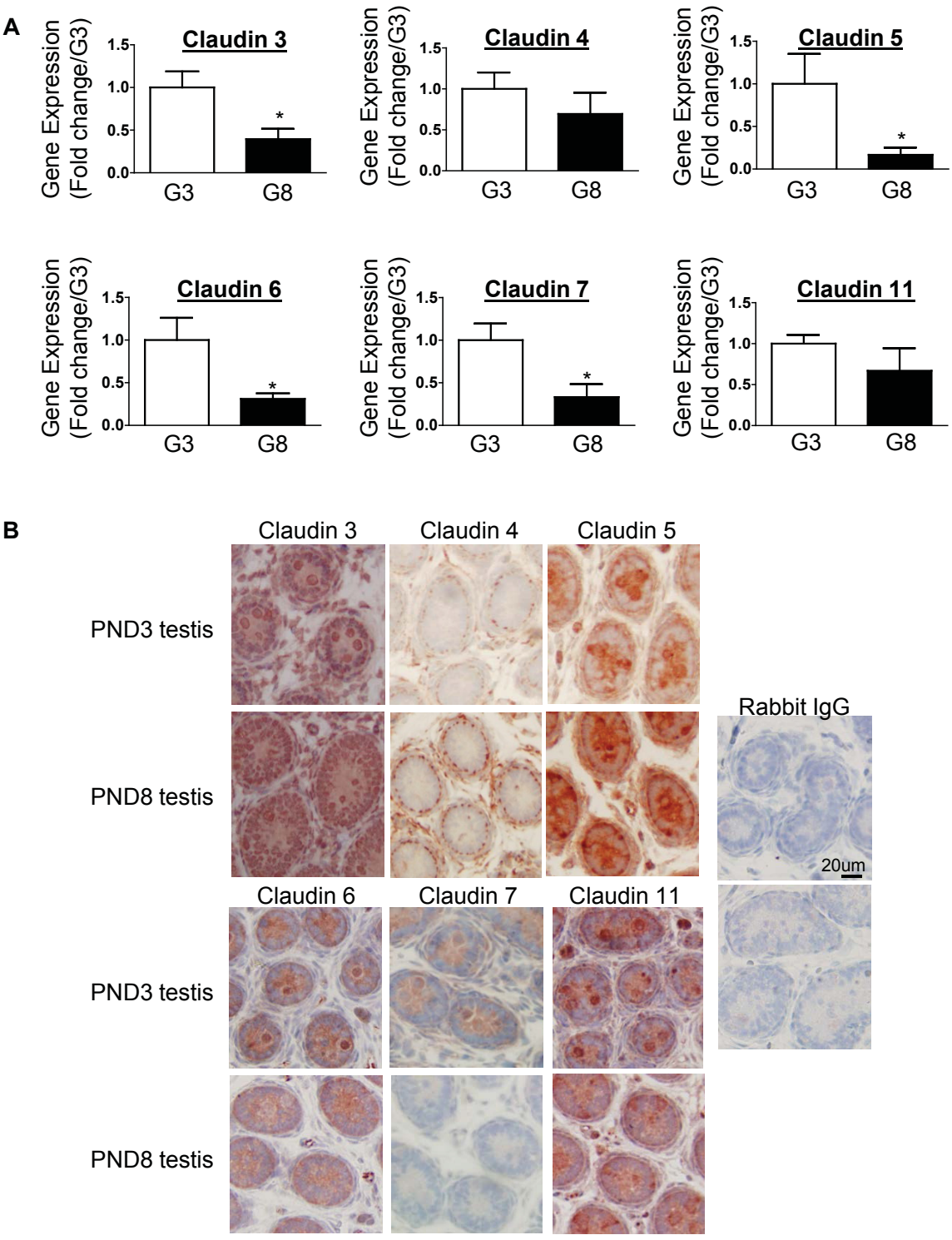


Figure 4.7: Claudin expression in normal and tumoral testicular tissue

(A) mRNA analysis of claudin 3, 4, 5, 6, 7, and 11 in normal testicular tissue and seminoma tumors is shown. Results are fold change values over expression levels in normal testicular tissue and are expressed as means ± SEM of data obtained from 3-7 independent human samples, each sample performed in triplicate. *P<0.05, ***P<0.005. (B) Claudin 6 protein expression in normal testicular tissue and various types of tumors. (i) normal testicular tissue, (ii-iv) seminoma tumors, (v-vi) carcinoma-in-situ, (vii) embryonal carcinoma, and (viii) yolk sac tumor. Background signal was determined by using rabbit IgG instead of primary antibody on each sample. Representative images are shown (x20). Inset: Hematoxylin and Eosin staining for each tissue type shown. (C) Claudin 7 protein expression in normal testicular tissue and various types of tumors. (i) normal testicular tissue, (ii-iv) seminoma tumors, (v-vi) carcinoma-in-situ, (vii) embryonal carcinoma, and (viii) yolk sac tumor. Background signal was determined by using rabbit IgG instead of primary antibody on each sample. Representative images are shown (x20). Arrow (1): cell membrane expression of Claudin 7, Arrow (2): nuclear expression of Claudin 7. Inset: Hematoxylin and Eosin staining for each tissue type shown.

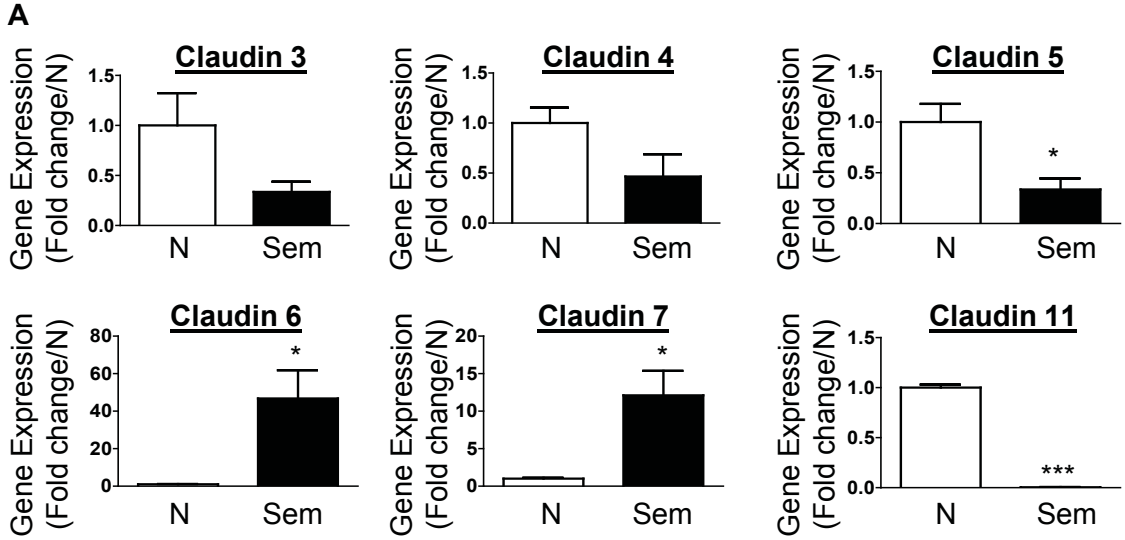
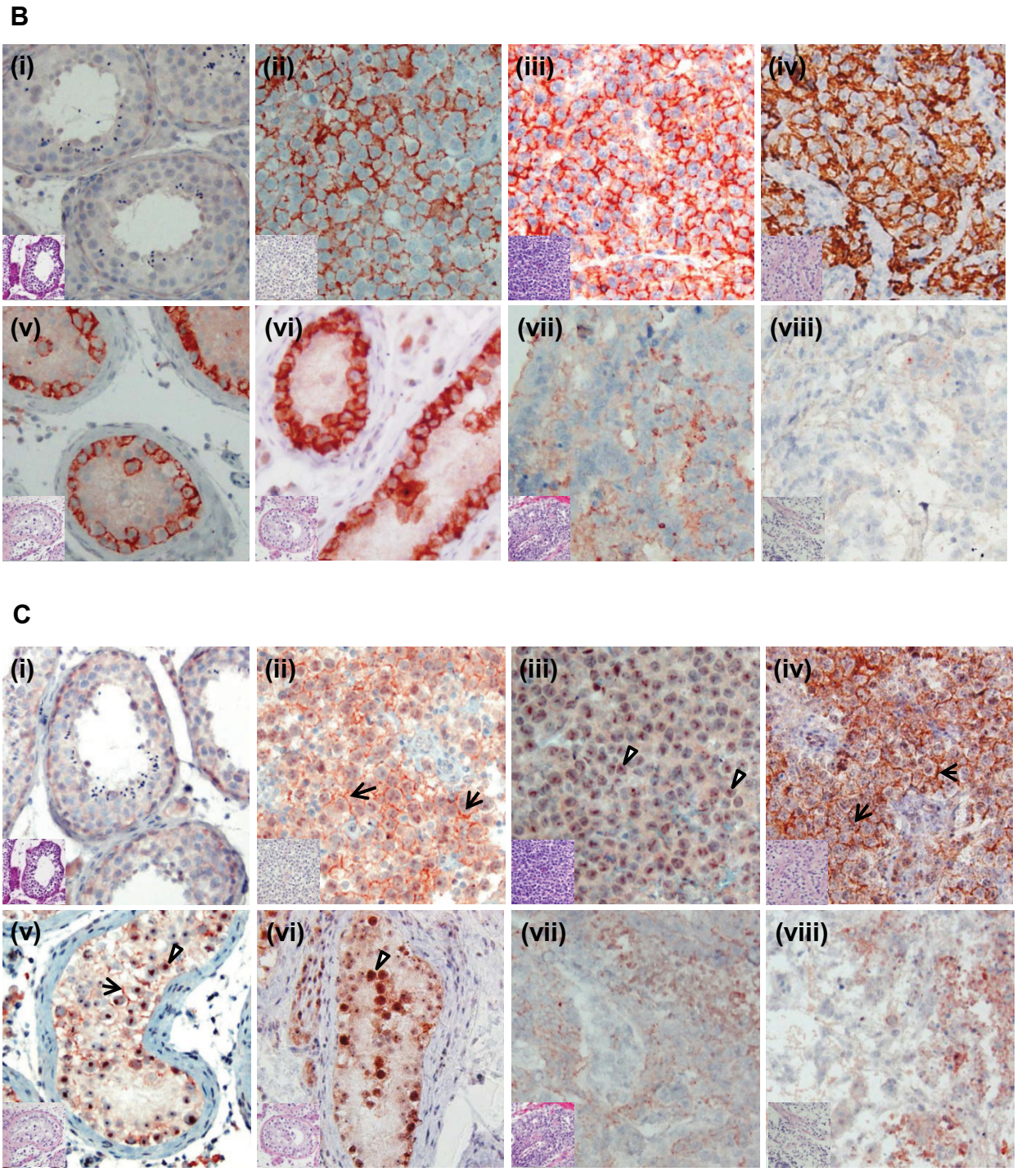
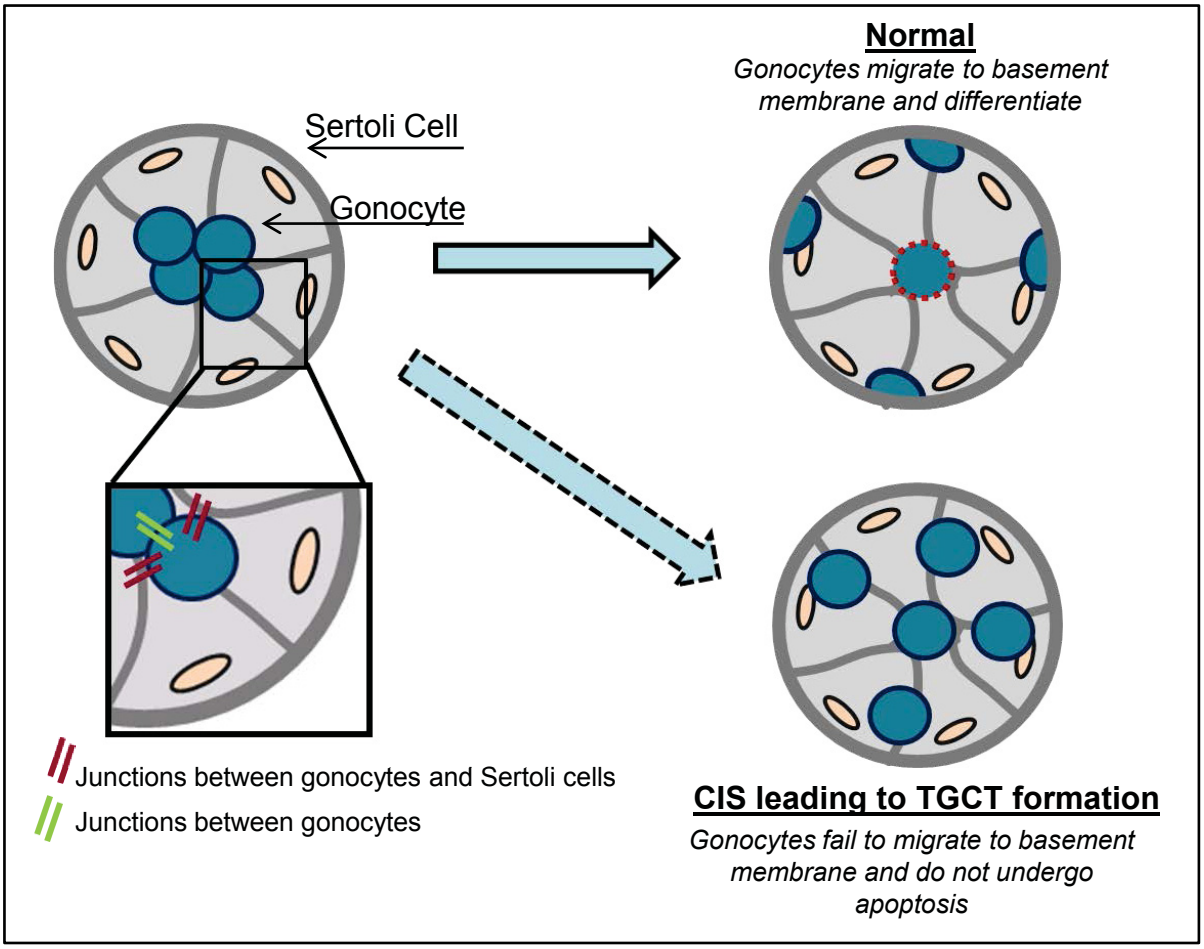


Figure 4.7: Claudin expression in normal and tumoral testicular tissue



Schematic 4.2: Possible role for claudins in both gonocyte development and tumor formation

Improper gonocyte differentiation would be possible due to a combination of the lack of proper migration of the gonocytes to the basement membrane and a lack of proper apoptosis of non-migrated gonocytes. A failure to downregulate of the claudin proteins throughout gonocyte development would prevent these cells from adequately undergoing migration to the basement membrane, thus hindering their differentiation, and possibly leading to TGCT formation. This failure to downregulate would also possibly explain why there is an upregulation of specific claudin proteins in seminomas.



4.8 Tables

Table 4.1: List of primers used for RT-PCR analysis

Rat

Gene	Accession no.	Primers (F/R)	Start BP
ANDROGEN RECEPTOR	NM_012502	CCCAGTCCCAGTTGTGTTAAA TAACATTTCCGGAGACGACAC	2482 2801
AP2γ (TFAP2C)	NM_201420	AAGTACAAAGTAACCGTGGCTGA GCTGAAATGAGACAAGCAGTTTT	930 1397
CD44	NM_012924	GCTATCTGTGCAGCCAACAA CAGGGTGCTCCCAATAAAGA	404 775
cKIT	NM_022264	AAAGGAGATCCGCAAGAATAGAC AGAGAGAATAGCTCCCAGAGGAA	2260 2641
DAZL	NM_001109414	GTTTTTGTTGGTGGAATTGATGT GGAGGATATGCCTGAACATACTG	322 707
ERα	NM_012689	TGCTGAACCACCTTTGATCTATT TTCAAGGTGCTGGATAGAAATGT	1187 1624
ERβ	NM_012754	TCCGACTGGCCAACCTCCTGAT GGCATTCGGTGGTACATCCTGGT	1639 2053
GFRα1	NM_012959	TGACCTGGAAGACTGCTTGAAAT AGCAGCCATTGATTTTGTGGTTA	1216 1573
INTEGRIN α6	XM_002729169	CTCTGGAAATAACGGTGACCA ACCACTTTTGCTTTCGCTGTA	2413 2770
INTEGRIN β1	NM_017022	GGACATTGATGACTGCTGGTT ACTTCGGATTGACCACAGTTG	2252 2553
INTEGRIN β3	NM_153720	ACTTCTCCTGTGTCCGCTACA CGTCAGTTCTTTCACCAGCTC	1660 2024
MILI (PIWIL2)	NM_001107276	AGCCTCCAACTCTGTCTCCA GCTCCAGGATCTTTGTCAGC	734 1117
MIWI2 (PIWIL4)	NM_001271133	TCCGCAATGAGACTGTTCTG TCAAGCGGAAGTCTGAGGTT	740 1123
NANOG	AB275459	ATTGCAGCTATTCTCAGGGCTAT AGAATTCACGGAGTAGTTCAGGA	542 921
NANOS2	NM_001108908	TACACTCAAGTATTGCCCACTCA CATCATCACCAGTCCCTCATAAT	400 746
NANOS3	NM_001105945	TCCAGAACGCCTATGTTCATT ATAGACACCTGCTGCTGCTTC	180 489
OCT 4 (POU5F1)	NM_001009178	GAGGAAGCTGACAACAACGAG AGGGACCGAGTAGAGTGTGGT	684 1070
PLZF (ZBTB16)	NM_001013181	TCTGTCTGCTGTGTGGGAA GTGGCAGAGTTTGCACTCAA	1632 1984
RET	NM_012643	CTTCTGAAACAAGTCAACCATCC AGATGTGATCGAAGAGAGACTCG	2348 2798
SOX2	NM_001109181	ACCAGCTCGCAGACCTACAT CCCTCCCAATTCCCTTGTAT	1000 1387
STELLA (DPPA3)	NM_001047864	AAAGGGTTCGTCTCCAGGTTA GGCAGAAAGTGCAGAGACATC	191 418
STRA8	XM_006236282	TGGATAATTTGCTGAAGCTCAA CCGTTCAAATTCCATGAGGT	2689 3005
THY-1	NM_012673	ACCCAGTCATCAGCATCACTC ACACATGTAGTCGCCCTCATC	99 406
VASA (DDX4)	NM_001077647	ACAAGTTTGAAGAAGGTGACAGC TTGGACCTTGAATATCACTGCTT	567 947
18S	X01117	TCCCAGTAAGTGCGGGTCAT GATCCTTCCGCAGGTTCAC	1648 1870

Human

Gene	Accession no.	Primers (F/R)	Start BP
ANDROGEN RECEPTOR	NM_000044	CAAGACCTACCGAGGAGCTTT CTGTGAAGGTTGCTGTTCCTC	1163 1469
AP2γ (TFAP2C)	NM_003222	TGAGATGGCAGCTAGGAAGAA TCTCCATTTTCTCCAGGGTTT	1272 1582
CD44 Std	NM_000610	GAGTTAAGTGCCTGGGGAGTC GAAGCATTGCCTGTTAAGCAC	3931 4254
CD44 V1	NM_000610	TTACCCACACACGAAGGAAAG CCTTCTTCGACTGTTGACTGC	2123 2459
CD44 V2	NM_001001389	CAGCACCATTTCAACCACAC GTTGCCAAACCACTGTTCCT	1220 1523
CD44 V3	NM_001001390	AAGGTGGAGCAAACACAACC CCACATTCTGCAGGTTCCTT	1582 1896
CD44 V4	NM_001001391	AAGGTGGAGCAAACACAACC CCACATTCTGCAGGTTCCTT	1186 1500
CD44 V5	NM_001001392	AATATAACCTGCCGCTTTGC TTGTTCACCAAATGCACCAT	507 815
CD44 V6	NM_001202555	CATCTACCCCAGCAACCCTA GGTTGTGTTTGCTCCACCTT	935 1409
CD44 V7	NM_001202556	GTGATCAACAGTGGCAATGG CAAGTGGGAACTGGAACGAT	1275 1710
CD44 V8	NM_001202557	TGAAGGCCACTCTCTCCCTA TTGGTTGCTTTCCAAAAACC	1472 1897
cKIT	NM_000222	TCTTCCCAAGGACTTGAGGTT CACCGTGATGCCAGCTATTAT	552 883
DAZL	NM_001190811	CTCCTCCACCACAGTTTCAGA AGATTTCTTTTGTGGGCCATT	749 1089
ERα (ESR1)	NM_000125	CCAAGGAGACTCGCTACTGTG AGGCCAGGCTGTTCTTCTTAG	770 1156
ERβ (ESR2)	NM_001437	TACGAAGTGGGAATGGTGAAG TTAACACCTCCATCCAACAGC	1096 1486
GFRα1	NM_005264	CACAGCTACGGAATGCTCTTC TCTAGGTCGTTCCCACTGTTG	1317 1666
INTEGRIN α6	NM_000210	CGAGGTTATGGAACAGCACAT AGCCTTGTGATATGTGGCATC	3064 3374
INTEGRIN β1	NM_002211	CTGCGAGTGTGGTGTCTGTAA TCTCCACAACATGAACCATGA	2057 2379
INTEGRIN β3	NM_000212	TGTGGCAGCTGTGTCTGTATC TGGGACACTCTGGCTCTTCTA	1845 2163
MILI (PIWIL2)	NM_018068	TGTCTCCCATAAGGTCATTCG CAGCAGGTTTCCCCTTTTAG	1285 1627
MIWI2 (PIWIL4)	NM_152431	TCGCTATGCAGATGACTTGC TGACCACAATCACCGACAGT	1995 2405
NANOG	NM_024865	AAAAACAACTGGCCGAAGAAT AGGACTGGATGTTCTGGGTCT	679 862
NANOS2	NM_001029861	AGAGATTGAGGAGCCAAGTCC ACACACGTAGTGCCTCAGGAT	184 379
NANOS3	NM_001098622	GAAAGAGGGTCCTGAAACCAG GTGGCTGTAGACGGAGGTGTA	66 423
OCT 4 (POU5F1)	NM_002701	AAGGAGAAGCTGGAGCAAAAC TCACTCGGTTCTCGATACTGG	449 797
PDGFRα (1.5kb)	NM_006206	GACTCAGGTTCCTCTGACATCTCG AATGATTCTGCCTGCCCACAG	INT 12 TCCE
PDGFRα (6.0kb)	NM_006206	GACCCGATGCAGCTGCCTTA AACTCCATTCCTCGGGCAACT	2057 2754
PLZF (ZBTB16)	NM_006006	GGCTGAGAATGCACTTACTGG AGCTGCCACAGAACTCACACT	1601 1924
RET	NM_020975	TGCCCAGTACCTACTCCCTCT TGACCCCTCCACTGTTACAAG	1401 1711
SOX2	NM_003106	AACCAAGACGCTCATGAAGAA GCGAGTAGGACATGCTGTAGG	782 1122

STELLA (DPPA3)	NM_199286	TCCCTTAGGCTCCTTGTTTGT ACAAATGCTCACCGAAGAAAA	123 441
STRA8	NM_182489	ACGATGGACCTTCTGACTGG TCGGAACCTCACTTTTGTCC	469 790
THY-1	NM_006288	GCTAACAGTCTTGCAGGTCTCC TCTGAGCACTGTGACGTTCTG	478 820
VASA (DDX4)	NM_024415	AATGCCATCAAAGGAACAGC TCTCTCTGTTCCCGATCACC	1503 1832
18S	NR_003286	CCCGAGATTGAGCAATAACA TCCTCGTTCATGGGGAATAA	1470 1640

Table 4.2: List of primers used for quantitative PCR analysis

Rat

Gene	Accession no.	Primers (F/R)	Start BP
ANDROGEN RECEPTOR	NM_012502	ATTCCTGGATGGGACTGATGGT cggtcGAGTTGACATTAGTGAAGGACCG	3158 3218
AP2γ (TFAP2C)	NM_201420	CGCCCATGTCACTCTCCTCAC cgggCAGACGTAGGCAAAGTCCCG	1103 1177
CD44	NM_012924	TTGCACACCGACCTTCCCACT cgggCAATAAAGAAGGCGTCATCCCG	704 764
cKIT	NM_022264	ACGTGGTAAAAGGAAATGCACG AAAATGCTCTCCGGTGCCA	2515 2572
CLAUDIN 3	NM_031700	cggcCTGTGCTCACCTTAGTGCCG GTTCCCATCTCTCGCTTCTGG	823 922
CLAUDIN 4	NM_001012022	cgtacatAGATGCAGTGCAAGATGTACG CCCACGATGATGCTGATGACC	373 463
CLAUDIN 5	NM_031701	CCAACATCGTAGTCCGGGAGTT cggtgCAGCTCGTACTTCTGAGACACCG	561 622
CLAUDIN 6	NM_001102364	GCTGATAGGTGGAGGGCTGCTA cgctatGGGACAGGCGAAGAATAGCG	653 745
CLAUDIN 7	NM_031702	GGTGGGTCTGATAGCGAGCAC cgacaGGGCTGTGATGATGTTGTCG	359 435
CLAUDIN 11	NM_053457	TGGAGTGGCCAAGTACAGG GACAATGGCGCAGAGAGC	531 603
DAZL	NM_001109414	cggttGATGTTAGGATGGATGAAACCG AGCCCTTCGACACACCAGTTC	340 442
ERα (ESR1)	NM_012689	TGAAAGGCGGGATACGAAAA TTCGGCCTTCCAAGTCATCT	976 1056
ERβ (ESR2)	NM_012754	CAACCAGTGGCTGGGAGT CATGGGACTCAGATGTAATGACTG	100 209
GFRα1	NM_012959	GAATGAGATCCCCACACACGTT TGAGCCTGCAAATTCGCAC	1408 1458
INTEGRIN α6	XM_006234351	TCAGAATATCAAGCTCCCTCATGC cggtgCGTCTTTGAGGGAAACACCG	3345 3408
INTEGRIN β1	NM_017022	TTGGGACGGATCTGATGAAT TCCACAAATGAGCCAAAGC	664 736
INTEGRIN β3	NM_153720	cgggCTAACCGACCAGGTGACCCG CTCGGTTACGTGACACACTCTGC	672 735
MILI (PIWIL2)	NM_001107276	GGGTCTCTTCTTGCTCGCTGA cggaaGACATCCAGTACAGAGTCATTCCG	1293 1356
MIWI2 (PIWIL4)	NM_001271133	ATGGCAGACAGGACTTCGTCGATT AGCTGCACAGGTATTCCGCTAGAA	161 260
NANOG	AB275459	ACGCTGCTCCGCTCCATAAC cgttgGGAGAAGTTTTGCTGCAACG	749 819
NANOS 2	NM_001108908	cgagacCAAGCACAACGGAGAGTCTCG CCTCAGGATGGGACACACTACC	271 355
NANOS 3	NM_001105945	cagcatAAGGCAAAGACACAGAATGCTG TGGAACCCGCATAGACACCTG	430 499

OCT 4 (POU5F1)	NM_001009178	cggttTTCGAGTGTGGTTCTGTAACCG GCGGCCTCATACTCTTCTCGT	880 961
PLZF (ZBTB16)	NM_001013181	CGCCCAGTTCTCAAAGGA AGACAGAAGACAGCCATGTCC	1561 1638
RET	NM_012643	cgggTGTAGCTCCTTCAAAGCCCG CCTGATGCGGAAGGACACAC	482 544
SOX2	NM_001109181	cggtcGGAAACTTTTGTCGGAGACCG ATCCGGGTGCTCCTTCATGT	605 690
STELLA (DPPA3)	NM_001047864	cggacTTTCCCAGGAGAAGGGTCCG CGAACAAATCTTCTCATCCTTGC	268 341
STRA8	XM_006236282	TGCTTTTGATGTGGCGAGCT GCGCTGATGTTAGACAGACGCT	3305 3367
THY-1	NM_012673	cggtcGTCAACCTTTTCAGTGACCG CATGTAGTCGCCCTCATCCTTG	326 403
VASA (DDX4)	NM_001077647	cgaatCAGTTTGGGCACTCAATTCG TCAGCCTCCCTGGAGTAGCAC	1430 1499
18S	X01117	cgggTGCTCTTAGCTGAGTGTCCCG CTCGGGCCTGCTTTGAACAC	768 842

Human

Gene	Accession no.	Primers (F/R)	Start BP
ANDROGEN RECEPTOR	NM_000044	GCCTTGCTCTCTAGCCTCAA GGTCGTCCACGTGTAAGTTG	3213 3315
AP2γ (TFAP2C)	NM_003222	cggctCTTTGCCTATGTCTGTGAAGCCG AGCTGCCATCTCATTTCGTCCT	1191 1284
CD44 Std	NM_000610	cggcTCGATTTGAATATAACCTGCCG CGAGAGATGCTGTAGCGACCA	499 571
CD44 V1	NM_000610	cggatACCCAAGCCATTCAAATCCG AAGCAGTGGTGCCATTTCTGTC	1417 1497
CD44 V2	NM_001001389	cggatACCCAAGCCATTCAAATCCG AAGCAGTGGTGCCATTTCTGTC	1288 1368
CD44 V3	NM_001001390	cggtgGAAGACAGTCCCTGGATCACCG TGTTTGGATTTGCAGTAGGCTGA	1053 1152
CD44 V4	NM_001001391	cggtgGAAGACAGTCCCTGGATCACCG TGGAATGTGTCTTGGTCTCTGG	1053 1120
CD44 V5	NM_001001392	cggcTCGATTTGAATATAACCTGCCG CGAGAGATGCTGTAGCGACCA	499 571
CD44 V6	NM_001202555	GACACCATGGACAAGTTTTGG CGGCAGGTTATATT	429 520
CD44 V7	NM_001202556	GACAGAATCCCTGCTACCAGAC TGTCCTTATAGGACCAGAGGTTG	1083 1160
CD44 V8	NM_001202557	GCAGAAAAATGCCCCTCAG GGTTGCTTTCCAAAAACCAG	1820 1895
cKIT	NM_000222	cggcGCTGTAAAGATGCTCAAGCCG GAGTTCAGACATGAGGGCTTCC	1948 2010
CLAUDIN 3	NM_001306	AGAACATCTGGGAGGGCCTGT gtacgaAGTGCCAGCAGCGAGTCGTAC	349 436
CLAUDIN 4	NM_001305	cggatCATCCAAGACTTCTACAATCCG ACGTAGAGCGAGGCACCCATC	768 836
CLAUDIN 5	NM_001130861	cgtaaaTTGTCGTCCGCGAGTTTTACG CTGCGCCCAGCTCGTACTTCT	1507 1569
CLAUDIN 6	NM_021195	cgatgaACGTGCCCTCTGTGTCATCG CCAGCAAGGTAGACCAGCAAGC	300 362
CLAUDIN 7	NM_001307	cgtgatCGGGTGACAACATCATCAcG GTGACGCAGTCCATCCACAG	1009 1069
CLAUDIN 11	NM_005602	CCCGGTGTGGCTAAGTACAG CAACAAGGGCGCAGAGAG	536 611
DAZL	NM_001190811	ATCGAACTGGTGTGTCCAAAGG cgtggTCTACTATCTTCTGCACATCCACG	569 641
ERα (ESR1)	NM_000125	TGTGTAGAGGGCATGGTGGAG cggttAGATTCATCATGCGGAACCG	1483 1553

ERβ (ESR2)	NM_001437	CAGTGTATGACCTGCTGCTGGA cggcGCTTTTACTGTCCTCTGCcG	1925 2028
GFRα1	NM_005264	CCTCCAAGCTGTGGTCTGAGC cgctcAAGATAATAGGGTGGACAGAGCG	2013 2079
INTEGRIN α6	NM_000210	CCCACGTCAGAAAGCAAGGAAG cgtccCTGGACCTTGGCTCTGGACG	516 576
INTEGRIN β1	NM_002211	CAATGAAAGACGATTTGGAGAATG cggacCCAAATCCAATTCTGAAGTCCG	682 775
INTEGRIN β3	NM_000212	cgggCTAACTGACCAGGTGACCCG CTCGGTTCCGTGACACACTCTG	684 747
MILI (PIWIL2)	NM_018068	cggaaGGTTTGGTCTCCATGTTCCG GGAAGCATTTCCCGTTTCAGAG	356 429
MIWI2 (PIWIL4)	NM_152431	AGTGGAAGAGCCCGAGTGA AGATCAACAGATCTAGGCAATGG	215 315
NANOG	NM_024865	GGCCTCAGCACCTACCTACCC cgactgACATTGGAAGGTTCCCAGTCG	720 802
NANOS2	NM_001029861	GTCTTCGCAGGCTCACCT ACCAGGGGGCATTGAAAG	1127 1193
NANOS3	NM_001098622	CGGGACCCAAGGATCAGAAG cgaacAGAAAGAGCACAGGCGTTCG	173 238
OCT4 (POU5F1)	NM_002701	CTGTACTCCTCGGTCCCTTTCC cgtcaGAGAGCCCAGAGTGGTGACG	1070 1139
RET	NM_020975	cggttCGGCCTCCTCTACCTTAACCG GTTGCGGACACTGAGCTTCTCC	466 529
STRA8	NM_182489	cgctgGGACCTTCTGACTGGCAGCG CTCCGAGAGGTTCTGCCACAG	474 570
VASA (DDX4)	NM_024415	CTGGTCGTTGTGGGAATACTGG cggatGGCTGTGCTAAATGGTTATCCG	1991 2063
18S	NR_003286	cggacTGTGATGCCCTTAGATGTCCG GTAGGGTAGGCACACGCTGAG	1494 1562

Table 4.3: List of antibodies used in immunohistochemical analysis

Antibody	Company	Catalog number	Species	IHC dilution
Claudin 3	Abcam	ab15102	Rabbit	1:75
Claudin 4	Santa Cruz	sc-376643	Mouse	1:50
Claudin 5	ARP	18855	Rabbit	1:100
Claudin 6	ARP	18865	Rabbit	1:100
Claudin 7	ARP	18875	Rabbit	1:100
Claudin 11	Santa Cruz	sc-28669	Rabbit	1:100
OCT 4	Santa Cruz	sc-5279	Mouse	1:200
PDGFRα	Abcam	ab61219	Rabbit	1:100
Biotin Anti Mouse	BD Pharmingen	550337	Goat	1:100
Biotin Anti Rabbit	BD Pharmingen	550338	Goat	1:100

Table 4.4: List of antibodies used in immunocytochemical analysis

Antibody	Company	Catalog number	Species	ICC dilution
AR	Fitzgerald	20R-AR012	Rabbit	1:100
cKIT	Santa Cruz	sc-1494	Goat	1:100
ERα	Thermo Scientific	MA1-310	Mouse	1:100
ERβ	Thermo Scientific	PA1-313	Rabbit	1:100
ERK1/2	Cell Signaling	4695	Rabbit	1:100
GFRα1	Santa Cruz	sc-6156	Goat	1:100
HSP90α	Enzo Life Sciences	ADI-SPS-771	Rabbit	1:100
OCT4	Santa Cruz	sc-5279	Mouse	1:100
PCNA	Santa Cruz	sc-56	Mouse	1:100
PDGF AB-1	CalBioChem	PC21	Rabbit	1:100
PDGFRα	Upstate	07-276	Rabbit	1:100
PDGFRβ	Santa Cruz	sc-432	Rabbit	1:100
STRA8	Abcam	ab49602	Rabbit	1:100
VASA	Santa Cruz	sc-48707	Goat	1:100
Biotin Anti Goat	Abcam	ab6740	Goat	1:50
Biotin Anti Mouse	BD Pharmingen	550337	Goat	1:100
Biotin Anti Rabbit	BD Pharmingen	550338	Goat	1:100

Table 4.5: Significantly altered cell adhesion molecules represented in KEGG pathway analysis

G3: PND3 gonocytes, G8: PND8 spermatogonia, N: normal testicular tissue, SE: seminoma tumors, EC: embryonal carcinoma tumors.

GONOCYTES and SPERMATOGONIA GENE ARRAY

CAMs upregulated in gonocytes

Gene	Accession no.	G3 (Ave)	G8 (Ave)	Fold (G3/G8)	P-value (G3/G8)
CLDN5	NM_031701	134.1 ± 20.5	18.9 ± 0.9	7.1	0.002
MYH6	NM_017239	126.3 ± 41.6	31.4 ± 7.0	4.0	0.044
CLDN4	NM_001012022	123.9 ± 3.7	39.9 ± 14.6	3.1	0.002
CLDN3	NM_031700	96.4 ± 17.0	36.8 ± 9.8	2.6	0.019

CAMs downregulated in gonocytes

Not Applicable

TCam-2 GENE ARRAY

CAMS upregulated in TCam-2 cells

Gene	Accession no.	N (Ave)	TCam2 (Ave)	Fold (TCam2/N)	P-value (TCam2/N)
CDH1	NM_004360	14.8 ± 2.1	770.3 ± 43.3	52.1	0.000
RAB3B	NM_002867	22.1 ± 7.7	933.1 ± 7.2	42.3	0.000
CLDN6	NM_021195	15.5 ± 6.9	434.7 ± 5.1	28.0	0.000
CLDN7	NM_001307	21.1 ± 5.4	245.3 ± 25.9	11.6	0.001
CDH3	NM_001793	37.9 ± 11.6	431.4 ± 11.7	11.4	0.000
ICOSLG	NM_015259	20.7 ± 0.9	150.7 ± 7.9	7.3	0.000
F11R	NM_016946	104.9 ± 5.5	715.5 ± 23.2	6.8	0.000
SDC2	NM_002998	114.4 ± 17.0	692.7 ± 10.8	6.1	0.000
OCLN	NM_002538	55.1 ± 8.0	331.2 ± 0.2	6.0	0.000
PARD6G	NM_032510	14.4 ± 0.8	84.8 ± 1.0	5.9	0.000
PTPRF	NM_002840	71.0 ± 4.5	402.7 ± 15.6	5.7	0.000
KRAS	NM_033360	99.7 ± 13.9	501.8 ± 15.9	5.0	0.000
TJP3	NM_014428	45.1 ± 5.3	211.5 ± 14.1	4.7	0.000

CAMS downregulated in TCam-2 cells

Gene	Accession no.	N (Ave)	TCam2 (Ave)	Fold (N/TCam2)	P-value (N/TCam2)
CLDN11	NM_005602	2977.9 ± 335.5	70.2 ± 9.1	42.4	0.003
JAM2	NM_021219	245.2 ± 29.5	6.9 ± 2.0	35.4	0.004
NCAM2	NM_004540	109.6 ± 24.3	6.5 ± 0.4	16.9	0.023
ITGB8	NM_002214	189.2 ± 39.3	21.9 ± 4.2	8.7	0.023
PVRL3	NM_015480	347.0 ± 11.5	54.9 ± 1.1	6.3	0.000
HLA-B	NM_005514	1210.6 ± 76.6	198.9 ± 35.0	6.1	0.001
HLA-A	NM_002116	1605.7 ± 106.7	318.2 ± 61.3	5.0	0.001
CADM1	NM_014333	155.8 ± 12.0	31.8 ± 4.0	4.9	0.002

Table 4.5: Significantly altered cell adhesion molecules represented in KEGG pathway analysis

SEMINOMA GENE ARRAY

CAMS upregulated in seminoma tumors

Gene	Accession no.	N (Ave)	SE (Ave)	Fold (SE/N)	P-value (SE/N)
CLDN6	NM_021195	15.3 ± 6.9	145.6 ± 21.4	9.5	0.002
HLA-DQA1	NM_002122	122.1 ± 36.8	1028.7 ± 305.4	8.4	0.022
PTPRC	NM_002838	21.7 ± 2.9	138.5 ± 47.5	6.4	0.037
HLA-DQA2	NM_020056	28.7 ± 5.5	179.2 ± 45.3	6.2	0.015
CDH3	NM_001793	37.6 ± 11.6	207.3 ± 31.4	5.5	0.004
ITGA4	NM_000885	13.6 ± 2.3	64.5 ± 8.9	4.7	0.003

CAMS downregulated in seminoma tumors

Gene	Accession no.	N (Ave)	SE (Ave)	Fold (N/SE)	P-value (N/SE)
CLDN11	NM_005602	2977.9 ± 335.5	93.7 ± 29.1	31.8	0.001
ITGB8	NM_002214	189.2 ± 39.3	11.9 ± 1.8	15.9	0.005
CNTN1	NM_001843	90.5 ± 19.4	6.9 ± 0.8	13.1	0.006
PVRL3	NM_015480	347.0 ± 11.5	32.2 ± 11.7	10.8	0.000
NCAM2	NM_004540	109.6 ± 24.3	11.4 ± 2.8	9.6	0.008
CD99	NM_002414	1147.4 ± 2.7	151.8 ± 32.4	7.6	0.000
JAM2	NM_021219	245.2 ± 29.5	41.1 ± 16.7	6.0	0.002
CADM1	NM_014333	155.8 ± 12.0	28.5 ± 4.4	5.5	0.000

EMBRYONAL CARCINOMA GENE ARRAY

CAMs upregulated in embryonal carcinoma tumors

Gene	Accession no.	N (Ave)	EC (Ave)	Fold (EC/N)	P-value (EC/N)
CLDN6	NM_021195	15.3 ± 6.9	195.6 ± 72.6	12.8	0.022
HLA-DQA1	NM_002122	122.1 ± 36.8	906.5 ± 1.5	7.4	0.000
HLA-DQB1	M60028	34.8 ± 6.0	251.3 ± 72.7	7.2	0.014
ITGA4	NM_000885	13.6 ± 2.3	89.1 ± 14.6	6.5	0.003
PTPRC	NM_002838	21.7 ± 2.9	141.1 ± 38.8	6.5	0.013
HLA-DQA2	NM_020056	28.7 ± 5.5	163.0 ± 3.9	5.7	0.000
CDH3	NM_001793	37.6 ± 11.6	203.3 ± 61.0	5.4	0.021
SELL	NM_000655	14.7 ± 0.6	75.5 ± 3.7	5.1	0.000
HLA-DRB3	NM_022555	219.7 ± 45.0	889.0 ± 48.8	4.0	0.001

CAMs downregulated in embryonal carcinoma tumors

Not Applicable

Table 4.6: <u>The top 100 genes of interest from both the rat and human gene array analysis</u>

The numbers represent the fold changes in each gene array category. Red values represent the fold change by which that particular gene is downregulated in either PND3 gonocytes (compared to PND8 spermatogonia) or TCam-2 cells, SE (seminoma) tumors, or EC (embryonal carcinoma) tumors (compared to normal testicular tissue). Green values represent upregulated genes in the same categories. If genes were not significantly altered within a specific array, the fold change was left blank. Fold change values shown are significant (p-value <0.05).

Table 4.6: The top 100 genes of interest from both the rat and human gene array analysis

Gene	Full name	Gonocytes	TCam-2	SE tumors	EC tumors
ADAM2	ADAM metallopeptidase domain 2		107.5	102.6	105.6
ADAM18	ADAM metallopeptidase domain 18		33.2	28.6	34.5
ADAM30	ADAM metallopeptidase domain 30		36.0	39.3	41.2
ADAM32	ADAM metallopeptidase domain 32		26.8		14.5
ADAMTS9	ADAM metallopeptidase with thrombospondin type 1 motif, 9		11.9		
ADAMTS19	ADAM metallopeptidase with thrombospondin type 1 motif, 19		8.9		
ALDH1A1	aldehyde dehydrogenase 1 family, member A1		124.8	11.8	8.0
ALDH1A2	aldehyde dehydrogenase 1 family, member A2		14.7	13.4	
CASC1	cancer susceptibility candidate 1		29.1	23.9	17.6
CCND1	cyclin D1	3.3			
CCND2	cyclin D2		12.4	6.3	4.6
CD24	CD24 molecule	2.3	23.2		
CD38	CD38 molecule		12.2	8.9	5.1
CD48	CD48 molecule			10.0	7.5
CD52	CD52 molecule			9.0	7.7
CD53	CD53 molecule			8.5	7.3
CD68	CD68 molecule			5.2	4.3
CDH3	cadherin 3, type 1, P-cadherin		11.5	5.5	5.4
CDKL2	cyclin-dependent kinase-like 2		10.6	15.8	19.7
CDKL3	cyclin-dependent kinase-like 3		8.3	10.9	10.6
CYP11A1	cytochrome P450, family 11, subfamily A, polypeptide 1	8.3	8.3	16.6	8.2
DAZ1	deleted in azoospermia 1		40.8	34.7	21.1
DAZ2	deleted in azoospermia 2		40.8	34.7	21.1
DAZ3	deleted in azoospermia 3		40.3	31.7	18.8
DAZ4	deleted in azoospermia 4		40.8	34.7	21.1
DAZL	deleted in azoospermia-like		67.5		
DDX4	DEAD (Asp-Glu-Ala-Asp) box polypeptide 4		64.8	10.4	18.2
DDX20	DEAD (Asp-Glu-Ala-Asp) box polypeptide 20			4.7	4.8
DDX25	DEAD (Asp-Glu-Ala-Asp) box helicase 25		27.3	25.7	20.4
DNMT3L	DNA (cytosine-5-)-methyltransferase 3-like	34.0	32.9		
DPPA4	developmental pluripotency associated 4		9.8	5.6	7.9
FBXO5	F-box protein 5		4.9		
FBXO15	F-box protein 15		8.3	8.2	9.0
FBXO24	F-box protein 24				6.2
FBXO25	F-box protein 25		7.7	8.0	5.0
FBXO31	F-box protein 31		13.4	14.5	15.3
FBXO43	F-box protein 43		6.4		4.7
FGF13	fibroblast growth factor 13		7.3		
FGF16	fibroblast growth factor 16		18.1		
GAPDHS	glyceraldehyde-3-phosphate dehydrogenase, spermatogenic		25.0	20.9	23.4
GDF3	growth differentiation factor 3			10.5	
GDF15	growth differentiation factor 15	2.3			
GPX2	glutathione peroxidase 2		9.0	12.7	6.4
GSG1	germ cell associated 1		34.1	22.8	22.4
JAK2	Janus kinase 2		5.1		
LIN28A	lin-28 homolog A		17.0	6.1	10.6
LIN28B	lin-28 homolog B		8.7		4.0
MAK	male germ cell-associated kinase		7.7	9.0	6.9
MMP3	matrix metallopeptidase 3	9.0			
MMP9	matrix metallopeptidase 9	2.7			5.3
MMP12	matrix metallopeptidase 12	5.4			

Table 4.6: The top 100 genes of interest from both the rat and human gene array analysis

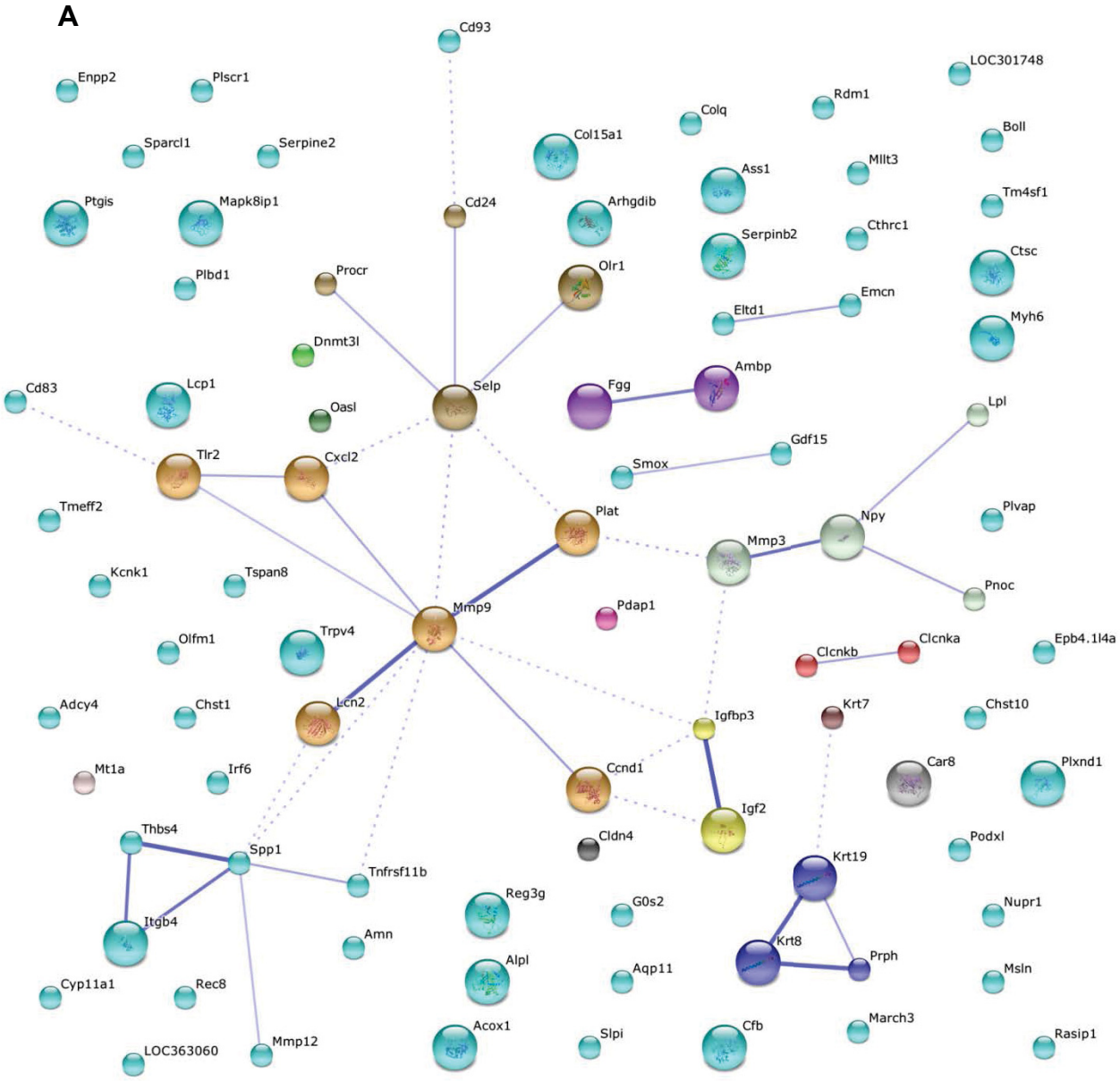
Gene	Full name	Gonocytes	TCam-2	SE tumors	EC tumors
NANOG	Nanog homeobox		15.8	10.5	9.3
NANOS3	nanos homolog 3 (Drosophila)	12.8		7.2	
PDGFA	platelet-derived growth factor alpha polypeptide		10.4		
POU5F1	POU class 5 homeobox 1		10.6	11.0	9.4
RNF17	ring finger protein 17		14.7		
RNF32	ring finger protein 32		16.1	9.8	10.5
RNF133	ring finger protein 133		24.1	19.4	17.4
RNF141	ring finger protein 141			5.2	5.1
RNF148	ring finger protein 148		22.2	17.7	19.8
RNF157	ring finger protein 157		11.6	13.1	7.9
RNFT1	ring finger protein, transmembrane 1		16.9	8.5	8.6
SERPINE 2	serpin peptidase inhibitor, clade E	2.3		4.1	
SOHLH2	spermatogenesis and oogenesis specific basic helix-loop-helix 2		12.4	4.6	6.2
SPA17	sperm autoantigenic protein 17		19.0	21.1	17.6
SPATA4	spermatogenesis associated 4		29.7	27.2	31.4
SPATA5	spermatogenesis associated 5	2.3			
SPATA8	spermatogenesis associated 8		54.0	61.6	72.6
SPATA16	spermatogenesis associated 16		37.6	34.2	38.4
SPATA17	spermatogenesis associated 17		38.7	20.9	27.2
SPATA19	spermatogenesis associated 19		15.5	11.8	13.9
SPATA20	spermatogenesis associated 20		14.5	9.0	
SPATA22	spermatogenesis associated 22		17.5	11.0	33.7
STAR	steroidogenic acute regulatory protein		29.0	16.2	16.3
TACSTD2	tumor-associated calcium signal transducer 2		16.3		
TDGF1	teratocarcinoma-derived growth factor 1		92.5		
TDRG1	testis development related 1		25.7	20.5	24.6
TEAD4	TEA domain family member 4		5.9		
TEX11	testis expressed 11		23.6	5.1	
TEX14	testis expressed 14		17.7	13.7	10.7
TEX15	testis expressed 15		24.8	32.3	10
TEX101	testis expressed 101		31.8	25.7	31.2
TIMP2	TIMP metallopeptidase inhibitor 2		8.0	8.7	9.1
TRIM36	tripartite motif containing 36		20.3	7.9	10.7
TRIM71	tripartite motif containing 71, E3 ubiquitin protein ligase		4.9		
TRIML2	tripartite motif family-like 2		10.2	6.2	4.6
TSPAN5	tetraspanin 5		9.4	4.9	
TSPAN8	tetraspanin 8	5.4			
TSPAN13	tetraspanin 13		5.3		
TSPAN16	tetraspanin 16		32.6	28.8	29.8
TSPAN18	tetraspanin 18		12.5		
TSPY1	testis specific protein, Y-linked 1		21.4	5.8	
TSPY2	testis specific protein, Y-linked 2		15.1	5.4	
UBE2C	ubiquitin-conjugating enzyme E2C		5.4		
UBE2U	ubiquitin-conjugating enzyme E2U		22.5	16.3	16.5
UBQLN3	ubiquilin 3			29.1	
USP2	ubiquitin specific peptidase 2		15.3	13.5	14.7
USP28	ubiquitin specific peptidase 28		7.2		
USP44	ubiquitin specific peptidase 44		7.1	20.7	
WT1	Wilms tumor 1		13.2	6.5	6.0

4.9 Appendix

Supplemental Figure 4.1: STRING analysis of predicted functional partners for upregulated genes

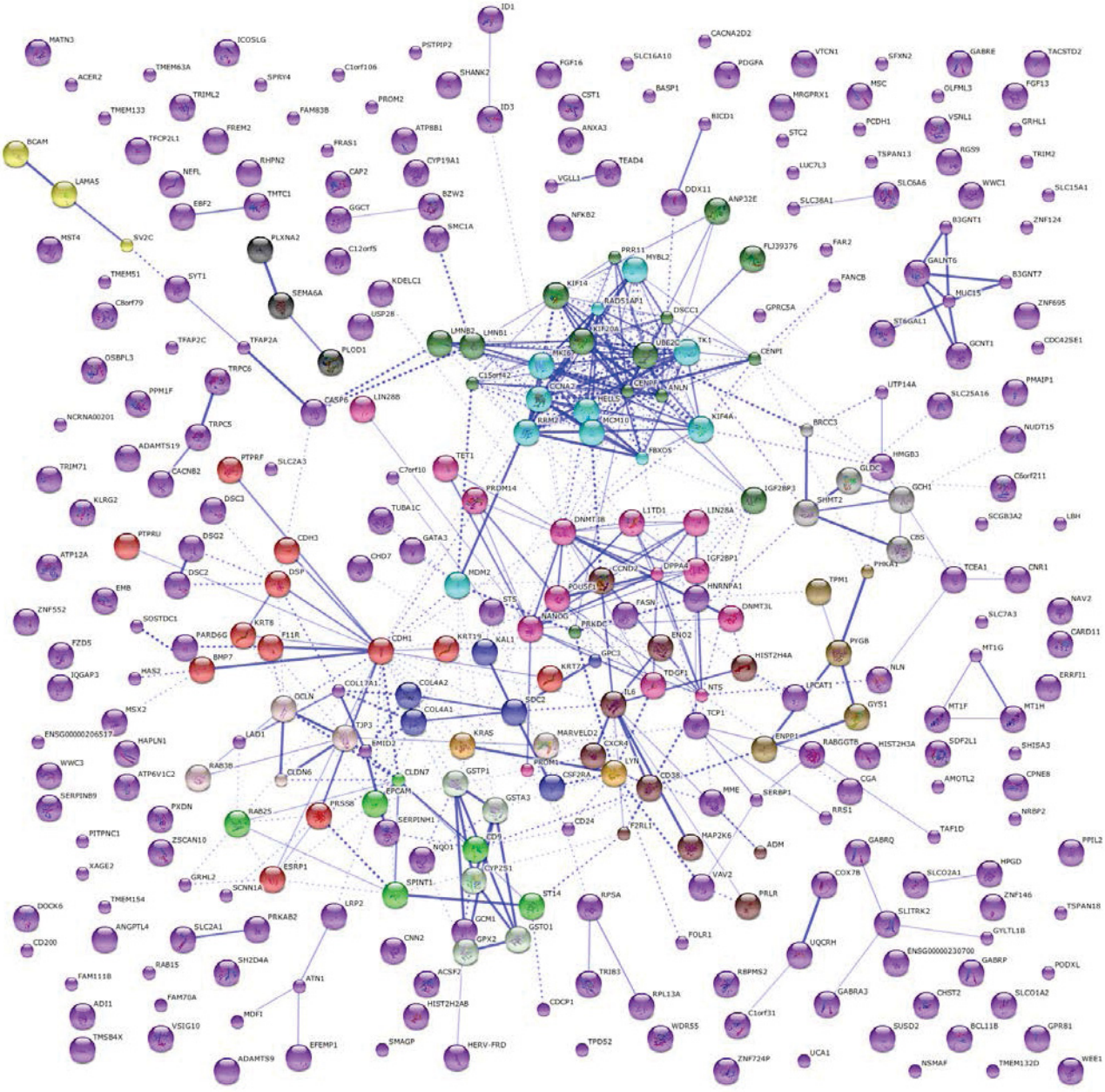
(A) STRING analysis of genes upregulated in PND3 gonocytes compared to PND8 spermatogonia, (B) genes upregulated in TCam-2 cells compared to normal testicular tissue, and (C) genes upregulated in Seminoma tumors compared to normal testicular tissue. Functional partner predictions were based on available experimental data, data bases, textmining, and homology. Genes were grouped into various clusters (represented by different colors). Clusters were formed based on the Kmeans method (a method commonly used in data analysis). The thickness of the lines connecting various genes represents the level of confidence with which functional partners can be predicted. Inter-cluster connections are represented using dotted-lines.

Supplemental Figure 4.1: <u>STRING analysis of predicted functional partners for upregulated genes</u>

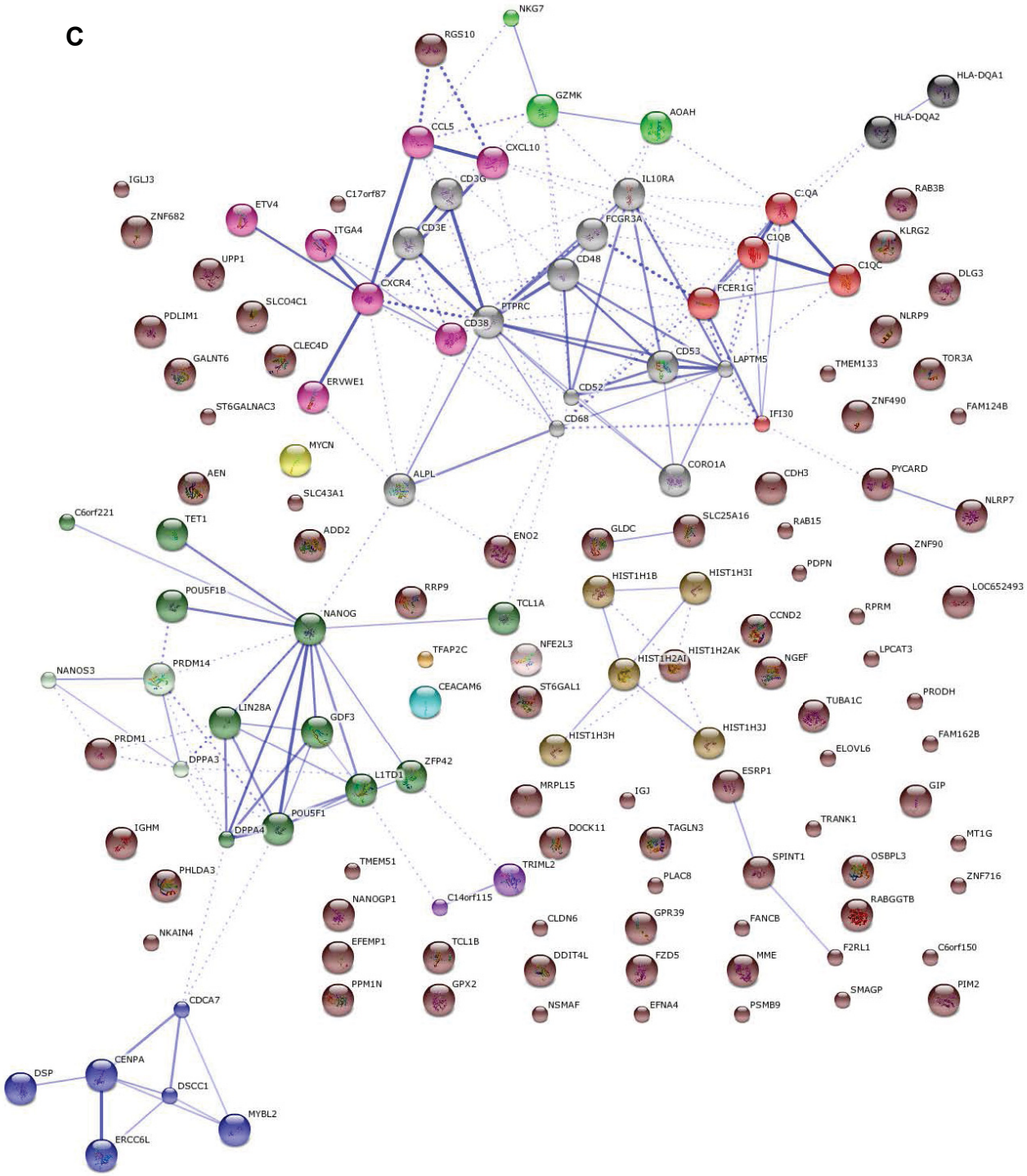


Supplemental Figure 4.1: <u>STRING analysis of predicted functional partners for upregulated genes</u>

B



Supplemental Figure 4.1: <u>STRING analysis of predicted functional partners for upregulated genes</u>



Supplemental Table 4.1: Ingenuity Pathway Analysis (IPA) of genes significantly altered in rat and human gene arrays

Based on p-value significance and number of genes present, the top two most-reproduction related conditions are listed. Within each condition, the top three most-reproduction related processes or pathologies have been listed. The number of genes, found by IPA, to be involved in each indicated condition/process/pathology is listed in parentheses.

Supplemental Table 4.1: Ingenuity Pathway Analysis (IPA) of genes significantly altered in rat and human gene arrays

Gene Array	Category	Condition	Process/Pathology
Upregulated in Gonocytes	Diseases and Disorders	Cancer (13)	• Benign neoplasia (4) • Tumorigenesis of carcinoma (2) • Hyperplasia of Leydig cells (1)
		Developmental Disorder (9)	• Sertoli cell hypoplasia (1) • Ectoderm hyperplasia (1) • Congenital atransferrinemia (1)
	Molecular/ Cellular Function	Cellular Growth and Proliferaton (16)	• Proliferation of cells (13) • Colony formation (2) • Cellular production (3)
		Cell Death and Survival (14)	• Necrosis/Apoptosis (13) • Cell death (of tumor cell-lines) (13) • Cytolysis of tumor cells/cell lines (2)
Downregulated in Gonocytes	Diseases and Disorders	Hereditary Disorder (2)	• High density lipoprotein deficiency (1) • Familial visceral amyloidosis (1) • Familial hypercholesterolemia (1)
		Inflammatory Response (1)	• Cellular phagocytosis (1) • Cellular activation (1) • Cell migration (1)
	Molecular/ Cellular Function	Cellular Growth and Proliferation (5)	• Cellular proliferation (4) • Induction of foam cells (1) • Neuroblastoma cell line formation (1)
		Cell-to-cell Signaling and Interaction (3)	• Signal transduction (3) • Cellular phagocytosis (1) • Cellular binding (1)
Upregulated in Seminoma	Diseases and Disorders	Cancer (72)	• Genital tumor (23) • Hematological neoplasia (19) • Testicular cancer (4)
		Reproductive System Disease (27)	• Germ cell and embryonal neoplasm (11) • Gonadal tumor (11) • Trophoblastic tumor (5)
	Molecular/ Cellular Function	Cellular Function and Maintenance (39)	• Cell homeostasis (10) • Phagocytosis of cells (10) • (Stem) cell maintenance/self-renewal (7)
		Cell-to-cell Signaling and Interaction (36)	• Activation of blood cells (22) • Phagocytosis (10) • Cell-cell adhesion (6)
Downregulated in Seminoma	Diseases and Disorders	Cancer (349)	• Solid tumor (314) • Testicular cancer (4) • Tumorigenesis of genital organ (3)
		Reproductive System Disease (85)	• Infertility (58) • Sperm disorder (51) • Asthenozoospermia (26)
	Molecular/ Cellular Function	Cell Morphology (110)	• (abnormal) germ cell morphology (63) • Cellular protusion formation (42) • Detachment of flagella (5)
		Cellular Development (82)	• Gametogenesis (64) • Spermatogenesis (60) • Spermiogenesis (20)

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Chapter 5

General Discussion

Spermatogenesis is an intricate process that comprises multiple timed events to ensure the continuous production of sperm throughout the lifetime of a male. Although the phases of spermatogenesis from the SSCs to the production of spermatozoa, as well as fertilization, are well studied, the earlier stages leading to the formation of the SSC pool are less studied. However, interest has arisen over the past years to decipher the early steps of germ cell development for two main reasons: 1) studies have demonstrated the possible use of SSCs as a potential source of embryonic stem cells; and 2) the belief that gonocytes, the precursor cells of SSCS, might be at the root of carcinoma-in-situ (CIS), the precursor pathology to testicular germ cell tumors (TGCTs), in view of their similarities in gene expression profiles and morphology (1). To add, for the past few decades, the incidence of TGCTs has been steadily increasing (2). Thus, understanding gonocyte development can provide invaluable insight into the mechanisms by which TGCTs, alongside other male reproductive pathologies such as infertility, can occur.

Given the limited knowledge of the early phases of spermatogenesis, this thesis focused on deciphering the mechanisms regulating gonocyte differentiation to spermatogonia. Although gonocytes themselves are not well studied, of the multiple phases of development gonocytes must undergo in both the fetal and neonatal periods, neonatal gonocyte differentiation might be the least characterized of all. Excluding the work presented in this thesis, previous information available regarding gonocyte differentiation included the findings that gonocyte differentiation is stimulated by retinoic acid (RA) (3, 4), that there is a first wave of spermatogenesis in which gonocytes directly differentiate into spermatogonia to contribute to the first wave of spermatozoa produced, bypassing the SSC stage (5, 6), and that gonocytes must first migrate to the basement membrane of the seminiferous tubules before undergoing differentiation (7, 8, 9). A recent study also observed that patients with undervirilisation syndromes presented premature gonocyte migration to the basement membrane and differentiation, further stressing the importance of understanding gonocyte development with regards to human pathologies and infertility (10). Given that improper gonocyte development might lead to TGCT formation and/or infertility, it is essential to better understand gonocyte physiology and identify genes that could play a role in their development and in germ cell pathologies.

This thesis includes three main research chapters (chapters 2-4) addressing different aspects of the mechanisms and genes involved in the regulation of gonocyte differentiation. Chapter 4

also identifies genes that are highly expressed in gonocytes and are upregulated in testicular germ cell tumors, representing new targets to study in relation with testicular cancer.

5.1 Role of retinoic acid and platelet-derived growth factor receptor crosstalk in the regulation of neonatal gonocyte and embryonal carcinoma cell differentiation

In chapter 2, we studied signaling pathways activated during RA-induced differentiation in the F9 mouse embryonal carcinoma cell line, an ESC-like cell line model (11), alongside the study of isolated PND3 gonocytes. Although F9 cells represent cells that exist earlier than gonocytes during development and have yet to undergo germline- and somatic lineage specifications, in an earlier study, we showed that they shared some of their molecular responses to RA with PND3 gonocytes (3). Moreover, F9 cells were originally generated from a testicular embryonal teratocarcinoma, a tumor type likely originating from CIS, which are themselves believed to arise from abnormal gonocytes (1). Neonatal (transitional) gonocytes are only present in rodents for a short time window, corresponding to the last trimester and first months of life in humans. There is no cell line model available that reproduces this developmental period, and the range of studies that can be performed on isolated gonocytes is limited by the small number of cells obtained after enrichment procedures. Moreover, due to the asynchronous nature of spermatogenesis, even in perinatal periods, germ cells isolated at any given time comprise more than one developmental phase, further limiting the available number of cells in each of these subsets (5, 11). Thus, using a cell line model such as F9 cells offered an alternative to studying RA-induced signaling pathways, which eventually unveiled interesting differences between the two developmental stages.

When gonocytes are treated with RA, there is a dose-dependent, significant increase in the expression of STRA8 (stimulated by retinoic acid 8) mRNA expression (3). In chapter 2 of the thesis, we showed that the inhibition of PDGFR kinase activity led to a significant decrease in Stra8 expression, used as an indicator of RA-induced differentiation, in both F9 cells and gonocytes, indicating a likely role of PDGFR activation during this process. However, when the most common downstream pathways of PDGFRs were inhibited, F9 cells and gonocyte differentiation were found to involve different downstream pathways. While the inhibition of MEK1/2 significantly reduced F9 cell RA-induced differentiation toward the somatic lineage, represented by collagen IV and laminin B1 gene expression (3, 12, 13), it increased the

expression of Stra8 in the same conditions, suggesting that the MEK1/2 pathway is involved in the lineage fate of F9 cells. Our F9 cell data suggest that MEK1/2 may act as a switch in ESCs by driving differentiation towards the somatic lineage when it is activated, while its inhibition would favor differentiation towards the germline lineage. By contrast, MEK1/2 was not involved in gonocyte differentiation, as we had previously shown its involvement in proliferation (14).

Interestingly, immunoblot analysis of F9 cells treated with RA revealed the presence of variant forms of this receptor. Interestingly, upon inhibition of either PDGFR or MEK1/2 activation, there was significant reduction in the expression levels of this 45kDa variant, concomitant with decreased expression of the somatic lineage markers, indicating the importance of these molecules in F9 cell differentiation. Although, by default, F9 cells are known to undergo differentiation towards the somatic lineage, they are also able to express germ cell markers such as STRA8, a classical marker for spermatogonia differentiation, upon RA-stimulation (3, 15, 16). Considering that STRA8 was first identified in F9 cells (15), it is surprising that no subsequent studies have explored the reason why RA upregulates STRA8 in these cells, and what prevents them to differentiate toward the germline lineage. It is possible that this tumor cell line has lost part of the molecular machinery driving germline fate, or that the environment in which they were cultured did not provide critical germline specification signals such as BMP4 and 8, normally produced by extraembryonic ectoderm cells to induce PGC formation.

In PND3 gonocytes, RA was also found to induce a dose-dependent increase in PDGFRα mRNA expression, similarly to STRA8 upregulation (3). Protein analysis revealed that there was very little, if any, full length PDGFRα present in gonocytes, and that instead, it is a variant form of PDGFRα of 65kDa, which is increased upon RA stimulation, indicating a possible role in differentiation. As seen in Figure 1.3, both Sertoli cells and germ cells possess the necessary enzymes to metabolize vitamin A into RA. Sertoli cells have been reported to be the main site of RA synthesis in the testis and it is believed that Sertoli cells are the source of RA ensuring spermatogonial differentiation (17, 18). However, it is also possible that Sertoli cells deliver retinol to the germ cells directly, where it would then be converted to RA, or that spermatogonia acquire retinol or RA directly from the serum, since they are located in the adluminal compartment, not protected by the blood-testis-barrier (18, 19). The exact pathway by which spermatogonia obtain retinol or RA is not clear, and the same thing is true for gonocytes. We

have shown that isolated gonocytes can differentiate in vitro when stimulated with RA directly, (3). Furthermore, Zhou Q et al have shown that when culturing PND2 mice testes, treatment with RA induces a significant upregulation in STRA8 mRNA expression, whereas stimulation with retinol does not (4). The lack of any significant induction of STRA8 expression by retinol in these studies indicated that neither Sertoli cells nor gonocytes responded to retinol, suggesting the inability of these cells to metabolize retinol to RA. This would indicate that in mice, RA, not retinol, can induce STRA8 mRNA expression during differentiation. This is an interesting finding as it is commonly believed that, within the testis, Sertoli cells are able to metabolize retinol into RA, which did not seem to be the case in this mouse study. It is possible that PND2 mouse testes do not express the necessary enzymes for this conversion, but that they acquire this capability later on. We have found that PND3 rat gonocytes abundantly express aldehyde dehydrogenase ALDH1A1 and cellular RA binding protein CRABP1 mRNAs, at higher levels than those measured in PND8 spermatogonia, PND3 and PND8 Sertoli cells (data not shown). Furthermore, retinol binding protein RBP1 and cytochrome P450 hydroxylase CYP26B1 (responsible for RA elimination) are also present in gonocytes, where they are expressed at similar levels as in spermatogonia and Sertoli cells (data not shown). These recent findings provide evidence that gonocytes might be able not only to respond to RA directly, but also to metabolize retinol to RA. However, measurement of protein levels and enzymatic activities will be needed to confirm this possibility.

Ours is not the first study reporting that RA can activate the expression of PDGFRα (20). This activation was shown to occur with or without the activation of RA receptors by their ligand (21). We examined which RA receptor was expressed in PND3 rat gonocytes, because there was conflicting results on which RARs were expressed in these cells (19, 22). In our studies, we found that both RARα and RARγ are abundantly expressed in PND3 gonocytes. Moreover, RARα inhibition significantly reduced RA-induced STRA8 upregulation, indicating that RARα activation is involved in gonocyte differentiation. Thus, our findings show that both PDGFRα and RARα activation are needed, supporting the existence of a crosstalk between RA/RAR and PDGFR during gonocyte differentiation. Although the RA-induced activation of PDGFRα has not been well studied, it seems likely to occur via a transcription factor intermediate (20). The literature provides examples and likely candidates for this role. GATA-4 is a member of the GATA transcription factor family and has been shown to be responsible for the positive

PDGFRα expression in the parietal endoderm, which F9 cells differentiate into upon stimulation with both RA and cAMP, and thus represents an example of transcription factor involvement (23). A likely candidate is OCT-4 (POU5F1), a POU domain transcription factor that has been shown to regulate the alternative PDGFRα promoter P2 that is responsible for the PDGFRα variant seen in human seminoma-type testicular tumors (24).

We then examined potential PDGFR downstream pathways activated in RA-induced differentiation and found that the SRC family of kinases, JAK2, and STAT5 were likely pathways involved in gonocyte differentiation. Although several SRC kinases had been reported to be present at various stages of spermatogenesis (25), to our knowledge, this is the first time that these proteins, as well as JAK2 and STAT5, are shown to be expressed in rat PND3 gonocytes. However, the present study did not provide evidence that these pathways were directly downstream of PDGFR activation, but although the factors described may not be the only ones involved in gonocyte differentiation, they likely play a significant role in this process.

Although the pathways crosstalking with RA in F9 cells were not the same as those involved in gonocyte differentiation, their study provided new insight into embryonal carcinoma and ESC differentiation, further unveiling conserved signaling elements between these cells and gonocyte differentiation. Among those are the formation of variant PDGFRα forms, present both in differentiating F9 cells and gonocytes. Although the present study did not fully sequence the F9 and gonocyte variant forms, initial experiments showed that in both cases, the variant PDGFRα likely contains nucleotide sequences between exon 12 and exon 23, and as a result, would function in a ligand-independent manner. However, the entire variant rat and mouse sequences needs to be confirmed before the function of these variants can be discovered. Once the full sequences are resolved, it will be possible to elucidate whether the variant present in gonocytes corresponds to the same sequence as the human variant found in seminomas. This will also allow overexpression and silencing experiments to be performed, to determine its physiological function, and examine if it plays an active role in tumor formation or if it is simply a by-product of failed differentiation. At this point, we cannot exclude that this variant is a proteolytic product of a larger form of PDGFRα, rather than the result of alternative splicing. However, the fact that inhibition of the protease Cathepsin L, produced by Sertoli cells and regulated by PDGF and germ cells (26, 27), did not have any effect on the levels of variant PDGFRα expressed in RA-

treated gonocytes, supports the hypothesis that the variant is produced by alternative mRNA splicing. The human PDGFRα variant form seen in seminomas was shown to result from the activity of an alternative promoter P2 located in intro 12 (28), but whether this is true for gonocytes is not yet known. Thus, as of now, there are indications supporting the possibility of alternative splicing, but proteolytic cleavage cannot be ruled out.

Future experiments will be needed to better understand the roles of activated SRC family of kinases, JAK2, and STAT5. Although JAK2 and STAT5 are commonly activated together, studies have shown that SRC is also able to activate STAT5 (29, 30). More studies will also be needed to confirm if the activations of SRC-related protein and/or JAK2, and STAT5 occur downstream of PDGFRs, or if they are activated independently of PDGFR activation, through other receptors such as VEGFR (vascular endothelial growth factor receptors) (31). We must also determine whether SRC family of kinases, JAK2, and STAT5 activation during gonocyte differentiation regulate the expression/activation of the variant PDGFRα. Preliminary results analyzing PDGFRα expression when gonocytes are treated with RA and the inhibitors for SRC, JAK2, and STAT5 suggest that likely, JAK2/STAT5 are involved in the regulation of the variant PDGFRα. To further our analysis, we could treat isolated gonocytes with the inhibitor of PDGFR and see if it had an effect on the phosphorylation status of SRC, JAK2, and STAT5 via immunoblot analysis. Furthermore, by accumulating fractions of control and RA-induced gonocytes in order to maximize cell numbers, we could perform co-immunoprecipitation experiments using a PDGFRα antibody, and then determine whether there is a physical binding/activation via phosphorylation of SRC or JAK2 to PDGFRα. For downstream molecules known to not have the SH2 domain, we could use immunoprecipitation experiments to first examine possible adaptor molecules, which could help the non-SH2 domain containing proteins to bind. Adaptor molecules can bind to phosphorylated tyrosines in PDGFRs and provide a docking/binding site for other downstream members of the signaling cascade that contain SH2 binding domain sequences (32). These studies would determine whether PDGFR activation resulted in activation of SRC, JAK2, and STAT5.

All in all, the studies of chapter 2 revealed that, independently or not, PDGFR, SRC, JAK2, and STAT5 activations all play a role in gonocyte differentiation. This is the first study describing these crosstalk mechanisms in the regulation of gonocyte differentiation. The use F9

mouse embryonal carcinoma cells as a comparison showed that both cell types acted in a similar manner by inducing the expression of variant PDGFRs and requiring PDGFR activation for their differentiation. However, the finding that gonocytes required SRC, JAK2, and STAT5 activation for differentiation, whereas F9 cells relied on MEK1/2 signaling for their differentiation to endoderm also showed where the two cell types diverged in their paths. The formation of a variant form of PDGFRα is quite striking as a PDGFRα variant form has also been shown to be present in seminoma-type testicular germ cell tumors (28, 33). Since this variant form is not found in normal human adult testicular tissue, it is possible that retention of this variant form during gonocyte development may be contributing to testicular tumor formation.

5.2 Expression of the Ubiquitin Proteasome System in neonatal rat gonocytes and spermatogonia: Role in gonocyte differentiation

Given that gonocyte development is a complicated process (5) that must occur within a finite amount of time and is associated with major reorganization of the cellular structure, changes in how gonocytes respond to nourishment factors released by Sertoli cells, their gene and protein expression profiles, and localization within the seminiferous tubules, we hypothesized that the Ubiquitin Proteasome System (UPS) was involved in gonocyte differentiation.

The UPS is the main system by which protein degradation occurs (34, 35, 36, 37). Besides its role in protein degradation, the UPS has also been shown to be involved in a plethora of other processes, including intercellular communication (38), cellular proliferation (39), and apoptosis (40). Studies have shown that the process in which the UPS will participate depends on the link between the ubiquitin molecules and the substrate being targeted (36, 41). Of note, alterations in the UPS have been shown to be involved in various types of cancers, neurological disorders, and infertility (34). Thus, the UPS plays an essential role, not only in protein degradation but also in a variety of different processes.

Our findings reported in chapter 3 represent the first description of UPS in PND3 gonocytes. However, the importance of the UPS and its various components has previously been shown in other stages of spermatogenesis (42-47). In chapter 3, we first showed that the proteasome itself is necessary for gonocyte differentiation, using the nonreversible, natural proteasome inhibitor Lactacystin, which, when added alongside RA, reduced in a significant dose dependent manner the RA-dependent expression of Stra8, a marker of differentiation. In an effort to search for

ubiquitin related genes expressed in PND3 gonocytes, we performed gene array analysis of PND3 gonocytes, in comparison to PND8 spermatogonia, and found that the E1 ubiquitin activating enzymes Uba1 and Uba6, and E3 ubiquitin ligases Huwe1, Trim47, and Rnf149 were significantly downregulated in PND8 spermatogonia. However, when isolated gonocytes were treated with RA, to identify genes similarly altered during in vitro and in vivo differentiation, we found that the E3 ligase Rnf149 was the only gene that was also statistically higher in undifferentiated gonocytes compared to gonocytes treated with RA. Thus, Rnf149 represents a candidate gene that is likely playing a role during gonocyte development or which needs to be downregulated in order for gonocyte differentiation to occur.

There is an ongoing project in our lab investigating the role Rnf149 is playing in gonocytes by using siRNA and overexpression techniques. Furthermore, co-immunoprecipitation experiments using isolated gonocytes and the RNF149 antibody could help identify key substrate proteins that must be degraded in order for gonocyte differentiation to occur. At this time, little is known about RNF149, except from the study of Nair VS et al which showed, by analyzing patients with non-small cell lung cancer, that RNF149 is associated with survival of this type of cancer by a function that remains to be elucidated (48). Furthermore, Hong SW et al have shown that RNF149 is the E3 ubiquitin ligase responsible for UPS-dependent degradation of BRAF (49). Interestingly, BRAF is a member of the RAF family of proteins involved in cellular proliferation, transformation, differentiation, and survival alongside ARAF and CRAF/RAF-1 (previously shown to be present in PND3 gonocytes) (14, 49). RAF proteins are known to activate MEK1/2, which is a pathway we have previously shown to be activated during gonocyte proliferation (14) and in chapter 2, we found that MEK1/2 acted as a switch regulating the differentiation potential of F9 mouse embryonal carcinoma cells. Thus, RNF149 represents an interesting gene to study forward.

The main function of the UPS is protein degradation. Interestingly, PDGFRs are known to be degraded by lysosomal degradation and the UPS, whereas RARs and RXRs are known to be degraded primarily by the UPS (50, 51). Although PDGFRs mainly undergo degradation via the lysosomal pathways, PDGFRβ has been shown to undergo ubiquitin-targeted degradation when bound to its ligand (52, 53). Furthermore, studies using human bone marrow-derived mesenchymal stromal cells (hMSCs) have shown that PDGFRα can also undergo ubiquitination

via Casitas B lineage lymphoma (Cbl), an E3 ubiquitin ligase (54). In this study, Severe N et al found that when the interaction between Cbl, PDGFRα, and FGF2 was inhibited using a mutant Cbl, there was a significant increase in hMSC differentiation (54). Using the mutant Cbl instead prevented the PDGFRα and FGF2 from being degraded and they found that there was an increase in PDGFRα expression during the differentiation of these cells, similar to what we see in RA-induced gonocyte differentiation in chapter 2. However, in this case, it was the MEK1/2 and PI3K signaling pathways that were activated downstream of PDGFRα (54), confirming that more than one downstream pathway can be activated during a complex process like cellular differentiation, as described in chapter 2.

In recent preliminary studies, we have seen that when isolated gonocytes are treated with RA and the proteasome inhibitor Lactacystin, there is no significant change in expression levels of PDGFRα and PDGFRβ, at both the mRNA and variant protein levels (data not shown). These results suggest three possibilities: (1) PDGFRs are not degraded by the UPS during differentiation, but rather at a later time point, once differentiation had been completed; (2) PDGFRs are degraded mainly via the lysosomal pathway rather than the proteasome in gonocytes; or (3) PDGFRs are needed for other processes during gonocyte development and retained in the cells. To note, the lysosomal pathway of protein degradation has not yet been studied in gonocyte development. However, northern blot analysis showed that the expressions of variant-PDGFRα transcripts and full length are maximal at PND3 and has noticeably decreased by PND21. Moreover, qPCR analysis of PND3 gonocytes and PND8 spermatogonia showed that PDGFRα expression was stronger in gonocytes (chapter 2), implying that PDGFRα is down-regulated some time after gonocyte differentiation. Because both PND3 gonocytes and seminoma-type TGCTs express variant forms of PDGFRα, it is possible that the retention of this variant form is linked to TGCT formation.

Overall, in chapter 3 we showed that activation of the ubiquitin proteasome system is necessary for proper gonocyte differentiation. Furthermore, we identified several genes highly expressed in gonocytes for which finding the function would be interesting. Among those, the E3 ligase RNF149 is a candidate gene that is highly expressed in gonocytes and is downregulated during both in vitro and in vivo differentiation, which we are presently studying.

5.3 Identification of genes overexpressed in testicular seminoma tumors and downregulated in during gonocyte differentiation as potential players in the origins of testicular cancer

The finding in chapter 2 that differentiating gonocytes express a variant PDGFRα similarly to seminomas, and the general belief that improper gonocyte development can lead to the formation of testicular germ cell tumors (TGCTs), led us to the third research chapter of this project. TGCT, a male reproductive pathology which is increasing steadily in incidence (1, 2), represents more than 95% of all testicular tumors, where the remaining 5% are made up of mainly Leydig cell and Sertoli cell tumors (55). Within TGCTs, there are multiple types of tumor types. When based on origins, TGCTs can be divided into two main categories: seminomas (deriving from the germ cells) and non-seminomas (56, 57, 58). Seminomas are the most common type of TGCTs (56, 57, 58). Seminoma tumors are thought to arise from a precursor pathology, carcinoma-in-situ (CIS) which are believed to develop from gonocytes unable to achieve proper differentiation (1).

Given the increasing prevalence of TGCTs over the past decades and the limited understanding of gonocyte development, the focus of our work in chapter 4 was to identify common genes or proteins between the developing normal germ cells and their tumoral counterpart, in hope to find genes that could possibly be involved in TGCT formation as a result of improper gonocyte differentiation. To this end, we not only performed a comprehensive gene array analysis of both gonocytes and human normal and tumoral testicular tissue, but to obtained a better understanding of seminoma-type tumors in general, we took advantage of the available human testicular seminoma cell line model, TCam-2 cells (59, 60).

The first goal of chapter 4 was to better characterize the TCam-2 cell line. Interestingly, similar to the variant PDGFRα form seen in gonocytes in chapter 2, we found that this seminoma cell line also express a variant form of PDGFRα. This is striking because the presence of a 1.5kb variant PDGFRα mRNA transcript has previously been described in seminoma-type tumors (28). Although it is also expressed in a subset of hematopoietic cells, this variant mRNA could potentially act as a diagnostic clinical marker for TGCT detection with concurrent expression of OCT-4 expression (a transcription factor which is a possible regulator of PDGFRα) (28). In view of their expression of variant PDGFRα, TCam-2 cells appear to be ideal for our studies.

The expression of factors commonly involved in gonocyte development was determined by immunocytochemistry in TCam-2 cells, indicating that these cells expressed more PDGFRα than PDGFRβ, ERβ and ERK1/2 and HSP90. Surprisingly, we also found that the androgen receptor was expressed in TCam-2 cells, both at the mRNA and protein level, reminiscent of other hormone sensitive cells expressing both ERs and AR, such as breast and prostate cancer cells (61). Moreover, similarly to PND3 gonocytes, upon RA treatment, there was a significant increase in STRA8 protein levels in TCam-2 cells. Although we have found these cells to respond to RA, other groups have shown that TCam-2 cell differentiation does not occur due to RA stimulation, but instead, via TGFβ, FGF4, and EGF (62). This apparent discrepancy is possibly due to a difference in the subset of TCam-2 cells used, since recent studies have shown that these cells include two TGCT types, seminoma and embryonal carcinoma, and that different culture conditions and environment can led them to adopt one phenotype more than the other (63). RT-PCR analysis of genes characteristic of gonocyte development and spermatogenesis further confirmed that TCam-2 cells and gonocytes have similar gene expression profiles for a number of key genes. These findings provide further evidence that seminomas, in this case TCam-2 cells, share common gene and protein expression profiles with their supposed cells of origin, the gonocytes.

Finally, to further our knowledge of TCam-2 cells, we treated the cells with varying amounts of fetal bovine serum (FBS), bovine serum albumin (BSA), and a variety of growth factors and inhibitors at varying doses for varying time periods (1-3 days). Growth factors tested included PDGF-AA, PDGF-AB, PDGF-BB, estradiol, leukemia inhibitory factor (LIF), glial-cell derived neurotrophic factor (GDNF), fibroblast growth factor (FGF2), epidermal growth factor (EGF), nerve growth factor (NGF), stem cell factor (SCF), insulin growth factor (IGF), insulin, TGFβ1, and GDNF family receptor alpha 1 (GFRα1). Inhibitors tested included AG370 and AG1295 (inhibitors for PDGFR activation), U0126 (MEK1/2 inhibitor), Wortmannin (PI3K inhibitor), U73122 (PLC inhibitor), ICI182780 (Estrogen Receptor inhibitor), PD173074 (FGF2 inhibitor), and SU5416 (GDNF inhibitor). Although some of the factors appeared to increase proliferation either using BrdU ELISA or MTT assays, none of these growth factors or inhibitors had a clear reproducible effect on TCam-2 proliferation over several experiments (data not shown). Considering our finding that these cells produce PDGF-AA (and potentially other growth factors not examined), together with the present knowledge of their plasticity and their ability to change

phenotype, the lack of reproducibility in our experiments is not unexpected, but may also indicate that these cells can respond to multiple factors to maintain growth. This possibility is supported by a study of Young JC et al who showed that, similarly to fetal germ cells, TCam-2 cells are able to respond to RA, BMP4, and TGFβ stimulation via the TGFβ receptor signaling pathway to induce cell proliferation and survival (64). Again, differences in cell sub-types, doses, culture time, and conditions are likely responsible for these differences.

Gene expression profiles were examined by gene array analysis, comparing rat PND3 gonocytes to PND8 spermatogonia, and normal human testicular tissues to seminoma and embryonal carcinoma biopsies. Here, similar to what we had done in chapter 3, we wanted to determine genes that were more highly expressed in gonocytes compared to spermatogonia because these genes would either possibly play a preferential role in gonocyte development, or need to be downregulated for gonocyte differentiation to occur. The goal here was to find genes that were preferentially expressed in rat gonocytes and upregulated in human seminoma tumors and then to determine whether common functional pathways or processes were upregulated in each array. Although not ideal, the inter-species approach was necessary because there is no rodent model for testicular seminoma tumors (65) and thus an all-rat approach could not be utilized. Furthermore, an all-human approach was also not feasible because gonocytes within the human exist from the fetal period until few months after birth, and we could not obtain such samples (5). Therefore, in order to determine common functional pathways or processes between gonocytes and TGCTs, an inter-species approach was necessary.

As described in chapter 4, functional analysis of genes significantly upregulated in PND3 gonocytes compared to PND8 spermatogonia determined that a large number of the genes differentially expressed were cell adhesion molecules. Interestingly, when comparing genes upregulated in both seminoma tumors and TCam-2 cells (compared to normal testicular tissue), we found that cell adhesion molecules were also the main genes that were upregulated. Among the upregulated cell adhesion molecules, the claudin family of proteins in particular, emerged as candidate genes.

Claudins have been commonly shown to be either upregulated or downregulated in a variety of tumors including breast cancer, ovarian cancer, and prostate cancer (66). Within the testes, claudins have not been that well described and only claudin 3, 5, and 11 have been shown to be

present in tight junctions of the blood testis barrier (67, 68, 69), which forms later during spermatogenesis and does not exist at the time when gonocytes are present; while claudin 7 has been shown to be weakly expressed in the seminiferous tubules (70). In our analysis, we confirmed our gene array results at both the mRNA and protein levels and found that claudins 6 and 7 were highly upregulated in gonocytes (compared to spermatogonia) and in seminoma tumors (compared to normal testicular tissue). Previously, claudins 6 and 7 have been shown to be expressed in some seminoma tumors in varying amounts but these proteins have not yet been reported in gonocytes (70, 71). Furthermore, positive claudin 6 expression has not yet been shown to be characteristic of any type of cancer whereas claudin 7 expression was altered in a variety of cancers, including ovarian cancer, where it promotes tumor invasiveness (72). We were able to obtain normal samples from testicular biopsies containing malignant lesions, by selecting areas of the testis that appeared to be normal when the pathology was analyzed, and from patients undergoing vasectomies. Due to the limitation in sample availability, we were not able to isolate a pure germ cell population for any of the human samples and thus, a whole testis analysis approach was undertaken. However, the group of RL Brinster has performed gene array analysis using isolated human spermatogonia. Analyzing their available gene array data, we found that claudins 6 and 7 had low levels of expression in human spermatogonia, as seen in our analysis of rat spermatogonia (73). However, the expression levels of claudins 6 and 7 in earlier human germ cells within spermatogenesis are not currently known, again likely due to limitations in sample availability.

Given that claudins 6 and 7 were highly expressed both in gonocytes and seminoma tumors, we propose that these claudins might be involved in cell-cell tight junctions between gonocytes and Sertoli cells, and that, in order for gonocytes to migrate, the junctions between the cells need to be broken. However, if proper downregulation of these proteins does not occur, this could lead to improper migration, and subsequent abnormal gonocyte differentiation, leading to tumor formation. Claudin 6 is expressed at the germ cell membrane in tumors, and thus this hypothesis is applicable. However, claudin 7 expression is seen as mainly nuclear. As surprising as it is to see nuclear cell adhesion molecule expression, this has been seen before (74, 75). Due to the overwhelming studies in which claudins were either up- or down-regulated in tumorigenesis, researchers began to believe that likely, claudins were playing a role in other molecular processes as well. As described in chapter 4, studies have shown that nuclear claudin expression

can be involved in oncogenic transformation, tumor invasion, signal transduction, and cellular proliferation (76, 77). The finding that nuclear claudin expression can be responsible for cellular proliferation is quite interesting.

Future studies must be conducted in order to determine the exact role of claudins 6 and 7 in gonocyte development (whether it be at the level of migration or differentiation) and in TGCT development. For gonocyte development, claudins 6 and 7 expression can be silenced or overexpressed and how the gonocytes develop in this new environment can be monitored. For tumor cells, TCam-2 cells can be used to determine how testicular tumors can be affected if claudins 6 or 7 are no longer present, in terms of invasiveness, survival or proliferative activity.

In terms of differentiation, it is possible that the claudins that are expressed at the germ cell membrane play a role in membrane permeability. As previously described in chapter 2, although isolated gonocytes are able to respond to RA and undergo partial differentiation in vitro, it is commonly believed that within the testis, retinol or RA is provided to the germ cells by the surrounding Sertoli cells. As of yet, Sertoli cells and germ cells are only known to communicate via gap junctions and desmosomes (78). However, it is possible that these claudin proteins are also responsible for the membrane permeability which allows for retinol or RA to travel, bound to their carrier binding proteins, from the Sertoli cells to the neighbouring germ cells. Furthermore, Kubota H et al have shown that during RA-stimulated F9 mouse embryonal carcinoma differentiation towards the visceral endoderm, RARγ and RXRα are able to mediate the expression of tight junction associated proteins which are important for the visceral endoderm, including claudins 6 and 7 (79). Given this finding and considering the multiple similarities between gonocytes and F9 cells, it is possible that the claudins expressed in gonocytes are also being mediating by the RARs and RXRs expressed in gonocytes.

In addition, studies have also shown that MAPK activation can lead to regulation of the expression and function of various claudins, and as previously mentioned, we have shown the MAPK pathway to be implicated in both gonocyte proliferation and F9 cell differentiation (66). To add, similarly to PDGFRs, claudins can also undergo phosphorylation (80). Although the phosphorylation of claudins is not that well studied, it has been reported that due to phosphorylation, there can either be an increase or a decrease in barrier permeability depending on which claudins are involved in the process (80). Thus, the possibility that claudins are able to

help regulate the actions of PDGFR (including cellular apoptosis, a process critical in tumor control) and related signaling pathways during gonocyte development may also be worth testing.

Finally, in chapter 3, we showed that the activation of the UPS is necessary for gonocyte differentiation and we identified the E3 ubiquitin ligase RNF149 as a candidate gene to study in the future. Although possible substrates for RNF149 are not currently known, claudin proteins could be possible substrates. As previously mentioned, claudins are cell adhesion molecules that play a role in tight junction formation. Tight junctions are not only able to control permeability and paracellular transport (81), but are able to coordinate molecules that are required for essential cellular processes (82). As a result, tight junctions and the proteins that they are made of must be tightly regulated, always maintained, and be able to respond to any cellular disturbance, which would require protein turnover and degradation. In this case, claudins have been shown to undergo degradation via the UPS and thus, represent a possible substrate for our candidate RNF149 (83, 84).

All in all, our findings in chapter 4 have not only provided further characterization of the TCam-2 human seminoma cell line, but have also provided evidence, for the first time, that claudins 6 and 7 are likely involved in gonocyte development and that improper downregulation of these proteins during gonocyte development could provide a link to TGCT formation.

5.4 The environment and the origins of testicular germ cell tumors

Although the exact cause of TGCT formation is unknown, due to their rapid increase in incidence, it has been suggested that environmental factors, especially the exposure to endocrine disruptors during embryogenesis, may be at fault (85). Endocrine disruptors are compounds that are usually environmental pollutants, including estrogenic compounds, pesticides and plasticizer phthalates, which are able to disrupt the endocrine system and alter homeostasis by modifying hormonal balance and changing gene regulation (86). Environmental importance has been seen in various population studies as discussed in chapter 1. In brief, there is a high incidence of TGCTs in Denmark and low rates are seen in Finland (87). When men from either of these countries migrated to Sweden, they maintained the relative incidence rates of TGCT from their country of origin. However, the first generation of men born in Sweden had the incidence rates seen in Swedish men, indicating the importance of their environment (88).

TGCTs are not the only pathology to be affected by endocrine disruptor exposure. TGCTs are a part of a larger set of male reproductive pathologies, termed testicular dysgenesis syndrome (TDS) (85), including impaired spermatogenesis, reduced semen quality, hypospadias (abnormally placed opening in the urethra), and cryptorchidism (undescended testis). Moreover, as the severity of TDS increases, the higher the risk becomes to developing TGCT (85).

Studies have shown that cryptorchidism is more highly concentrated in geographical areas where there are high levels of agricultural activities (89). Furthermore, not only can cryptorchidism lead to TGCT formation, but it can also be a significant cause of infertility (90). Thus, it is possible that cryptorchidism and TGCTs share common gene expression profiles. Using data generously provided to us by Dr. Robert Sullivan at Université de Laval, we examined whether genes we had found to be important in gonocyte development and possibly leading to CIS formation, were also altered in cryptorchid patients. This gene array was performed using an Affymetrix Gene Chip Human Gene 1.0 ST array which included 28869 genes. The data was obtained using samples from five patients without cryptorchidism and four patients with cryptorchidism. These patients varied in ages from 23-53 years old. When comparing normal versus cryptorchid patients, we found that both PDGFRα and PDGFRβ were significantly upregulated in cryptorchid testes. This is interesting because, in chapter 2, we had found a variant PDGFRα to be likely involved in gonocyte differentiation and had hypothesized that retention of this variant form could possibly lead to TGCTs. Here, although it is not known whether the PDGFRα that is significantly upregulated in cryptorchid testes is the full length receptor or a variant form, it is possible that the retention of PDGFRα (variant or full length) can be a result of impaired germ cell development as it is often seen in cryptorchidism, which in turn could be linked to the increased risks of developing TGCTs. When analyzing RNF149, claudin 6, and claudin 7 gene expression, there was no significant change between normal and cryptorchid samples detected and claudins 6 and 7 were expressed in relatively low levels in both sample types. The low expression seen in claudins 6 and 7 further confirms that the upregulation seen in TGCT (as described in chapter 4) is likely specific to seminoma type tumors within the testis. Interestingly, although claudins 6 and 7 were not significantly altered when comparing normal and cryptorchid patients, functional gene analysis revealed that both cell adhesion molecules and genes related to pathways in cancer were significantly upregulated in cryptorchid

patients. Thus, this would confirm that both cryptorchidism and TGCTs have some commonality in their gene expression profiles.

Among the main types of endocrine disruptor compounds are estrogenic compounds such as bisphenol A (BPA), a component of polycarbonate plastics, the phytoestrogen genistein, and diethylstilbesterol (DES), a man-made strong estrogen agonist, and phthalates, plasticizers produced in very high amounts and added to PVC plastics, cosmetics, medical devices, which are found in our environment (91, 92). While BPA, genistein and DES act mainly by binding on estrogen receptors (ERs), phthalates disrupt androgen homeostasis independent of the androgen receptors (ARs), whereas a number of pesticides act as AR antagonists (89). Interestingly, we have seen that TCam-2 cells (human testicular seminoma cell line) express high levels of AR (chapter 4). Furthermore, we have previously shown that when rats were treated in utero with either BPA and genistein, there was a significant increase in PDGFRs and the MAPK pathway mRNA and protein levels in the neonatal testis, suggesting alterations in the MAPK signaling pathway upon estrogenic exposures (93, 94). This is interesting as we have previously shown the importance of the MAPK signaling pathway in PDGF and 17β-estradiol-induced gonocyte proliferation, and thus, dysregulation of MAPK signaling could lead to uncontrolled gonocyte proliferation (14). To add, we have found that prenatal exposure to estrogenic compounds can lead to altered HSP90 expression and given that HSP90 can interact with several different signaling molecules, including ERs and Raf-1, alterations in HSP90 expression could cause alterations in gonocyte development (95). There are also many studies indicating how phthalates can induce TDS and disrupt short and long term testosterone production (96, 97). Moreover, studies have shown that mono-(2-ethylhexyl) phthalate (MEHP) can promote the invasion and migration of testicular embryonal carcinoma cells (98) thus suggesting that phthalate exposure could lead to TGCT formation. Interestingly, it has recently been reported that phytoestrogens (estrogen-like compounds deriving from plants that can act as agonists or antagonists for the ERs) can cause anti-proliferative effects on testicular tumors cells such as TCam-2, causing the downregulation of stem cell factors NANOG and POU5F1, and the increase in genes important for tumoral cell differentiation and oncogenic inhibition (99). Although this is the effect reported using phytoestrogens *Belamcanda chinensis* extract (BCE) and tectorigenin, estradiol itself has been shown to induce TCam-2 tumoral cell proliferation (100).

All in all, it is clear that the increased exposure to endocrine disruptor compounds within our environment is likely causing the increase seen in TGCT rates, possibly by affecting the various signaling pathways and cellular processes that are regulating gonocyte development.

5.5 Clinical applications

The focus of the work presented in this thesis has been to better understand the processes involved in gonocyte differentiation and how they could possibly lead to carcinoma-in-situ formation, which is the precursor pathology to TGCTs. Testicular cancer is one of the most common types of cancer in young men today and for the past few decades, the incidence have been steadily inclining (2). As discussed in chapter 1, testicular cancer is a curable disease with a high survival rate and thus, is often overlooked as a pathology that needs to be further studied (101). However, because testicular cancer in often diagnosed in young men, it raises questions regarding the quality of life and fertility issues after treatment. Like any chemotherapeutic regimen, the BEP (bleomycin, etoposide, and cisplatin) strategy used for the treatment of testicular cancer has many side effects including pulmonary, vascular, neurological, renal, and cardiovascular toxicity, alongside increased risks for subsequent tumor formation (102, 103). Although the data available for side effects in humans are limited and not always clear, studies have shown persistent genetic damage in the sperm genome before and after chemotherapy treatment for testicular cancer (104, 105, 106). As a result, it is not surprising that the next generation fathered by these treated men would present higher congenital abnormalities than control children (107). BEP chemotherapy has been shown to induce azoospermia (lack of sperm) but this is only transient and a large portion of survivors will be able to recover fertility once the treatment has been completed (108). Studies have also shown that there is a defect in sperm DNA integrity, as measured by the DNA fragmentation index, in men with childhood cancers (109). Finally, although there is conflicting data whether chemotherapy treatment affects a patient's ability to conceive after treatment or to have healthy children afterwards, a recent study has shown that post-testicular cancer treatment, there was a decrease in fertility, but of the babies that were born, there was no obvious immediate malformations (110). The negative effects on sperm quality via BEP treatment in the rat model have been shown but although there are some, albeit limiting, human studies available, it is currently unclear whether future fertility and child health is affected with this chemotherapeutic treatment. However, as unclear as the

available data may seem, it is safe to say that BEP treatment has a large number of side effects and although it results in a high survival rate, the quality of life of these patients is not ideal, and could definitely be improved by developing new, more targeted therapeutics.

To avoid the problems of fertility, it is believed that one solution is sperm banking before cancer treatment (111). However, at the time of detection, it is likely that the sperm produced might be affected due to the illness itself, such as a defect in SSCs leading to abnormal germ cell maturation. Furthermore, the age at which young boys are presenting TGCTs is becoming younger , and thus, for boys who are of a pre-pubertal age, sperm banking would not even be a viable option (111). Thus, there is a need for other types of approaches to protect the future reproductive health of these young boys. Options include extracting the germ cells before chemotherapy treatment, obtaining a testicular biopsy prior to treatment and transplanting back into the testis after treatment, and more popularly, using the patient's own spermatogonial stem cells (SSCs) from testicular biopsies prior to treatment, to re-populate the testis (111).

Improper gonocyte development can also lead to infertility. Although not a focus of this thesis, infertility is also on the rise in the developed world, with more and more children being born by using assisted reproductive technologies (ARTs) such as in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) (112, 113). Infertility is a difficult pathology to study because the underlying causes are not always known. However, about 50% of infertility occurs due to male factors (113). Thus, better understanding gonocyte development would not only provide a better understanding of TGCT formation, but also of how infertility may arise.

The findings from our thesis have identified potential targets for future drug development. In chapter 2, we described the presence of a variant PDGFRα in gonocytes that was increased during gonocyte differentiation. Previously, the presence of a PDGFRα variant has been described in seminomas (28) and, it is possible that the retention of this variant form is involved in tumor formation. Thus, the variant PDGFRα could be targeted to design new drugs for TGCTs, with fewer side effects. Receptor tyrosine kinases are commonly used as druggable targets and Imatinib is a classic example. Imatinib is known to inhibit PDGFR and cKIT and is clinically used for the treatment of chronic myeloid leukemia (CML) (114). In CML, Imatinib binds to the BCR-ABL kinase domain, preventing activation. As a result, proliferative signals to the nucleus are blocked and apoptosis is induced (114). Similarly, a specific inhibitor for variant-

PDGFRα could be developed. In a disease-prevention manner, this inhibitor could ensure the inhibition of the variant-PDGFRα once gonocyte differentiation had occurred in high risk individuals so that there would not be variant form retention, that is possibly leading to tumor formation. However, in cancer therapy, this inhibitor could prevent the expression of the variant PDGFRα in seminoma tumors, and act as a treatment towards the already existing tumor.

Furthermore, in chapter 4, we established the possible importance of claudins 6 and 7 in both gonocyte differentiation and in TGCT as these two claudins were highly expressed in gonocytes and seminoma tumors. Interestingly, claudins have also been shown to be druggable targets, which is ideal due the prevalence of claudin upregulation and downregulation seen in a multitude of cancers (115). To begin, specific drugs could be developed in order to allow for proper downregulation of the claudins to occur during gonocyte development so that proper gonocyte development, and then spermatogenesis, could progress as expected. Furthermore, studies have shown that taking advantage of the cell adhesion molecule properties and cell barrier properties of claudins would allow for higher drug absorption (115, 116, 117). To add, multiple claudins have the ability to bind to CPE, *Clostidium perfringens* enterotoxin, which allows for cytolysis in mammalian cells because it effects membrane permeability (115). Originally, only claudins 3 and 4 had been shown to have the ability to form this complex, however, more recently, it has been reported that claudin 6 can also act as a receptor for CPE, thus having the potential for CPE based therapy (116, 118). To add, claudins have two extracellular loops that have been suggested to be targeted for antibody-based therapy for various cancers (119). All in all, claudins 6 and 7 also represent possible targets for developing more targeted drugs for TGCT treatment.

Although developing a better cure for testicular cancer and proper gonocyte differentiation is important, there are situations in which a lack of proper spermatogenesis is beneficial. It is well known that the options for male contraceptives are limited. Because spermatogenesis, including gonocyte differentiation, relies on vitamin A and its metabolite RA, it has been suggested that enzymes that metabolize vitamin A into RA can be targeted for male contraceptive development (120). Not only RA related targets, but targets such as PDGFRα and claudins 6 and 7, that could ideally be targeted for cancer therapy, could also possibly be targeted for male contraception.

In chapter 3, we found that activation of the ubiquitin proteasome system is necessary for gonocyte differentiation to occur and that likely, the E3 ubiquitin ligase RNF149, is playing a role. The UPS is not only important during gonocyte differentiation, but has also been shown to play an important role in later stages of spermatogenesis. Thus, given its importance, it is possible that the UPS can be targeted by cancer therapy, but can also be targeted for male contraception. Targeting the ubiquitin proteasome system for drug development is not a novel idea, as even one of the inhibitors used in our analysis, Bortezomib, is used for multiple myeloma treatment (121, 122). However, it has been suggested that instead of targeting the actual proteasome, more specific and more targeted drug development could occur by targeting an enzyme upstream of the proteasome, such as an E3 ligase or a de-ubiquitinating enzyme, to control which substrates could be prevented from degradation, as anti-cancer therapy (36, 37).

All in all, throughout this thesis, we have found druggable targets that could possibly be used for infertility treatment, male contraception, and most importantly, testicular cancer treatment.

5.6 Final conclusions

The findings presented in this thesis have contributed to a better understanding of neonatal gonocyte development. Although later stages of spermatogenesis have been well studied, it was the limited knowledge on gonocyte development that motivated our studies to focus on these cells, the necessary step for spermatogonial stem cell formation. From describing the signaling pathways involved in differentiation, to determining the importance of molecular processes such as the UPS and identifying new molecules potentially critical for gonocyte function, such as the claudins, this thesis provides original data allowing for a more complete understanding of the gonocyte, a cell that has been neglected until recently. The findings in this body of work have set up the ground work for future projects that will offer a more complete view of gonocyte differentiation. Until then, we hope that our work here highlights key elements in gonocyte differentiation and provides a better understanding of genes that may provide a link between improper gonocyte differentiation and testicular germ cell tumor formation.

5.7 References

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