EXPRESSION AND ROLE OF THE ORPHAN NUCLEAR RECEPTOR NR5A2 IN MOUSE EMBRYOGENESIS AND FEMALE REPRODUCTIVE FUNCTION.

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Abstract

The orphan nuclear receptor NR5A2 is implicated in a multitude of biological processes including cholesterol homeostasis and development. Its role in cholesterol metabolism and cell proliferation is now well established *in vitro* and *in vivo*. Both *in vitro* and gene expression studies have suggested a role for NR5A2 in ovarian function. In this study, we provide *in vivo* evidence for its involvement in reproductive function by demonstrating that heterozygosity for a null mutation of *NR5A2* leads to a reduction in female fertility. Furthermore, we showed that *NR5A2* +/- females display a severe reduction in ovarian progesterone production and that progesterone supplementation can rescue the *NR5A2* +/- subfertiliy phenotype. We also provide evidence that one of the mechanisms by which NR5A2 regulates ovarian progesterone production is through modulating the expression of StAR, which controls one of the rate-limiting steps of progesterone synthesis.

A targeted disruption of the *NR5A2* gene in the mouse leads to early lethality *in utero* between embryonic days 6.0 and 7.5, showing that NR5A2 plays a crucial role during early embryogenesis. The molecular mechanisms underlying this early lethality, however, are poorly understood. In this study, we used a morphological and marker gene analysie to characterize the *NR5A2* -/- embryonic phenotype and showed that although initial axis specification occurs in *NR5A2* -/- embryos, primitive streak and mesoderm fail to form. Using a chimeric approach, we

demonstrated a requirement for NR5A2 function in the visceral endoderm (VE), an extra-embryonic tissue, for proper primitive streak morphogenesis and gastrulation. Our results also indicate a reduction in the expression of VE marker genes involved in the nutritive function of this tissue, suggesting that NR5A2 play a dual role in the VE, being implicated in the mediation of both its patterning and nutritive activity.

Taking advantage of the LacZ knock-in approach used to inactivate the *NR5A2* gene, we also demonstrated that NR5A2 is expressed during craniofacial and nervous system development, suggesting a novel role for NR5A2 in head formation and neural development.

Résumé

Le récepteur nucléaire orphelin NR5A2 joue un rôle prépondérant dans divers processus biologiques, notamment aux niveaux du métabolisme du cholestérol et du développement embryonnaire. Son implication dans la régulation de l'homéostasie du cholestérol et de la prolifération cellulaire est clairement établie, autant *in vitro* que *in vivo*. Des études récentes ont clairement démontré que *NR5A2* est fortement exprimé dans l'ovaire et qu'il joue un rôle dans le contrôle de la steroïdogenèse *in vitro*. Afin d'évaluer le rôle potentiel de NR5A2 au niveau des fonctions ovariennes *in vivo*, nous avons utilisé des souris *NR5A2* +/- et démontré que les femelles hétérozygotes pour une mutation du gène *NR5A2* ont une diminution de fertilité. Nos résultats indiquent que le taux de progestérone est sévèrement réduit chez les souris *NR5A2* +/- et qu'une supplémentation en progestérone conduit au sauvetage du phénotype de sous-fertilité.

Nous avons également trouvé que l'un des mécanismes par lequel NR5A2 affecte la production de progestérone est par la régulation de l'expression de StAR, une protéine régulant une des étapes limitantes dans la biosynthèse de la progestérone.

L'inactivation ciblée du gène *NR5A2* conduit à une mort *in utero* entre les jours embryonnaires 6.0 et 7.5, démontrant un rôle important pour NR5A2 au cours de l'embryogenèse précoce. Afin de déterminer la fonction de NR5A2 durant cette période, nous avons entrepris une

analyse de marqueurs et démontré que bien que la spécification initiale de l'axe antéropostérieur soit induite, la formation de la ligne primitive et du mésoderme échoue. Des expériences de sauvetage par génération d'embryons chimériques ont démontré que la fonction de NR5A2 est requise dans l'endoderme viscéral (EV), un tissue d'origine extra-embryonnaire, pour la morphogenèse de la ligne primitive et l'accomplissement de la gastrulation. De plus, nos résultats indiquent une diminution importante du niveau d'expression de deux marqueurs de l'EV importants pour sa fonction nutritive. Ces résultats suggèrent donc que NR5A2 joue un double rôle dans l'EV, agissant comme médiateur des deux fonctions de ce tissu, influençant la régionalisation et la nutrition de l'embryon.

En prenant avantage de la stratégie d'inactivation du gène *NR5A2* par insertion du gène rapporteur codant pour la β-galactosidase en fusion et en phase avec le gène *NR5A2* endogène, nous avons défini son patron d'expression post-gastrulation. Nous avons montré que NR5A2 est exprimé au cours du développement craniofacial et neural, suggérant l'implication de NR5A2 au niveau de la formation de la tête et du système nerveux lors de l'embryogenèse.

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Abbreviations

Aa: amino acid

ABST: apical sodium-dependent bile salt transporter

AF: activation function

AFP: alpha-fetoprotein

A-P: anterior-posterior

AVE: anterior visceral endoderm

BMP: bone morphogenetic protein

C/EBP: CCAAT/enhancer-binding protein

CL: corpus luteum

CPF: CYP7A promoter-binding factor

CREB: cAMP response element-binding protein

CTE: carboxy-terminal extension

CYP7A1: cholesterol 7α-hydroxylase

CYP8B1: sterol 12α-hydroxylase

CYP11A1: cytochrome P450 side-chain cleavage

CYP19: cytochrome P450 aromatase

Dax-1: dosage-sensitive, sex-reversal adrenal hypoplasia congenital critical region on the X chromosome gene 1

DBD: DNA-binding domain

Dkk-1: Dickkopf 1

DNA: deoxyribonucleic acid

Dpc: day post-coitum

D-V: dorso-ventral

E: embryonic day

Eomes: Eomesodermin

ER: estrogen receptor

ES: embryonic stem

FGF: fibroblast growth factor

FTF: fetoprotein transcription factor

FSH: follicle stimulating hormone

FSHR: FSH receptor

Ftz-F1: fushi tarazu- factor 1

GnRH: gonadotropin-releasing hormone

hB1F: human B1-binding factor

HDL: high-density lipoprotein

Hh: hedgehog

HNF: hepatocyte nuclear factor

HRE: hormone response element

3β-HSD: 3β-hydroxysteroid dehydrogenase

ICM: inner cell mass

Ihh: indian hedgehog

Kb: kilobase

LBD: ligand-binding domain

LDL: low-density lipoprotein

LH: luteinizing hormone

LMC: lateral motor column

LRH-1: liver receptor homolog-1

LXR: liver X receptor

MAPK: mitogen- activated protein kinase

MesD: mesodermin

MMC: medial motor column

MRP3: multidrug resistance protein 3

NR: nuclear receptor

PBS: phosphate buffer saline

PCR: polymerase chain reaction

PDX-1: pancreatic-duodenal homeobox 1

PG: prostaglandin

PHR-1: pancreatic homolog receptor 1

PI3K: phosphatidyl inositol-3 kinase

PKA: protein kinase A

PKC: protein kinase C

PR: progesterone receptor

PRL: prolactin

RXR: retinoid X receptor

SF-1: steroidogenic factor 1

Shh: sonic hedgehog

SHP: small heterodimer partner

SR-B1: scavenger receptor type B1

StAR: steroidogenic actute regulatory protein

SUMO: small ubiquitin-related modifier

T: brachyury

TCF: T cell factor

TR: thyroid hormone receptor

TGFβ: transforming growth factor β

VE: visceral endoderm

Publications

1. **Labelle-Dumais C**, Paré JF, Bélanger L, Farookhi R, and Dufort D. Mice haploinsufficient for the nuclear receptor NR5A2 are subfertile due to impaired ovarian progesterone synthesis. *Submitted*

2. **Labelle-Dumais C**, Jacob-Wagner M, Paré JF, Bélanger L, and Dufort D (2006). The nuclear receptor NR5A2 is required for proper primitive streak morphogenesis. *Dev Dyn* 235, 3359-3369.

3. **Labelle-Dumais C**, Paré JF, Bélanger L, and Dufort D. The expression pattern of NR5A2 during mouse embryogenesis reveals a potential role during craniofacial and nervous system development. *To be submitted*

4. Mohamed OA, Jonnaert M, **Labelle-Dumais C**, Kuroda K, Clarke H, and Dufort D (2005). Uterine Wnt/β–catenin signaling is required for implantation. *Proc. Nalt. Acad. Sci. USA* 102, 8579-8584. (Not included in this thesis)

Contributions of authors

1. **Labelle-Dumais C**, Paré JF, Bélanger L, Farookhi R, and Dufort D. Mice haploinsufficient for the nuclear receptor NR5A2 are subfertile due to impaired ovarian progesterone synthesis. *Submitted*
Jean- Francois Paré and Dr. Luc Bélanger generated and provided the *NR5A2* +/- mice. Dr. Riaz Farookhi participated in the experimental design, data analysis, and manuscript revision. I wrote the manuscript and performed all the experiments presented in this study.

2. **Labelle-Dumais C**, Paré JF, Jacob-Wagner M, Bélanger L, and Dufort D (2006). The nuclear receptor NR5A2 is required for proper primitive streak morphogenesis. *Dev Dyn* 235, 3359-3369.
Jean- Francois Paré and Dr. Luc Bélanger generated and provided the *NR5A2* +/- mice. Mariève Jacob-Wagner performed the quantitative real time PCR. Dr. Daniel Dufort participated in the generation of tetraploid chimeras. I wrote the manuscript and performed all the other experiments.

3. **Labelle-Dumais C**, Paré JF, Bélanger L, and Dufort D. The expression pattern of NR5A2 during mouse embryogenesis reveals a potential role during craniofacial and nervous system development. *To be submitted.*
Jean- Francois Paré and Dr. Luc Bélanger generated and provided the *NR5A2* +/- mice. I wrote the manuscript and performed all the experiments presented in this study.

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In this study, I participated in the experimental design, generation of embryoid bodies, animal maintenance and genotyping, and to the revision of the manuscript.

CHAPTER 1: GENERAL INTRODUCTION

INTRODUCTION

The survival of a species relies on its reproductive efficiency. In mammals, as in other sexually reproducing organisms, reproduction involves the production and fusion of two distinct parental haploid cells that will lead to the generation of a diploid cell, called the zygote, which will develop into a new and distinct individual.

The fusion of the maternal gamete, the egg, and the paternal gamete, the sperm, therefore constitutes the first step in the generation of a new individual and depends on the production of functional gametes. The process of gametogenesis is under tight hormonal regulation and takes place in the gonads. In response to hormonal signals, somatic gonadal cells proliferate and differentiate to support germ cell maturation, to promote gamete release, and to produce steroid hormones required to coordinate reproductive functions and behaviors. For instance, in placental mammals, steroid hormones are involved the preparation of the uterine environment for embryo implantation and subsequent maintenance of pregnancy. Hormonal regulation is therefore central for the orchestration of reproductive functions that will allow the generation and development of a new organism.

The advent of sexual reproduction in multicellular organisms led to the apparition of higher and more complex life forms. This increasing complexity of life forms raised one of the central questions of modern biology: how can a single cell generate all the different tissues that will

give rise to a complete and complex multicellular organism. The process by which this single cell, the fertilized egg or zygote, generates an embryo that will eventually become a functional adult organism is referred to as embryogenesis. The creation of a multicellular organism therefore begins with the fertilization of the egg, which will subsequently divide mitotically to produce all cell types essential for the elaboration of the body. Embryonic development of multicellular organisms does not merely involve cell division but also proper integration and regulation of cell differentiation, cell growth and morphogenetic events that will lead to the formation and organization of the different tissues and organs that constitute a functional organism. All these events depend on the concerted actions of extracellular signals and intercellular communication that modulate the activity of various intracellular signaling pathways, which transduce their effects via their actions on transcription factors.

Transcription factors are nuclear proteins that regulate gene expression, which ultimately results in a specific cellular response such as proliferation, differentiation or migration. Identification of mutations leading to impaired embryonic development has revealed that many important genes code for transcription factors, indicating that transcriptional regulation is of crucial importance in controlling embryogenesis. A remarkable diversity of cell types that carry out a variety of different functions and display various morphologies must be produced during development, and each cell type appears to be characterized by a

particular gene expression profile. A given qualitative and quantitative gene expression pattern therefore reflects the activity of specific transcription factors or combinations of them.

One family of transcription factors that is implicated in the regulation of both reproduction and embryogenesis, the two major biological processes involved in the generation of a new individual, is the nuclear receptor superfamily. Members of the nuclear receptor superfamily have been shown to alter cell fate in response to hormonal and extracellular signals at multiple levels along the reproductive–developmental axis, ensuring the reproductive success of a species and the production of healthy offspring.

1.1 DESCRIPTION OF NUCLEAR RECEPTORS

1.1.1 General concepts about the nuclear receptor superfamily of transcription factors

The nuclear receptor superfamily is one of the largest families of transcription factors, comprised of 48 and 49 members in human and mice, respectively (Laudet and Gronemeyer, 2002; Maglich et al., 2003). This family of transcription factors has been implicated in a multitude of biological processes, including development, differentiation, metabolism, reproduction, homeostasis, and disease. Nuclear receptors (NRs) can be defined as transcription factors capable of directly modulating gene expression in the nucleus in response to various extracellular signals and

liphophilic molecules (Mangelsdorf et al., 1995). The wide repertoire of actions of NRs and the fact that their activity is usually modulated by small lipophilic molecules, including hormones, metabolites, as well as natural and synthetic ligands, make them appealing pharmacological targets of therapeutic significance. The NR superfamily was initially divided into 3 main classes of receptors: type I receptors (steroid hormone receptors), which comprise the glucocorticoid and estrogen receptors; type II receptors, which include retinoic acid, thyroid and vitamin D receptors; and type III receptors which are referred to as orphan nuclear receptors (Mangelsdorf et al., 1995). Subsequently, a detailed study of the evolutionary relationships between NRs led to the assignment of these transcription factors to seven well defined subfamilies referred to as NR0 to NR6, resulting in a unified nomenclature system for nuclear receptors (Laudet, 1997; Nuclear Receptors Nomenclature Committee, 1999).

1.1.1.2 Structure of nuclear receptors

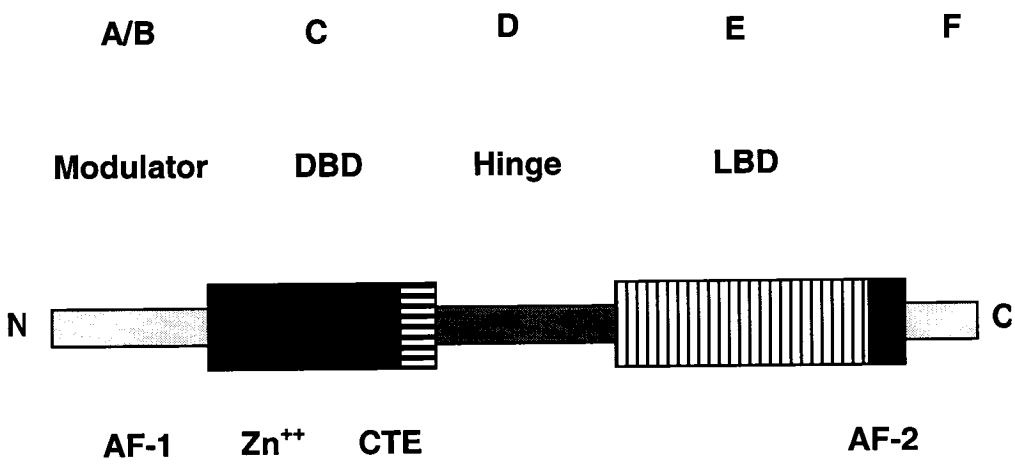
Despite the large number of NRs and their vast spectrum of biological actions, all members of this superfamily are defined by a characteristic and conserved modular structure (Figure 1). The modular structure shared by NRs is characterized by five to six distinct domains that have been assigned specific functions (Mangelsdorf et al., 1995; Giguère, 1999; Renaud and Moras, 2000). These functional modular domains include: the modulator A/B domain also known as the N-terminal

Figure 1. Canonical modular structure of a nuclear receptor

Nearly all nuclear receptors contain two highly conserved domains: the DNA binding domain (DBD) or C domain, which is composed of two zinc finger motifs (Zn^{++}) and a carboxy-terminal extension (CTE), and the ligand-binding domain (LBD) or E domain, which contains a ligand-dependent activation function (AF-2). Nuclear receptors also comprise variable domains: the modulator A/B domain, which contains a ligand-independent activation function (AF-1), the hinge region or D domain, and the F-domain, which is not always present.

C= carboxy-terminus, N= amino-terminus

Figure 1. Canonical modular structure of a nuclear receptor



activation domain, the DNA binding domain (DBD) or C domain, the hinge region or D domain, the ligand-binding domain (LBD) or E domain, and an F domain that is not found in all NRs and whose function is poorly understood.

Amongst the NRs, the modulator A/B domain exhibits the most variability in both sequence and length and usually contains a ligand-independent activation function referred to as AF-1. This modulator domain possesses promoter and cell context-dependent activities. For instance, the modulator domain cooperates with cell-specific promoter-bound transcription factors and other co-factors to modulate the transcriptional activity of NRs. Moreover, phosphorylation of the A/B domain by specific kinases, activated in a particular cell-context, can enhance the transcriptional activation function of NRs. The A/B domain of NRs is therefore responsible for mediating the cooperation of NRs with cell-specific co-regulators and co-factors to elicit a particular cellular response in a ligand-independent manner (Giguère, 1999; Chambon, 2005).

The DBD is the most highly conserved domain among members of the nuclear receptor superfamily. This domain contains two zinc fingers in tandem that encompass around 70 amino acid residues and is directly implicated in DNA recognition of the nuclear receptor. These DNA recognition sites are referred to as hormone response elements (HREs). HREs consist of a recognition motif derived from the archetypal AGGTCA

(half-site) sequence and contain one or two consensus core half-site sequences exhibiting different configurations: direct, inverted or everted repeats. NRs can bind to their target genes as monomers, homodimers or heterodimers, and therefore, a given organization of the HRE will be recognized by specific NRs or a combination of them (Glass, 1994). Although half-site sequences can deviate from the consensus sequence, a single conserved half-site is usually sufficient to confer high affinity binding for dimeric forms of NRs. The region of the DBD that is involved in generating DNA-binding specificity is known as the P-box and is contained in the first zinc finger motif; whereas the second zinc finger motif is responsible for dimerization through recognition of half-site spacing and orientation (D-box). The DBD carboxy-terminal extension or CTE is involved in protein-DNA and protein-protein interaction.

The D domain of NRs also displays variability in length and sequence and displays high flexibility since it acts as a hinge between the DBD and the LBD. The hinge region allows rotation of the DBD to accommodate binding to HRE. Although the D domain possesses an important structural function, recent studies suggest that this region also plays a functional role. For instance, the hinge domain was shown to be subject to post-translational modifications, including phosphorylation, which can potentiate the transcriptional activity of NRs and can stimulate co-factor recruitment (Hammer et al., 1999; Lee et al., 2006). Further

supporting a role for the hinge domain in modulating NR function, it was shown to act as a docking site for co-regulators, notably for co-repressors (Chen and Evans, 1995; Horlein et al., 1995). The hinge region thus appears to influence both the structure and the transactivation ability of NRs.

Despite the fact that the LBD of NRs exhibit sequence variability, crystallographic analyses have revealed that NR LBDs display a canonical structure, primarily helical, that forms a single protein domain (Wurtz et al., 1996). This domain is created by the folding of twelve α–helices (H1 to H12) into a three-layered antiparallel sandwich with a conserved β-turn that forms a hydrophobic ligand-binding pocket, which comprises two halves (Laudet and Gronemeyer, 2002; Renaud and Moras, 2000; Nagy and Schwabe, 2004). Sequence alignment analysis revealed that the two halves of NR LBDs are composed of a conserved hydrophobic core in the upper region and of a hypervariable region corresponding to the ligand-binding site located on the lower half (Wurtz et al., 1996). Since the LBD of all NRs display a similar structure, the LBD constitutes a signature motif for NRs. The LBD not only serves as a ligand-binding cleft but also mediates other functions including dimerization, protein interactions, and transactivation. For instance, ligand-dependent transcriptional activation relies on the presence of a highly conserved motif known as the ligand-dependent activation function-2, or AF-2, located in the LBD.

1.1.1.3 Mechanisms of action of nuclear receptors

Although there is some variability in the mechanisms of action among NRs, there is a generally accepted model to explain the molecular mechanisms underlying their mode of action (Nagy and Schwabe, 2004). The activation of NRs is normally induced by binding of their cognate lipophilic ligand or by post-translational modifications induced by extracellular signals. Ligand binding or post-translational modifications of NRs then induce global stabilization of the LBD, and promote positioning of helix 12 (H12) in the active conformation. Stabilization of H12 in the active conformation is associated with the release of co-repressors and recruitment of co-activators. Co-activator binding then facilitates the initiation of transcription by promoting opening of the chromatin which increases the accessibility of the target DNA to the basal transcriptional machinery.

Although the model explaining the general mechanism of action of NRs is mainly based on the existence of an activatory ligand, many members of the nuclear receptor family have no known ligand. These so-called orphan nuclear receptors may be activated by as yet unidentified ligands or may not require ligand binding to be transcriptionally active (Mangelsdorf et al., 1995).

1.1.2 Orphan nuclear receptors

Orphan nuclear receptors constitute about 75% of the nuclear receptor superfamily (Willy and Mangelsdorf, 1998). Originally, classic members of the NR superfamily were characterized on the basis of their ability to transduce the biological effects of a given ligand by directly modulating gene expression. New members of the NR superfamily, however, were mainly cloned on the basis of their sequence and structural homology to known receptors and were therefore not associated with a specific hormone or ligand. Thus, these putative nuclear receptors were named orphan nuclear receptors (Willy and Mangelsdorf, 1998). Many orphan receptors have now been "adopted", but there remains orphan nuclear receptors for which no ligand have been found (Sablin et al., 2003; Wang et al., 2003).

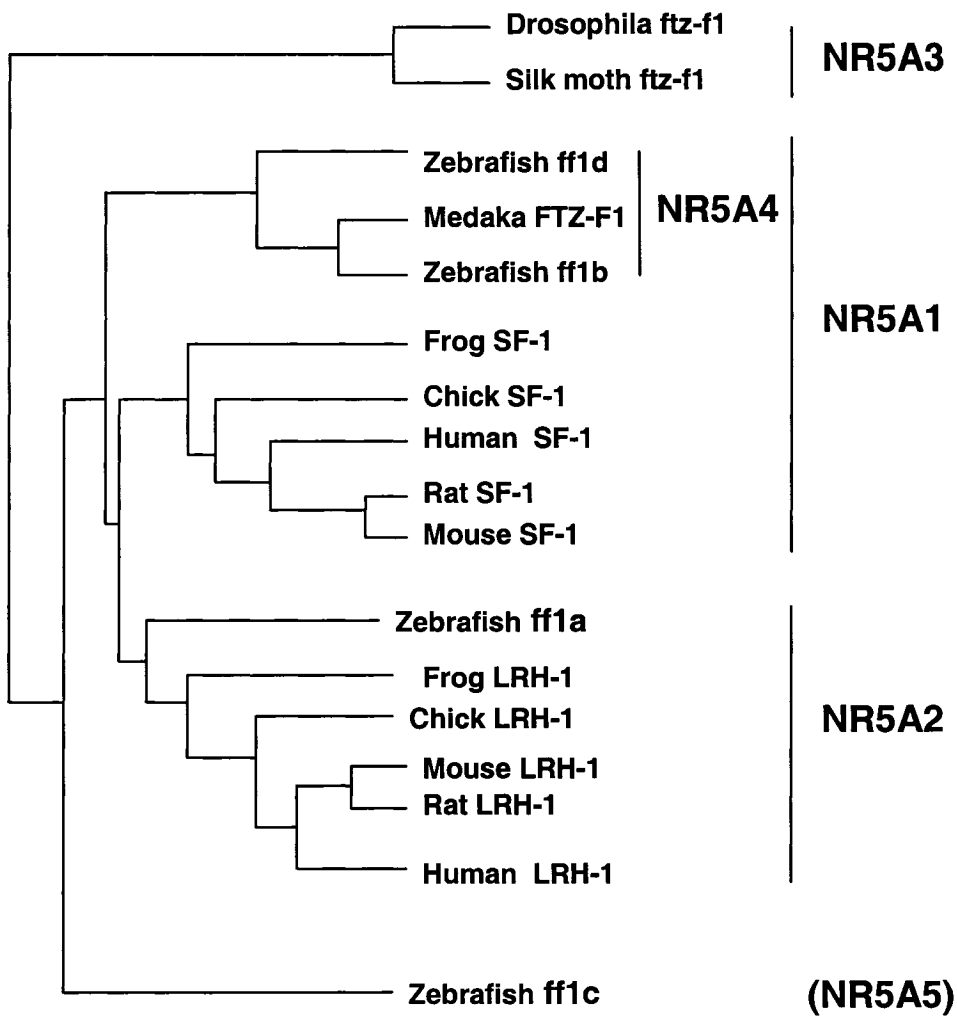
Orphan receptors are found in several subfamilies of nuclear receptors, and although putative ligands have not yet been identified for many of them, functional studies have implicated them in a multitude of biological processes such as development, detoxification and metabolism (Giguère, 1999). Orphan nuclear receptors of the NR5A subgroup have been shown to be involved in several physiological functions, including cholesterol homeostasis, reproduction, and development and will be the focus of this thesis.

1.1.3 The NR5A subfamily of nuclear receptors

The mammalian NR5A subfamily of nuclear receptors is often referred to as the FTZ-F1 subfamily since it shares homology with the Drosophila fushi tarazu factor-1 (FTZ-F1) (Ueda et al., 1990; Lavorgna et al., 1991). FTZ-F1 was isolated as a regulator of the fushi tarazu homeobox gene (ftz), which plays a pivotal role in segmentation (Kuroiwa et al., 1984; Wakimoto et al., 1984). FTZ-F1 was also shown to be involved in metamorphosis (Lavorgna et al., 1993, Broadus et al., 1999; Yamada et al., 2000). Genes homologous to the Drosophila FTZ-F1 have been subsequently identified in several species including frog, zebrafish, horse, chicken, mouse, rat and human, where they appear to play a variety of roles in development and adult physiology (Ellinger-Ziegelbauer et al., 1994; Nakajima et al., 2000; Liu et al., 1997; Boerboom et al,. 2000; Kudo and Sutou, 1997; Tugwood et al., 1991; Galarneau et al., 1996; Becker-André et al., 1993; Li et al., 1998). The high conservation of the NR5A subfamily of NRs through evolution suggests a predominant role for these receptors. Figure 2 illustrates the phylogenetic distribution of NR5A receptors.

Unlike the vast majority of nuclear receptors that bind DNA as homo- or hetero-dimers, NR5A nuclear receptors bind DNA as monomers to the consensus DNA recognition sequence 5'-YCA AGG YCR-3', where Y is any pyrimidine and R any purine (Lavorgna et al., 1991; Ueda et al., 1992; Ikeda et al., 1993; Galarneau et al., 1996). In the NR5A subfamily,

Figure 2. Phylogenetic tree of the NR5A subfamily of nuclear receptors



Simplified and adapted from Fayard et al., 2004 and von Hofsten and Olsson, 2005.

DNA binding specificity is conferred by the FTZ-F1 box present in the C-terminus of the DBD, which encompasses 26 amino acids and constitutes a unique feature of members of the NR5A subfamily (Ohno et al., 1994). The FTZ-F1 box specifically targets the YCA motif located upstream of the AGG YCR half-site sequence of the consensus site (Fayard et al., 2004).

There are two distinct but closely related NR5A members present in the mammalian genome: NR5A1 and NR5A2, which have evolved from the same ancestral gene (Ellinger-Ziegelbauer et al., 1994). NR5A1 is also known as steroidogenic factor-1 (SF-1) which was identified as an important regulator of steroidogenic genes expression (Lala et al., 1992; Honda et al., 1993; Lynch et al., 1993) and was shown to be a crucial factor involved in the development and function of endocrine and steroidogenic organs (Val et al., 2003). The mammalian NR5A2 was first isolated in the mouse as liver receptor homolog (LRH-1) (Tugwood et al., 1991) and was given different names as it was independently isolated by different groups; pancreatic homolog receptor 1 (PHR-1) (Becker-André et al., 1993), fetoprotein transcription factor (FTF) (Galarneau et al., 1996), human B1-binding factor (hB1F) (Li et al., 1998), and CYP7A promoter binding factor (CPF) (Nitta et al., 1999). NR5A2 has been implicated in various biological processes including development, metabolism and steroidogenesis (Fayard et al., 2003).

Although the two closely related members, NR5A1 and NR5A2, share a high degree of homology and bind to the same DNA consensus site, they display distinct expression patterns, which correlate with their respective determined or suggested functions. NR5A1 is predominantly expressed in the developing and adult steroidogenic organs and in tissues of the hypothalamic-pituitary-adrenal axis, which is consistent with its central role in the development and function of these tissues. NR5A2, on the other hand, displays a more widespread and dynamic expression pattern in the mouse. NR5A2 is detected in multiple areas during early embryogenesis starting from ubiquitous expression during early pre-implantation stages (Paré et al., 2004). As development proceeds, NR5A2 is expressed in a more restricted fashion, predominantly in the primitive and definitive endoderm and endoderm-derived organs, but also in growing bones, neural crest cells, lung mesenchyme, and developing gonads (Rausa et al., 1999; Paré et al., 2004; Hinshelwood et al., 2005). In the adult, NR5A2 is mainly expressed in the digestive and reproductive systems (Fayard et al., 2004).

The expression pattern and function of NR5A1 are well established (Val et al., 2003). The exact roles played by NR5A2, however, remain speculative since most of its proposed functions rely on evidences obtained from gene expression and *in vitro* studies. This thesis will therefore concentrate on better characterizing the roles of NR5A2 in the mouse.

1.2 DESCRIPTION OF THE MOUSE NR5A2

1.2.1 Genomic organization of murine *NR5A2*

In the mouse, the gene coding for NR5A2 is located on chromosome 1 and comprises 9 exons (Paré et al., 2001). There are two *NR5A2* transcripts resulting from alternative promoter usage, a 3.6 and a 5.2 kilobase (kb) transcript. The 3.6 kb transcript is present in embryonic stem cells and is expressed throughout embryogenesis and in some adult tissues. The 5.2kb transcript is first detected in embryos on day 10 of gestation and is present in many adult tissues (Galarneau et al., 1996; Gao et al., 2006). The predicted size of the protein generated from these two transcripts are 499 amino acids (aa) and 560 aa, respectively, with the shorter form lacking 61 residues at the N-terminal domain leading to a truncation of the A/B domain. Three distinct isoforms have been identified in mouse liver extracts and are generated in the protein N-terminal A/B domain by alternative usage of translation initiation codons and by different promoter usage. All three mouse NR5A2 isoforms exhibit similar binding abilities and presumably similar transactivation capacities since functional deletion analysis showed that the NR5A2 A/B domain does not possess particular activation function (Galarneau et al., 1996).

1.2.2 Regulation of *NR5A2* expression

A wide variety of extracellular signals and nuclear factors have been implicated in the regulation of *NR5A2* expression. For instance,

NR5A2 expression was shown to be stimulated by several extracellular factors including follicle-stimulating hormone (FSH) and prostaglandin E2 (PGE2) and to be modulated by activation of several intracellular signaling pathways such as PKA, PKC, MAPK and PI3K (Falender et al., 2003; Clyne et al., 2004; Zhou et al., 2005; Yu et al., 2005; Weck and Mayo, 2006). *NR5A2* expression is not only subject to positive but also to negative regulation by extracellular signals. For instance, pro-inflammatory tumor necrosis factor α (TNF-α)-induced signaling was shown to both reduce *NR5A2* expression in the intestine and up-regulate hepatic *NR5A2* expression (Schoonjans et al., 2005; Bohan et al., 2003).

In addition to its regulation by extracellular signaling molecules, *NR5A2* expression is also directly modulated by transcription factors. Characterization and functional analysis of the 5' regulatory sequences of the *NR5A2* gene led to the identification of functional binding sites for multiple transcription factors. Notably, several transcription factors involved in the development and function of the digestive system, including GATA factors, HNF4α, NR5A2, basic helix-loop-helix factors, pancreatic-duodenal homeobox1 (Pdx1), LXR-RXR and TR-RXR nuclear receptor dimers have been shown to regulate *NR5A2* expression (Paré et al., 2001, Annicotte et al., 2003, Paré et al., 2004). *NR5A2* expression was also shown to be stimulated by estrogen via direct binding and activation of the estrogen receptor α (ER α) (Annicotte et al., 2005).

Expression of *NR5A2* is therefore subject to regulation by several signaling pathways and transcription factors that exhibit specific spatio-temporal expression or activation, suggesting complex cell context-dependent *NR5A2* transcriptional regulation. Adding to this complexity, some of these factors have been shown to synergize in the activation of *NR5A2* expression (Paré et al., 2001). *NR5A2* expression therefore appears to depend on the concerted action of multiple extracellular and intracellular factors acting indirectly or directly on its promoter.

1.2.3 Regulation of NR5A2 activity

The mere presence of the nuclear receptor mRNA and protein product does not necessarily correlate with its activity. Indeed, NR activity, including that of NR5A2, appears to be regulated by many different mechanisms. Notably by ligand binding, post-translational modifications, such as phosphorylation, co-factor interactions, and subcellular localization, which are common means of altering the activity of transcription factors.

Although the vast majority of nuclear receptors require ligand binding to activate transcription, crystallographic analysis showed that NR5A2 adopts a transcriptionally active conformation in the absence of ligand (Sablin et al., 2003). The constitutive ligand-independent active conformation of NR5A2 is accounted for by the presence of an extended helix 2 (H2), which leads to stabilization of helix 12 (H12) in its active

position in the absence of ligand and co-activators. Further structural analysis revealed that although phospholipids can act as ligands for human NR5A1, mouse NR5A1 and human NR5A2, they do not act as ligand for mouse NR5A2, demonstrating that a late evolutionary event altered ligand-binding ability in the rodent NR5A2 lineage (Krylova et al., 2005). Although these results do not exclude the existence of NR5A2 ligands in the mouse, they indicate that they are not required for basal activity of this receptor.

NR5A2 transcriptional activity has been shown to be modulated by post-translational modifications, including phosphorylation and sumoylation. For instance, PKA- and MAPK-mediated phosphorylation of NR5A2 were shown to stimulate its transcriptional activity on different promoters (Bouchard et al., 2005; Lee et al, 2006). While PKA stimulates phosphorylation of the LBD, MAPK signaling promotes phosphorylation of the hinge region to potentiate NR5A2 transcriptional activity. Another post-translational modification that was shown to affect NR5A2 activity is sumoylation. Covalent attachment of the small–ubiquitin-related modifier (SUMO) to the hinge domain of NR5A2 was demonstrated to repress its transcriptional activity by sequestering it to specific nuclear compartments, thereby preventing access to its target genes (Chalkiadaki and Talianidis, 2005; Lee et al., 2005). These findings demonstrate that post-translational modifications of the LBD and hinge region of NR5A2 are involved in positive and negative regulation of its transcriptional activity.

The transactivation capacity of NR5A2 was also shown to be influenced by its interaction with other proteins. Notably, NR5A2 was shown to interact with various transcriptional co-regulators that do not bind DNA directly but that possess enzymatic activity to remodel chromatin structures and / or interact directly with the basal transcriptional apparatus. For instance, NR5A2 has been shown to interact with co-activators of the p160 and CBP/p300 families, which promote histone acetylation leading to loosening and opening of the chromatin structure (Lee and Moore, 2002; Sablin et al., 2003; Fayard et al., 2004). The interaction between DNA-bound NR5A2 and these co-activators therefore leads to the opening of the chromatin, rendering the target gene promoter accessible to the basal transcriptional machinery, ultimately resulting in transcriptional activation. The interaction between NR5A2 and these co-activators was shown to be dependent on the AF-2 domain and, in contrast to other NRs, this AF-2 mediated co-factor interaction occurs in the absence of ligand.

NR5A2 also interacts with co-regulators that repress its transcriptional activity. Since NR5A receptors adopt an active conformation in the absence of ligands, their interaction with co-repressors constitute a means to repress their constitutive basal activity. Notably, members of the NR0B subfamily of nuclear receptors, Dosage-sensitive, sex-reversal adrenal hypoplasia congenital critical region on the X chromosome gene 1 (Dax-1; NR0B1) and the small heterodimer partner (SHP; NR0B2) have been identified as tissue-specific co-repressors of

NR5A2 (Suzuki et al., 2003; Lee and Moore, 2002). Dax-1, which is predominantly expressed in steroidogenic tissues, was shown to interact directly with NR5A1 and NR5A2 to repress their transcriptional activity by competing with co-activator binding and recruiting co-repressors that promote histone deacetylation (Ito et al., 1997; Crawford et al., 1998; Suzuki et al., 2003). Histone deacetylation is associated with a compact chromatin structure that prevents interaction between the target gene promoter and the basal transcriptional machinery, inhibiting transcription. SHP, which is mainly expressed in the digestive system, specifically interacts with the NR5A2 AF-2 domain to repress NR5A2 transcriptional activity. SHP was shown to suppress NR5A2-mediated transactivation by a two-step mechanism that involves competition with co-activator binding and direct transcriptional repression through its C-terminal autonomous repression function (Lee and Moore, 2002).

Adding to the complexity of the regulatory mechanisms modulating NR5A2 activity, NR5A2 was shown to cooperate with other transcription factors to control target gene expression. For instance, NR5A2 can act as a competence factor, whereby it facilitates activation of target genes by other transcription factors. This is the case during the process of bile acid synthesis where NR5A2 potentiates LXRα–mediated expression of the metabolic enzyme cholesterol 7α-hydroxylase (CYP7A1) (Goodwin et al., 2000; del Castillo-Olivares and Gil, 2000). NR5A2 was also shown to act as a competence factor to facilitate PKA induced cytochrome P450

aromatase (CYP19) expression in pre-adipocytes (Clyne et al., 2002). In addition, NR5A2 can form a complex with the cyclic AMP response element binding protein (CREB) and co-activators on the promoter of the inhibin α subunit gene, increasing its transcription in response to FSH agonists (Weck and Mayo, 2006). Another example of cooperative interaction between NR5A2 and other transcription factors is that NR5A2 physically interacts and synergizes with GATA transcription factors to regulate CYP19 and 3β-HSD expression in breast cancer and steroidogenic cells, respectively (Bouchard et al., 2005; Martin et al., 2005).

Altogether these findings indicate that NR5A2 transcriptional activity is regulated by a multitude of mechanisms that must be carefully coordinated in a cell-context specific manner.

1.2.4 Roles of NR5A2

Given the widespread expression of NR5A2 during development and in adult tissues, it is not surprising that this orphan nuclear receptor has been assigned a variety of different functions. Indeed, NR5A2 has been implicated in a wide spectrum of biological processes, including cell proliferation, metabolism, reproduction, and development.

1.2.4.1 NR5A2 in cell proliferation and tumorigenesis

A role for NR5A2 in the regulation of cellular proliferation and tumorigenesis has recently emerged. NR5A2 was shown to cooperate with the β-catenin/ T cell factor (TCF) 4 signaling pathway to stimulate cell proliferation via activation of genes coding for factors involved in the regulation of the cell cycle, including cyclin E1, cylin D1 and c-myc, which have all been implicated in gastrointestinal cancer (Botrugno et al., 2004). This synergistic interaction between NR5A2 and β-catenin has implicated NR5A2 as a potential regulator of intestinal crypt cell renewal. Further supporting a role for NR5A2 in the regulation of cell proliferation, heterozygosity for a null mutation of *NR5A2* was shown to significantly reduce intestinal tumorigenesis in two different mouse models of intestinal cancer (Schoonjans et al., 2005). In addition, inhibition of NR5A2 expression using RNA interference in hepatocellular carcinoma cells results in cell cycle arrest through down regulation of cyclin E1 (Wang et al., 2005).

Further evidence supporting the involvement of NR5A2 in the regulation of cell proliferation and tumorigenesis, NR5A2 was shown to be expressed in breast cancer cells and tissues (Clyne et al., 2002; Clyne et al., 2004; Zhou et al., 2005; Annicotte et al., 2005). In normal breast adipose tissue, NR5A2 expression correlates with the state of pre-adipocyte differentiation. NR5A2 expression is elevated in pre-adipocytes but is progressively lost as adipocytes differentiate. These findings

suggest that NR5A2 might play a role in pre-adipocyte function or differentiation during normal breast development and tumorigenesis. 50% of breast cancers are hormone-dependent carcinomas in which estrogen plays a primary role in promoting tumor growth (Lippman et al., 1986). Interestingly, NR5A2 was shown to stimulate estrogen production in breast tumors by activating the expression of the estrogen-synthesizing enzyme CYP19 (Clyne et al., 2002; Zhou et al., 2005). In addition, NR5A2 was shown to be specifically expressed in estrogen-responsive breast cancer cell lines, where it was found to be a direct downstream target of estrogen signaling (Annicotte et al., 2005). Further evidence supporting the involvement of NR5A2 in breast cancer, inhibition of NR5A2 expression significantly decreased estrogen-dependent proliferation of breast cancer cells in culture (Annicotte et al., 2005). Collectively, these studies suggest a dual role for NR5A2 in breast carcinogenesis, acting as an upstream activator and downstream effector of estrogen signaling to promote tumor growth and cancer cell proliferation.

1.2.4.2 NR5A2 and cholesterol homeostasis

Cholesterol is central for a diversity of biological processes such as the formation of cellular membranes, as well as for steroid hormones and bile acid biosynthesis. Impairment in cholesterol homeostasis lead to the development of multiple diseases, notably atherosclerosis, indicating that proper regulation of cholesterol levels is crucial for the maintenance of

physiological homeostasis, and therefore health. Cholesterol homeostasis requires coordinated control of its biosynthesis, transport, storage and catabolism, and thus involves multiple levels of regulation. Several studies have underscored the importance of NR5A2 in the complex regulatory network of cholesterol homeostasis. In fact, NR5A2 appears to be involved in reverse cholesterol transport, cholesterol catabolism and bile acid synthesis, and bile acid enterohepatic circulation (Fayard et al., 2004 and figure 3).

In mammals, extra-hepatic or peripheral cells, including endothelial cells, are unable to metabolize cholesterol and must therefore export their excess cholesterol to the liver, where it is converted to bile acids or secreted into the bile, a process referred to as reverse cholesterol transport. The reverse cholesterol transport pathway relies on the activity of high-density lipoprotein (HDL), which is composed of cholesterol, cholesteryl ester, triglyceride, phospholipids, and alipoproteins. Then, cholesterol uptake by hepatocytes is mediated by the scavenger receptor class B type I (SR-BI) cell surface receptor. Interestingly, NR5A2 was shown to regulate the expression of two key molecules involved in the process of reverse cholesterol transport, namely SR-BI and apolipoprotein AI, which is released from the liver an intestine to promote HDL biogenesis (Schoonjans et al., 2002; Delerive et al., 2004). Confirming the involvement of NR5A2 in reverse cholesterol transport *in vivo*, *NR5A2* +/- mice display a reduced SR-BI expression (Schoonjans et al., 2002).

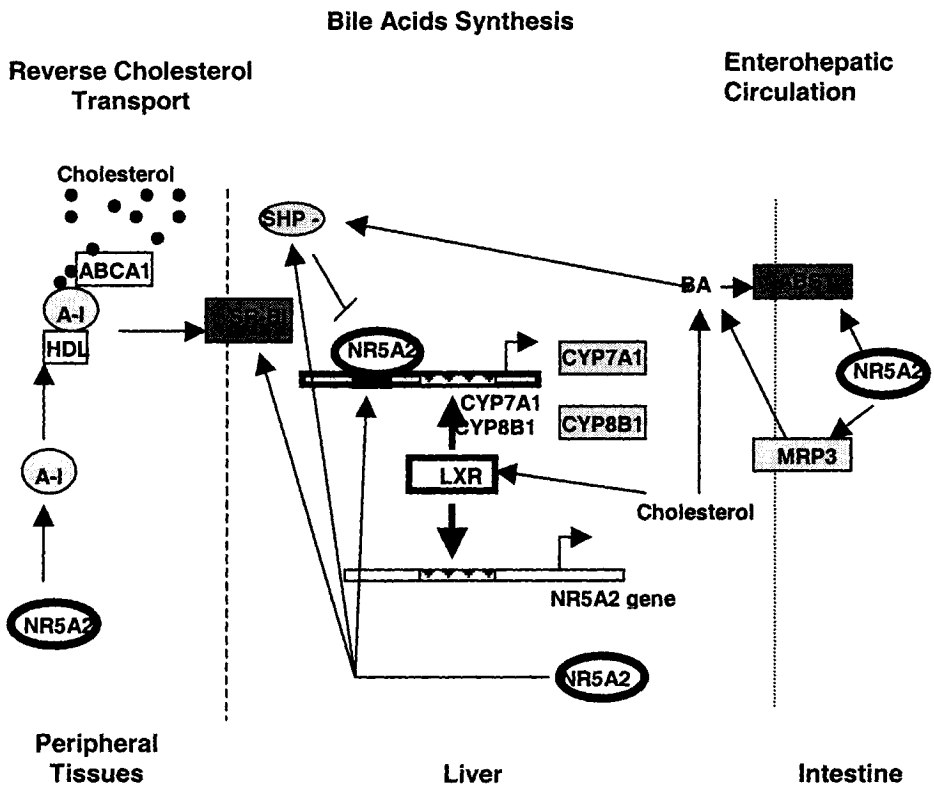
Figure 3. Model for NR5A2 function in cholesterol homeostasis

(Adapted from Fayard et al., 2004 and Paré et al., 2004)

NR5A2 is implicated at multiple levels in the regulation of cholesterol homeostasis. In reverse cholesterol transport, NR5A2 regulates the expression of alipoprotien I (AI) and SR-B1, which is involved in cholesterol cellular uptake. In the liver, NR5A2 regulate the expression of two key enzymes, CYP7A1 and CYP8B1 which are involved in bile acid synthesis. The co-repressor SHP, negatively regulate NR5A2-mediated CYP7A1 and CYP8B1 expression by physically interacting with NR5A2. NR5A2 also facilitate enterohepatic circulation by modulating the expression of two bile acid transporter: ABST and MRP3.

ABST: apical sodium-dependent bile salt transporter, CYP7A1: cholesterol 7α-hydroxylase, CYP8B1: sterol 12α-hydroxylase, HDL: high-density lipoprotein, LXR: liver X receptor, MRP3: multidrug resistance protein 3, SR-B1: scavenger receptor type B1.

Figure 3. Model for NR5A2 function in cholesterol homeostasis



Upon entry into the liver, cholesterol can be stored as cholesteryl esters and secreted directly into the bile, or can be eliminated through bile acid synthesis by a highly coordinated multienzyme process. Interestingly, in the context of high cholesterol concentrations, NR5A2 has been shown to act in concert with liver X receptor (LXR) to positively regulate the expression of two key enzymes involved in the conversion of cholesterol to bile acid, cholesterol 7α-hydroxylase (CYP7A1) and sterol 12α-hydroxylase (CYP8B1) (Nitta et al., 1999; del Castillo-Olivares and Gil., 2000; Goodwin et al., 2000; Fayard et al., 2004). Moreover, LXR was shown to promote *NR5A2* expression in response to high cholesterol levels, creating a positive metabolic feedback loop (Paré et al., 2004). Conversely, in the context of elevated bile acid levels, NR5A2 has been implicated in the repression of *CYP7A1* and *CYP8B1* expression through its interaction with SHP (del Castillo-Olivares and Gil, 2000; Goodwin et al., 2000; Fayard et al., 2004). NR5A2 therefore appears to be a central player in the regulation of cholesterol metabolism and bile acid synthesis, being involved in both positive and negative regulation of CYP7A1 and CYP8B1 expression.

In addition to its role in bile acid synthesis, NR5A2 act as a key player in bile acids recycling. Up to 95% of bile acids are reabsorbed in the intestine and return to the liver, whereas only 5% is excreted. This enterohepatic circulation of bile acids relies on the activity of various proteins that facilitate bile acid transport of which two were shown to be

regulated by NR5A2: the apical sodium-dependent bile acid transporter (ASBT) and multidrug resistance protein 3 (MRP3) (Chen et al., 2003; Bohan et al., 2003). ABST is expressed in enterocytes where it promotes intestinal re-uptake of bile acids, whereas MRP3 is present in both the liver and the intestine where it facilitates bile acid export from hepatocytes and enterocytes to the blood.

Further confirming the importance of NR5A2 in cholesterol homeostasis, *in vivo* studies have demonstrated that overexpression of NR5A2 in mice enhanced *CYP7A1* expression and cholesterol metabolism (Paré et al., 2004; Delerive et al., 2004), whereas *NR5A2* +/- mice are hypocholesterolemic due, at least in part, to an increase in *CYP7A1* and *CYP8B1* expression (Paré et al., 2004; del Castillo-Olivares et al., 2004).

Collectively, these findings implicate NR5A2 in reverse cholesterol transport, bile acids synthesis, and enterohepatic circulation and therefore clearly underscore a central role for this factor in cholesterol homeostasis.

1.2.4.3 NR5A2 in steroidogenesis and reproductive function

Until recently, NR5A1 was considered the only member of the NR5A subfamily to be present in endocrine tissues and to be involved in the regulation of steroidogenic gene expression. Recent studies, however, indicate that NR5A2 is also expressed in steroidogenic tissues. NR5A2 expression is detected in pre-adipocytes (Clyne et al., 2002), the ovary

(Boerboom et al., 2000; Schoonjans et al., 2002; Hinshelwood et al., 2003; Falender et al., 2003; Liu et al., 2003; Sirianni et al., 2002; Hinshelwood et al., 2005) and the testis (Sirianni et al., 2002, Pezzi et al., 2004, Hinshelwood et al., 2005). Interestingly, the highest level of NR5A2 expression is detected in the ovary, suggesting that NR5A2 may play an important role in this organ (Falender et al., 2003). NR5A2 was also shown to activate the expression of multiple genes involved in steroidogenesis (Sirianni et al., 2002; Clyne et al., 2002; Hinshelwood et al., 2003; Peng et al., 2003; Kim et al., 2004; Pezzi et al., 2004; Kim et al., 2005). This finding is not surprising since NR5A2 and NR5A1 bind to the same DNA element. The gonadal expression of NR5A2 and its ability to regulate the expression of genes coding for factors involved in steroidogenesis therefore suggest that NR5A2 may be involved in the regulation of ovarian function (Falender et al., 2003; Liu et al., 2003; Hinshelwood et al., 2003; Saxena et al., 2004; Yu et al., 2005).

1.3 NR5A2 AND OVARIAN FUNCTION

1.3.1 The ovary

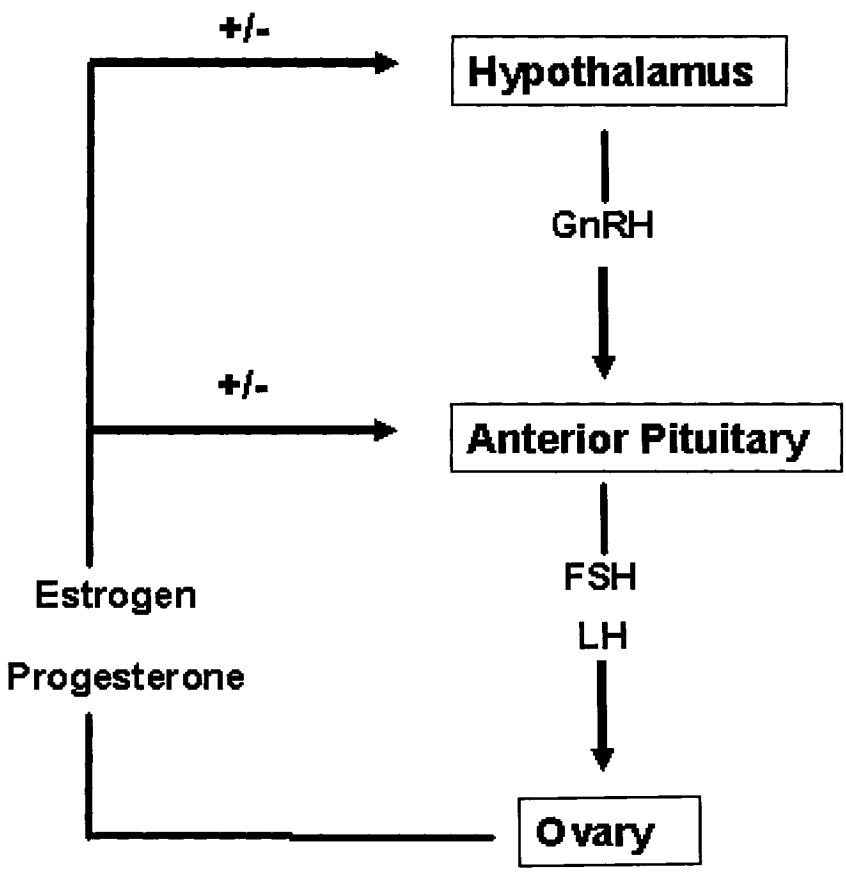
The ovary serves two distinct but interrelated physiological functions essential for female reproduction; the production and release of mature oocytes for fertilization and the synthesis of steroid hormones (McGee and Hsueh, 2000). Steroid hormones are crucial for follicular development, oocyte maturation, establishment of the estrous cycle, and

for the maintenance and function of the reproductive system (Hirshfield, 1991). Although the gametogenic potential of the ovary is established during embryonic development, its endocrine function only appears at the onset of puberty.

1.3.2 NR5A2 and the ovarian cycle

The ovarian cycle comprises two distinct phases, the follicular and luteal phases, which are differentially regulated by tropic hormones and characterized by the production of two different steroid hormones, estrogen and progesterone, respectively. During the early stages of folliculogenesis, follicular development is independent of gonadotropins, and involves mainly intraovarian factors (Kol and Adashi, 1995; Epifano and Dean, 2002; Choi and Rajkovic, 2006). At puberty, endocrine regulation of ovarian function is mediated by the hypothalamic-pituitary axis. Under the influence of hypothalamic gonadotropin releasing hormone (GnRH), pituitary gonadotropins, FSH and luteinizing hormone (LH), are secreted to regulate the progression of the ovarian cycle both directly and indirectly through their influence on steroid hormone biosynthesis. The production and release of pituitary gonadotropins are in turn modulated by cyclic ovarian events, via feedback mechanisms (Figure 4).

Figure 4. The endocrine hypothalamic-pituitary-ovarian axis



GnRH: Gonadotropin releasing hormone

FSH: Follicular stimulating hormone

LH: Luteinizing hormone

1.3.2.1 Follicular phase of the ovarian cycle

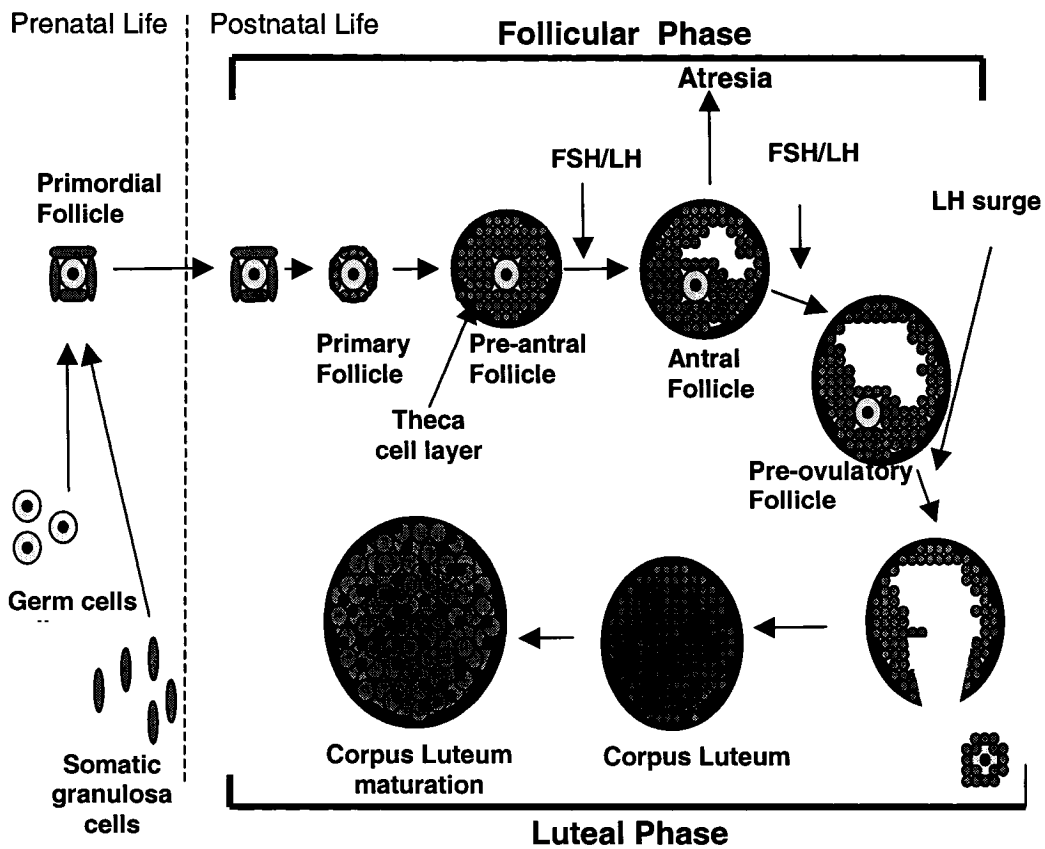
In the mouse, folliculogenesis initiates during fetal life with the formation of primordial follicles, which consist of a single oocyte surrounded by a single layer of flattened somatic granulosa cells. Although the organization of primordial follicles and initiation of follicular growth can take place in the absence of pituitary gonadotropins, small growing follicles are responsive to FSH and final development of pre-ovulatory follicles absolutely requires FSH (Greenwald and Roy, 1994). In the cycling ovary, growing follicles are composed of an oocyte surrounded by granulosa cells and an outer layer of theca cells. In the mouse, the circulating levels of FSH are modestly high at the beginning of the ovarian cycle but are then diminished during most of the follicular phase. Early follicular maturation is thought to rely on the initially elevated serum levels of FSH. FSH acts specifically on granulosa cells, the only ovarian cell type bearing the FSH receptor (FSHR), to induce their proliferation and differentiation. FSH- induced granulosa cell proliferation and differentiation promote growth of the antral follicle, which is characterized by the formation of a fluid-filled antral cavity called the antrum (Richards et al., 2001). FSH-stimulated granulosa cell differentiation is marked by an increase in the expression of CYP19, which regulates the rate-limiting step of estrogen biosynthesis that involves the conversion of androgens to estrogens. The increase in CYP19 expression leads to an augmentation of estrogen synthesis, which further promotes granulosa cell differentiation

and proliferation during follicular maturation (Richards et al., 2001). Estrogen production by the early maturing follicle leads to the feedback inhibition of pituitary gonadotropin secretion, resulting in low circulating levels of FSH and LH during most of the follicular phase. Low levels of circulating FSH are essential to potentiate granulosa cell proliferation and differentiation, whereas low levels of LH are important to stimulate androgen production from theca cells. In the growing antral follicle, estrogen, together with FSH, stimulate the expression of genes involved in granulosa cell proliferation, notably *cyclin D2*, and activate the expression of *LH receptor* (*LHR*) to promote differentiation of granulosa cells, which did not possess LHR until this point (Richards, 2001). The acquisition of LHR on granulosa cells is critical for successful transformation of the expanded antral follicles into pre-ovulatory follicles. Pre-ovulatory or Graafian follicles are the only follicles that have the potential to rupture and release an oocyte upon hormonal stimulation and their formation marks the end of the follicular phase of the ovarian cycle (Figure 5).

1.3.2.2 Luteal phase of the ovarian cycle

As antral follicles progress to the pre-ovulatory follicular stage, increasing levels of estrogen are produced and despite its initial feedback inhibition on gonadotropin release, once estrogen levels reach a peak at the late antral stage, its sustained action on the hypothalamic-pituitary axis

Figure 5. The ovarian cycle



FSH: Follicular stimulating hormone

LH: Luteinizing hormone

stimulates a surge of gonadotropin secretion. The resulting elevated levels of LH stimulate theca cells and granulosa cells to produce progesterone and induce ovulation of the pre-ovulatory follicle. Once the oocyte has been released, the residual theca and granulose cells of the post-ovulatory follicle differentiate to form a specialized structure called the corpus luteum, marking the initiation of the luteal phase of the ovarian cycle.

1.3.2.2.1 The corpus luteum

The corpus luteum (CL) is an endocrine gland that is transient in nature and whose main function is to produce high levels of progesterone which are critical for successful pregnancy (Niswender et al., 2000). In the mouse, elevated progesterone production by the CL is required throughout pregnancy to ensure its reproductive success (Niswender et al., 2000; Conneely et al., 2003). During luteal development, follicular theca and granulosa cells are transformed into small and large luteal cells, respectively, and change their repertoire of steroidogenic gene expression so that each of these cell types now mainly produce progesterone. In rodents however, low levels of estrogen are also produced by luteal cells. The small and large luteal cells of the CL coexist with other types of cells including fibroblasts, endothelial cells and cells coming from the blood stream (Bachelot and Binart, 2005). CL development is marked by extensive remodeling where follicular theca cells invade granulosa cell

layers to generate an intermixed cellular population. This cellular intermingling is accompanied by migration and proliferation of endothelial cells leading to neovascularization and elaboration of an extensive capillary network essential for providing sufficient blood supply to the CL. An important aspect of early luteal development is the rapid growth of the CL, which mainly stems from an increase in the size of large luteal cells and an increase in the number of small luteal cells. As luteal development proceeds, however, luteal cells stop proliferating and focus on progesterone production (Robker and Richards, 1998).

1.3.2.3 NR5A2 expression and regulation during the ovarian cycle

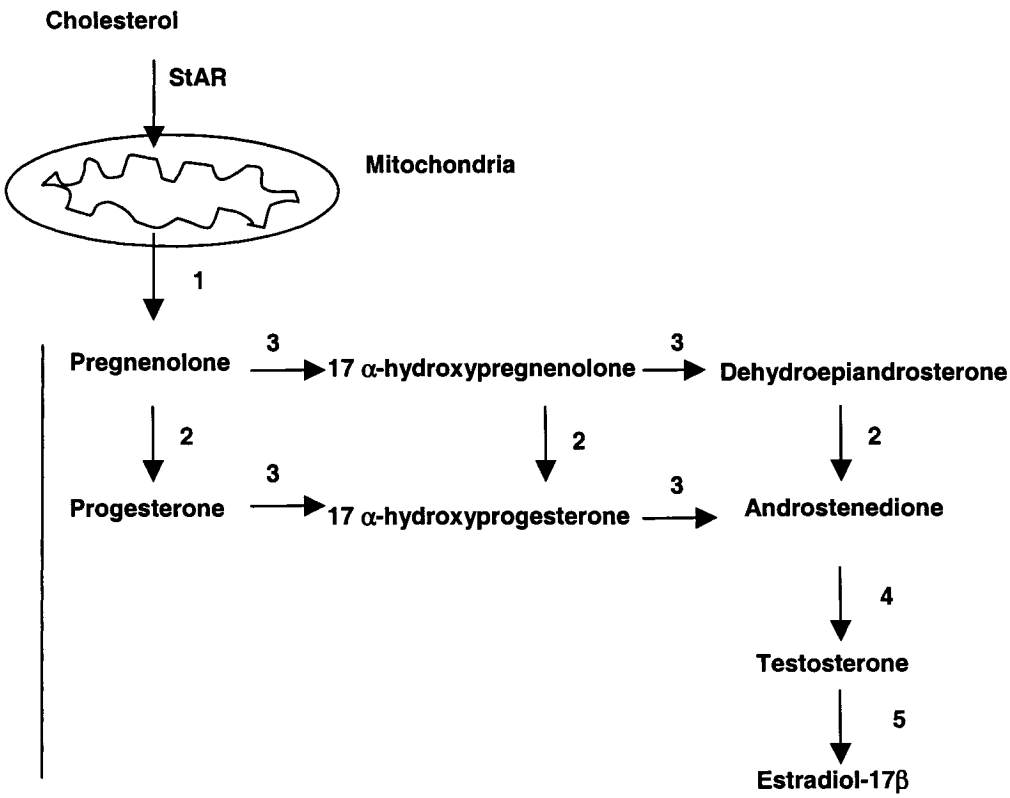
Although FSH and LH are of critical importance in coordinating the ovarian cycle, the identities of the nuclear factors transducing their effects in somatic gonadal cells still need to be clarified. One potential transcription factor that might work downstream of pituitary gonadotropin signaling to regulate the expression of genes involved in the regulation of the ovarian cycle is the nuclear receptor NR5A2. During folliculogenesis *NR5A2* is specifically expressed in granulosa cells, where its expression is positively regulated by FSH signaling (Falender et al., 2003; Liu et al., 2003; Yu et al., 2005). LH agonist-stimulated signaling was also reported to promote *NR5A2* expression in ovarian follicles *in vivo* (Liu et al., 2003). NR5A2 is not only expressed in the ovarian follicles but also in the corpus luteum of cycling and pregnant females (Falender et al., 2003;

Hinshelwood et al., 2003; Liu et al., 2003). Together, these findings suggest a potential role for NR5A2 in follicular development and/or corpus luteum formation or function.

1.3.3 Ovarian steroidogenesis

In the ovary, three main steroid hormones, namely progestins, androgens and estrogens, are synthesized in a sequential manner by theca and granulosa cells (Drummond et al., 2002). Ovarian steroidogenesis is initiated *de novo* from cholesterol, which is provided by high- or low- density lipoprotein (HDL or LDL). In the ovary, the scavenger receptor SR-B1 mediates the selective cellular uptake of cholesterol from HDL (Christenson and Devoto, 2003; Acton et al., 1996). Once in the cytoplasm of ovarian somatic cells, cholesterol must be transported to the outer mitochondrial membrane where the steroidogenic acute regulatory protein (StAR) promotes the transfer of cholesterol from the outer to the inner membrane of the mitochondria. Cholesterol transfer from the outer to the inner mitochondrial membrane constitutes one of the rate limiting steps in the ovarian steroidogenic pathway (Christenson and Devoto, 2003). Then, cholesterol is converted to progesterone, androgen, and estrogen through a complex mutlienzyme process taking place in the endoplasmic reticulum (Figure 6).

Figure 6. Biosynthetic pathway of steroid hormones



1.Cytochrome p450 side chain cleavage(CYP11A1)

2. 3β-hydroxysteroid dehydrogenase (3β-HSD)

3. Cytochrome p450 17α-hydroxylase/C17-20 lyase (CYP17)

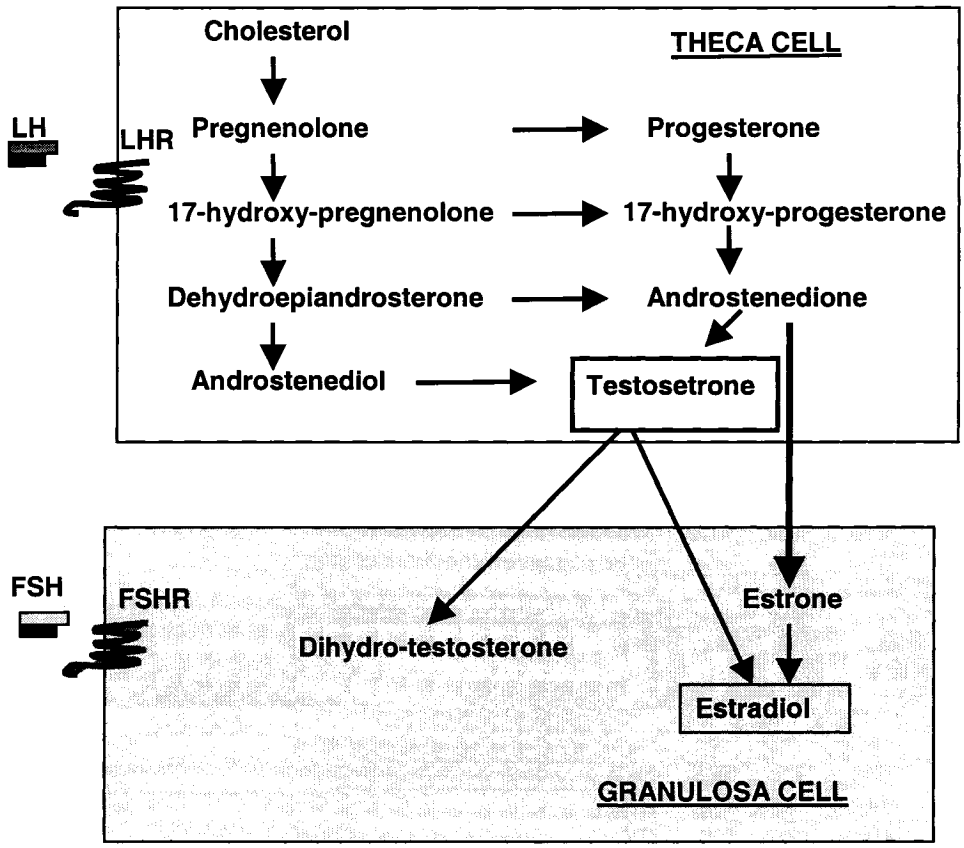
4. 17β-hydroxysteroid dehydrogenase (17β-HSD)

5. Cytochrome p450 aromatase (CYP19)

The follicular phase of the ovarian cycle is characterized by a predominance of estrogen production, which requires the concerted action of theca and granulosa cells. Currently, the process of estrogen biosynthesis during folliculogenesis is described as a two-cell, two-gonadotropin model, stressing the importance of the communication between the different ovarian compartments for successful ovarian function (Figure 7). In the growing follicle, theca cells express the enzymes required to convert cholesterol to androgens, but lack the enzyme necessary to convert androgens to estrogens (CYP19) and are responsive to LH but not to FSH. Conversely, granulosa cells produce progesterone but are unable to convert pregnenolone to androgen. Granulosa cells, however, express CYP19 and are only responsive to FSH during the initial stages of folliculogenesis. Thus, in response to LH, theca cells synthesize androgens that can be aromatized to estrogens in FSH-stimulated granulosa cells, demonstrating the importance of cell-cell interactions for proper ovarian function.

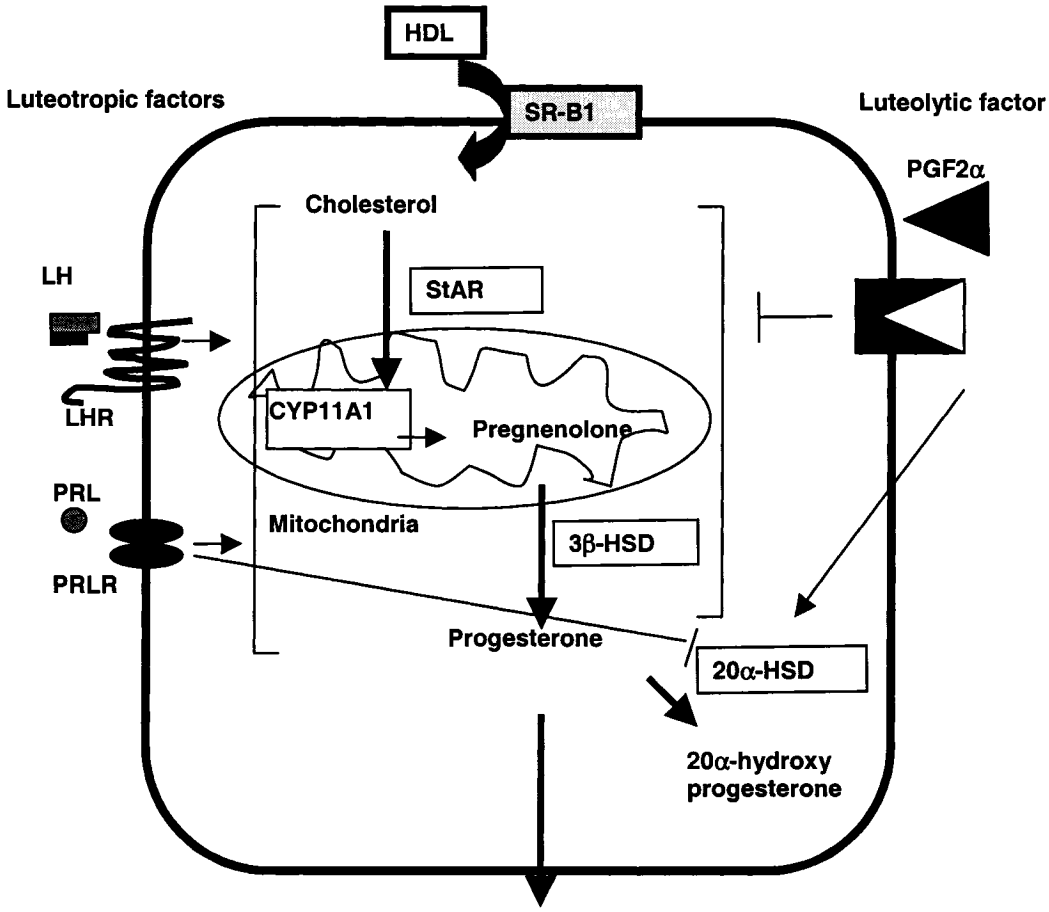
During the luteal phase of the ovarian cycle, differentiating theca and granulosa cells are characterized by a dramatic change in their repertoire of steroidogenic enzyme expression. The LH surge promotes luteinization by inducing a shift from a predominantly estrogen-producing follicle to a predominantly progesterone-producing corpus luteum. This LH-induced switch in steroids production is mediated by the concomitant

Figure 7. The two-cell, two-gonadotropin model of ovarian steroid biosynthesis



increase in the expression of progesterone-synthesizing enzymes and decrease in the expression of estrogen- and androgen- synthesizing enzymes. The resulting increase in progesterone leads to feedback inhibition of LH production to ensure a constant low level of circulating LH essential to maintain luteal functions. In the mouse, granulosa cells of the post-ovulatory follicle initiate corpus luteum formation and start producing important levels of progesterone in response to LH. In the absence of copulation, granulosa cells begin to express 20α-HSD, which inactivates progesterone, and undergo apoptosis leading to corpus luteum regression. If mating occurs, coital stimulation induces the release of prolactin (PRL) from the anterior pituitary, which represses the activity of 20α–HSD and rescues the corpus luteum, allowing sustained luteal progesterone production required for pregnancy maintenance (Freeman et al., 2000). During pregnancy, luteotropic factors promote maintenance of CL function by stimulating the expression and activity of factors involved in progesterone biosynthesis and by inhibiting the progesterone-metabolizing enzyme 20α-HSD (Niswender et al., 2000; Niswender, 2002). Corpus luteum survival, and therefore successful pregnancy, depends on the balance between luteotropic factors and luteolytic factors that induce CL demise (Figure 8). In rodents, well established luteotropic factors are LH, PRL, and estrogen, whereas prostaglandin F2α appears to be the major luteolytic factor. Proper regulation of follicular and luteal ovarian

Figure 8. Model for progesterone biosynthesis and metabolism in a generic luteal cell



steroidogenesis therefore relies on a complex interplay between a variety of hormones and signaling factors.

In addition to their critical requirement in regulating the ovarian cycle, ovarian steroids are essential for modulating the dynamic uterine changes that allow successful pregnancy to occur. In the mouse, the concerted action of estrogen and progesterone is important to coordinate endometrial capillary permeability and uterine cell proliferation and differentiation, which are associated with implantation and decidualization (Carson et al., 2000). Estrogen is known to stimulate uterine cell proliferation and promote hyperemia (Martin et al., 1973a; Quarmbyand and Korach, 1984; Kachkache et al., 1991; De et al., 1990). In contrast, progesterone induces uterine stromal cell proliferation and differentiation, which are essential for the decidualization process (De et al., 1990; Lydon et al., 1995; Lydon et al., 1996), and inhibits estrogen-mediated epithelial cell proliferation and hyperemia (Martin et al., 1973b; De et al., 1990; Lydon et al., 1995; Lydon et al., 1996). Ovarian steroid hormones are therefore essential to realize the full reproductive potential of the female, being involved in the control of the ovarian cycle and uterine function.

1.3.3.1 NR5A2 and ovarian steroidogenesis

Ovarian steroidogenesis is not only regulated by extracellular factors but also by intrinsic factors. One nuclear factor that might play a central role in the transcriptional regulation of steroidogenic genes in the

ovary is NR5A2. Indeed, NR5A2 has been shown to stimulate the expression of *StAR* and of multiple genes coding for steroidogenic enzymes including *CYP19*, *CYP11A1,* and *3β-HSD* (Kim et al., 2004; Siranni et al., 2002; Kim et al., 2005; Clyne et al., 2002; Hinshelwood et al., 2003; Kim et al., 2005; Peng et al., 2003; Saxena et al., 2004). Along with its ability to modulate the expression of genes involved in steroidogenesis and its regulation by gonadotropins, the high expression level of NR5A2 in the ovary strongly suggest that it might act as a key regulator of ovarian steroidogenesis.

1.3.4 Proposed roles for NR5A2 in the ovary

Although *in vivo* evidence for a role for NR5A2 in ovarian function is still lacking, there are currently two different theories to explain its potential function in the ovary. Since ovarian estrogen synthesis mainly relies on CYP19 and NR5A2 has been implicated in the regulation of *CYP19* expression (Hinshelwood et al., 2003; Clyne et al., 2002), it was proposed that the main function of NR5A2 was to regulate estrogen production in response to gonadotropins. Further evidence supporting this theory is the co-localization of *NR5A2* and *CYP19* in ovarian cells (Hinshelwood et al., 2003; Hinshelwood et al., 2005; Liu et al., 2003). Alternatively, based on the ability of NR5A2 to activate the expression of genes involved in progesterone production, including *StAR*, *CYP11A1* and *3β-HSD* (Sirianni et al., 2002; Peng et al., 2003; Kim *et al.,* 2004; Kim et al., 2005) and its

strong expression in the corpus luteum of cycling and pregnant females, it was proposed that NR5A2 might rather be involved in luteal function. Supporting this idea, *NR5A2* expression was shown to be positively regulated by the luteotropic factor PRL (Falender et al., 2003). Moreover, a recent study by Saxena et al. (2004) has provided evidence for a direct role for NR5A2 in progesterone biosynthesis, but not in estrogen production, during granulosa cell differentiation. Furthermore, it was demonstrated that the endogenous species binding to the *CYP19* promoter in rodent granulosa cells was NR5A1, and not NR5A2 (Falender et al., 2003), suggesting that NR5A2 might be more important for corpus luteum function.

1.4 NR5A2 AND EMBRYONIC DEVELOPMENT IN THE MOUSE

In adult animals, NR5A2 has been implicated in the maintenance of biological functions, including metabolism and reproduction. The role of NR5A2, however, is not limited to the regulation of adult physiology. NR5A2 exhibits a widespread and dynamic pattern of expression during embryogenesis, suggesting its involvement in many developmental processes. NR5A2 is expressed from the early stages of mouse embryogenesis and, as development proceeds, its expression is strongly detected in the embryonic endoderm and its derivatives. NR5A2 is also expressed in the yolk sac, neural crest cells, ossifying zones, lung

mesenchyme, developing gonads, as well as in the developing head (Rausa et al., 1999, Paré et al., 2004, Hinshelwood et al., 2005).

1.4.1 NR5A2 and early embryogenesis in the mouse

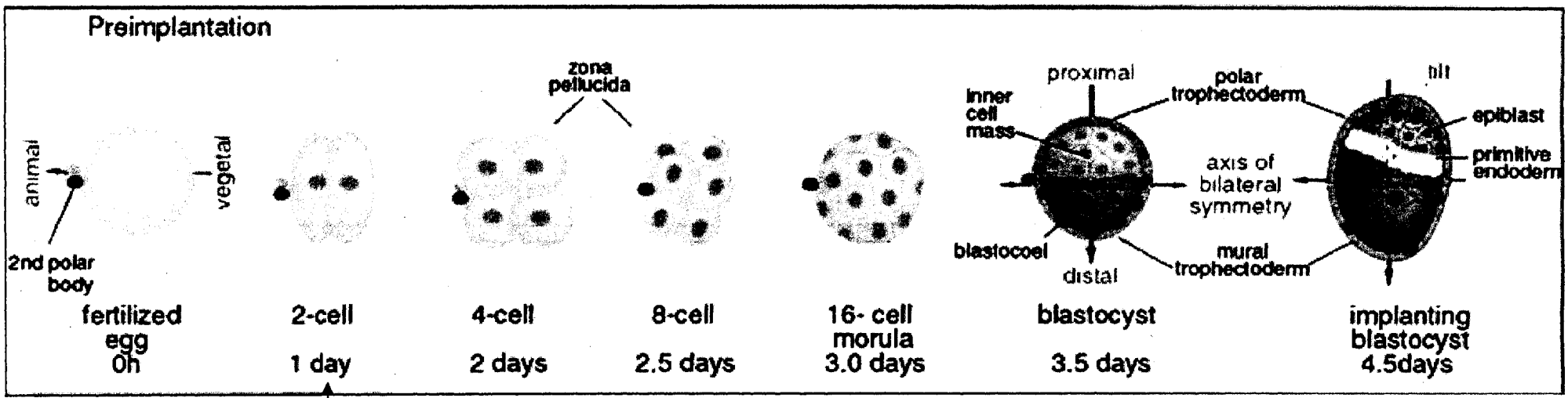
Targeted disruption of the *NR5A2* gene in the mouse results in early embryonic lethality between embryonic day (E) 6.0 and E7.5, demonstrating a requirement for NR5A2 function during early mouse embryogenesis (Paré et al., 2004; Gu et al., 2005; Schoonjans et al., 2002).

1.4.1.1 Early mouse embryogenesis

Mammalian embryogenesis is the process by which a single cell gives rise to a complete and complex organism and depends on a series of highly orchestrated events (Beddington and Robertson, 1999; Lu et al., 2001, Figures 9 and 10). In the mouse, embryogenesis begins by the fertilization of the egg by the sperm. Upon fertilization, the mouse egg, now referred to as the zygote, undergoes a series of cleavage divisions to give rise to a mass of 16 cells, referred to as the morula, three days post-fertilization. Once the embryo reaches the two-cell stage, within twenty four hours after fertilization, the zygotic genome is activated and maternal mRNAs are degraded. Cleavage leads to blastulation around 3.5 days post-coitum (dpc) when the morula undergoes cavitation to generate the blastocyst, which is composed of two mutually exclusive tissues: the

Figure 9. Pre-implantation development in the mouse

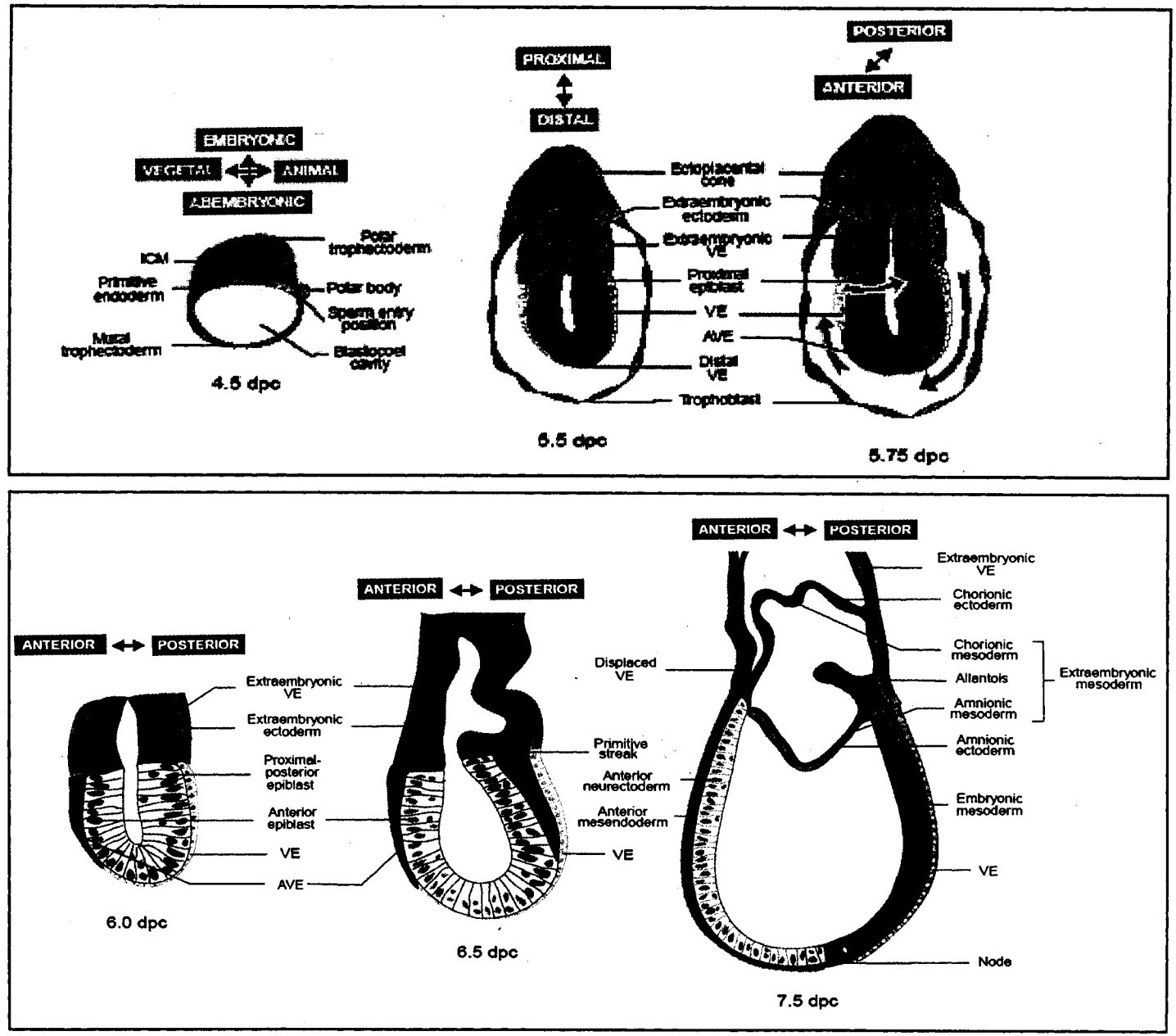
Fertilization **Implantation**



Zygotic Genome Activation

Adapted from Beddington and Robertson, 1999

Figure 10. Early post-implantation development in the mouse



Adapted from Lu et al., 2001

trophectoderm (TE) and the inner cell mass (ICM). TE cells give rise to trophoblast and extra-embryonic ectoderm that will contribute exclusively to placental tissues, whereas the ICM is fated to generate the entire fetus as well as the extra-embryonic mesoderm and endoderm. Blastocyst formation confers the initial proximal-distal polarity of the conceptus with the ICM marking the proximal end of the conceptus and the blastoceal cavity the distal end. In contrast to many animal species where embryonic patterning relies on pre-existing regionalization of morphogenetic determinants in the egg, establishment of the body plan in the mouse is not pre-determined and occurs only at later developmental stages (Gardner, 1999; Gardner, 2001; Rossant and Tam, 2004).

At the time of implantation, around 4.5 dpc, when trophectodermal cells of the blastocyst invade the uterine tissue, ICM cells in contact with the blastoceal cavity differentiate to form the primitive endoderm that will give rise to the parietal and visceral endoderms. The remaining portion of the ICM epithelializes to form the epiblast, a tissue from which the three definitive germ layers of the fetus: ectoderm, endoderm and mesoderm, are derived. TE then undergoes extensive cellular proliferation to generate the extra-embryonic ectoderm that pushes the epiblast and overlying visceral endoderm (VE) distally. Interplay between apoptotic and survival signals emanating from the VE leads to the formation of a cavity, called the proamniotic cavity, at the center of the epiblast (Coucouvanis and Martin, 1995) to give rise to a cup-shaped conceptus around 5.5dpc. At

that time, the mouse embryo has reached the egg-cylinder stage and has well delineated extra-embryonic and embryonic regions covered by a layer of VE cells, further defining the proximal-distal polarity of the conceptus with the extra-embryonic ectoderm marking the proximal end and the epiblast, the distal end. From the blastocyst stage to the egg-cylinder stage, the mouse embryo is ovaloid in shape and displays bilateral symmetry. The morphological bilateral symmetry of the mouse conceptus is conserved until the initiation of the gastrulation process.

1.4.1.1.1 Gastrulation in the mouse

In the mouse, the gastrulation process starts around 6.5 dpc and is responsible for the generation of the three definitive germ layers and for the specification of the three major body axes: anterior-posterior (A-P), dorsal-ventral (D-V), and left-right (L-R). The ultimate goal of gastrulation is to establish a basic body plan that will serve as a blueprint for subsequent embryonic morphogenesis (Tam and Behringer, 1997). It is at this developmental stage that the first morphological sign of embryonic asymmetry can be detected, when a subpopulation of cells located at the proximo-posterior region of the epiblast undergoes epithelial to mesenchymal transition to give rise to the primitive streak. The site of primitive streak formation defines the prospective posterior side of the embryo. During gastrulation, epiblast cells ingress into the primitive streak to lead to the formation of the embryonic endoderm and mesoderm,

whereas the remaining epiblast will generate the embryonic ectoderm. The ectoderm will produce the epidermis and the nervous system. The endoderm will generate the lining of the digestive tract and endoderm-derived organs including the liver, the pancreas and the lungs. The mesoderm will give rise to several organs such as the heart, the kidneys, the gonads, as well as to blood cells and connective tissues, including muscles, bones and blood vessels.

As gastrulation progresses, the primitive streak elongates distally until 7.5dpc. During this extension process, epiblast cells are recruited for ingression as the primitive extend to their vicinity. Fate map and lineage tracing studies have shown that the order of epiblast cell ingression correlate with their fate and have demonstrated that the primitive streak can be divided into three distinct functional regions (Tam and Behringer, 1997). Mesodermal cells emerging from the proximal streak region give rise to germ cells and extra-embryonic mesoderm of the yolk sac; mesodermal cells arising from the middle streak region produce paraxial, intermediate and lateral plate mesoderm of the trunk, whereas mesodermal cells exiting from the distal streak region generate cardiac mesoderm and node-derived axial mesendoderm (Lawson et al., 1991). Thus, the primitive streak not only defines the A-P axis but also the D-V axis since epiblast cells ingressing into the streak at different proximo-distal levels generate tissues of different dorso-ventral identities. The formation of the L-R axis is dependent on the prior orientation of the A-P

and D-V axes (Beddington and Robertson, 1999). Mutational analysis has implicated three major signaling pathways in primitive streak formation and morphogenesis, namely Nodal, Wnt and FGF. These pathways have been shown to genetically interact to promote primitive streak induction and subsequent mesoderm formation (Brennan et al., 2001; Liu et al., 1999; Hsieh et al., 2003; Morkel et al., 2003; Kelly et al., 2004; Ciruna and Rossant, 2001)

Following primitive streak elongation, a specialized structure called the node arises from the anterior-distal end of the streak. The node is referred to as the mouse organizer because it organizes the body plan. The node was shown to induce the formation of an ectopic body axis upon transplantation and to pattern pre-existing neural and mesodermal tissues (Lemaire and Kodjabachian, 1996). However, in contrast to other organisms where organizer transplantation induces the formation of a complete body axis, the ectopic body axis generated by node transplantation lacks anterior structures (Beddington, 1994). This finding indicates that the mouse node acts as a trunk organizer and suggests the existence of an additional signaling centre for the induction of anterior structures in the mouse. Upon differentiation, the node gives rise to the gut endoderm and to the axial mesendoderm, including the notochord and prechordal plate that act as signaling centres to pattern the neural tube along the A-P and D-V axes (Lemaire and Kodjabachian, 1996).

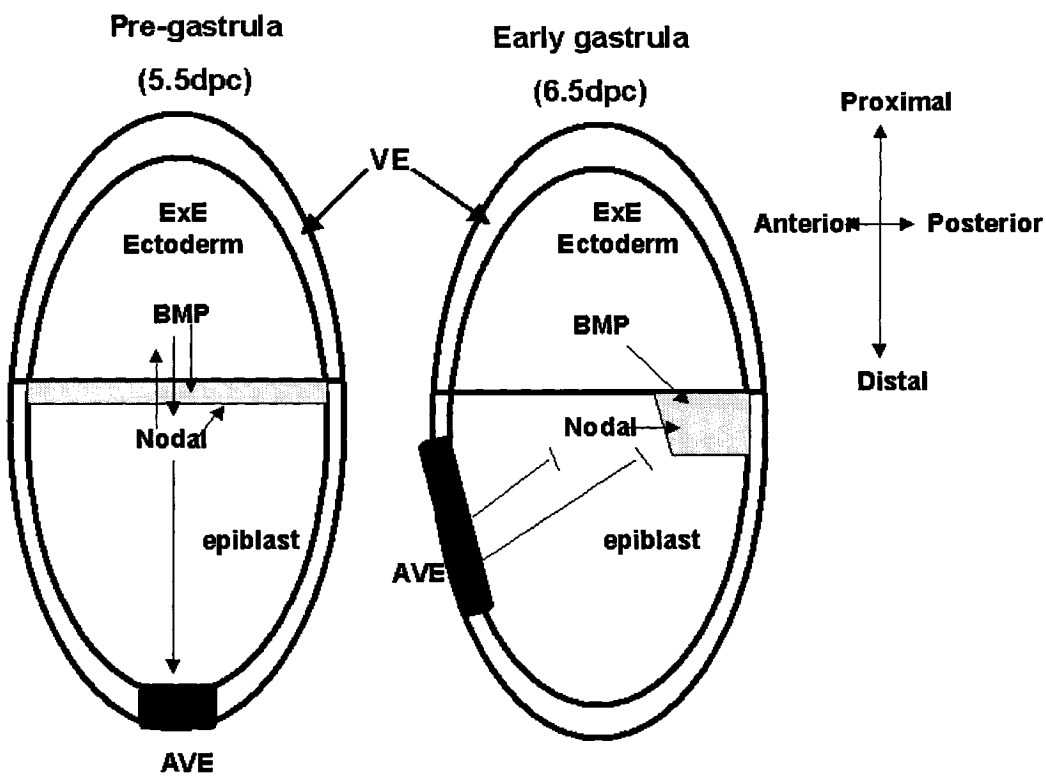
Following gastrulation, the three germ layers are established and the major body axes are laid down, allowing cell-cell interactions and morphogenetic rearrangement to occur to initiate the process of organogenesis, which is characterized by the formation and organization of tissues and organs.

1.4.1.1.2 Axis formation in the mouse

Although primitive streak induction constitutes the first overt morphological sign of A-P axis formation, molecular signs of axis specification are detected before gastrulation and rely on the integrity of two extra-embryonic tissues, the extra-embryonic ectoderm and the VE. This suggests that although extra-embryonic tissues do not contribute to the embryo proper, they play a predominant role in embryo patterning.

In the mouse, axis specification begins one day prior to the initiation of gastrulation (5.5 dpc), when the proximal–distal (P-D) axis is established (Figure 11). The current model for axis specification in the mouse indicates that the extra-embryonic ectoderm signals to the subjacent epiblast to induce the expression of proximo-posterior genes that are crucial for the establishment of the posterior identity of the embryo. At the same time, a subpopulation of specialized visceral endodermal cells located at the distal tip of the conceptus, referred to as the anterior visceral endoderm (AVE), produce inhibitory signals to counteract the activity of these proximo-posterior marker genes in the

Figure 11. Model of axis specification in the mouse



AVE: Anterior visceral endoderm

ExE: Extra-Embryonic

VE: Visceral Endoderm

underlying epiblast, promoting anterior specification. By 6.5 dpc, the P-D axis is converted to the A-P axis by migration of the AVE to the prospective anterior side of the embryo, leading to the establishment of the anterior identity in the subjacent epiblast (Srinavas et al., 2004). This anterior migration of the AVE is associated with a concomitant shift in the proximal expression of proximo-posterior genes to the posterior region of the epiblast where primitive streak formation initiates. As gastrulation proceeds, a population of anterior mesendodermal cells derived from the node that share AVE gene expression, displaces the AVE proximally and laterally. These anterior mesendodermal cells now act as a signaling centre in the epiblast to pattern the neuroectoderm (Lu et al., 2001).

Interestingly, the extra-embryonic ectoderm itself displays polarity prior to gastrulation. It was shown that *BMP4* is expressed asymmetrically in the extra-embryonic ectoderm prior to gastrulation, where it is restricted to the extra-embryonic ectoderm adjacent to the proximal epiblast (Lawson et al., 1999). In addition to its molecular proximal-distal polarity, the extra-embryonic ectoderm expression of *BMP4* was shown to be of crucial importance for inducing the expression of posterior marker genes in the underlying epiblast around 5.5 - 6.0 dpc, and hence for the establishment of the posterior identity (Lawson et al., 1999).

The T-box gene *Eomesodermin (Eomes)* is another gene specifically expressed in the extra-embryonic ectoderm prior to gastrulation that might play a role in the induction of the posterior identity

in the underlying epiblast (Russ et al., 2000; Ciruna and Rossant, 1999). During gastrulation, *Eomes* is also expressed in the primitive streak and nascent mesoderm, and loss of *Eomes* function in the epiblast demonstrated that although Eomes might play a role in the extra-embryonic ectoderm to promote posterior epiblast cell fate, it is also required in the epiblast for primitive streak and mesoderm formation (Russ et al., 2000).

At 5.5 - 6.0 dpc, the proximal epiblast is characterized by the expression of posterior marker genes including the T-box mesoderm-inducing gene *Brachyury (T)* and *Wnt3*, whose expression shifts posteriorly at the time of primitive streak induction. A targeted mutation of *Wnt3* has shown a requirement for this signaling molecule in the establishment of the posterior identity and primitive streak formation (Liu et al., 1999). Transplantation and explant culture experiments have demonstrated that the extra-embryonic ectoderm can induce distal epiblast cells, normally fated to become neuroectoderm, to differentiate into primordial germ cells and extra-embryonic mesoderm, cell types that are normally associated with the proximal epiblast (Tam and Zhou, 1996; Yoshimizu et al., 2001). Further supporting a role for the extra-embryonic ectoderm in posterior patterning, physical removal or specific loss of this tissue results in a failure in the establishment of posterior identity and primitive streak formation (Donninson et al., 2005; Rodriguez et al., 2005; Georgiades and Rossant, 2006). Collectively, these findings clearly

indicate that the extra-embryonic ectoderm fulfills an inductive function by signaling to the subjacent epiblast to promote posterior cell fates prior to gastrulation. In addition, it was demonstrated recently that the extra-embryonic ectoderm is not only involved in promoting posterior identity in the epiblast, but also in restricting AVE formation, suggesting a broader role for this tissue in patterning the embryo (Rodriguez et al., 2005; Georgiades and Rossant, 2006).

Importantly, there appears to be a reciprocal cross-talk between the epiblast and the extra-embryonic ectoderm for proper posterior patterning of the epiblast. This reciprocal interaction requires the action of the TGF-β-related signaling molecule Nodal. At 5.0 dpc, *Nodal* is expressed ubiquitously in the VE and the epiblast. During the next 24 hours, *Nodal* expression is extinguished in the VE and becomes progressively restricted to the proximo-posterior epiblast, marking the prospective site of primitive streak formation. Downregulation of *Nodal* expression in the anterior-distal epiblast is a result of AVE inhibitory actions (Lu et al. 2001). As gastrulation occurs, *Nodal* expression is abolished until node formation. Targeted mutagenesis has shown that the early expression of Nodal in the epiblast is essential to interpret signals emanating from the extra-embryonic ectoderm to induce posterior cell fate in the epiblast, and to maintain patterning of the extra-embryonic ectoderm (Brennan et al., 2001). In *Nodal* null mutants, the expression of two extra-embryonic marker genes, *BMP4* and *Eomesodermin* (*Eomes*), is not maintained and

consequently, the posterior identity is not established in the epiblast. Indeed, in *Nodal* -/- embryos the expression of the proximo-posterior genes *Wnt3* and *T*, and the posterior marker *FGF8* are absent from the epiblast, leading to a failure in axis specification and gastrulation.

As mentioned above, the VE is another extra-embryonic tissue that exhibits molecular asymmetry prior to gastrulation. The VE is characterized by the asymmetric expression of the homeobox gene *Hex* around 5.0-5.5 dpc (Thomas et al., 1998). *Hex* expression is present in a subpopulation of distal VE cells, which moves proximally to form the AVE that marks the prospective anterior side of the embryo (Thomas et al., 1998; Beddington and Robertson, 1998; Lu et al., 2001; Rossant and Tam, 2004). The AVE was shown to be required for the induction of the anterior identity in the underlying epiblast since its physical removal results in embryos lacking anterior structures (Thomas and Beddington, 1996). The AVE therefore appears to act as the head organizer in the mouse (Thomas and Beddington, 1996; Beddington and Robertson, 1998). Although the AVE is critical for the initiation of anterior development in the mouse, the axial mesendoderm derived from the node is essential to maintain and further define the anterior identity of the epiblast (Lu et al., 2001). The AVE is characterized by the specific expression of several marker genes including various transcription factors such as *Hex*, *Foxa2*, *Lim-1*, and *Otx2*, and various secreted molecules such as *Cer-l*, *Dkk-1*, and *Lefty-1* (Beddington and Robertson, 1998; Lu et al., 2001; Perea-

Gomez et al., 2001). The AVE appears to promote formation of anterior structures by protecting the anterior region of the epiblast from posterior inducing signals, and thereby preventing posterior development in the anterior epiblast (Perea-Gomez et al., 2001). Consistent with this idea, Cer-l, Dkk-1 and Lefty-1 act as secreted inhibitors of posteriorizing signals including BMPs, Wnts, and Nodal. Mutational analysis has shown that the expression of *Otx2*, *Lim-1* and *Foxa2* transcription factors in the VE is important for the induction of anterior neural characters and for the repression of posterior marker genes in the underlying epiblast (Perea-Gomez et al., 2001). Interestingly, the expression of *Cer-l* and *Lefty-1* is altered in embryos lacking *Otx2* or *Lim-1* and *Foxa2* in the VE, suggesting that these transcription factors control the expression of posterior signal antagonists to protect the subjacent epiblast from posteriorizing signals, allowing anterior development.

Although the role of the VE in anterior patterning of the epiblast is well established, much less is known about its role in posterior patterning. Recent evidence, however, supports a role for the VE in posterior patterning of the epiblast. For instance, explant culture experiments have demonstrated that the VE from early streak-stage embryos produced secreted molecules that can induce the anterior epiblast, normally destined to form neuroectoderm, to differentiate into hematopoietic and endothelial cells which normally arise from the proximo-posterior epiblast (Belaoussoff et al., 1998). The signaling molecule Indian hedgehog (Ihh),

which is expressed in the VE during the peri-gastrulation period, was shown to substitute for VE tissue to activate hematopoietic and endothelial development of isolated anterior epiblast tissue (Dyer et al., 2001). However, gene targeting studies have shown that Ihh is not essential for proximo-posterior cell fate specification, indicating that if it is involved in the posterior patterning activity of the VE, other signals can compensate for its loss during gastrulation (Byrd et al., 2002). Further evidence supporting a role for the VE in posterior patterning, the *Amnionless* (*Amn*) gene, which encodes a type I transmembrane protein exclusively expressed in the VE around the time of gastrulation, was shown to be required for the formation of middle primitive streak derivatives (Tomihara-Newberger et al., 1998; Kalantry et al., 2001). *Amn* was shown to specifically localize to the apical surface of the VE opposite to the epiblast (Kalantry et al., 2001). A recent study demonstrated that amn is important for the apical localization of the multi-ligand scavenger receptor Cubulin in the VE, suggesting that amn might be involved in endocytosis and/or transcytosis of ligands in the VE that promote proper growth and patterning of the embryo (Strope et al., 2004). Interestingly, amn contains a cysteine rich domain that can interact with BMP molecules suggesting that it might also act as a co-receptor for BMP signaling molecules (Kalantry et al., 2001). Supporting the potential involvement of BMP signaling from the VE in posterior patterning of the embryo, the type I BMP receptor, Alk2, which is also located on the apical surface of the VE, was

shown to be required in this tissue to promote proper primitive streak and mesoderm formation (Gu et al., 1999). Furthermore, BMP2, which is highly expressed in the posterior VE during gastrulation, was also shown to be important for primordial germ cell and allantois formation (Ying and Zhao, 2001).

Another signaling pathway that could potentially be involved in the posterior patterning activity of the VE is the Wnt pathway. Before the initiation of gastrulation, *Wnt3* is expressed in the proximo-posterior epiblast and associated VE and was shown to be required for primitive streak formation (Liu et al. 1999). However, whether Wnt3 is required in the VE to promote posterior specification of the epiblast is not clear. Interestingly, there is a requirement for MesD, a Wnt co-receptor chaperone, in extra-embryonic tissue for primitive streak formation (Hsieh et al., 2003). It is still unknown, however, if the gastrulation defects seen in *MesD* -/- embryos results form loss of *MesD* in the VE or in the extra-embryonic ectoderm. Although increasing evidence support the involvement of the VE in posterior patterning of the epiblast much, work remains to be done to elucidate its exact role.

The complex interplay between the epiblast and two extra-embryonic tissues; the extra-embryonic ectoderm and the VE, is of central importance for the initial patterning of the mouse embryo. The reciprocal cross-talk between these three tissues must therefore be carefully coordinated and orchestrated to establish the initial body plan that will be

used for subsequent morphogenesis of the embryo. During embryogenesis, the integrity of tissue interactions is not only important for embryonic patterning but also for embryonic growth, which mainly relies on the nutritive function of the VE.

1.4.1.1.3 The nutritive function of the visceral endoderm

During mouse embryogenesis, the VE does not only display a patterning activity but also exerts an important nutritive function. In fact, the traditional role ascribed to this extra-embryonic tissue was to mediate nutrient uptake and transport (Bielinska et al., 1999). For instance, the VE produces several proteins that facilitate endocytosis, digestion, and secretion of nutrients. Moreover, by promoting blood cell differentiation and blood vessel development in the underlying epiblast, the VE ensures the efficacy of materno-fetal nutrient and gas exchange. Interestingly, the VE displays a gene expression profile that recapitulates the endodermal program of gene expression indicating that, during early mouse embryogenesis, the VE fulfills endodermal functions including metabolism and nutrient uptake and transport. Of interest, several genes involved in hepatic development and function, including *HNF4* and *Foxa2*, are expressed in the VE during early embryogenesis.

HNF4 is a member of the nuclear receptor superfamily whose expression is first detected in the VE of 5.5 dpc embryos (Duncan et al., 1994). Later in gestation, *HNF4* is expressed in the developing liver and

gut endoderm (Duncan et al., 1994). Homozygous inactivation of *HNF4* leads to early embryonic lethality around the gastrulation period (Chen et al., 1994; Duncan et al., 1997). *HNF4* -/- embryos display excessive cell death in the epiblast, delayed primitive streak and mesoderm formation, and severe impairment in the development of all embryonic structures due to loss of *HNF4* expression in the VE (Chen et al., 1994; Duncan et al., 1997). The VE of *HNF4* -/- embryos exhibit an altered expression pattern characterized by the absence or reduction of genes involved in nutrient uptake and transport (Duncan et al., 1997). Altogether, these results indicate that the early embryonic lethality of *HNF4* -/- embryos stems from a general nutritional deficiency caused by impairment in VE function.

Foxa2 is a member of the winged-helix family of transcription factor sstrongly expressed in VE from E6.0 as well as in several epiblast-derived tissues later on during development (Ang and Rossant, 1994; Farrington et al., 1997). A targeted disruption of the gene coding for Foxa2 leads to embryonic lethality between 6.5 and 9.0 dpc, and mutant embryos are characterized by altered primitive streak morphogenesis, absence of node and notochord, and exhibit various patterning defects and impaired yolk sac development (Ang and Rossant, 1994). Although the VE forms in *Foxa2* -/- mutants, it displays a decreased expression of genes involved in nutrient transport (Farrington et al., 1997). This is consistent with the fact that Foxa2 acts as an activator of genes involved in VE nutritive function (Duncan et al., 1998). Chimeric analysis showed that *Foxa2* function is

required in the VE for proper primitive streak morphogenesis (Dufort et al., 1998). Foxa2 therefore appears to be involved in both the patterning and nutritive functions of the VE.

Endoderm-specific transcription factors of the GATA family, GATA4 and GATA6, are also expressed in the VE during early post-implantation development, GATA6 being expressed first and acting as an activator of *GATA4* and *HNF4* expression. Targeted disruption of *GATA6* leads to embryonic death between 5.5 and 7.5 dpc (Morrissey et al., 1998; Koutsourakis et al., 1999). *GATA6* -/- embryos display impaired VE development, growth retardation and extensive cell death, suggesting that GATA6 is required in the VE to provide nutritive support to the embryo. *GATA4* -/- embryos undergo gastrulation but exhibit defects in both ventral morphogenesis and heart tube formation leading to embryonic death between 8.5 and 10.5 dpc (Kuo et al., 1997, Molkentin et al., 1997). Although this finding suggests that GATA4 is not required for early VE function, *in vitro* differentiation studies have clearly established the involvement of this factor in VE formation (Soudais et al., 1995). Since *GATA6* expression is elevated in the VE of *GATA4* -/- embryos, it is likely that GATA6 can compensate for the loss of GATA4 function during early post-implantation stages (Kuo et al., 1997, Molkentin et al., 1997).

Thus, during early embryogenesis the VE is not only involved in patterning the epiblast, but also exerts an important nutritive function that promotes embryonic growth (Bielinska et al., 1999).

1.4.1.2 NR5A2 expression and function during early mouse embryogenesis

Recently, *NR5A2* was shown to be expressed early during mouse embryogenesis (Paré et al., 2004; Gu et al., 2005). *NR5A2* is ubiquitously expressed during the pre-implantation period. There are conflicting reports, however, on the expression pattern of NR5A2 during early post-implantation stages. Paré et al. (2004) have demonstrated that shortly after implantation, *NR5A2* transcripts are present exclusively in the VE, whereas Gu et al. (2005) detected ubiquitous NR5A2 expression during this developmental period. Despite this discrepancy in the expression pattern of *NR5A2* during early post-implantation development, targeted mutagenesis has clearly demonstrated a requirement for NR5A2 during early embryogenesis. Indeed, consistent with its early expression pattern, a null mutation of *NR5A2* leads to embryonic lethality around gastrulation (Paré et al., 2004; Gu et al., 2005). Gu et al. (2005) have shown that *NR5A2* -/- embryos display a reduction in the level of expression of the Pou-domain transcription factor Oct4 around the gastrulation period. Oct4 is a marker of pluripotency normally expressed in pluripotent ICM and epiblast cells. NR5A2 was therefore suggested to be important to maintain epiblast cells in an undifferentiated state (Gu et al., 2005). Alternatively, it is possible that the reduction in Oct4 expression results from cell death in the embryo undergoing resorbtion.

1.4.2 NR5A2 and development of digestive organs

NR5A2 was initially identified as an activator of the α–fetoprotein (AFP) gene, a marker of VE and hepatic specification (Galarneau et al., 1996). *NR5A2* expression was subsequently found to be regulated by endodermal transcription factors of the GATA family and shown to activate the expression of various liver-specific genes (Paré et al, 2001). During embryogenesis, the embryonic endoderm differentiates into endoderm-derived organs including the liver, pancreas, and intestine. Early embryonic endoderm determination and morphogenesis occurs shortly after gastrulation and is marked by the expression of GATA4 and GATA6 (Zaret, 1999). Inactivation of *GATA4* in the mouse was shown to block endoderm development, showing a requirement for GATA4 during endoderm determination (Kuo et al., 1997; Molkentin et al., 1997). Around 8.0-8.5 dpc, endodermal cells of the ventral foregut are induced by fibroblast growth factor (FGF) and bone morphogenetic protein (BMP) signals coming from the cardiac mesoderm to initiate the hepatic differentiation program to generate a morphologically distinguishable liver around E10.5 (Zaret, 2002). Endoderm differentiation into hepatocytes is characterized by the activation of a specific transcriptional program reflected by the expression of various hepatocyte nuclear factor (HNF) transcription factors including HNF1, Foxa2 formerly known as HNF3, and HNF4 and by the expression of the CCAAT/enhancer-binding protein (C/EBP) transcription factor (Xanthopoulos et al., 1991). During

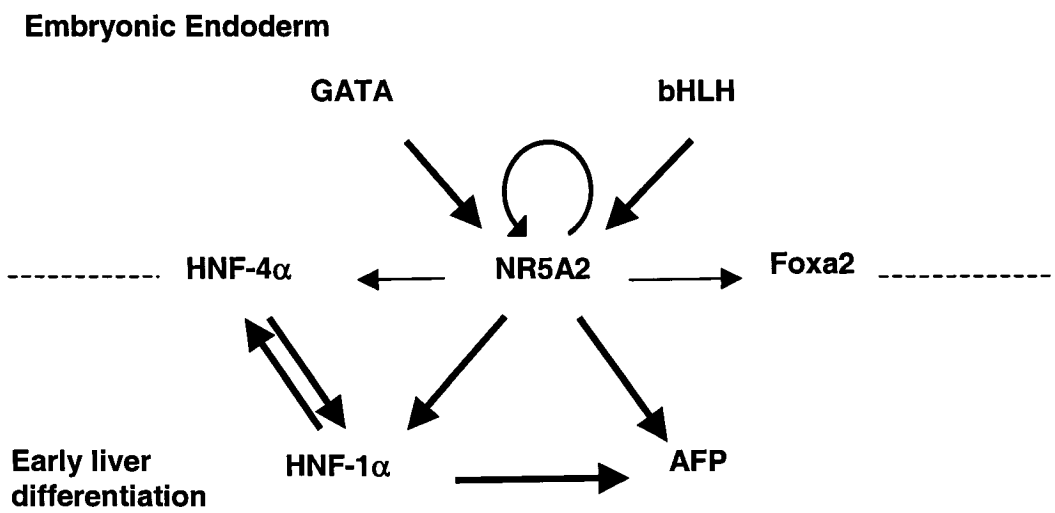
embryogenesis, *NR5A2* expression is detected in the endoderm as well as the developing liver and pancreas, and is believed to play a pivotal role in hepatic determination and development (Rausa et al., 1999; Paré et al., 2001). The position of NR5A2 in the transcriptional cascade leading to liver formation makes it an appealing candidate as a liver determination factor. *NR5A2* is activated by GATA factors and in turn, regulates the expression of HNF transcription factors and AFP, one of the earliest markers of endoderm specification to liver (Bélanger et al., 1994). NR5A2 therefore appears to act as an intermediary between endoderm differentiation and liver specification (Paré et al., 2001, figure 12).

In addition, NR5A2 was also demonstrated to be co-expressed with and activated by pancreatic-duodenal homeobox 1 (PDX-1), a transcription factor required for pancreas formation (Annicotte et al., 2003). The PDX-1-mediated activation of *NR5A2* expression during pancreas development *in vivo* suggests a broader role for NR5A2 in the development of the digestive system, where NR5A2 appears to act as a central player governing the differentiation of various endoderm-derived organs (Paré et al., 2004; Rausa et al., 1999; Annicotte et al., 2003).

Figure 12. Relationship between NR5A2 and other transcription factors involved in early hepatic differentiation

Simplified pathway showing the relationship between NR5A2 and other transcription factors involved in early hepatic differentiation. GATA factors act in concert with basic helix-loop-helix (bHLH) or homeodomain factors, that play important roles in differentiation in various cell lineages, to promote the expression of *NR5A2*, which in turn activates transcription of genes associated with early hepatic determination and liver functions. This results in the enhancement of the transcriptional cross-regulatory networks leading to the establishment of the hepatic phenotype. (Adapted from Paré et al., 2001)

Figure 12. Relationship between NR5A2 and other transcription factors involved in early hepatic differentiation



1.5 RATIONALE AND OBJECTIVES OF THIS THESIS

The nuclear receptor NR5A2 has been implicated in the regulation of multiple physiological and developmental processes in the mouse. The roles of NR5A2 in metabolism and cell proliferation are now well established. A role for NR5A2 in female reproductive function has also been suggested by gene expression and *in vitro* studies. In this thesis, we used a genetic mouse model to provide *in vivo* evidence supporting a role for NR5A2 in female ovarian function. A better characterization of the role of NR5A2 in the mouse ovary has potential therapeutic implications for female fertility since NR5A2 is also expressed in human ovaries (Sirianni et al., 2002).

Although NR5A2 is definitely essential for early mouse embryogenesis, its exact role is poorly understood. In an attempt to clarify NR5A2 function in early embryonic development, we have performed a molecular characterization of the embryonic phenotype resulting from *NR5A2* inactivation and determined the tissue requirement for NR5A2 function during the gastrulation period.

The early embryonic lethality of *NR5A2* -/- embryos precludes the identification of later developmental functions for this transcription factor. To infer potential roles of NR5A2 later during embryogenesis, we characterized its expression pattern during mid to late gestation.

Therefore, the three studies presented in this thesis will characterize the roles of NR5A2 along the reproductive-developmental

axis, which ultimately determines the efficiency of offspring production and therefore, species survival.

CHAPTER 2: MANUSCRIPT I

Mice haploinsufficient for the nuclear receptor NR5A2 are subfertile due to impaired ovarian progesterone synthesis

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PREFACE

Although the role of NR5A2 in cholesterol homeostasis and development is well established, its potential role in ovarian function remains speculative. The high level of NR5A2 expression in the ovary and its ability to regulate the expression of multiple genes involved in steroidogenesis strongly suggest that it might be implicated in female reproductive function. The objective of the following study was to decipher the potential role of NR5A2 in reproductive function *in vivo* using *NR5A2* +/- mice.

ABSTRACT

The role of the orphan nuclear receptor NR5A2 in cholesterol metabolism and embryogenesis has been well established. The expression pattern of NR5A2 in the ovary and its involvement in the regulation of steroidogenic gene expression suggest a role in female reproductive function. *In vivo* evidence for a role for NR5A2 in fertility, however, is lacking. In order to address this possibility, we used *NR5A2* +/- mice to demonstrate that heterozygosity for a null mutation of *NR5A2* leads to a decreased fertility in females. We show that the *NR5A2* +/- subfertility phenotype stems from a reduction in circulating progesterone concentrations and can be rescued by exogenous progesterone supplementation. Furthermore, we demonstrate that the *NR5A2* +/- impaired progesterone production arises, in part, through diminished ovarian StAR expression. This study therefore provides the first *in vivo* evidence for a role of NR5A2 in reproductive function and steroidogenesis.

INTRODUCTION

The orphan nuclear receptor NR5A2, also known as fetoprotein transcription factor (FTF) and liver receptor homolog-1 (LRH-1), plays an important role in embryogenesis and hepatic metabolism. A targeted mutation of the gene coding for NR5A2 in the mouse is embryonic lethal around the gastrulation period, indicating a requirement for this nuclear receptor early in development (Schoonjans et al., 2002; Falender et al., 2003; Paré et al., 2004; Gu et al., 2005). Although the early embryonic lethal phenotype of *NR5A2 -/-* mice precludes the identification of later developmental functions for this nuclear receptor, a significant body of evidence underscores a potential role for this factor during development of endoderm-derived organs. The strong expression pattern of NR5A2 in the embryonic endoderm and its derivatives, the developing liver, intestine, and pancreas, and its well established role in the transcriptional cascade leading to hepato-pancreatic specification, suggest a role for NR5A2 in endodermal differentiation during organogenesis (Rausa et al., 1999; Paré et al., 2001; Annicotte et al. 2003; Paré et al., 2004). In adult animals, *NR5A2* expression is predominant in endoderm-derived organs namely the liver, the intestine and the pancreas (Galarneau et al., 1996; Rausa et al., 1999; Fayard et al., 2004). Consistent with its enterohepatic expression pattern, NR5A2 has been shown to act as a key regulator of cholesterol homeostasis and to be involved in intestinal crypt cell proliferation *in vivo* (Paré et al., 2004, Botrugno et al. 2004; Fayard et al.,

2004). NR5A2 has been implicated in reverse cholesterol transport, bile acid synthesis and enterohepatic circulation (Fayard et al., 2004), and mice haploinsufficient for, or overexpressing NR5A2, exhibit altered cholesterol metabolism (Paré et al., 2004; Delerive et al. 2004; del Castillo-Olivares et al., 2004).

In addition to its role in metabolism and development, recent evidence points to a role for NR5A2 in fertility. *NR5A2* has been shown to be expressed in steroidogenic tissues such as pre-adipocytes (Clyne et al., 2002), the ovary (Boerboom et al., 2000; Schoonjans et al., 2002; Hinshelwood et al., 2003; Falender et al., 2003; Liu et al., 2003; Sirianni et al., 2002; Hinshelwood et al., 2005) and the testis (Sirianni et al., 2002; Pezzi et al., 2004; Hinshelwood et al., 2005). In fact, the highest level of *NR5A2* expression is detected in the ovary where its expression is restricted to the granulosa cells of developing follicles and to the luteal cells of the corpus luteum (Hinshelwood et al., 2003; Liu et al., 2003; Falender et al., 2003). In ovarian follicles, NR5A2 appears to be regulated by FSH and LH, whereas in the corpus luteum its expression is modulated by prolactin (Falender et al., 2003; Liu et al., 2003). NR5A2 has also been shown to control steroid hormone biosynthesis by regulating the expression of genes involved in steroidogenesis including steroidogenic acute regulatory protein (StAR) (Sirianni et al., 2002; Kim et al., 2004), cholesterol side-chain cleavage (CYP11A1) (Sirianni et al., 2002; Saxena et al., 2004; Kim et al., 2005), 17α- hydroxylase, 17,20 lyase (CYP17)

(Sirianni et al., 2002), 3β–hydroxysteroid dehydrogenase type II (HSD3B2) (Sirianni et al., 2002; Saxena et al., 2004; Peng et al., 2003), 11 β-hydroxylase (CYP11B1) (Sirianni et al., 2002), and P450 aromatase (CYP19) (Clyne et al., 2002). Therefore, the elevated gonadal expression of NR5A2 and its ability to regulate the expression of genes involved in steroidogenesis point to a role for NR5A2 in reproductive function. *In vivo* evidence for a role for NR5A2 in fertility, however, is lacking.

To evaluate the potential involvement of NR5A2 in reproductive function *in vivo*, we used mice heterozygous for a null mutation of the *NR5A2* gene and showed that *NR5A2* haploinsufficiency leads to a reduction in female fertility, thereby establishing, for the first time, a role for NR5A2 in female reproductive function. Furthermore, the results presented herein clearly indicate that the *NR5A2* +/- subfertility results from impaired ovarian progesterone production.

MATERIALS AND METHODS

Maintenance, mating and manipulation of mice

All animal care and experimental procedures were approved by the Animal Care Committee of the Royal Victoria Hospital and were in accordance with the regulations established by the Canadian Council on Animal Care. The generation of *NR5A2* +/- mice was described previously in Paré et al. (2004). All mice were housed in filtered-topped isolator cages under a 12 hour light-dark cycle (7h00 am-7h00 pm). Successful fertilization or mating events were assessed by the presence of a vaginal plug. Hormonal stimulation and superovulation of immature (twenty-one to twenty-four day-old) *NR5A2* +/- and wild-type MF1 females were performed by intra-peritoneal injection of 7.5 I.U. of gonadotropin from pregnant mare serum (PMSG) (Sigma, cat# G4877) alone or followed by intra-peritoneal injection of 5 I.U. of human chorionic gonadotropin (hCG, Sigma cat # CG10) 46 hours later.

To determine the frequency of pregnancy for the *NR5A2* +/- mating study, successful pregnancy was evaluated by allowing the females to go to term. To determine when the pregnancy loss takes place in *NR5A2* +/- females, we sacrificed *NR5A2* +/- females at different gestation times and found that pregnancy loss occurs prior to 6.5 days post coitum (dpc) and in 59% of females. Therefore, for the progesterone supplementation

assay, mice were sacrificed at 8.5dpc and the frequency of pregnancy was assessed by the presence of well developed deciduas and embryos.

Genotyping of mice

Immature mice (twenty-one day-old) obtained from *NR5A2* +/- crosses were genotyped using polymerase chain reaction (PCR) on tail DNA. The targeted *NR5A2* allele was amplified using oligonucleotides for either the neomycin resistant cassette or the LacZ reporter gene used to disrupt the *NR5A2* gene (Paré et al., 2004). LacZ primers sequence: LacZ forward: CAG TGG CGT CTG GCG GAA AAC CTC, LacZ reverse: GGC GGC AGT AAG GCG GTC GG, Neo primer sequences: Neo forward: GGC TAT GAC TGG GCA CAA CAG ACA ATC, Neo reverse: AGC TCT TCA GCA ATA TCA CGG GTA GC. The sequences of the oligonucleotides used to amplify the wild-type NR5A2 allele were: FTF forward: TAC AGC CTC CAA ATT TTG CC and FTF reverse: TAT CGC CAC ACA CAG GAC AT. The PCR conditions were as follows: denaturation at 94°C for 60 seconds, annealing at 58°C for 60 seconds, elongation at 72°C for 60 seconds for 30 cycles for tail DNA.

Ovarian Histological Analysis

Ovaries were fixed overnight with 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C. Fixed ovaries were then dehydrated through a graded series of ethanol, cleared in xylene (two times 15 minutes) and

embedded in paraffin. Paraffin-embedded ovaries were sectioned (8 to 10 um thick), and stained with hematoxylin and eosin.

Cell extracts

Whole cell protein extracts were prepared by homogenization of ovaries in lysis buffer (50mM Tris-Cl pH6.8, 100mM dithiothreitol, 2% SDS, 10% glycerol, and 1% protease inhibitor cocktail from Sigma, cat # P8340) followed by a brief sonication and subsequent centrifugation (14 000rpm for 5 minutes at 4°C) to collect soluble proteins. Prior to gel electrophoresis, proteins from whole cell extracts were denatured for 5 minutes at 90°C in gel loading buffer (50mM Tris-Cl pH6.8, 100mM dithiothreitol, 2% SDS, 10% glycerol, and 0.2% bromophenol blue).

Immunoblotting

Approximately 30ug of ovarian extracts were subjected to SDS-PAGE. Proteins were resolved on 11% acrylamide gels. Resolved proteins were transferred electrophoretically to PVDF membranes (Amersham Biosciences, GE Healthcare) and blocked for at least one hour at room temperature in Tris-buffered saline containing 0.1% Tween-20 (TBST) and 0.2% blocking reagent (Roche). Blots were incubated overnight at 4°C with a 1:1000 dilution of rabbit anti-StAR antibody (generously provided by Dr. Stocco, Department of Cell Biology and Biochemistry, Texas Tech University Health Sciences Center, U.S.A.) or with 1:1000 dilution of goat

anti-actin antibody (Santa Cruz Biotechnology, CA, cat # Sc1616) in blocking buffer. Subsequently, blots were washed three times (15 minutes) in TBST at room temperature after which they were incubated with a 1:2000 dilution of goat anti-rabbit antibody conjugated to horseradish peroxidase (Sigma, cat # A0545) or with a 1:4000 dilution of donkey anti-goat antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology, CA, cat # Sc2020) for one hour, washed 3 times (15 minutes) in TBST. Immunoreactive bands were revealed by chemiluminescence using the ECL plus kit (Amersham Biosciences, GE Healthcare). Blots were exposed to Kodak photographic films (Amersham Biosciences, GE Healthcare). Actin-normalized quantification of the intensity of the fluorescence of immunoreactive bands was performed by scanning the blots with a densitometer (Storm Scanner 840; Amersham Biosciences, GE Health care), and analyzing the data using the ImageQuant V5.2 software (Amersham Biosciences, GE Health Care).

Silastic Progesterone implants

Progesterone implants were prepared as described previously (Lefebvre et al., 1994). Briefly, progesterone implants consisted of 6 mm long pieces of Silastic medical grade tubing (id, 0.132 in.;od, 0.183 in. ; Dow Corning, Midland, MI) packed with crystalline progesterone (Steraloids). Empty or progesterone-packed implants were inserted subcutaneously on the third

day of gestation and mice were sacrificed and evaluated for pregnancy at 8.5 dpc.

Measurement of serum steroid concentrations

Mice were anesthetized by inhalation of 1-chloro-2,2,2-trifluoroethyl difluoromethyl ether (Baxter Corporation, Toronto, Ontario) and blood samples were collected by cardiac puncture and serum was obtained by centrifugation using microtainer serum separator tubes (Becton Dickinson) 48 hours after PMSG treatment, 24 hours after PMSG/hCG treatment and 24 or 48 hours after saline treatment, 5 days post-hCG injection for luteinized ovaries, and after 6.0 or 8.5 (implants) days of natural pregnancy. Serum concentrations of 17 β-estradiol, and progesterone in immature females following gonadotropin stimulation were initially determined in duplicates by ELISA and were performed by the Centre for Bone and Periodontal Research (McGill University, Royal Victoria Hospital, Montreal) and progesterone levels were confirmed by RIA using the service provided by Dr. Bruce D. Murphy (Centre de Recherche en Reproduction Animale, Faculté de Médecine Vétérinaire, Université de Montréal, St-Hyacinthe, Québec, Canada). Serum levels of progesterone during natural pregnancy, following normally-induced luteinization or progesterone supplementation were measured by RIA in duplicates using the service provided by Dr. Bruce D. Murphy.

Statistical Analysis

All data are presented as the mean +/- SEM. The analysis of variance for the progesterone rescue experiment in *NR5A2* +/- females was performed using the Kruskal-Wallis nonparametric test. All other statistical analyses of variance were done using the two-tailed student t-test, except for figure 5 b, where a one-tailed student t-test was performed. A p value < 0.05 was considered to be significant. Each western blot experiment was repeated at least twice. Otherwise stated, n is = or > 3.

RESULTS

NR5A2 heterozygous mice exhibit a reduction in fertility

The targeted disruption of the *NR5A2* gene in embryonic stem (ES) cells and subsequent generation of *NR5A2* +/- mice has been described previously (Paré et al., 2004). Mice heterozygous for the *NR5A2* null mutation are viable and appear morphologically normal. In an attempt to examine the potential implication of NR5A2 in reproductive function *in vivo*, we evaluated the frequency of pregnancy resulting from different crosses using *NR5A2* +/- mice. We reasoned that since *NR5A2* is expressed in both the ovary (Boerboom et al., 2000; Schoonjans et al., 2002; Hinshelwood et al., 2003; Falender et al., 2003; Liu et al., 2003; Sirianni et al., 2002; Hinshelwood et al. 2005) and the testis (Hinshelwood et al.; 2005, Pezzi et al.; 2004, Sirianni et al., 2002; Boerboom et al., 2000), NR5A2 could be involved in either female or male reproductive functions. Table 1 shows that although 100% of wild-type females mated with either wild-type or *NR5A2* +/- males led to successful pregnancies, only 56% of *NR5A2* +/- females mated with wild-type males had successful pregnancies. Some *NR5A2* +/- females that did not support pregnancy after the first breeding event were able to generate offspring on subsequent matings with wild-type males. Conversely, some *NR5A2* +/- females that had a successful pregnancy after the first mating event were not always able to sustain successful pregnancy in subsequent mating events. Collectively, these results clearly indicate that heterozygosity for a

null *NR5A2* mutation leads to a subfertility phenotype in females, providing evidence for the involvement of NR5A2 in female reproductive function.

Morphological phenotype of *NR5A2* +/- ovaries

Having demonstrated that *NR5A2* +/- females display a reduction in fertility, we next wanted to determine the underlying cause for this phenotype. The *NR5A2* ovarian expression pattern suggests that NR5A2 might play a role in either folliculogenesis or corpus luteum formation or both. To assess if defects in folliculogenesis and/or corpus luteum formation were responsible for the subfertility observed in *NR5A2* +/- mice, we performed a histological analysis of *NR5A2* +/- ovaries with or without hormonal stimulation. No obvious morphological defects were observed in *NR5A2* +/- ovaries when compared to their wild-type counterparts (figure 1). Moreover, no difference was detected between the weight of *NR5A2* +/- and wild-type ovaries in either the absence or presence of hormonal treatment (data not shown). The presence of follicles at all stages of development (figure 1c and d) and corpora lutea (figure 1e and f) suggest that folliculogenesis and corpus luteum formation occur properly in *NR5A2* +/- mice, indicating that the *NR5A2* +/- subfertility does not result from defects at the ovarian level per se, but rather from defects in pregnancy support and maintenance. To confirm the absence of defects in follicular development and function, we analyzed the ovulation process in *NR5A2* +/- females. The number of oocytes released upon gonadotropin

stimulation did not differ between wild-type and *NR5A2* +/- females (Figure 2a). In agreement with this finding, the average litter size was not significantly different between *NR5A2* +/- females that sustained pregnancy and wild-type females (figure 2 b). The similarity in litter size between wild-type females mated with wild-type and *NR5A2* +/- males is consistent with the absence of fertility problems in *NR5A2* +/- males.

Steroid hormone production in *NR5A2* +/- females

The establishment and maintenance of pregnancy rely on the integrity of ovarian steroidogenesis. Despite the absence of morphological ovarian abnormalities and ovulatory defects in *NR5A2* +/- mice, it is possible that impaired steroidogenesis underlie the subfertility seen in these females. Since NR5A2 has been implicated in the regulation of genes involved in steroidogenesis (Clyne et al., 2002, Peng et al., 2003, Kim et al., 2004, Kim et al., 2005), we examined this possibility by evaluating the serum concentrations of two steroid hormones crucial for female reproductive function, estrogen and progesterone, in response to hormonal stimulation. The levels of circulating estrogen in *NR5A2* +/- females were similar to those observed in wild-type females in the absence or presence of hormonal treatment (figure 3a). Indeed, both wild-type and heterozygous females displayed low levels of circulating estrogen in response to saline whereas estrogen levels were significantly increased upon PMSG hormonal stimulation, and decreased following

hCG treatment. This finding demonstrates that a single *NR5A2* allele is sufficient to maintain appropriate levels of ovarian estrogen synthesis.

We next evaluated the serum levels of progesterone in *NR5A2* +/- females in the absence or presence of gonadotropin stimulation. The levels of circulating progesterone were low to undetectable in *NR5A2* +/- and wild-type female in the absence of hormonal stimulation (figure 3b). Following PMSG treatment, progesterone levels increased in both *NR5A2* +/- and wild-type females. This increase, however, was significantly lower in *NR5A2* +/- mice. When PMSG stimulation was followed by hCG treatment, the levels of circulating progesterone increased and reached levels similar to those of wild-type females. Thus, *NR5A2* +/- females display an altered progesterone production in response to gonadotropin stimulation. This finding is supported by assessment of the weight of the uterus. In the absence of gonadotropin stimulation both *NR5A2* +/- and wild-type uterine weights were similar. In response to hormonal treatment, the uterine weights of *NR5A2* +/- females were significantly higher, consistent with the reduced level of circulating progesterone (figure 4). Collectively, these results indicate that haploinsufficiency for *NR5A2* leads to altered progesterone production, and more specifically identify a defect in the *NR5A2* +/- ovarian progesterone response to PMSG.

To further characterize the impairment in progesterone synthesis, the level of circulating progesterone was assessed in wild-type and *NR5A2* +/- females on the 6thday of gestation, the period when pregnancy

loss was normally observed in *NR5A2* +/- females that display decreased fertility. The average progesterone level of *NR5A2* +/- female after 6 days of gestation was significantly lower than that observed in wild-type females (figure 5a). This finding indicates that progesterone production is altered in *NR5A2* +/- females during the course of natural pregnancy.

The reduction in the level of progesterone during natural pregnancy suggested that NR5A2 might be important for luteal function and corpus luteum maturation. To test this possibility, we determined the serum level of progesterone following hormonal induction of luteinization. The level of circulating progesterone was dramatically reduced in *NR5A2* +/- females compared to their wild-type littermates during luteinization (figure 5b). Taken together, these results clearly indicate impairment in progesterone production in *NR5A2* +/- females and suggest that this altered progesterone synthesis might be responsible for the subfertility phenotype.

Ovarian expression of StAR in *NR5A2* +/- females

Although our results indicated that ovarian progesterone production was impaired in *NR5A2* +/- females, the molecular mechanism by which NR5A2 regulated progesterone synthesis *in vivo* was unclear. Since NR5A2 has been shown to modulate the expression of genes involved in progesterone synthesis, including StAR, which regulates the rate-limiting step of progesterone production (Stocco, 2001), we next determined the expression of StAR in *NR5A2* +/- ovaries. To this end, western blot

analyses were performed on various ovarian extracts. The level of StAR protein expression was similar between *NR5A2* +/- and wild-type immature ovaries in the absence or presence of gonadotropin stimulation (figure 6). *NR5A2* +/- cycling females, however, exhibit a significant decrease (approximately eight fold) in ovarian StAR expression compared to their wild-type littermates (figure 6a, lanes 7 and 8). The level of StAR protein expression was not altered in *NR5A2* +/- females on the 6^{th} day of gestation (figure 6b). Conversely, there was a dramatic and significant reduction of ovarian StAR protein expression following hormonally-induced luteinization in *NR5A2* +/- mice (approximately 6-fold) which correlated with the decrease in progesterone levels (figure 7). Collectively, these results suggest that altered ovarian StAR expression may contribute to the impairment in progesterone production observed in *NR5A2* +/- mice.

Progesterone supplementation rescues pregnancy

The decrease in progesterone production in *NR5A2* +/- females following PMSG stimulation and during natural pregnancy strongly suggested that defective progesterone synthesis might underlie the reduced reproductive ability of *NR5A2* +/- females. To further examine the potential role of altered progesterone production in the *NR5A2* +/- subfertility phenotype, we undertook a progesterone supplementation experiment. If impaired progesterone synthesis was responsible for the reduced fertility seen in *NR5A2* +/- females, we would expect that

administration of progesterone implants at the beginning of pregnancy would rescue the subfertility phenotype. Table 2 shows that administration of either an empty or progesterone implant does not interfere with normal pregnancy in wild-type female and that administration of a progesterone implant, but not of en an empty one, restores the frequency of pregnancy to the wild-type level in *NR5A2* +/- females. Moreover, the progesterone levels following administration of an empty implant were significantly lower in *NR5A2* +/- animals. Conversely, administration of a progesterone implant restored the level of circulating progesterone to wild-type levels in *NR5A2* +/- mice. Thus, the rescue of the *NR5A2* +/- subfertility phenotype by progesterone supplementation indicates that the impairment in progesterone production seen in *NR5A2* +/- females is responsible for the reduction in fertility.

DISCUSSION

In an attempt to elucidate the potential role of NR5A2 in reproductive function *in vivo*, we used *NR5A2* haploinsufficient mice and established different crosses to show that heterozygosity for *NR5A2* leads to a significant reduction in female fertility, with pregnancy loss occuring prior to 6.5 dpc. This finding, along with the well established ovarian expression pattern of NR5A2 in granulosa cells and luteal cells, suggested that NR5A2 might be involved in folliculogenesis or corpus luteum formation /function *in vivo*. We performed a morphological analysis and showed that *NR5A2* +/- ovaries do not display obvious defects in response to hormonal stimulation. The presence of corpora lutea in *NR5A2* +/- ovaries, the similar numbers of oocytes released, and similar litter sizes all demonstrated that ovulation occurred in *NR5A2* +/- females. Furthermore, the serum levels of estradiol in heterozygous females were similar to those of wild-type mice in the absence or presence of hormonal stimulation. Collectively, these results indicate that the reduction in fertility observed in *NR5A2* +/- is not a consequence of an ovarian defect per se, but rather arises from defects in pregnancy support, suggesting that NR5A2 might play a role in luteal function.

Proper corpus luteum maturation and function is central to the establishment and maintenance of pregnancy. The primary role of the corpus luteum is to produce progesterone to support pregnancy. Our results are consistent with a role for NR5A2 as a regulator of progesterone

synthesis. We demonstrated that *NR5A2* +/- displayed significantly altered progesterone levels. Interestingly, we found that progesterone production in response to hormonal stimulation was affected in a cell-specific manner in *NR5A2* +/-. Progesterone synthesis was reduced in *NR5A2* +/- female following PMSG stimulation but when PMSG stimulation was followed by hCG treatment, circulating progesterone levels were similar to those of wild-type animals. Since PMSG exerts predominantly an FSH-like activity in rodents (Murphy and Martinuk, 1991), the stimulation of steroidogenesis appears to be limited to granulosa cells which are the only FSH target cells in the ovary. HCG, on the other hand, stimulates steroidogenesis in granulosa cells and theca cells. The latter do not express *NR5A2* (Falender et al., 2003; Hinshelwood et al., 2003). This observation suggests that NR5A2 is involved in the regulation of progesterone synthesis in granulosa cells, but not in theca cells during the hormonally regulated peri-ovulation period, and that NR5A2-independent regulation of progesterone production in theca cells following hCG stimulation can compensate for the decreased progesterone production in *NR5A2* +/- ovaries. One likely factor to regulate progesterone biosynthesis in theca cells is NR5A1 which is expressed in these cells (Falender et al., 2003; Hinshelwood et al., 2003). The altered progesterone production in response to gonadotropin stimulation in *NR5A2* +/- mice is also indicated by an impaired uterine response. PMSG stimulation induced an increase in uterine weight, which results from estrogen-dependent water imbibition

and increase in cell proliferation. Progesterone is known to counteract and limit the effects of estrogens on the uterus. In *NR5A2* +/- females, however, the increase in uterine weight in response to hormonal stimulation was greater than that observed in wild-type females, which is consistent with a reduction in progesterone production.

The diminished levels of progesterone in pregnant *NR5A2* +/- animals and *NR5A2* +/- that fails to maintain pregnancy points to an important role for luteinized granulosa cells in early corpus luteum function. The corpora lutea of most mammals, including rodents, are formed by contributions from theca and granulosa cells (Murphy, 2003). The differential role, if any, of these two contributing cell populations in corpus luteum function is not clear. Granulosa lutein cell hypertrophy is an important aspect of the initial corpus luteum growth, which is followed in some species (e.g. sheep) by proliferation of theca lutein cells (Smith et al., 1999). Our results suggest that the role of granulosa lutein cells may extend beyond being only a contributing cell population to an important role in establishing corpus luteum function. Goyeneche et al. (2003) have shown that progesterone itself is involved in corpus luteum survival in the rat. The diminished capacity of the granulosa cells of *NR5A2* +/- mice in progesterone production may underlie their deficient luteal progesterone capability. Alternatively, granulosa lutein cells may provide factors involved in theca lutein function and/or survival. The factors and molecular

processes involved in luteinization of theca cells, however, are not well known.

Our results clearly show that progesterone production is dramatically reduced during luteinization and natural pregnancy in *NR5A2* +/- mice compared to their wild-type littermates and that the reduction in ovarian progesterone production observed in *NR5A2* +/- mice was responsible, most likely, for the subfertility phenotype. To pursue this possibility, we performed a progesterone supplementation experiment and showed that administration of a progesterone implant at the beginning of pregnancy rescues the fertility defects observed in *NR5A2* +/- females.

As a first step in elucidating the molecular mechanisms by which NR5A2 regulates ovarian progesterone, we evaluated the level of ovarian StAR expression in *NR5A2* +/- females. StAR regulates the rate-limiting step of progesterone synthesis and its gene was shown to be a NR5A2 target (Sirianni et al., 2002; Kim et al., 2004). We showed that the level of ovarian StAR expression in *NR5A2* +/- mice is not affected by gonadotropin stimulation but that it is significantly reduced in cycling *NR5A2* +/- females. This supports the idea that follicular development and initial corpus luteum formation is not affected in *NR5A2* +/- mice but that corpus luteum maintenance and/or function is altered. Despite the reduction in progesterone production observed in naturally pregnant females however, there was no difference in the level of ovarian StAR expression. This could be explained by the fact that during natural

pregnancy, the ovary is composed of a heterogeneous population of cells, in which StAR protein expressed by theca and interstitial cells could mask the reduction in StAR expression from granulosa cells and their derivatives, the granulosa lutein cells of the corpus luteum. Alternatively, StAR might not be the only factor involved in mediating NR5A2-induced progesterone synthesis. To further examine the potential role of StAR in mediating NR5A2 effects on luteal function, we hormonally-induced luteinization in wild-type and *NR5A2* +/- immature female to obtain a homogenous ovarian population mainly composed of established corpora lutea. Our results show that StAR expression is significantly diminished in *NR5A2* +/- luteinized ovaries, indicating that NR5A2-induced progesterone production in maturing corpora lutea is mediated, at least in part, by activation of StAR expression.

Since *NR5A2* and its closest mammalian relative *NR5A1* are both expressed in the ovary and share the same DNA consensus binding site, the respective roles of these two closely related nuclear receptors in ovarian steroidogenesis have been extensively debated. There are two major hypotheses regarding the putative role of NR5A2 in the regulation of ovarian function. First, since ovarian estrogen synthesis mainly relies on CYP19 activity and that NR5A2, as its closely related member NR5A1, has been implicated in the regulation of *CYP19* expression (Clyne et al., 2002; Hinshelwood et al., 2003; Pezzi et al., 2004), it was proposed that the main function of NR5A2 was to regulate ovarian estrogen production

in response to gonadotropins. Co-localization of *NR5A2* and *CYP19* in ovarian cells (Hinshelwood et al., 2003; Hinshelwood et al., 2005; Liu et al. 2003) also supports this hypothesis. Alternatively, based on the ability of NR5A2 to activate the expression of genes involved in progesterone production including *StAR*, *CYP11A1* and *3ß-HSD* (Sirianni et al., 2002; Peng et al., 2003; Kim et al., 2004; Kim et al., 2005) and the fact that NR5A2, but not NR5A1, displays a high level of expression in the corpus luteum, it has also been proposed that NR5A2 might play a preponderant role in progesterone synthesis (Falender at al., 2003; Liu et al., 2003; Hinshelwood et al., 2003). *NR5A2* was shown to be expressed in follicular granulosa cells, in newly formed corpora lutea as well as in mature corpora lutea throughout pregnancy. Conversely, *NR5A1* was shown to be expressed in follicular theca and granulosa cells but absent from corpora lutea of cycling and pregnant females (Liu et al. 2003, Falender et al., 2003; Hinshelwood et al., 2003). Interestingly, hypophysectomized females treated with estradiol, FSH and hCG exhibit NR5A1 expression in their corpora lutea, albeit at low levels (Falender et al., 2003). This finding suggests that while prolactin stimulates *NR5A2* expression, it might induce a concomitant inhibition of *NR5A1* expression during luteinization, indicating that NR5A1 and NR5A2 may play different roles in the ovary, in part through functional compartmentalization.

A few recent studies have brought support to the second hypothesis. For instance, Saxena et al. (2004) provided a direct role for

NR5A2 in progesterone biosynthesis but not estrogen during granulosa cell differentiation. In addition, a recent report demonstrated that NR5A1, and not NR5A2, is the endogenous species binding to the *CYP19* promoter in rodent granulosa cells, which suggests that NR5A1 is the most important factor regulating estrogen synthesis in granulosa cells (Falender et al., 2003). This finding also supports the notion that NR5A2 may play a more predominant role in luteal cells. In agreement with this view, it was shown that granulosa cell-specific knockout of *NR5A1* leads to sterility accompanying an ovarian phenotype resembling the estrogen α receptor *(ERα)* and *CYP19* knock out phenotypes (Jeyasuria et al., 2004). Thus, if NR5A2 was important for estrogen biosynthesis in granulosa cells, we would expect not to see this drastic granulosa cell-specific *NR5A1* knock out phenotype. Further supporting these recent findings, the results presented herein clearly indicate that NR5A1 and NR5A2 perform distinct functions in the ovaries *in vivo,* where NR5A1 is important for regulating theca cell steroidogenesis and estrogen production in granulosa cells, whereas NR5A2 plays a more important role in progesterone biosynthesis in differentiating granulosa cells and in corpora lutea.

In conclusion, this study provides the first *in vivo* evidence for a requirement for NR5A2 in ovarian function. More specifically, we demonstrate that heterozygosity for *NR5A2* leads to a reduction in female reproductive ability, and that this decreased fertility results from impaired progesterone production. Therefore, based on our results, we propose

that NR5A2 is a regulator of corpus luteum maturation and function and that although a single copy is sufficient to initiate its formation, loss of a second allele should lead to a more severe phenotype. If this is correct, an ovarian-targeted deletion of *NR5A2* should result in defective corpus luteum formation.

Acknowledgments

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Table 1. *NR5A2* +/- females display a reduction in fertility

Matings	Successful pregnancy	Absence of pregnancy	Percentage of successful
NR5A2 +/+ female X *NR5A2* +/+ male	17	0	100%
NR5A2 +/+ female X *NR5A2* +/- male	21	0	100%
NR5A2 +/- female x *NR5A2* +/+ male	23	18	56.1%

Table 2. Progesterone supplementation rescued the *NR5A2* +/- subfertility phenotype

Genotype	# of mice	Type of implant	% successful pregnancy	Progesterone level 8.5dpc (ng/ml)
NR5A2 +/+	4	Empty	100%	45.2
NR5A2 +/+	5	Progesterone	100%	60.1
NR5A2 +/-	8	Empty	62.5%	20.8
NR5A2 +/-	11	Progesterone	100%	58.2

Figure 1. Morphology of *NR5A2* +/- ovaries

Haematoxylin/eosin staining was performed on ovarian sections from *NR5A2* +/- (b,d,f) and wild-type ovaries (a,c,e) obtained from immature mice (21 to 24 day-old) 48 hours after saline injection (a, b), 48 hours after PMSG injection (c,d), and at 24 hours post-hCG treatment (e, f). Arrows indicate corpus luteum.

Figure 1. Morphology of *NR5A2* +/- ovaries

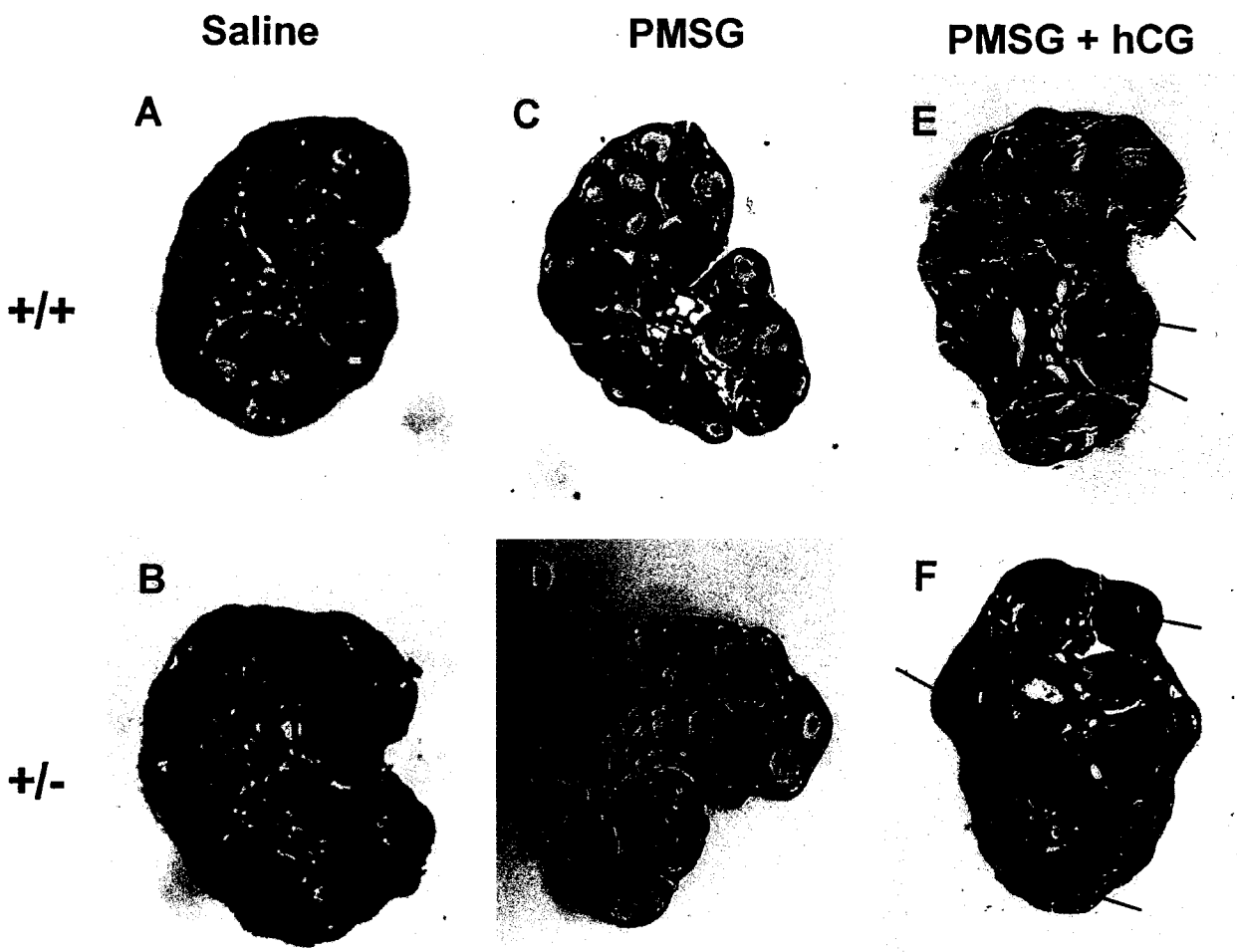


Figure 2. Normal ovulation and litter size in *NR5A2* +/- females

a. Number of oocytes recovered from the oviduct of wild-type (+/+) or *NR5A2* +/- (+/-) females upon superovulation with PMSG stimulation followed by hCG injection. b. Number of pups born per litter from various crosses as indicated.

Figure 2. Normal ovulation and litter size in *NR5A2* +/- females

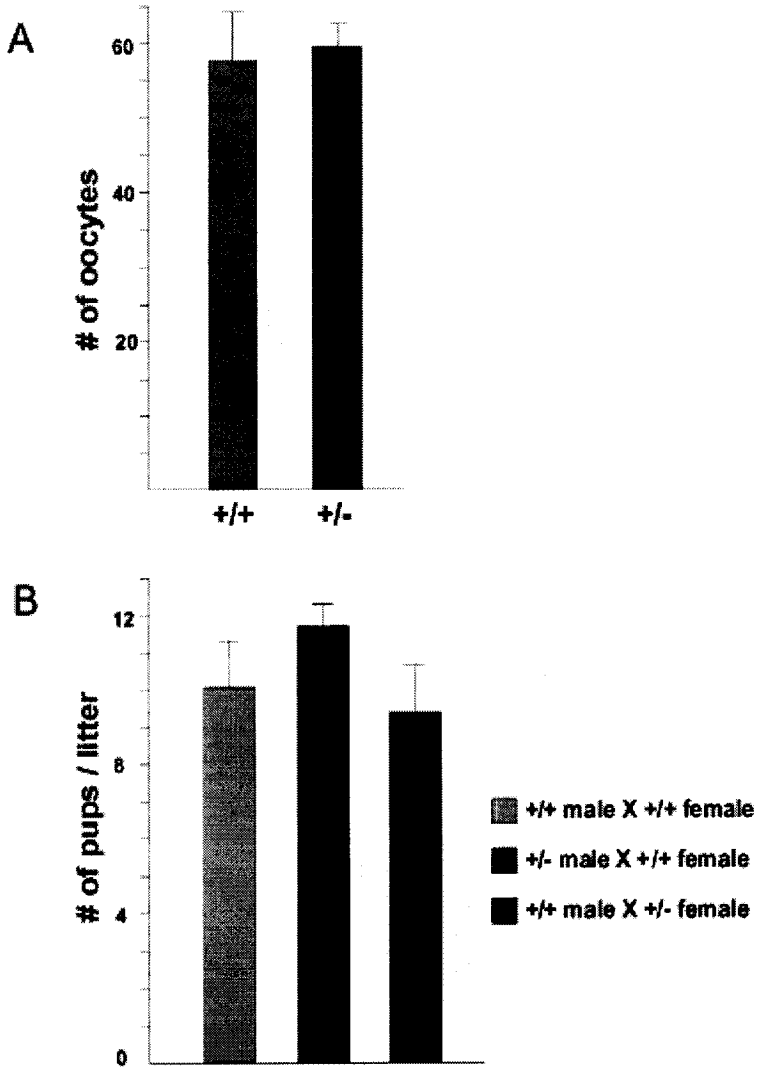


Figure 3. Steroid hormone levels in *NR5A2* +/- females in response to gonadotropin stimulation

a. Serum concentrations of estrogen in immature *NR5A2* +/- and wild-type (*NR5A2* +/+) females in absence or presence of hormonal stimulation. b. Serum levels of progesterone in immature *NR5A2* +/- and wild-type (*NR5A2* +/+) female in absence or presence of hormonal stimulation. * indicates $p<0.05$.

Figure 3. Steroid hormone levels in *NR5A2* +/- females in response to gonadotropin stimulation

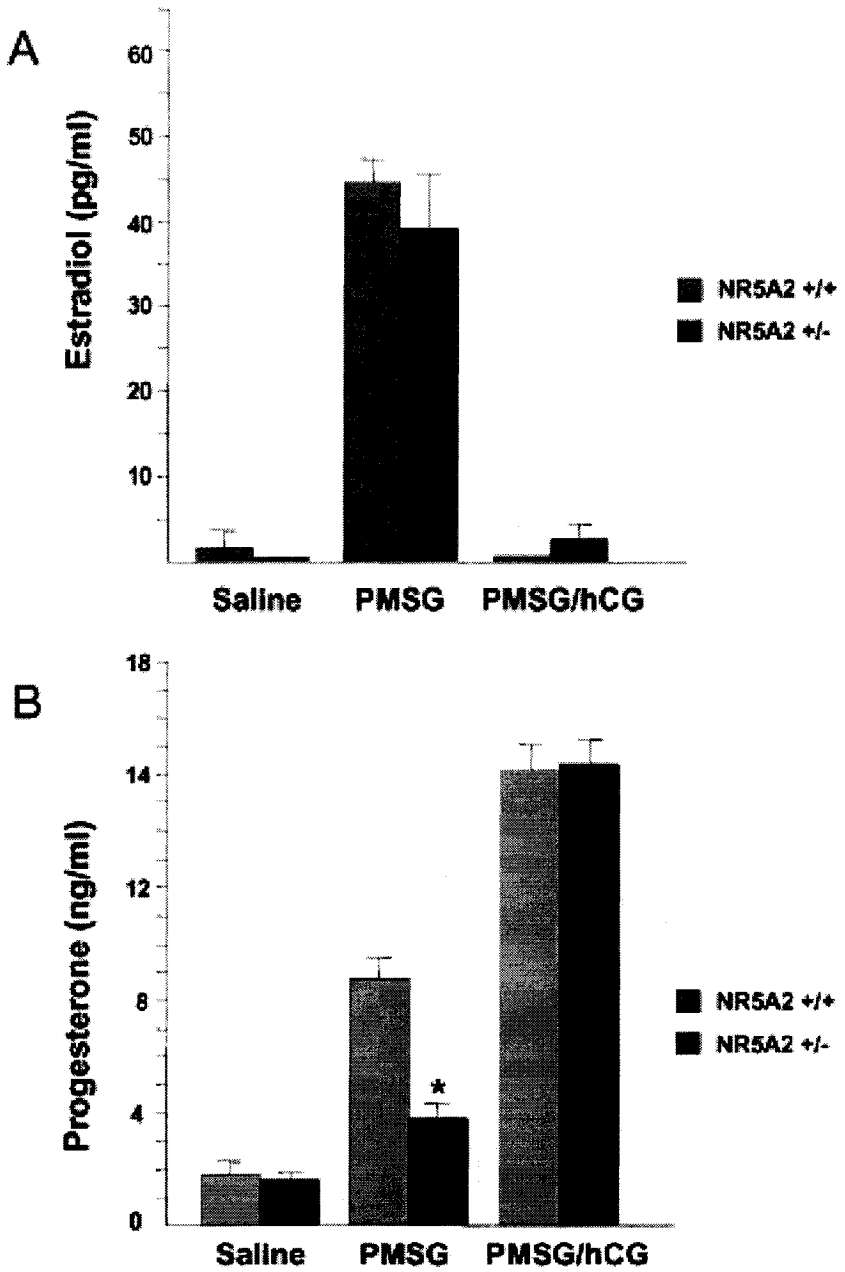


Figure 4. Uterine weight in response to hormonal stimulation

The uterine wet weight was evaluated in immature wild-type (*NR5A2* +/+) and *NR5A2* +/- immature females in absence or presence of hormonal stimulation. (Note that the wet and dry uterine weights exhibited a similar pattern, suggesting that changes in cellular proliferation, rather then water imbibition, is responsible for the uterine weight difference observed between wild-type and *NR5A2* +/- uteri, data not shown). * indicates $p<0.05$.

Figure 4. Uterine weight in response to hormonal stimulation

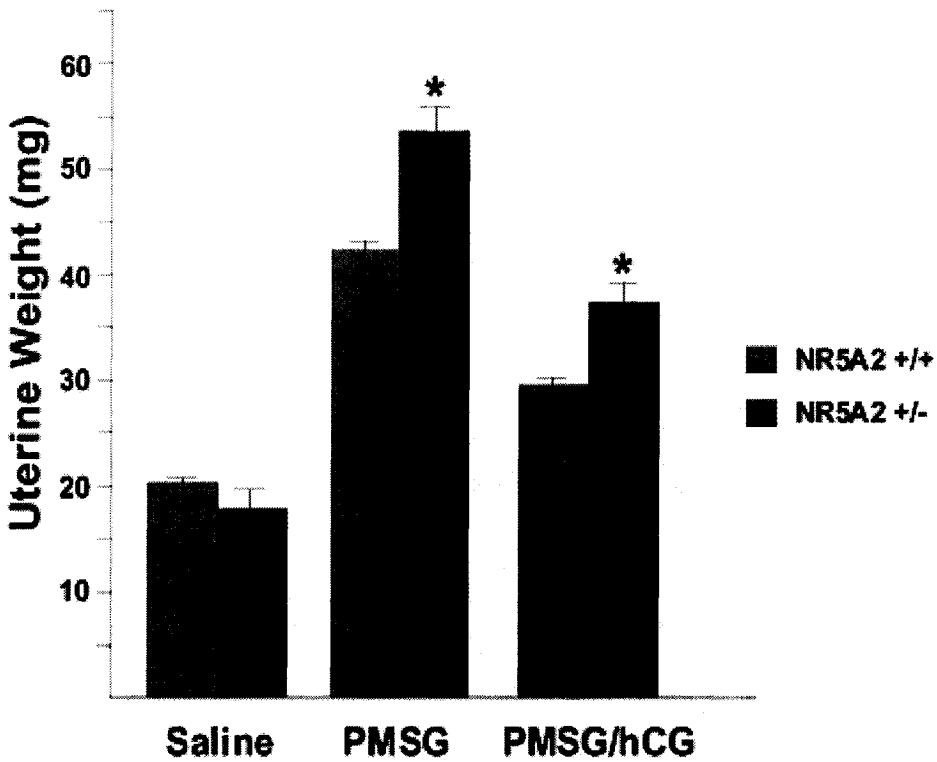


Figure 5. Decreased progesterone levels during natural pregnancy and hormone-induced luteinization in *NR5A2* +/- mice

a. Serum concentrations of progesterone in wild-type and *NR5A2* +/- females during natural pregnancy as determined by radio-immunoassay (RIA) on day 6 of gestation. b. Serum levels of progesterone in immature wild-type and *NR5A2* +/- females following hormone-induced luteinization as determined by radio-immunoassay (RIA) 5 days post-hCG injection. * indicates $p<0.05$.

Figure 5. Decreased progesterone levels during natural pregnancy and hormone-induced luteinization in *NR5A2* +/- mice

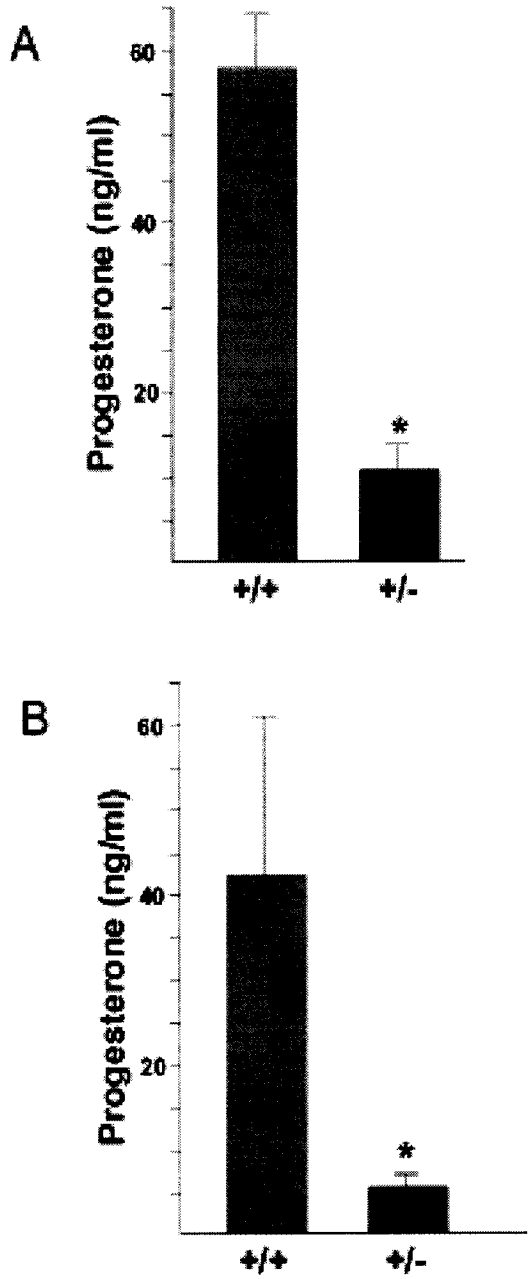


Figure 6. Ovarian StAR expression in wild-type and *NR5A2* +/- females

a. Western blot analysis for StAR protein expression performed on whole ovarian extracts from immature wild-type (+/+) and *NR5A2* +/- (+/-) females treated with either saline, PMSG, or PMSG and hCG. Each experiment was repeated twice (n=2 for each genotype of each group). The level of ovarian StAR expression was also evaluated in cycling wild-type (+/+) and *NR5A2* +/- (+/-) ovarian extracts. Extracts were obtained from 4 to 5 pooled ovaries for each genotype and was repeated 3 times with independent ovarian extracts (n=3 for each genotype). b. Western blot analysis for StAR protein expression performed on whole ovarian extracts from 6 days pregnant wild-type (+/+) and *NR5A2* +/- (+/-) females.

Figure 6. Ovarian StAR expression in wild-type and *NR5A2* +/- females

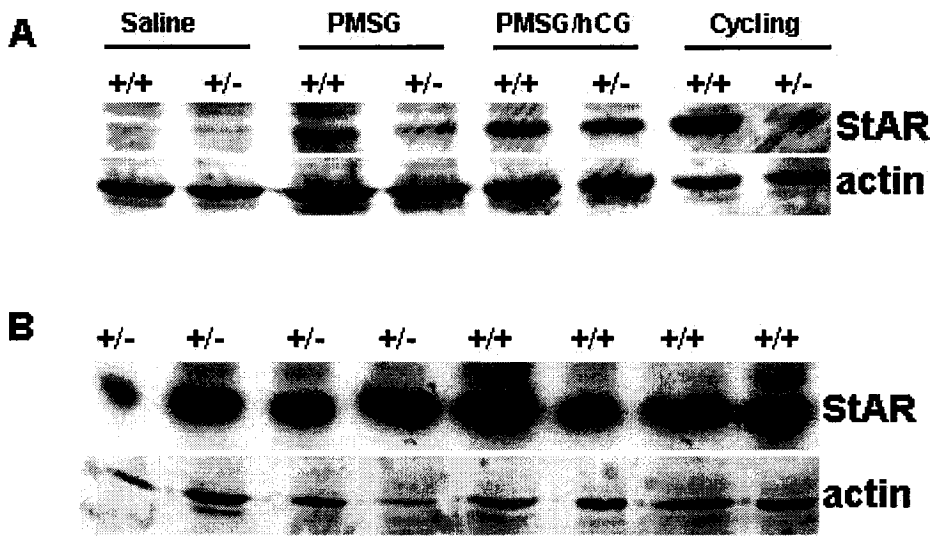
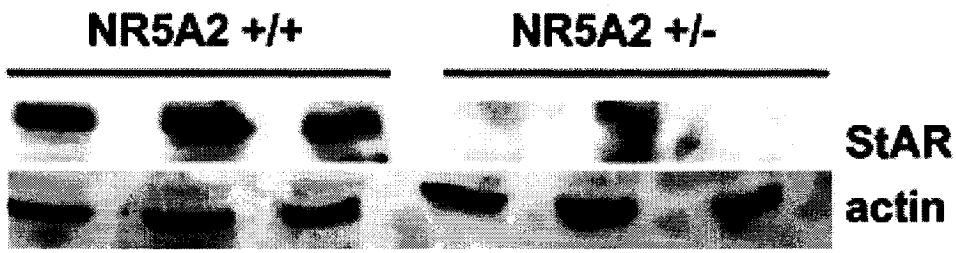


Figure 7. Ovarian StAR expression in hormonally-induced luteinized ovaries

Western blot analysis for StAR protein expression was performed on whole ovarian extracts from hormonally-induced luteinized ovaries obtained form wild-type (*NR5A2* +/+) and *NR5A2* +/- immature females 5 days post-hCG.

Figure 7. Ovarian StAR expression in hormonally-induced luteinized ovaries



CHAPTER 3: MANUSCRIPT II

The nuclear receptor NR5A2 is required for proper primitive streak morphogenesis

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Short title: NR5A2 in mesoderm and streak formation

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Key Words: NR5A2, nuclear receptor, primitive streak, mesoderm, gastrulation, visceral endoderm

PREFACE

Targeted mutagenesis has clearly established a requirement for NR5A2 function during early mouse embryogenesis. The molecular mechanisms underlying the early embryonic lethality resulting from homozygous *NR5A2* inactivation, however, remain poorly understood. In an attempt to better characterize the role of NR5A2 during early embryogenesis, we performed a molecular characterization of the *NR5A2* -/- embryonic phenotype and determined the tissue requirement for NR5A2 function during early mouse development.

ABSTRACT

NR5A2, also known as liver receptor homologue 1 (LRH-1) and fetoprotein transcription factor (FTF), is an orphan nuclear receptor involved in the regulation of cholesterol metabolism and steroidogenesis in the adult. NR5A2 was also shown to be expressed during early mouse embryogenesis. Consistent with its early expression pattern, a targeted disruption of this gene leads to embryonic lethality around the gastrulation period. To characterize the embryonic phenotype resulting from *NR5A2* loss of function, we undertook morphological and marker gene analyses and showed that *NR5A2* -/- embryos display growth retardation, epiblast disorganization, a mild embryonic-extra-embryonic constriction, as well as abnormal thickening of the proximo-posterior epiblast. We demonstrated that although initial specification of the anterior-posterior axis occurred in the absence of NR5A2, primitive streak formation was impaired and neither embryonic nor extra-embryonic mesoderm was generated. Moreover, although the visceral endoderm does not show major morphological abnormalities in *NR5A2* -/- embryos, a decrease in the expression level of *HNF4* and *GATA4* was observed. Aggregation experiments demonstrated that, in the presence of wild-type tetraploid cells, *NR5A2* mutant cells in the epiblast are capable of undergoing normal gastrulation. Therefore, our results suggest a requirement for NR5A2 in extra-embryonic tissues and identify a novel role of this gene in proper primitive streak morphogenesis.

INTRODUCTION

The orphan nuclear receptor NR5A2, also known as fetoprotein transcription factor (FTF) and liver receptor homologue-1 (LRH-1), belongs to the FTZ-F1 or NR5A subfamily of nuclear receptors. NR5A2 was first isolated as a transcriptional activator of the alpha-fetoprotein (*AFP*) gene, which encodes a factor that plays a crucial role in hepatic specification (Galarneau et al., 1996). NR5A2 has also been shown to regulate the expression of other liver-specific genes such as *Foxa2*, *HNF4*, and *HNF1α* (Paré et al., 2001; Rausa et al., 1999). In turn, the expression of *NR5A2* is controlled by early endodermal transcription factors including GATA4, GATA6 and HNF4, as well as by other developmental transcription factors from the nuclear receptor, basic helix-loop-helix and homeodomain families (Paré et al., 2001; Annicotte et al., 2003). NR5A2 therefore acts downstream of transcription factors important for endoderm specification and upstream of genes involved in hepato-pancreatic development, suggesting a central role for this nuclear receptor in the transcriptional cascade leading to endoderm differentiation and hepatic determination.

In adult mammals, *NR5A2* is predominantly expressed in endoderm-derived organs such as the liver, the pancreas, and the intestine (Galarneau et al., 1996; Rausa et al., 1999; Fayard et al., 2004). Consistent with its expression profile in the enterohepatic system, NR5A2 was shown to act as a key regulator of cholesterol and bile acid

homeostasis (Fayard et al., 2004). In addition, a role for NR5A2 as a modulator of intestinal crypt cell renewal and proliferation has recently emerged (Botrugno et al., 2004). Therefore, NR5A2 clearly appears to be a crucial factor that promotes proper development and function of the enterohepatic system (Botrugno et al., 2004; Paré et al., 2004). In the adult, *NR5A2* is not only expressed in tissues of endodermal origins but also in steroidogenic tissues, such as pre-adipocytes (Clyne et al., 2002), the ovary (Boerboom et al., 2000; Falender et al., 2003; Hinshelwood et al., 2003; Liu et al., 2003; Schoonjans et al., 2002; Sirianni et al., 2002), and the testis (Sirianni et al., 2002, Pezzi et al., 2004). High gonadal expression levels of *NR5A2* and its ability to regulate the expression of genes involved in steroidogenesis point to a role for NR5A2 in reproductive functions.

Recently, *NR5A2* was shown to be expressed early during mouse embryogenesis (Paré et al., 2004; Gu et al., 2005). Indeed, *NR5A2* is ubiquitously expressed during pre-implantation stages of development. Shortly after implantation, *NR5A2* transcripts are detected in the visceral endoderm (VE), an extra-embryonic tissue of crucial importance for proper growth and patterning of the embryo (Paré et al., 2004; Bielinska et al., 1999). Consistent with the early expression pattern of *NR5A2*, a targeted mutation of this nuclear receptor leads to embryonic lethality around the gastrulation period (Paré et al., 2001; Paré et al., 2004; Falender et al., 2003; Gu et al., 2005).

Although targeted mutagenesis has shown that NR5A2 is essential for early embryogenesis in the mouse, its exact role is poorly understood. To better characterize the role of this nuclear receptor during embryonic development, we performed morphological and extensive marker gene analyses and showed that although the initial specification of the A-P axis occurs in *NR5A2* -/- embryos, primitive streak morphogenesis and mesoderm formation are impaired. Moreover, using a chimeric approach, we demonstrated that in the presence of wild-type cells in the extra-embryonic tissues, *NR5A2* -/- cells in the epiblast have the ability to undergo gastrulation and form mesoderm, suggesting a requirement for NR5A2 in the extra-embryonic lineage for gastrulation to proceed.

MATERIALS AND METHODS

Maintenance, mating, and manipulation of mice

The generation of *NR5A2* +/- mice was described previously in Paré et al. (2004). All mice were housed in filtered-topped isolator cages under a 12 hour light-dark cycle (7h00 am-7h00 pm). For staging of embryos, the day of fertilization, assessed by the presence of a vaginal plug, was considered to be 0.5 day post-coitum (dpc). Superovulation of *NR5A2* +/- and wild-type CD-1 females was done by intra-peritoneal injection of 7.5 I.U. of gonadotropin from pregnant mare serum (PMSG-Sigma cat# G-4877) followed by intra-peritoneal injection of 5 I.U. of human chorionic gonadotropin (hCG, Sigma cat# CG-10) 46 hours later. Superovulated *NR5A2* +/- and wild-type CD-1 females were mated with fertile *NR5A2* +/- or wild-type CD-1 males, respectively, and 2 cell stage (CD-1 matings) and morulae stage (*NR5A2* +/- mating) embryos were then collected for aggregation experiments. Pseudopregnant females used for chimeric embryo transfer were obtained by mating wild-type CD-1 females with vasectomized males. Embryo transfer was performed with 2.5 dpc pseudopregnant females.

Genotyping of mice and embryos

21 day-old mice obtained from *NR5A2* +/- crosses were genotyped using polymerase chain reaction (PCR) on tail DNA. Genotyping of embryos obtained from *NR5A2* +/- crosses was done by PCR and by morphological

analysis. The targeted *NR5A2* allele was amplified using oligonucleotides for either the neomycin (Neo) resistance cassette or the LacZ reporter gene used to disrupt the *NR5A2* gene (Paré et al., 2004). LacZ primers sequence: LacZ forward: CAG TGG CGT CTG GCG GAA AAC CTC, LacZ reverse: GGC GGC AGT AAG GCG GTC GG, Neo primer sequences: Neo forward: GGC TAT GAC TGG GCA CAA CAG ACA ATC, Neo reverse: AGC TCT TCA GCA ATA TCA CGG GTA GC. The sequences of the oligonucleotides used to amplify the wild-type *NR5A2* allele were: FTF forward: TAC AGC CTC CAA ATT TTG CC and FTF reverse: TAT CGC CAC ACA CAG GAC AT. The PCR conditions were as follows: denaturation at 94°C for 60 seconds, annealing at 58°C for 60 seconds, elongation at 72°C for 60 seconds for 30 cycles for tail DNA and for 35 cycles for embryos.

Histology Analysis

Deciduas from *NR5A2* +/- matings were dissected between 6.0 and 7.5 dpc and were fixed overnight with 4% paraformaldehyde in PBS at 4°C. Fixed deciduas were then dehydrated through a graded series of ethanol, incubated twice in xylenes and embedded in paraffin. Paraffin-embedded deciduas were then subjected to serial transverse or saggital histological sectioning (7um) and stained with nuclear fast red solution (Sigma- cat# N-3020).

In situ hybridizations

For whole mount *in situ* hybridizations, embryos were processed as described in Henrique et al. (1995). Briefly, embryos were rehydrated in PTW (0.1% Tween 20 in 1 X PBS pH 7.3) and digested in proteinase K (10 ug/ml proteinase K in PTW) for 6 minutes (6.0- 6.5 dpc embryos) or 7 minutes (7.0- 7.5 dpc embryos) at room temperature. Embryos were washed twice in PTW at room temperature and post-fixed in 4% paraformaldehyde with 0.1% glutaraldehyde in PBS. Embryos were washed and hybridized overnight at 70°C with approximately 200 ng/ml of digoxigenin-labeled riboprobe in hybridization solution (50% formamide, 1.3 X SSC, 5 mM EDTA, 50 ug/ml yeast RNA, 0.2% Tween-20, 0.5% CHAPS, and 100 ug/ml heparin). The embryos were rinsed three times at 70°C with fresh hybridization solution, and washed twice for 30 minutes at 70°C with fresh hybridization solution. Embryos were then washed once in hybridization mix : MABT (0.1% Tween-20 in MAB) for 20 minutes at 70°C and four times for one hour at room temperature in fresh MABT. Embryos were incubated in 2% Boehringer blocking reagent (BBR) in MABT for 1 hour at room temperature, in 2% BBR, 10% heat inactivated sheep serum in MABT at room temperature for 2 hours and with anti-digoxigenin alkaline phosphatase antibody in 2% BBR, 10% heat inactivated sheep serum in MABT (1 in 2000 dilution) overnight at 4°C. Embryos were rinsed three times with MABT, washed 4 times for 1 hour in MABT at room temperature, and twice in NTMT buffer (100 mM NaCl, 100 mM Tris-HCL

pH9.5, 50 mM MgCl2, and 1% Tween-20). Embryos were stained in NBT and BCIP in NTMT overnight at room temperature in dark conditions.

Plasmids used for DIG-labeled RNA probes used in this study were obtained from: *GATA4* (Mona Nemer, University of Montreal, Quebec, Canada), *Cerberus-like* (Eddy M. De Robertis, Howard Hughes Medical Institute, University of California, Los Angeles, USA), *mSnail*, *mEomes* and *BMP4* (Janet Rossant, Samuel Lunenfeld Research Institute Toronto, Ontario, Canada), *Otx2* (Ang et al., 1996), Brachyury (Herrmann, 1991), *Fgf8* (Crossley and Martin, 1995), *Wnt3* (Elizabeth J. Robertson, Department of Molecular and Cellular Biology, Harvard University, Cambridge, Boston, USA), *Lim-1* (Richard Behringer, University of Texas M. D. Anderson Cancer Center, Houston, Texas,USA). The *Dkk1*, *Nodal* and *HNF4* DNA fragments used to generate probes were amplified by PCR and cloned in pKS.

Generation of chimeric embryos

To determine the tissue requirement for NR5A2 for gastrulation, a tetraploid aggregation approach was used. To assess the role of NR5A2 in extra-embryonic tissues, we generated chimeric mouse embryos composed of wild-type extra-embryonic tissue and *NR5A2* mutant epiblast as previously described in Dufort et al. 1998, with slight modifications. Briefly, wild-type tetraploid CD1 embryos were generated by electrofusion

of 2 cell stage embryos and allowed to develop *in vitro* until they reached the morulae stage. They were then aggregated with morulae stage embryos obtained from *NR5A2* heterozygote matings. Aggregated chimeric embryos were then cultured *in vitro* until they reached the blastocyst stage and then transferred in the uterus of pseudopregnant females. Transferred tetraploid embryos aggregation chimeras were dissected around 9.0 dpc. PCR analysis was performed to genotype the embryonic portion of the chimeric embryos using PCR primers described above.

Real time quantitative RT- PCR

Isolation and purification of total RNA were accomplished using RNeasy Micro Kits (Qiagen). For reverse transcription-PCR (RT-PCR), total RNA was reverse-transcribed using Superscript II RNase H RT (Invitrogen) with oligo-dT. Real Time RT-PCR was used to quantify the relative levels of *GATA4*, *HNF4* and *GAPDH*. Real Time RT-PCR was performed in duplicate using 1% of the RNA obtained by reverse transcription in a 10 µl reaction volume of Platinum SYBR Green qPCR superMix UDG (Invitrogen) on the Light Cyclr 1.2 (Roche Applied Science). The relative expression was analyzed using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). GAPDH was used as a reference control. Primer sequences were designed as follows: *GATA4* forward primer TCCATGTCCCAGACATT; *GATA4* reverse primer GTGACAGGAGATGCATAG; *HNF4* forward

primer: CTG GAG GAT TAC ATC AAC G, *HNF4* reverse primer: ATG AAC TGG ATC TGT TCG, *GAPDH* forward primer: ACC CAG AAG ACT GTG GAT GG, *GAPDH* reverse primer: CAC ATT GGG GGT AGG AAC AC.

RESULTS

Morphological phenotypes of *NR5A2* -/- embryos.

Although *NR5A2* heterozygous mice are viable, homozygous disruption of the *NR5A2* gene leads to early embryonic lethality around the gastrulation period, between embryonic day (E) 6.0 and E7.5 (Paré et al., 2004; Gu et al., 2005). *NR5A2* homozygous mutant embryos appear morphologically normal up to E6.0. Thereafter, *NR5A2* mutant embryos begin to show various degrees of growth retardation, a mild constriction at the extra-embryonic-embryonic junction, and a disorganization of the epiblast compared with their wild-type and heterozygous littermates (figure 1 a and Paré et al., 2004). Furthermore, an abnormal thickening in the proximal-posterior region of the epiblast was also present in approximately 25% of *NR5A2* -/- embryos that reached the early gastrula stage (figure 1b). Histological analysis also demonstrated that a morphologically discernible primitive streak and mesodermal wings were absent in *NR5A2* -/- embryos compared to their wild-type or heterozygous littermates (figure1 c-f). In addition, the chorion of *NR5A2* -/- embryos that reached 7.5dpc appeared distorted compared to their wild-type and heterozygous littermates (figure 1 g and h). Moreover, although the amnion of wild-type embryo normally contains both ectodermal cells and extra-embryonic mesodermal cells derived from the epiblast at this stage, this structure is composed of only ectodermal cells in *NR5A2* -/- embryos (figure 1 g and h). Although some cells located in the posterior extra-embryonic region of

7.5 dpc mutant embryo exhibit a mesenchymal morphology, they were negative for the expression of the extra-embryonic mesoderm marker gene *flk-1* (figure 1 h and data not shown).

The absence of a morphologically distinguishable primitive streak was further confirmed by examining the expression of *Brachyury* (*T*), a molecular marker of primitive streak formation. In wild-type E7.0 embryos, *T* was expressed throughout the streak, whereas in *NR5A2* -/- embryos, *T* expression was not detectable at E7.0 (figure 1e and f), suggesting that either primitive streak formation does not occur, or that this structure is not maintained in the absence of NR5A2. In agreement with the defects in primitive streak morphogenesis seen in *NR5A2* -/- embryos, *Foxa2* expression was reported to be selectively lost in the epiblast of *NR5A2* -/- embryos, thereby indicating that the anterior primitive streak tissue and its derivative, the node, are absent (Paré et al., 2004). Collectively, these results clearly demonstrate that the gastrulation process is severely impaired in *NR5A2* -/- embryos. To further characterize the gastrulation defects exhibited by *NR5A2* -/- embryos, we next analyzed the expression of an extensive panel of marker genes.

Initial A-P axis specification occurs in *NR5A2* -/- embryos

A failure in primitive streak formation can result from prior defects in the proper specification of the anterior- posterior (A-P) axis. To assess this possibility, the expression of various anterior and posterior marker genes

was analyzed in *NR5A2* mutant embryos. The initial establishment of the anterior identity occurs prior to gastrulation and relies on proper formation and localization of the anterior visceral endoderm (AVE). *Dkk-1*, *Cer-l* and *Otx2* are all expressed in the AVE of wild-type embryos and mutational analysis has revealed that *Otx2* is required in the visceral endoderm for proper development of anterior structures in the mouse (Perea-Gomez et al., 2001; Rhinn et al., 1998). In *NR5A2* -/- embryos, all three anterior markers exhibited a pattern of expression similar to the one observed in their wild-type littermates (figure2 a-c), indicating that the AVE is specified and that it localized to the prospective anterior region of *NR5A2* -/- embryos. However, there was an expansion in the expression domain of both *Otx2* and *Cer-l* in *NR5A2* mutant embryos (figure 2 b and c).

To determine if the posterior identity was properly established in *NR5A2* -/- embryos, the expression of early posterior marker genes was examined. Shortly after implantation, *Nodal* is initially expressed throughout the epiblast, and as the AVE forms, its expression becomes restricted to the prospective posterior side of the embryonic ectoderm (figure 2 d) (Conlon et al., 1994). In the absence of *NR5A2*, *Nodal* expression was also confined to the caudal portion of the epiblast prior to gastrulation (figure 2 e). In wild-type embryos, *Fgf8* expression was restricted to the posterior side of the epiblast before the onset of gastrulation, marking the prospective site of primitive streak formation (figure 2 f) (Crossley and Martin, 1995). In *NR5A2* -/- embryos, the *Fgf8*

expression pattern was also confined to the presumptive posterior side (figure 2 g). *Wnt3*, a gene essential for primitive streak induction and formation, is initially expressed in the proximal epiblast around E6.0 and its expression is then shifted posteriorly by E6.5, at the time of primitive streak induction (figure 2 h) (Liu et al., 1999). In *NR5A2* -/- embryos, *Wnt3* expression was detected in the proximal epiblast around E6.5 and was more pronounced on the posterior side (figure 2 i). Thus, the posterior expression pattern of *Nodal*, *Fgf8* and *Wnt3* in pre-gastrulation *NR5A2* -/- embryos indicated that the posterior identity was specified and that primitive streak induction was initiated in the absence of *NR5A2*. To further confirm that the posterior identity is established in *NR5A2* -/- embryos, early expression of *T* was also examined. In wild-type embryos, *T* is expressed at the proximal posterior region of the epiblast around E6.25, marking the site of primitive streak induction and mesodermal precursor cells. Although no *T* expression was detected in *NR5A2* -/- embryos at E7.0 (figure1 h), weak expression was detected at E6.25, indicating that even though induction of the primitive streak occurs, its formation is abrogated in the absence of *NR5A2* (figure 2 j and k). Collectively, these results demonstrate that the impaired primitive streak morphogenesis seen in *NR5A2* mutants does not result from prior defects in the initial establishment of the A-P identity.

Mesoderm formation in *NR5A2* -/- embryos

To further confirm that primitive streak formation and function was impaired in *NR5A2* -/- embryos, we tested the ability of these mutant embryos to form mesoderm. Mesoderm formation relies on distinct molecular events beginning with the initial specification of prospective mesoderm cells, which then undergo an epithelial to mesenchymal transition (EMT), allowing them to migrate through the streak to adopt a mesodermal cell fate. To determine if prospective mesodermal cells were present in *NR5A2* -/- embryos, *Lim-1* expression, which marks epiblast cells fated to become mesoderm prior to primitive streak formation, was analyzed (Shawlot and Behringer, 1995). As shown by proper *Lim-1* expression in the proximal posterior region of the epiblast, mesoderm precursor cells were specified in *NR5A2* -/- embryos, which is consistent with early *T* expression detected in the same region of *NR5A2* -/- embryos (figure 3 a and b). Moreover, as in wild-type embryos, *Lim-1* is expressed in the VE of *NR5A2* -/- embryos. To determine if EMT occurs in *NR5A2* mutant embryos, the expression of *Snail (Sna)* was examined. At the onset of gastrulation, *Sna* is expressed in the proximal-posterior region of wild-type embryos and marks the initiation of the EMT process. In *NR5A2* -/- embryos, the expression pattern of *Sna* recapitulated the one observed in wild-type embryos, which indicates that EMT was initiated in these embryos at the beginning of gastrulation (figure 3 c and d). Mesoderm formation was then assessed by examining *Eomesodermin* (*Eomes*)

expression in mutant embryos. During early post-implantation stages, *Eomes* expression is restricted to the extra-embryonic ectoderm. Around the gastrulation period, *Eomes* expression is still detected in the extra-embryonic ectoderm but it is also present in the nascent mesoderm (Ciruna and Rossant, 1999; Russ et al., 2000). In *NR5A2* mutant embryos, *Eomes* expression was detected in the extra-embryonic ectoderm but was absent from the epiblast, showing that mesoderm does not form (figure 3 e-h). Therefore, although mesodermal precursor cells were specified and EMT initiated, mesoderm failed to form in *NR5A2* -/- embryos. Together, these results further support the notion that NR5A2 is required for proper primitive streak formation and function. Since the extra-embryonic ectoderm is also important for specification of mesodermal cell fate, the molecular integrity of this tissue was further confirmed by examining *BMP4* expression. As in wild-type embryos, *BMP4* was expressed in the extra-embryonic ectoderm adjacent to the epiblast of *NR5A2* -/- embryos (figure 3 i and j). Proper expression of *BMP4* and *Eomes* in the extra-embryonic ectoderm suggests that, although this tissue displays some morphological abnormalities in the absence of NR5A2, its molecular integrity is maintained.

Visceral endoderm marker gene expression is reduced in the absence of *NR5A2*.

Since *NR5A2* is expressed in the VE prior to gastrulation, it raises the possibility that the phenotype observed in *NR5A2* -/- embryos might result from defects in the formation and/or function of this tissue. To test this, the expression of two VE marker genes, *HNF4* and *GATA4*, which have been shown to be important for the formation and/or function of this tissue, was examined by whole mount *in situ* hybridization (Chen et al., 1994; Duncan et al., 1994; Soudais et al., 1995). Prior to the onset of gastrulation, *GATA4* was expressed in the VE layer overlying the embryonic-extra-embryonic junction, whereas *HNF4* was expressed throughout the VE tissue covering both the embryonic and extra-embryonic portions of the conceptus (figure 4 a and c). In *NR5A2* -/- embryos, these two VE markers exhibited an expression pattern similar to the one observed in wild-type embryos (figure4 b and d). However, by *in situ* hybridization, *NR5A2* -/- embryos appeared to show a decrease in the level of expression of both *GATA4* and *HNF4* compared to their wild-type and heterozygous littermates. To quantify the relative levels of *GATA4* and *HNF4*, we performed real time qPCR on 29 *NR5A2* +/+ or +/- and 8 *NR5A 2* -/- embryos. *NR5A2* -/- embryos showed a two fold reduction in *GATA4* expression and a three fold reduction in *HNF4* expression (Figure 4e). Despite the reduction in the expression level of these two marker genes in

NR5A2 -/- embryos, the VE did not show major morphological abnormalities.

Presence of wild-type cells in the extra-embryonic tissues rescues the gastrulation defects of *NR5A2* mutant embryos.

Since marker gene expression in the VE was reduced and given the conflicting reports on the expression pattern of *NR5A2* during early post-implantation development (Gu et al., 2005; Paré et al., 2004), we next wanted to determine the tissue requirement for *NR5A2* function during the gastrulation process. To this end, tetraploid chimeric embryos were generated by aggregating wild-type tetraploid morulae with diploid morulae stage embryos obtained from *NR5A2* heterozygous crosses. This chimeric approach takes advantage of the developmental bias of cells from tetraploid embryos to contribute preferentially to extra-embryonic tissue, whereas morulae stage embryos derived from *NR5A2* heterozygous matings can generate both embryonic and extra-embryonic tissues (MacKay and West, 2005, and see Appendix I). Thus, if NR5A2 is required in the extra-embryonic lineage to promote proper primitive streak morphogenesis and mesoderm formation in the underlying epiblast, the presence of wild-type tetraploid cells in the extra-embryonic lineage should rescue the gastrulation defects observed in *NR5A2* -/- embryos. PCR analysis performed on the embryonic portion of chimeric embryos, devoid of all extra-embryonic tissues, at E 9.0 showed that 2 out of 26

(7.6%) embryos were homozygous for the *NR5A2* mutation (figure 5a) These *NR5A2* mutant embryos were indistinguishable from wild-type embryos (figure 5 b and c). Therefore, *NR5A2* -/- cells are capable of undergoing gastrulation and form mesoderm in the presence of wild-type extra-embryonic tissues. This demonstrates that *NR5A2* is not required cell autonomously in the epiblast since homozygous cells are capable of forming mesoderm. This supports the notion that NR5A2 may be required in extra-embryonic tissues to promote proper primitive streak morphogenesis.

DISCUSSION

We demonstrated that homozygous inactivation of *NR5A2* leads to early embryonic lethality between 6.0 and 7.5 dpc, which correspond to the gastrulation period, and that *NR5A2* -/- embryos display several characteristic developmental defects. These included various degrees of growth retardation and epiblast disorganization, a mild constriction at the embryonic–extra-embryonic junction, and absence of morphologically distinguishable primitive streak, node and mesoderm. In addition, an abnormal accumulation of cells at the proximo-posterior region of the embryo was also observed in 25% of *NR5A2* mutant embryos that survived to a later developmental stage (E7.0 to 7.5). Since the absence of a primitive streak suggested axis specification defects and impaired gastrulation in *NR5A2* -/- embryos, we performed a marker gene analysis to further characterize the gastrulation defects observed in *NR5A2* mutant.

The AVE has been shown to be essential for anterior specification of the epiblast and its formation constitutes the first sign of A-P asymmetry. We have demonstrated that, in *NR5A2* -/- embryos, the AVE is specified and properly localized to the prospective anterior side. Furthermore, the restriction of *Nodal* expression to the prospective posterior aspect of the embryo prior to gastrulation suggests that the AVE is functional in *NR5A2* mutants. Although the AVE is properly specified, we showed that there is an expansion in the domain of expression of two anterior marker genes in embryos lacking *NR5A2* function. Indeed, the

expression of *Cer-l* and *Otx2* extend caudally in E6.5 mutant embryos. There are several possible explanations for the expansion in the domain of expression of these genes. In the mouse, restriction of anterior marker gene expression is dependent upon posteriorizing signals and on the presence of a functional primitive streak (Ang et al., 1994; Lu et al., 2001). Thus, it is possible that the abrogation of primitive streak formation seen in *NR5A2* mutant embryos may be responsible for the rostral expansion of the expression domain of *Cer-l* and *Otx2*. Indeed, although the AVE is specified before the posterior identity, the presence of a primitive streak maybe important to maintain the restriction of anterior marker genes at the time of gastrulation. Alternatively, it has recently been demonstrated that the extra-embryonic ectoderm is essential for restricting AVE formation, and anterior specification of the epiblast (Rodriguez et al. 2005, Donnison et al. 2005, Georgiades and Rossant, 2006). Indeed, these studies have demonstrated that loss of this tissue led to ectopic AVE formation and anteriorization of the epiblast. Therefore, it is possible that the expansion in anterior marker gene expression observed in *NR5A2* -/- embryos may result from defects in extra-embryonic ectoderm function. Conversely, it is possible that the expansion of anterior marker gene expression prevent primitive streak formation in the prospective posterior side of *NR5A2* -/- embryos.

Nodal and *Fgf8*, two early posteriorizing signals, are present in the prospective posterior region of *NR5A2* -/- embryos prior to gastrulation.

However, *Wnt3* expression was confined to the proximal epiblast of *NR5A2* -/- embryos, and although it was stronger on the posterior side it failed to become localized to the presumptive primitive streak region around the time of gastrulation. Similarly, *T* transcripts were detected in the proximo-posterior epiblast of *NR5A2* -/- embryos at E6.25 but were absent from the prospective posterior region of E7.0 *NR5A2* -/- embryos. Thus, the expression of *T*, *Wnt3* and *Lim-1* in the proximo-posterior region of pre-gastrula stage *NR5A2* -/- embryos demonstrates that cells fated to become mesoderm are initially specified and that primitive streak and mesoderm-inducing signals are present in *NR5A2* mutant embryos. However, although initiation of primitive streak formation occurs in the proximo-posterior region of *NR5A2* -/- embryos, there is a failure in subsequent primitive streak morphogenesis, elongation and/or maintenance.

In agreement with the absence of a primitive streak in *NR5A2* -/- embryos, our marker gene analysis has demonstrated that the expression of *Eomes* is absent from the epiblast of *NR5A2* -/- embryos. Interestingly, embryos lacking *Eomes* expression in the epiblast exhibit a phenotype closely resembling the one observed in *NR5A2* -/- embryos (Russ et al., 2000). Indeed, NR5A2 -/- embryos and embryos lacking *Eomes* function in the epiblast exhibit impaired primitive streak and mesoderm formation without affecting initial establishment of the A-P axis or mesoderm induction. Moreover, the absence of *Eomes* in the epiblast leads to an

abnormal posterior thickening with morphological signs of EMT with no emergence of embryonic or extra-embryonic mesoderm (Russ et al., 2000). This phenotype results from migratory defects of epiblast cells and resembles the cellular accumulation present in the posterior epiblast of *NR5A2* -/- embryos. The presence of *Sna* transcripts in *NR5A2* -/- embryos demonstrates that EMT is initiated and suggests that the posterior thickening of the epiblast results from defects in migration or final mesenchymal differentiation that will interfere with primitive streak morphogenesis and subsequent mesoderm formation. Further supporting this idea is the fact that although cells in the posterior extra-embryonic region do show signs of epithelial to mesenchymal transition, none of embryonic or extra-embryonic mesoderm markers used in this study were expressed in these cells. Thus, the specific loss of *Eomes* in the embryonic lineage of *NR5A2* -/- embryos and the similarity between the phenotypes resulting from loss of NR5A2 function or loss of epiblastic Eomes function suggest that NR5A2 is required for the induction of *Eomes* expression in the embryonic ectoderm. Therefore, the loss of *Eomes* in the epiblast of *NR5A2* -/- embryos, is likely responsible for the impairment in mesoderm formation. However, since the phenotype of *NR5A2* -/- embryos is more severe than the one resulting from lack of epiblastic *Eomes*, it suggests that NR5A2 may play additional roles during early embryogenesis. Indeed, although *NR5A2* -/- embryos die prior to 7.5 dpc,

embryos lacking epiblastic *Eomes* expression can develop to 8.5dpc (Russ et al., 2000).

Previous studies based on the expression pattern of *NR5A2* and on both EMSA and transient transfection assays have implicated NR5A2 as a key factor involved in the transcriptional cascade leading to endoderm specification and liver determination. During early embryogenesis, the VE displays a gene expression profile that recapitulates the endodermal program of gene expression and fulfills endodermal functions (Bielinska et al. 1999). As a number of liver-specific transcription factors, *NR5A2* is expressed in the VE prior to and during gastrulation. Although this tissue does not show major morphological abnormalities in *NR5A2* -/- embryos, real time quantitative RT-PCR demonstrated that there is a decrease in the expression level of at least two VE markers, *HNF4* and *GATA4*. The decreased expression of these two VE genes supports a role for NR5A2 in the hepatic transcriptional hierarchy involved in the regulation of *HNF4* and *GATA4* in the VE *in vivo*. However, the fact that *HNF4* and *GATA4* are still expressed in *NR5A2* mutants indicates that other transcriptional factors are involved in the regulation of these genes. Despite the reduction in the expression level of these two VE markers, the phenotype resulting from the loss of either of these factors is distinct from the one observed in *NR5A2* -/- embryos. *GATA4* -/- embryos undergo gastrulation but exhibit defects in both ventral morphogenesis and heart tube formation leading to embryonic death between E8.5 and E10.5 (Kuo et al., 1997, Molkentin et

al., 1997). Loss of *HNF4* function leads to embryonic lethality during gastrulation and mutants display excessive cell death in the epiblast, delayed primitive streak and mesoderm formation, and severe impairment in the development of all embryonic structure (Chen et al., 1994, Duncan et al., 1994). Therefore, although the reduced expression of *HNF4* and *GATA4* may contribute to the phenotype of *NR5A2* -/- embryos, the differences between *HNF4* -/-, *GATA4* -/- and *NR5A2* -/- embryonic defects indicate that the phenotype resulting from *NR5A2* inactivation is unlikely to result exclusively from a reduction in *HNF4* or *GATA4* expression.

Since there are conflicting reports on the expression pattern of *NR5A2* during early post-implantation development (Gu et al., 2005; Paré et al., 2004), and that we demonstrated a decrease in VE gene expression, we performed preliminary experiments to determine the tissue requirement for NR5A2 function during the gastrulation process. We have taken advantage of the developmental bias of cells originating from tetraploid embryos to be preferentially excluded from the embryonic lineage and to contribute predominantly to extra-embryonic tissues. We aggregated wild-type tetraploid embryos with diploid embryos derived from *NR5A2* heterozygous crosses. Genotyping of the complete embryonic portion of embryos derived from tetraploid aggregations demonstrated that *NR5A2* mutant embryos could develop until day 9 and were indistinguishable from wild-type embryos. Although it was initially believed

that tetraploid cells contributed exclusively to extra-embryonic tissues, recent evidence suggests that some tetraploid cells are still present in the epiblast at the time of gastrulation (Eakin et al., 2005, MacKay and West, 2005). Thus, there are at least two possible explanations for how tetraploid cells can rescue the *NR5A2* mutant phenotype. It is possible that upon aggregation of the wild-type tetraploid embryo with the *NR5A2* mutant embryo, the initial presence of wild-type tetraploid cells in the ICM of the peri-implantation embryos, caused by the intermingling of cells, as well as the presence of a few wild-type tetraploid cells in the epiblast might be sufficient to compensate for the absence of NR5A2. Alternatively, since tetraploid cells contribute mostly to extra-embryonic tissues at the time of gastrulation, it is possible that NR5A2 function is required in these tissues to promote gastrulation. Although it still needs to be determined exactly how wild-type tetraploid cells rescue the phenotype, our results clearly demonstrate that mutant *NR5A2* cells are capable of undergoing gastrulation since mutant embryos completely derived from *NR5A2* mutant cells were isolated at E9.0. This demonstrates that NR5A2 function is required non-cell autonomously in the epiblast since mutant cells can undergo normal gastrulation in the presence of wild-type tetraploid cells.

Although our results suggest that the function of NR5A2 is required in extra-embryonic tissues to promote gastrulation, the use of tetraploid aggregation chimeras does not allow one to distinguish between the requirement for NR5A2 function in the extra-embryonic ectoderm or the

VE. NR5A2 function could therefore be required in either or both of these tissues. The extra-embryonic ectoderm plays a predominant role in the induction of proximo-posterior gene expression and primitive streak formation (Donnison et al., 2005; Beck et al., 2002; Fujiwara et al., 2002; Winnier et al., 1995). However, since *NR5A2* -/- embryos exhibit a decrease in VE marker gene expression but show normal expression pattern for both *BMP4* and *Eomes* in the extra-embryonic ectoderm, this suggests that NR5A2 function may be required in the VE for the gastrulation process. The fact that induction of proximo-posterior gene expression occurs in the proximal epiblast of *NR5A2* -/- embryos also supports the idea that the extra-embryonic ectoderm is functional.

Although a role for the VE in anterior patterning of the embryo is well established, much less is known about its role in patterning the posterior epiblast. However, explant culture assays have shown that the VE provide signals inducing posterior cell fate in the epiblast (Belaoussoff et al., 1998). To our knowledge, only a few genes have been shown to be required in the VE to specifically induce/establish posterior structure in the underlying epiblast. Targeted mutagenesis has demonstrated that the function of the *amnionless* (*amn*) gene is required in the VE for proper patterning of the primitive steak. Indeed, in *amn* -/- embryos there is a specific loss of middle streak derivatives (Kalantry et al., 2001; Tomihara-Newberger et al., 1998). Alk2 function was also shown to be required in the VE to promote proper mesoderm formation (Gu et al., 1999) and

Foxa2 has been shown to be required in the VE for proper primitive streak elongation (Dufort et al., 1998). In addition, there is a requirement for *MesD* gene function in extra-embryonic tissues for primitive streak formation (Hsieh et al., 2003). However, it is still unknown if the gastrulation defects seen in *MesD* -/- embryos results from loss of MesD in the VE or in the extra-embryonic ectodem. Interestingly, phenotypes resulting from loss of function of any of these genes resemble the *NR5A2* -/- phenotype. Indeed, inactivation of any of these genes specifically leads to impairment in posterior patterning (or loss of posterior structure) and do not exhibit any defects in initial A-P axis specification or extra-embryonic ectoderm development aside from *Foxa2*.

Taken together, our results identify a new role for NR5A2 in primitive streak morphogenesis and mesoderm formation. In addition, our chimeric analysis demonstrates that NR5A2 function is required non-cell autonomously for primitive streak and mesoderm formation in the epiblast and suggests that its function may be required in extra-embryonic tissues to promote gastrulation.

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Figure 1. Morphological defects of *NR5A2* -/- embryos

(A) *NR5A2* -/- embryos at E7.0 displayed a reduced size compared to *NR5A2* +/- and wild-type littermates, mild embryonic-extra-embryonic constriction, epiblast disorganization. Arrow points to the embryonic-extra-embryonic constriction. Note that due to a dissection artifact, there is a dimple at the anterior distal end of the *NR5A2* -/- embryo. (B) *NR5A2* -/- embryos that developed to E7.0 often displayed an accumulation of cells on the posterior region. (C, D) Nuclear fast red-stained transverse-sections of wild-type (C) or *NR5A2* -/- (D) embryos, demonstrating a cellular accumulation at the proximo-posterior epiblast of E7.0 *NR5A2* -/- embryos as well as the absence of mesodermal wings. (E,F) At E7.25, *NR5A2* -/- embryos (F) do not form a primitive streak as confirmed by whole mount *in situ* hybridization for *T*, note that the extra-embryonic portion of this embryo was mistakenly dissected out (three E7.0-7.5 *NR5A2* -/- embryos examined); whereas a well defined primitive streak is clearly present in wild-type and *NR5A2* +/- embryos at this developmental stage (E).(G,H) Nuclear fast red-stained sagittal sections of wild-type (G) or *NR5A2* -/- (H) embryos, showing epiblast disorganization and collapsing, thickening at the proximo-posterior region of the epiblast and absence of a discernible primitive streak. Red arrow points to the cellular accumulation at the proximo-posterior region of *NR5A2* -/- embryos. Am: amnion, Acc: accumulation of cells, c: chorion, MW: mesodermal wings, PS: primitive streak. Scale bar = 100 microns

Figure 1. Morphological defects of *NR5A2 -/-* embryos

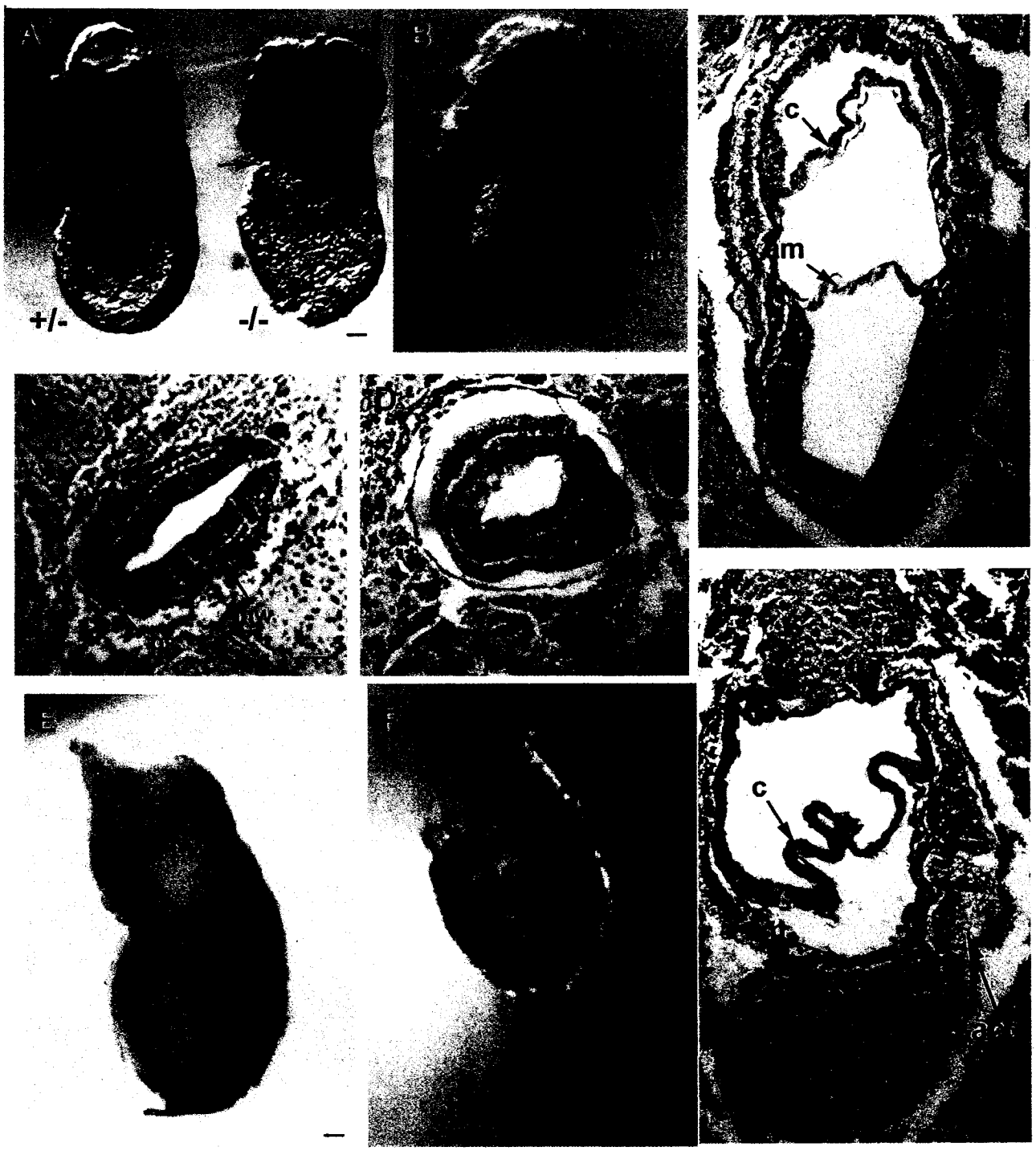


Figure 2. Initial specification of the A-P axis prior to gastrulation in *NR5A2* -/- embryos

Whole mount *in situ* hybridizations for anterior (*Dkk-1*, *Cer-l*, *Otx2*) and posterior (*Nodal*, *Fgf8*, *Wnt3* and *T*) marker genes in pre- to early- gastrula stage embryos.

(A) *Dkk-1* is expressed in the AVE of wild-type and *NR5A2* -/- embryos. (B) *Cer-l* expression in wild-type and *NR5A2* -/- embryos. (C) *Otx2* expression in wild-type and *NR5A2* -/- embryos. (D, E) *Nodal* expression is restricted to the posterior region of wild-type (D) and *NR5A2* -/- (E) embryos. (F, G) *Fgf8* expression in the posterior region of wild-type (F) and *NR5A2* -/- (G) embryos. (H,I) *Wnt3* expression in the proximo-posterior region of wild-type (H) and *NR5A2* -/- embryos (I). Early *T* expression in wild-type (J) and *NR5A2* -/- (K) embryos. For anterior marker gene expression, a total of three *NR5A2* -/- embryos were analyzed for *Dkk-1* expression, five for *Otx2*, and four for *Cer-l*. For posterior marker gene expression, three *NR5A2* -/- embryos were examined for *Nodal* expression, three for *Fgf8*, four for *Wnt3*, and six for *T*.

Figure 2. Initial specification of the A-P axis prior to gastrulation in *NR5A2* -/- embryos

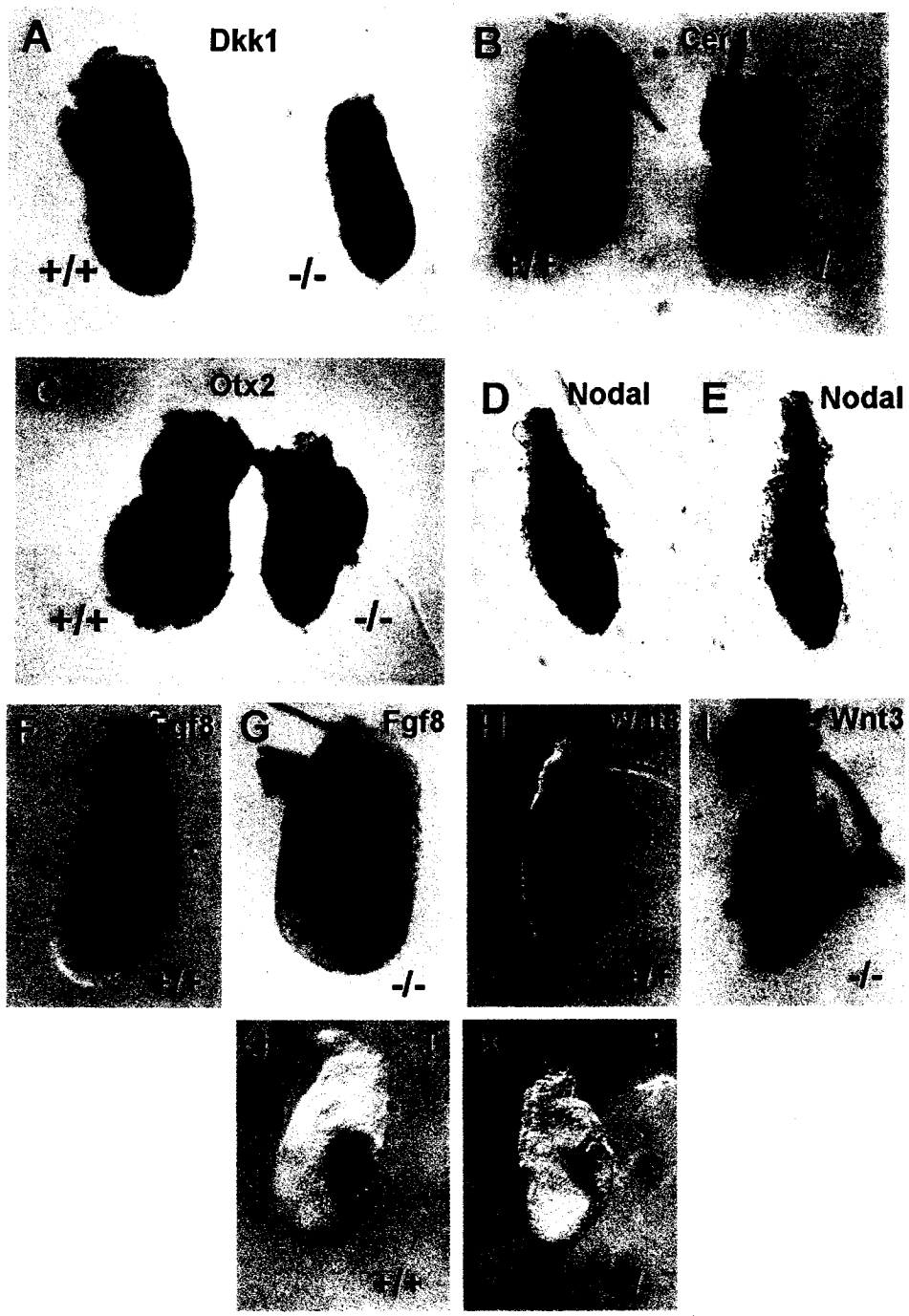


Figure 3. Induction and formation of mesoderm in *NR5A2 -/-* embryos

Whole mount *in situ* hybridizations for prospective and definitive mesoderm and extra-embryonic ectoderm marker genes in pre-gastrula stage embryos. (A,B) *Lim-1* expression in wild-type (A) and *NR5A2 -/-* embryos (B). (C,D) *Snail* expression in wild-type (C) and *NR5A2 -/-* (D) embryos. (E, F, G, H) *Eomes* expression in E6.25 and E7.25 wild-type (E, G) and *NR5A2 -/-* embryos (F,H). (I, J) *BMP4* expression in wild-type (I) and *NR5A2 -/-* (J) embryos. A total of three *NR5A2 -/-* embryos were examined for *Lim-1* expression, three for *Snail,* four for *Eomes*, and six for *BMP4*.

Figure 3. Induction and formation of mesoderm in *NR5A2* -/- embryos

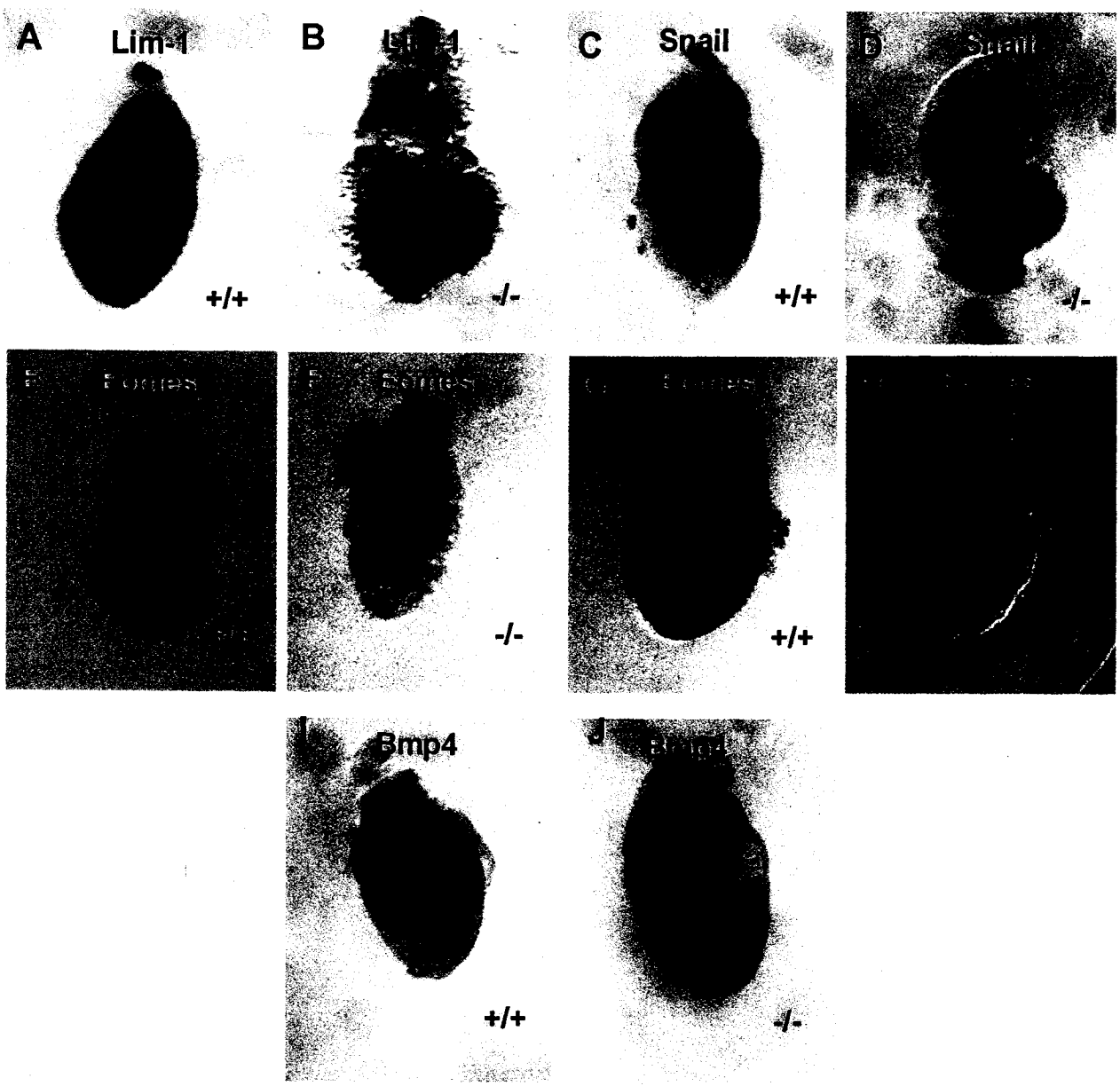


Figure 4. Visceral endoderm marker gene expression in *NR5A2* -/- embryos

Whole mount *in situ* hybridizations for *HNF4* and *GATA4* in pre-gastrula stage embryos. (A, B) *HNF4* expression in the VE of wild-type (A) and *NR5A2* -/- (B) embryos. (C, D) *GATA-4* expression in the VE overlying the embryonic-extra-embryonic junction of wild-type (C) and *NR5A2* -/- (D) embryos. A total of three *NR5A2* -/- embryos were analyzed for *GATA4* expression and four for *HNF4*. (E) Real time quantitative PCR on 21 +/+ or +/- and 8 -/- *NR5A2* embryos for *GATA4* and *HNF4*. A two fold decrease in *GATA4* expression was observed in *NR5A2* -/- embryos, chi square test $p<0.02$. A three fold decrease in *HNF4* expression in *NR5A2* -/- embryos, chi square test $p<0.001$.

Figure 4. Visceral endoderm marker gene expression in *NR5A2* -/- embryos

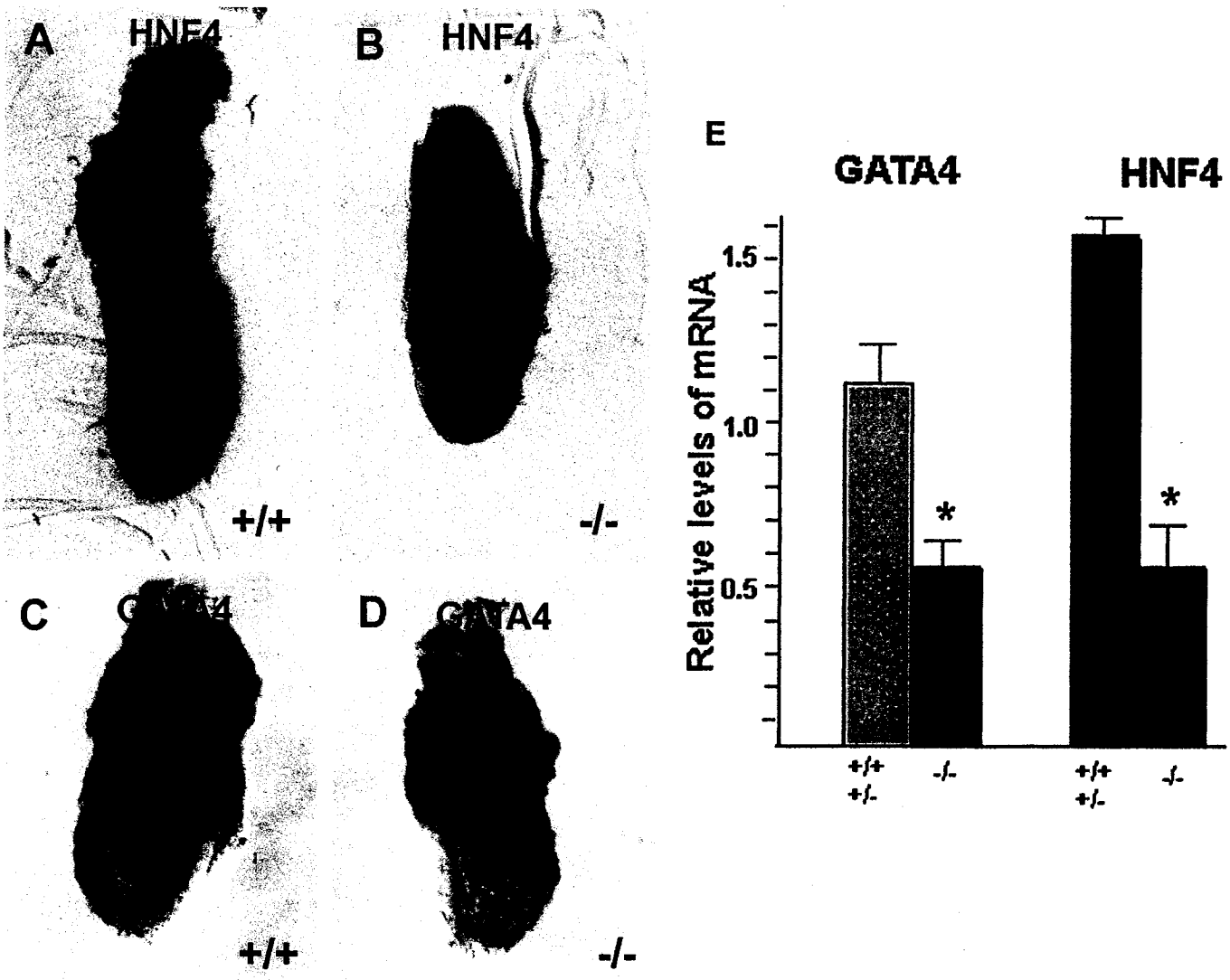
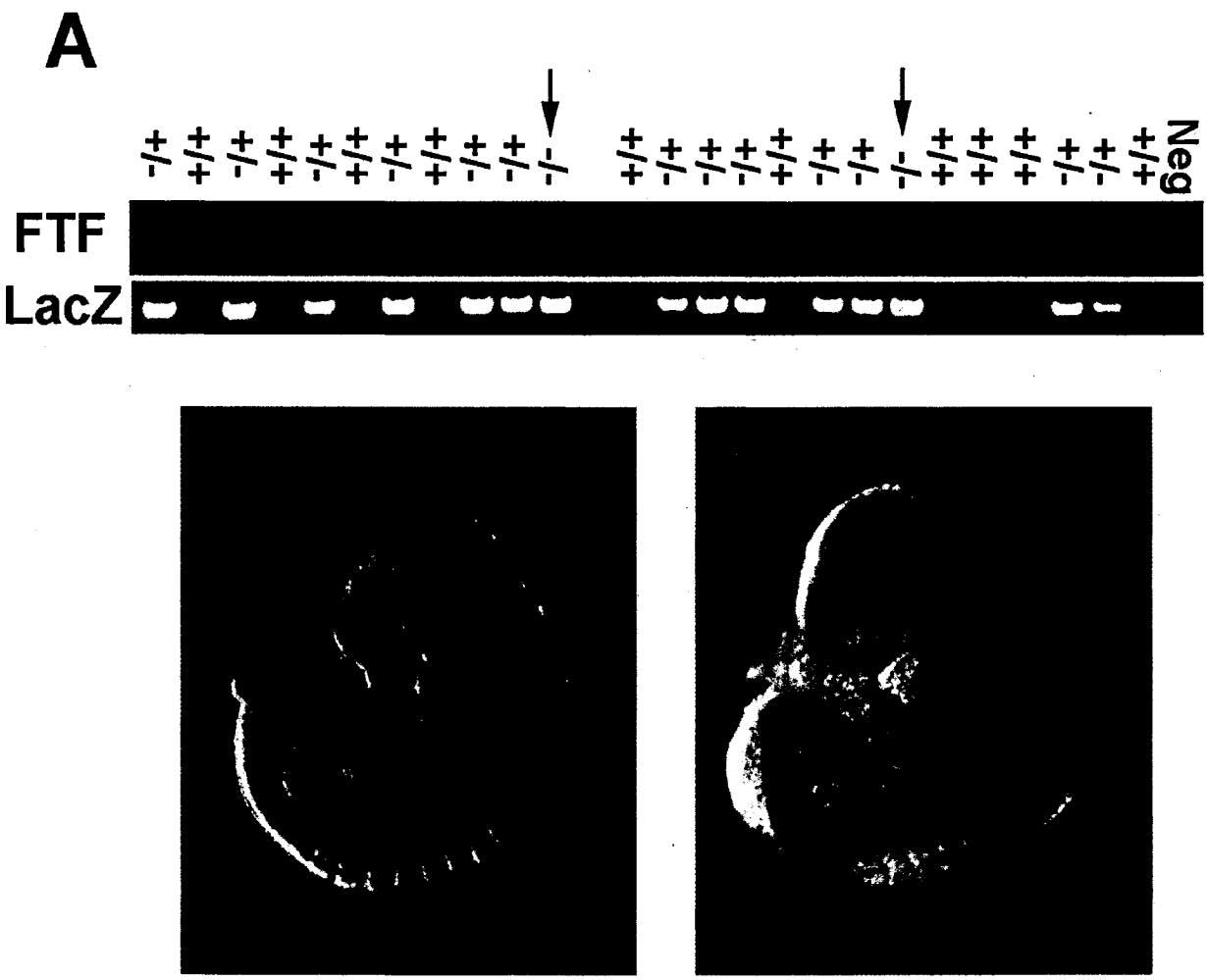


Figure 5. NR5A2 is required in the extra-embryonic lineage for the gastrulation process

Chimeric embryos were generated by tetraploid aggregations and isolated at E9.0. The embryonic portion of the embryo was used for genotyping. (A) PCR genotyping of embryos derived from aggregations. (B) E9.0 tetraploid aggregated chimeric embryo composed of wild-type extra-embryonic and embryonic tissues. (C) E9.0 tetraploid aggregated chimeric embryo composed of wild-type extra-embryonic tissues and *NR5A2* -/- embryonic tissues.

Figure 5. NR5A2 is required in the extra-embryonic lineage for the gastrulation process



CHAPTER 4: MANUSCRIPT III

The expression pattern of the nuclear receptor NR5A2 during mouse embryogenesis reveals a potential role in craniofacial and nervous system development

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To be submitted

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PREFACE

Due to our inability to generate homozygous *NR5A2* null embryonic stem (ES) cells for the generation of tetraploid embryos, chimeric embryos were generated using aggregation between wild-type tetraploid embryos and diploid embryos coming from *NR5A2* +/- crosses (as described in the previous chapter). This chimeric approach is much more exhaustive and does not allow the production of a high number of *NR5A2* -/- embryos rescued from the early embryonic lethality that would enable us to assess later developmental functions of NR5A2. In an attempt to identify potential requirements for NR5A2 function post-gastrulation, we took advantage of the LacZ knock-in approach used to generate *NR5A2* +/- mice. In *NR5A2* +/- mice, β-galactosidase expression was shown to recapitulate that of the endogenous *NR5A2* allele, allowing us to better define the *NR5A2* expression pattern during mid to late gestation. The determination of the expression pattern of a given factor is the first step in elucidating its potential roles and therefore serves as a starting point for subsequent functional analyses.

ABSTRACT

The orphan nuclear receptor NR5A2 plays important roles in adult physiology, notably in cholesterol homeostasis, steroidogenesis and cell proliferation. Targeted inactivation of the *NR5A2* gene leads to early embryonic lethality, demonstrating a requirement for this transcription factor during early embryogenesis and precluding the assessment of later developmental roles for NR5A2. In this study, we took advantage of the LacZ knock-in approach used to generate the *NR5A2* null mutation to better define the embryonic expression pattern of NR5A2, with an emphasis on mid to late gestational development. Our results show that NR5A2 displays a widespread and dynamic expression pattern during mouse embryogenesis and that NR5A2 is expressed abundantly during nervous system and craniofacial development. More specifically, NR5A2 exhibits high levels of expression in the ventral spinal cord and dorsal root ganglia, suggesting that NR5A2 may play a role in the development and/or specification of motor and sensory neurons.

INTRODUCTION

In adult animals, the nuclear receptor NR5A2 plays important roles in a multitude of biological processes including cholesterol homeostasis, steroidogenesis, and cell proliferation (Fayard et al., 2004; Botrugno et al., 2004; Annicotte et al., 2005; Wang et al., 2005). Targeted disruption of the *NR5A2* gene has demonstrated a central role for this transcription factor during early embryogenesis. *NR5A2* -/- embryos die around the gastrulation period and display defects in primitive streak morphogenesis (Paré et al., 2004; Gu et al., 2005, and Labelle-Dumais et al., 2006). The early embryonic lethality resulting from *NR5A2* inactivation precludes the identification of NR5A2 developmental functions post-gastrulation. Since the expression pattern of a given gene can provide insights on its potential roles, we took advantage of the LacZ knock-in approach used to disrupt the *NR5A2* allele to characterize the NR5A2 expression pattern during mouse embryogenesis, focusing mainly on the mid to late gestation period (Paré et al., 2004).

In this study, we show that NR5A2 exhibits a widespread and dynamic expression pattern throughout mouse embryogenesis, starting from the two-cell stage. More specifically, we demonstrate that NR5A2 is expressed in various craniofacial areas as well as in the developing nervous system. In the nervous system, NR5A2 expression is detected in motor neurons located in the ventral spinal cord and in the sensory

neurons of the dorsal root ganglia, suggesting a potential role for NR5A2 in the development of the sensory-motor nervous system.

MATERIALS AND METHODS

Animal maintenance and mating

The generation of *NR5A2-LacZ* +/- mice was previously described in Paré et al. (2004). All mice were housed in filtered-topped isolator cages under a 12 hour light-dark cycle (7h00 am-7h00 pm). Successful fertilization/ mating events were assessed by the presence of a vaginal plug. For embryo staging, the morning on which the vaginal plug was observed was considered 0.5 day post-coitum (dpc).

Genotyping of mice

21 day-old mice obtained from *NR5A2* +/- crosses were genotyped using polymerase chain reaction (PCR) on tail DNA. The targeted *NR5A2* allele was amplified using oligonucleotides for either the neomycin resistant cassette or the LacZ reporter gene used to disrupt the NR5A2 gene (Paré et al., 2004). LacZ primers sequence: LacZ forward: CAG TGG CGT CTG GCG GAA AAC CTC, LacZ reverse: GGC GGC AGT AAG GCG GTC GG, Neo primer sequences: Neo forward: GGC TAT GAC TGG GCA CAA CAG ACA ATC, Neo reverse: AGC TCT TCA GCA ATA TCA CGG GTA GC. The sequences of the oligonucleotides used to amplify the wild-type *NR5A2* allele were: FTF forward: TAC AGC CTC CAA ATT TTG CC and FTF reverse: TAT CGC CAC ACA CAG GAC AT. The PCR conditions were as follows: denaturation at 94°C for 60 seconds, annealing at 58°C

for 60 seconds, elongation at 72°C for 60 seconds for 30 cycles for tail DNA and for 35 cycles for embryos.

Detection of β-galactosidase activity

Post-implantation embryos obtained from *NR5A2* +/- matings were dissected in phosphate buffer saline (PBS) and pre-implantation embryos were collected by flushing the oviducts or uteri of pregnant females. Embryos were rinsed in 100 mM sodium phosphate, pH 7.3, and fixed in 0.2% glutaraldehyde, 2 mM $MgCl_2$, 5 mM EGTA, and 100 mM sodium phosphate, pH 7.3, for 15 minutes at room temperature. Embryos were washed three times in wash buffer (0.02% Nonidet P-40, 0.01%deoxycholate, 2 mM $MgCl_2$, and100 mM sodium phosphate, pH 7.3) for 15 min at room temperature. To reveal β-galactosidase activity, embryos were incubated in X-gal staining solution (1 mg/ml X-gal, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 0.02% Nonidet P-40, 0.01% deoxycholate, 2 mM $MgCl_2$, and100 mM sodium phosphate, pH 7.3) overnight at 37°C. Stained embryos were then rinsed with wash buffer and PBS and post-fixed overnight in 4% paraformaldehyde in PBS at 4°C.

Histology Analysis

Post-fixed embryos stained for β-galactosidase activity were dehydrated through a graded series of ethanol, incubated twice in xylene and embedded in paraffin. Paraffin-embedded X-gal stained embryos were

sectionned (10 μm thick) and stained with nuclear fast red solution (Sigma- cat# N-3020).

Immunofluorescence Confocal Microscopy

Embryos obtained from *NR5A2* +/- matings were fixed in 2% PLP (2% paraformaldehyde, 0.1M Na2HPO4, 10mM sodium periodate, and 70mM L-lysine) for 2 to 4 hours at 4°C. Fixed embryos were incubated for 24 hours in 30% sucrose solution at 4°C and embedded in Shandon Cryomatrix medium (Thermo Electron Corporation) before freezing in liquid nitrogen, and stored at -80°C. 15μm sections were cut on cryostat, mounted on Superfrost slides, air-dried, and stored at -80°C. Representative tissue sections were equilibrated at room temperature, rinsed 5 minutes in PBS, washed three times 15 minutes in 0.1% Triton-X in PBS at room temperature. Tissue sections were then incubated for 1 hour at room temperature in blocking solution manufactured specifically for detecting mouse primary antibodies on mouse tissue (M.O.M. kit; Vector Laboratories, Burlingame, CA, USA). Tissue sections were incubated overnight at 4°C with two different combinations of primary antibodies: mouse anti-Lim1-2 (1:1000) and rabbit anti-β-galactosidase (1:2000) or mouse anti-mouse-Islet 1 (1:25) and rabbit anti-mouse-β-galactosidase (1:2000) and 20 minutes at room temperature with appropriate secondary antibodies. The following secondary antibodies were used for double labelling: Cy3-conjugated goat anti-mouse and

FITC-conjugated anti-rabbit (1:500, Jackson Immunoresearch, West Grove, PA, USA). All primary and secondary antibodies used in this study were generously provided by Dr. Stifani (Centre for Neuronal Survival, Montreal Neurological Institute, Montreal, Quebec, Canada). Sections were then mounted in mowiol (Calbiochem) and examined using a Zeiss 510 confocal scanning laser microscopic imaging system.

RESULTS

NR5A2-LacZ expression during pre-implantation development

During the pre-implantation period, NR5A2-LacZ expression was assessed by β-galactosidase staining and was detected as early as the two-cell stage, at the time of zygotic genome activation (figure 1a). As shown in figure 1b, NR5A2-LacZ displayed strong and ubiquitous expression at the morula stage. As development proceeded, NR5A2 expression was still ubiquitous at the blastocyst stage being present in both the inner cell mass (ICM) and the trophectoderm at 3.5 dpc and 4.5 dpc (figure 1 c and d). It should be noted that NR5A2-LacZ expression declined to low levels in 4.5 dpc blastocysts. NR5A2-LacZ therefore exhibits a ubiquitous expression pattern throughout the pre-implantation period, as suggested previously (Paré et al., 2004; Gu et al., 2005).

NR5A2-LacZ expression during mid to late gestation

To better characterize the expression pattern of NR5A2 during mid to late gestation, we examined NR5A2-LacZ expression in NR5A2-LacZ +/- embryos between 9.5 dpc and 15.5 dpc. As shown in figure 2 a, strong NR5A2-LacZ expression was detected in the embryonic endoderm, the hepato-pancreatic primordium and the branchial arches around 9.5 dpc, which is consistent with previous reports on the *NR5A2* expression pattern (Rausa et al., 1999; Annicotte et al., 2003; Paré et al., 2004). At 11.5 dpc, NR5A2-LacZ expression was still high in the developing digestive system

and was present in the rib primordia, the spinal cord, and the dorsal root ganglia (figure 2 b and c, and data not shown). Elevated NR5A2-LacZ expression levels persisted in the developing spinal cord and dorsal root ganglia throughout embryogenesis (figure 2 d, f and g, figure 3 d, and data not shown). During mid to late gestation, NR5A2 expression was also detected in the lung mesenchyme (figure 2 f), the developing endodermal organs, including the liver (figure 2 e), and in the umbilical cord (figure 2 d and e). In the developing head, NR5A2-LacZ was strongly expressed in the midbrain region (figure 3 b and c, figure 4 g and h), the developing jaw (figure 3 a), the developing ear (figure 3 c), the ocular muscles (figure 3 c), in the trigeminal ganglion (figure 3 a and b), as well as in the thyroid and submandibular glands (figure 3 d, figure 4 e and f).

NR5A2-LacZ expression during nervous system development

To further characterize the expression pattern of NR5A2 in the developing spinal cord, we analyzed transverse tissue sections and demonstrated that NR5A2-LacZ was initially expressed at 10.5 dpc, the time at which various neuronal cell types are generated in the spinal cord (figure 4a). Interestingly, NR5A2–LacZ expression was restricted to the ventral region of the spinal cord, throughout its rostro-caudal extent, at all stages examined (figure 4 a, b, c, d, e, f and data not shown).

To determine if NR5A2 was expressed in spinal motor neurons, which are confined to the ventral spinal cord, we performed co-

immunofluorescence experiments with antibodies against two different spinal motor neuron marker genes; Islet-1 (isl-1) and Lim1. In addition to being expressed in motor neurons, Isl-1 is also present in the sensory neurons of the dorsal root ganglia (DRG), located on either side of the spinal cord. As depicted in figure 5 a, NR5A2-LacZ expression overlapped with the expression of isl-1 in medial motor neurons of the lateral motor column (LMC) and medial motor column (MMC), and in the dorsal root ganglia at the level of the forelimbs. Figure 5b shows that NR5A2-LacZ expression co-localized with that of Lim1 in the lateral region of the lateral motor column of the spinal cord at the level of the forelimbs. These results indicate that NR5A2 is expressed in different types of motor neurons in the developing spinal cord, and suggest that NR5A2 might be involved in the determination of motor neuron identity. The expression of NR5A2-LacZ in the DRGs also suggests that NR5A2 might be implicated in the development of sensory neurons in the peripheral nervous system.

DISCUSSION

In this study, we took advantage of the LacZ knock-in approach used to inactivate the *NR5A2* gene to better define the embryonic expression pattern of *NR5A2* and to infer its potential developmental roles post-gastrulation. Using this NR5A2-LacZ reporter strategy, we showed that NR5A2 embryonic expression initiates earlier then reported previously (Paré et al., 2004; Gu et al., 2005). Indeed, our results indicate that NR5A2 is expressed as early as the 2 cell-stage, which corresponds to the time of zygotic genome activation. In agreement with previous studies, NR5A2-LacZ exhibits a ubiquitous expression pattern during the pre-implantation period (Paré et al., 2004; Gu et al. 2005). However, our results demonstrate a decline in the level of NR5A2-LacZ expression from the 3.5 dpc to the 4.5 dpc blastocyst stage, suggesting that NR5A2 expression is down-regulated prior to implantation. It should be noted that the NR5A2 protein may not be present anymore at the late blastocyst stage but that residual β-galactosidase activity may still remain. Although NR5A2-LacZ is highly expressed during pre-implantation development in the mouse, targeted mutagenesis has demonstrated that its function is not required during this developmental period (Paré et al., 2004; Gu et al. 2005). It is possible that NR5A2 may be playing a role during pre-implantation development but that the presence of factors provided by the uterine environment might compensate for its embryonic loss at this stage. Since *NR5A2* transcripts are not present in oocytes, it is unlikely that

maternal contribution could compensate for *NR5A2* embryonic loss during early development (Falender et al., 2003; Paré et al., 2004).

Consistent with previous findings, our results show that NR5A2-LacZ is strongly expressed in the developing digestive system (Rausa et al., 1999; Annicotte et al., 2003; Paré et al., 2004). NR5A2-LacZ expression is not only detected in tissues of endodermal origins but displays a widespread and dynamic pattern of expression during the mid to late gestation period. For instance, during head formation, NR5A2-LacZ is expressed in various craniofacial areas including the branchial arches, the trigeminal ganglion, the orofacial prominences and developing jaw, the developing ear, the ocular muscles and the submandibular and thyroid glands. Many of these head structures such as the branchial arches, trigeminal ganglion, and the developing jaw and ear are derived, at least in part, from cranial neural crest cells in which *NR5A2* was shown to be expressed (Rausa et al., 1999). In addition to its craniofacial expression, NR5A2-LacZ is strongly expressed in the ventral region of the spinal cord and in the DRGs, suggesting that NR5A2 might be involved in nervous system development.

In the mouse, nervous system development begins with the formation of the neural plate, which arises from the dorsal ectoderm of the gastrulating embryo around 8.5 dpc (figure 6). The neural plate then folds to form the neural tube by 9.5 dpc. The most anterior portion of the neural tube will generate the brain, whereas the posterior neural tube will form

the spinal cord at the level of the trunk. Around the time of neural tube closure, neural crest cells are found on the dorsal side of the neural tube and migrate ventrally to eventually give rise to neurons and glial cells of the peripheral nervous system, including sensory neurons of the DRGs that form on either side of the neural tube. Around the same time, a variety of motor neurons and interneurons are generated along the dorso-ventral axis of the neural tube, leading to the formation of the spinal cord. Sonic hedgehog signaling is crucial for the induction of motor neurons in the ventral most region of the spinal cord and for the generation of several classes of ventral interneurons at positions dorsal to motor neurons. Conversely, BMP signaling promotes dorsal interneuron cell fates (Tanabe and Jessell, 1996; Jessell, 2000; Lee and Jessell, 1999). The specification of sensory and motor neurons along the dorso-ventral axis of the developing neural tube is important for the establishment of the sensory-motor nervous system involved in processing and integrating sensory information to generate an appropriate motor response.

To better define the expression pattern of NR5A2 in the developing nervous system, we performed a co-immunofluorescence analysis and demonstrated that NR5A2-LacZ is expressed in different types of spinal motor neurons as well as in the dorsal root ganglia. Our results show that NR5A2-LacZ is co-expressed with isl-1 in medial motor neurons of the MMC and LMC and with Lim1 in lateral motor neurons of the LMC at the forelimb level. NR5A2-LacZ expression also co-localized with that of isl-1

in the dorsal root ganglia, which arise from ventrally migrating trunk neural crest cells. Interestingly, NR5A2-LacZ expression is also detected in regions of the spinal cord that are negative for isl-1 and lim1 expression, suggesting that NR5A2 is expressed in additional spinal neuronal cell types. To address this possibility, it would be interesting to perform co-immunofluorescence studies with markers for different types of spinal interneurons.

The expression of NR5A2-LacZ in trunk neural crest cell derivatives (DRGs) and cranial neural crest cell derivatives in the developing head suggest that NR5A2 may be important for the development and differentiation of neural crest cells. Supporting this idea, NR5A2 was shown to be expressed during neural crest formation prior to neural tube closure (Rausa et al., 1999).

The expression pattern of NR5A2-LacZ in motor neurons of the central nervous system and sensory neurons (DRGs) of the peripheral nervous system during mouse embryogenesis suggests a potential role for NR5A2 in the formation of the sensory-motor nervous system. Of interest, the zebrafish NR5A2 nuclear receptor, ftz-f1a, is not only expressed in endoderm-derived organs but also in spinal neurons and in various head regions, including the trigeminal ganglion and mandibular arches, during development (Lin et al., 2000; Kuo et al., 2005). The conserved expression pattern of NR5A2 in the developing head and nervous system in different organisms further supports a requirement for NR5A2 function

in craniofacial development and neurogenesis. Interestingly, a recent study has established that hedgehog (hh) signalling positively regulates NR5A2 expression during skeletal muscle organization in zebrafish (Sheela et al., 2005). It is therefore possible that shh signaling could also activate NR5A2 expression in the developing neural tube to specify ventral motor neural cell fate.

Since the establishment of spinal neuronal subtype identity relies on the interplay between extracellular signals and cell-intrinsic programs, the identification of signals and transcription factors involved in this process is central to unravel the molecular mechanisms implicated in generating neuronal diversity in the spinal cord. In this study, we have used an expression analysis approach to demonstrate that NR5A2 might be an important transcription factor involved in the generation of spinal neuronal identity.

Acknowledgments

We would like to thank Dr. S. Stifani (Centre for Neuronal Survival, Montreal Neurological Institute, Montreal, Quebec, Canada) for helpful discussion and suggestions and for providing us with primary and secondary antibodies used for the co-immunofluorescence study. We would also like to thank Kevin Ebata for helpful discussion.

Figure 1. NR5A2-LacZ expression during pre-implantation development in the mouse

Pre-implantation-stage embryos obtained from *NR5A2* +/- matings were collected and stained for β-galactosidase activity. Top embryos are *NR5A2* +/- or *NR5A2* -/-, bottom embryos are *NR5A2* +/+. A) 2-cell stage embryos, B) morula-stage embryos, C) 3.5 dpc blastocysts, and D) 4.5 dpc blastocysts. ICM: inner cell mass, TE: trophectoderm

Figure 1. NR5A2-LacZ expression during pre-implantation development in the mouse

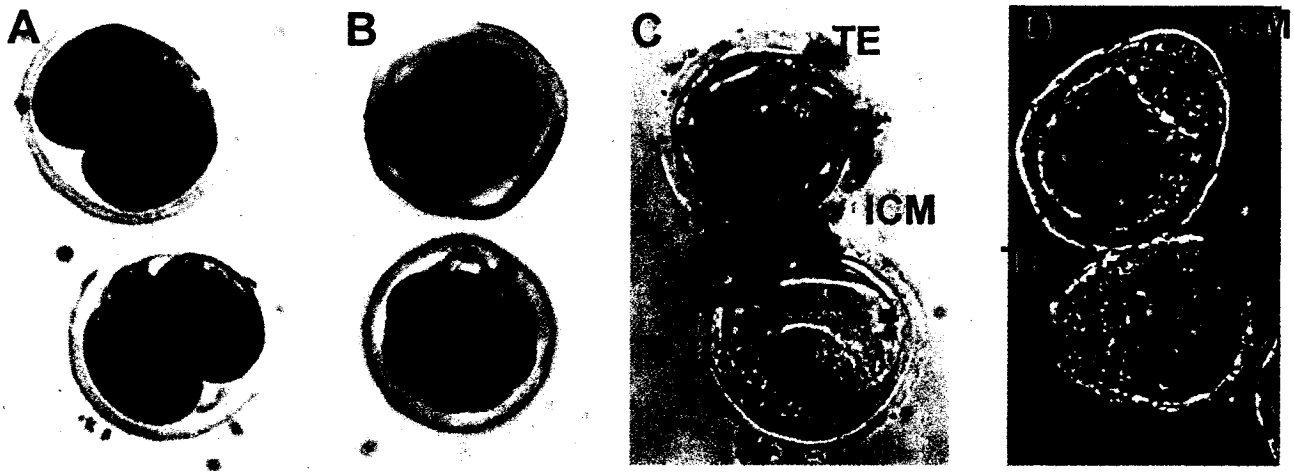


Figure 2. NR5A2-LacZ expression from 9.5 dpc to 15.5 dpc

Different stage embryos were obtained from *NR5A2* +/- crosses and stained for β-galactosidase activity. Lac-Z stained *NR5A2* +/- embryos at 9.5 dpc (A, side view), 11.5 dpc (B, side view and C, dorsal view), 13.5 dpc (D, side view), 14.5 dpc (E, frontal view), and 15.5 dpc (F and G, top view). Ba: branchial arches, drg: dorsal root ganglia, ee: embryonic endoderm, hpp: hepato-pancreatic primordium, l: liver, lm: lung mesenchyme, sc: spinal cord, ub: umbilical cord.

Figure 2. NR5A2-LacZ expression from 9.5 dpc to 15.5 dpc

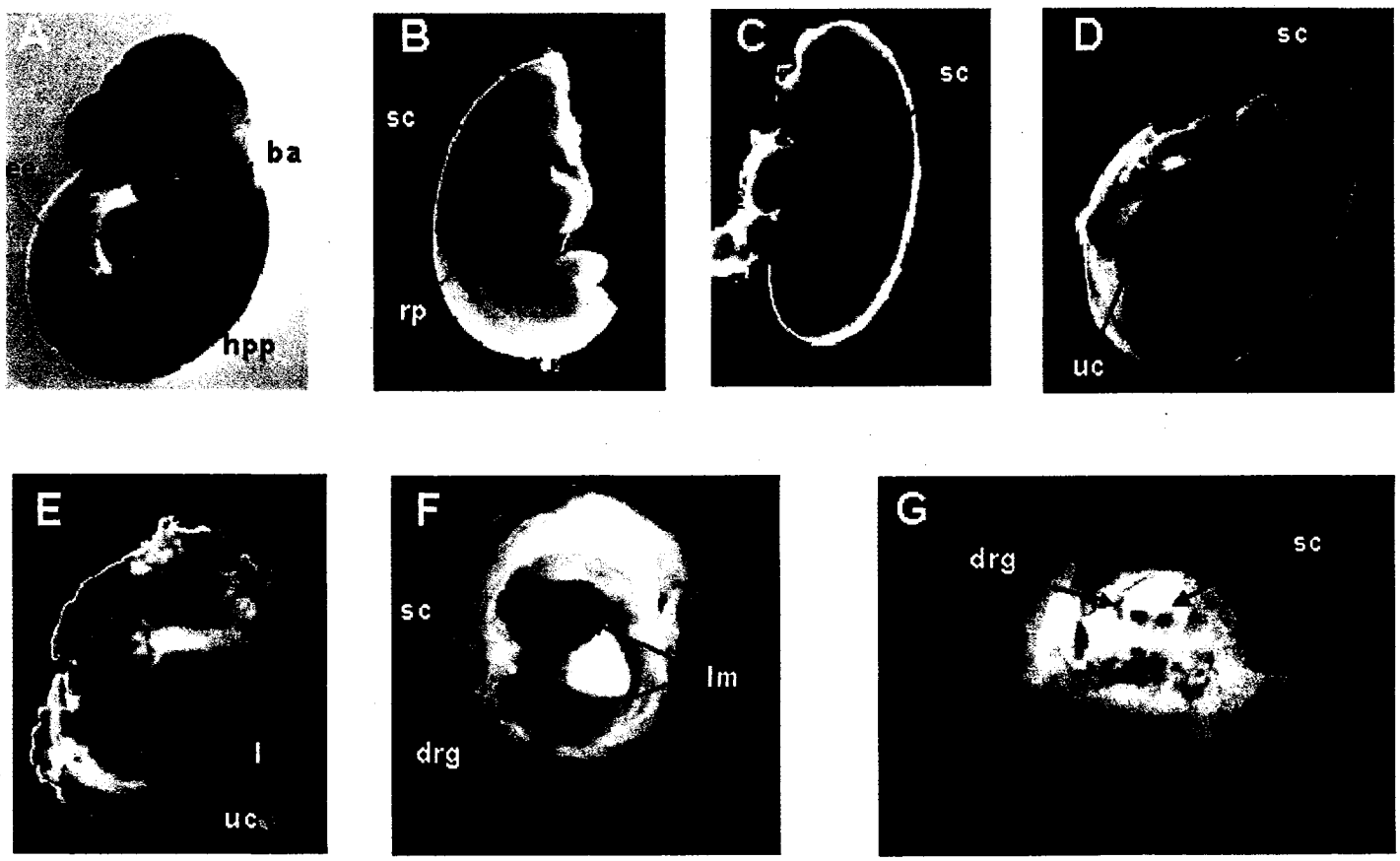


Figure 3. NR5A2-LacZ expression during cranio-facial development

Heads from *NR5A2* +/- embryos were isolated at different developmental stages and stained for β-galactosidase activity. Heads from LacZ stained *NR5A2* +/- embryos at 11.5 dpc (A), 13.5 dpc (B), 14.5 dpc (C), and 15.5 dpc (D). Drg: dorsal root ganglia, Mb: midbrain, mc: meckel's cartilage, om: ocular muscles, smg: submandibular gland, tg: thyroid gland, TgG: trigeminal ganglion, vsp: ventral spinal cord. The black circle demarcates the mandibular component of the first branchial arch.

Figure 3. NR5A2-LacZ expression during craniofacial development

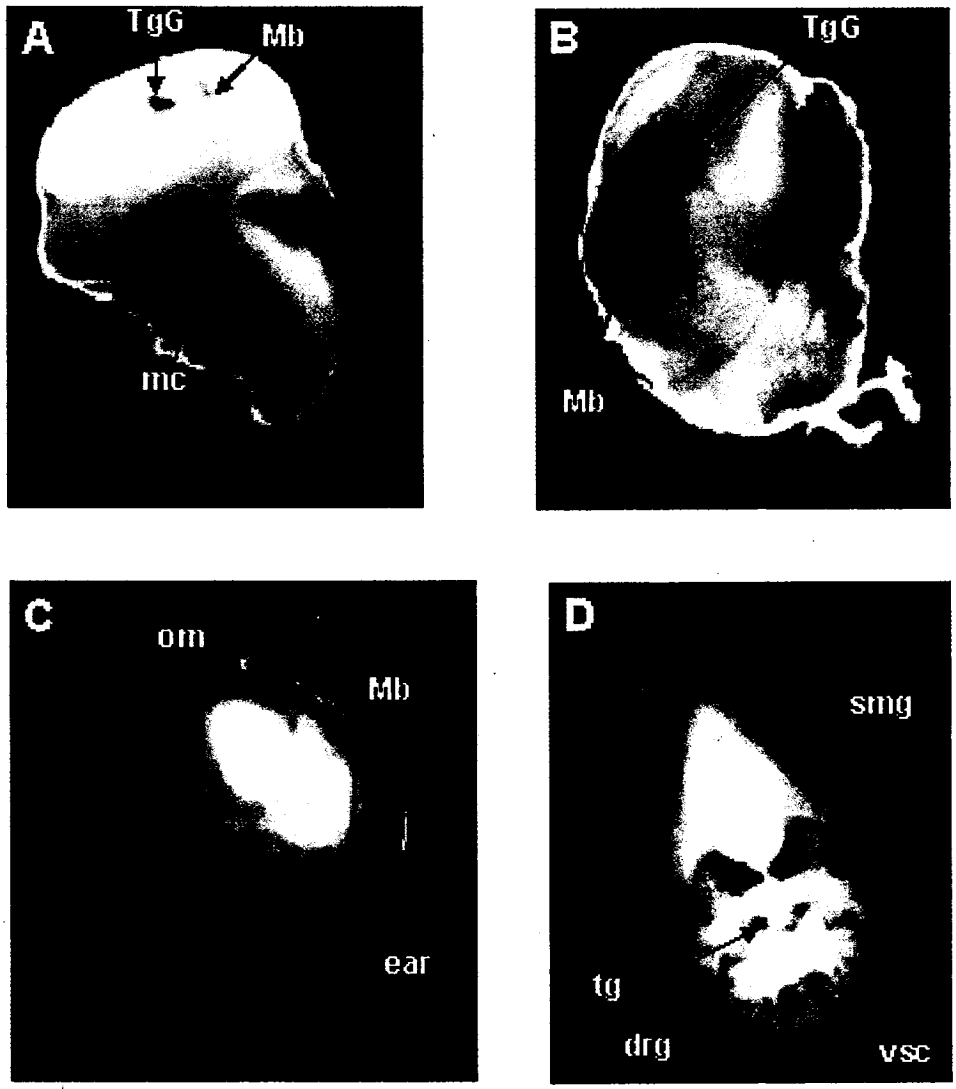


Figure 4. NR5A2 expression in the developing spinal cord

NR5A2 +/- embryos isolated at different developmental stages (10.5 dpc to 14.5 dpc) were stained for β-galactosidase activity and sectioned. Transverse sections were counterstained using nuclear fast red solution. All embryonic sections are presented with the dorsal side to the left and ventral side to the right. Transverse sections through the spinal cord of *NR5A2* +/- embryos at 10.5 dpc (A), 11.5 dpc (B), 12.5 dpc (C), 13.5 dpc (D). Transverse sections through the head region of *NR5A2* +/- embryos at 14.5 dpc (E, G, and H) and 13.5 dpc (F). Mb: midbrain, sc: spinal cord, smg: submandibular gland, tg: thyroid gland.

Figure 4. NR5A2 expression in the developing spinal cord

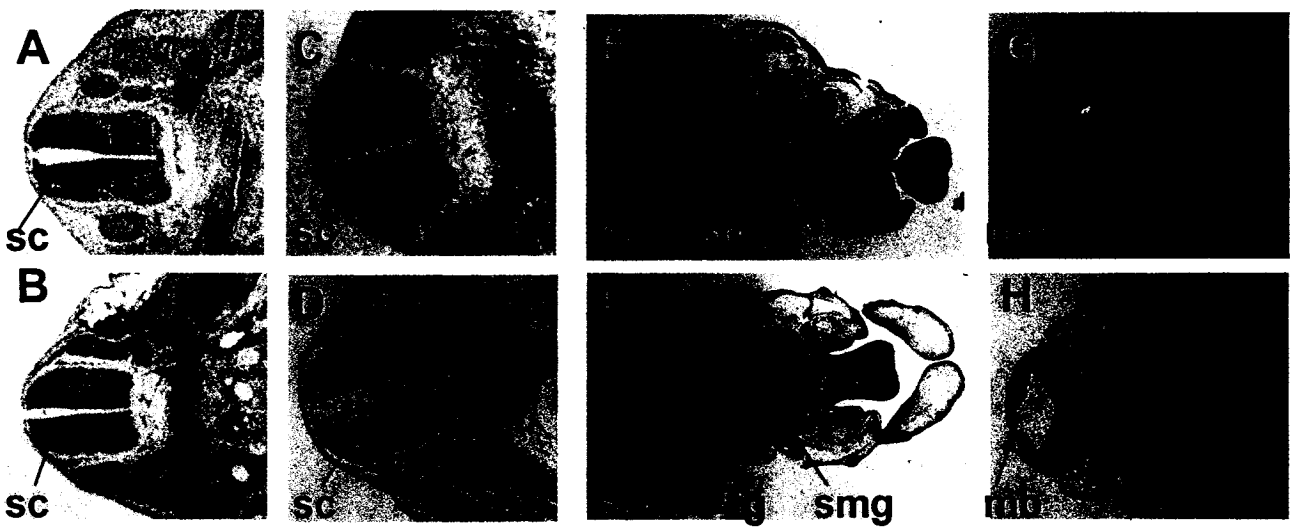


Figure 5. NR5A2 is expressed in motor neurons and dorsal root ganglia during nervous system development

Co-immunofluorescence experiments were performed on transverse cryosections from 13.5 dpc *NR5A2* +/- embryos at the level of the forelimbs with antibodies against β-galactosidase (green) and islet-1 (red) (A) and with antibodies against β-galactosidase (green) and Lim1-2 (red) (B). D: dorsal, drg: dorsal root ganglia, l: lateral, LMC: lateral motor column, m: medial, MMC: medial motor column, sc: spinal cord, V: ventral.

Figure 5. NR5A2 is expressed in motor neurons and dorsal root ganglia during nervous system development

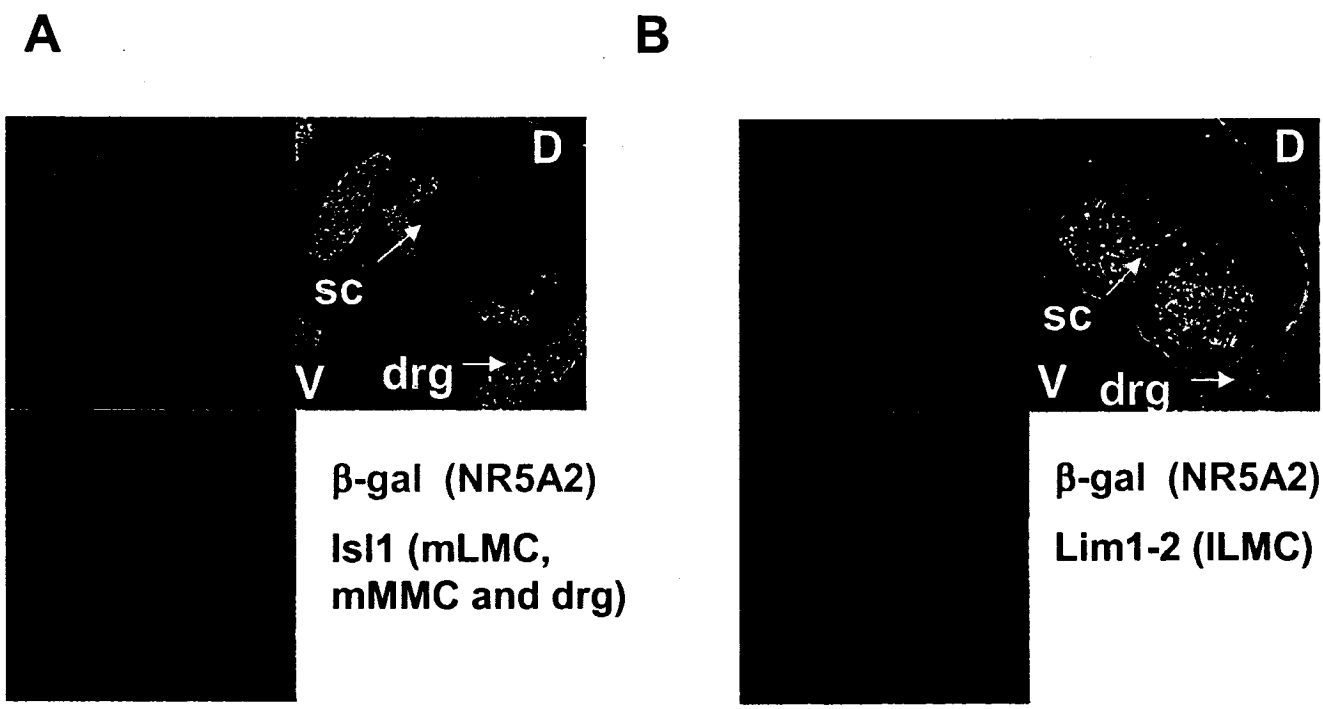
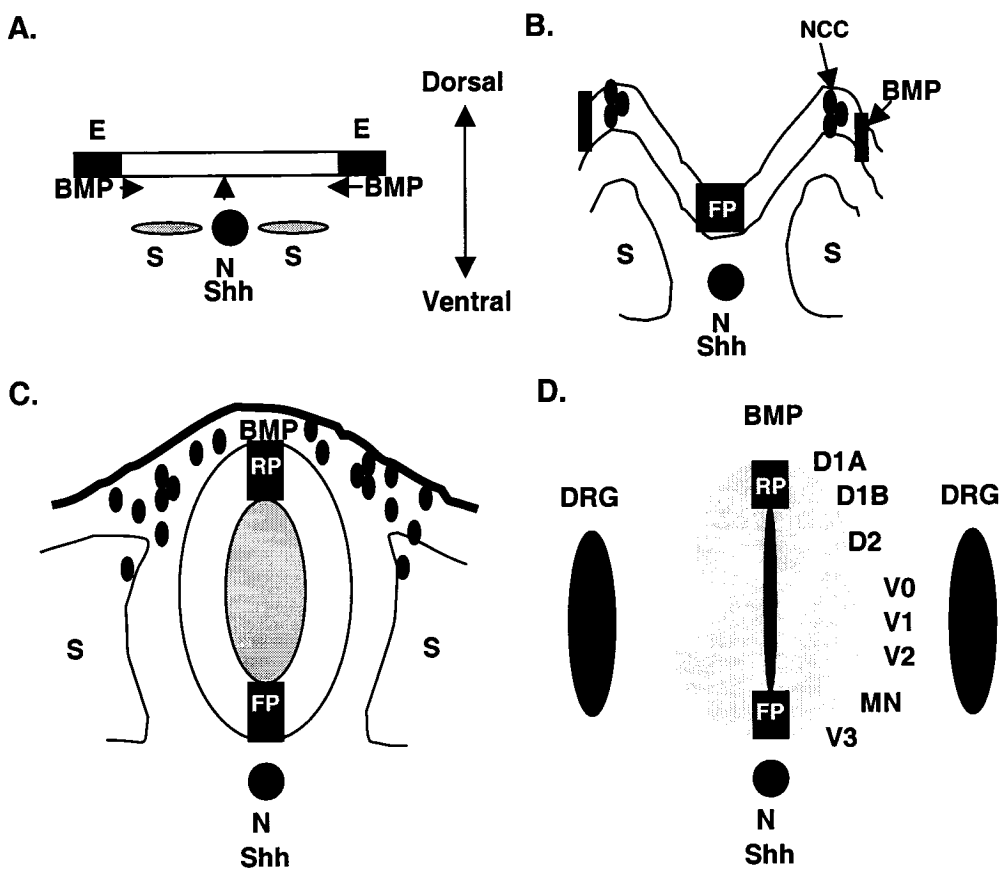


Figure 6- Early nervous system development

A) The spinal cord is derived from the neural plate, an epithelial layer overlying the notochord (N) and somitic mesoderm (S) and surrounded by epidermal ectoderm (E). The neural plate forms around 8.0-8.5 dpc in the mouse. Dorso-ventral patterning of the neural tube is controlled by sonic hedgehog (shh) signaling emanating from the notochord, which induces ventral cell fate, and by dorsalizing BMP signaling coming from the epidermal ectoderm. B) Around 8.5dpc-9.0dpc, which corresponds to neural fold stage, BMP signaling from the epidermal ectoderm promotes the formation of neural crest cells (NCC). At that stage, a specialized signaling centre, the floorplate, starts secreting dorsalizing shh signals required to promote spinal ventral cell fate C) At the time of neural tube closure, around 9.5dpc in the mouse, NCC emigrate dorsolaterally to form the melanocytes and ventromedially to give rise to neural and glial cells of the peripheral nervous system. At the same time, a specialized signaling center, the roof plate, is generated at the dorsal midline of the neural tube. Roof plate cells secrete BMP signals to induce spinal dorsal cell fate. At the time of neural tube closure, the concerted action of dorsalizing BMP signaling and ventralizing shh signaling pattern the neural tube along the dorso-ventral axis. D) During spinal cord development, around 10.5 dpc, different neuronal cell types are generated along the dorso-ventral axis. Dorsal interneurons (D1A, D1B, and D2) differentiate in the dorsal horn of the spinal cord, whereas motor neurons (MN) and ventral intereneurons (V0, V1, V2, V3) develop in the ventral half of the spinal cord. Dorsal root ganglia differentiate from neural crest cells progenitors and forms on either side of the spinal cord.

Figure 6. Early nervous system development



CHAPTER 5: GENERAL DISCUSSION

DISCUSSION

The generation of a new mammalian organism relies on a series of highly orchestrated events beginning with proper regulation of male and female reproductive functions. Then, fertilization must take place for embryonic development to begin. In placental mammals, an intimate dialogue between the developing embryo and the maternal uterus is required for embryogenesis to proceed. In turn, the process of embryogenesis must be carefully regulated in a spatio-temporal manner for the generation and organization of all the organs and tissues that will compose the body of the newly formed individual. The generation of offspring is therefore dependent on the proper regulation of events that take place at different levels along the reproductive-developmental axis. Successful reproductive functions and embryogenesis depends on the concerted action of a variety of extracellular and intrinsic factors. In this thesis, we have identified the nuclear receptor NR5A2 as playing major roles at different levels along this reproductive-developmental axis. More specifically, we have examined the involvement of NR5A2 in female reproductive function and during embryogenesis in the mouse.

5.1 Involvement of NR5A2 in female reproductive function, perspectives and implications.

In this thesis we have established, for the first time, that the transcription factor NR5A2 is important for ovarian function *in vivo*. Using *NR5A2* +/- mice, we have shown that heterozygosity for a null mutation of *NR5A2* leads to a subfertility phenotype in females, owing to a reduction in progesterone production. We also demonstrated that progesterone supplementation can rescue pregnancy loss in *NR5A2* +/- females, further supporting the involvement of NR5A2 in progesterone biosynthesis. Although NR5A2 is expressed in granulosa cells during follicular development, haploinsufficiency for *NR5A2* specifically results in altered corpus luteum function and subsequent pregnancy loss, whereas follicular development, estrogen production, ovulation, and initial corpus luteum formation are unaffected. The results presented in this study thus clearly identify a specific role for NR5A2 in corpus luteum function during pregnancy.

It is important to keep in mind, however, that these studies were done using *NR5A2* heterozygous mice, and that the presence of one functional allele may hinder a requirement for NR5A2 in other ovarian processes. For instance, it is possible that NR5A2 might also be involved in estrogen production during folliculogenesis, but that a single functional *NR5A2* allele is sufficient to perform this function. In light of recent studies, however, this is very unlikely since it was demonstrated that NR5A1,

rather than NR5A2, was the endogenous NR5A species binding to the *CYP19* promoter in granulosa cells (Falender et al., 2003) and that a granulosa cell-specific *NR5A1* knock out leads to sterility with an ovarian phenotype resembling that of *ERα* and *CYP19* knockouts (Jeyasuria et al., 2004).

In *NR5A2* +/- females, corpus luteum formation occurs normally and corpora lutea are present during the course of pregnancy, as well as in *NR5A2* +/- females experiencing pregnancy loss. *NR5A2* haploinsufficiency therefore appears to interfere specifically with corpus luteum function but not with corpus luteum development. A role for NR5A2 in corpus luteum formation *per se*, however, cannot be ruled out since the presence of only one functional *NR5A2* allele may be sufficient to induce corpus luteum formation even though its proper function necessitates a higher NR5A2 expression, especially during pregnancy when high levels of progesterone are required. To investigate this possibility, it would be noteworthy to generate an ovarian-specific *NR5A2* knock out. Loss of both *NR5A2* alleles in the ovary should lead to a more severe phenotype and could reveal additional roles for NR5A2 in female reproductive function. Given the importance of NR5A2 in ovarian progesterone production, its complete absence in the ovary should lead to a more pronounced reduction in progesterone synthesis compared to *NR5A2* +/- ovaries, and therefore to a more severe phenotype. Progesterone is known to modulate its own production and has been proposed to act as an

intracrine luteotropic factor that promotes luteal development and sustains luteal function during pregnancy (Stouffer, 2003). In rodents, progesterone has been shown to promote corpus luteum survival in the absence of its cognate receptor, underscoring the importance of the nongenomic action of this steroid hormone (Goyeneche et al., 2003). *NR5A2* ovarian deficiency could lead to defects in the initial luteinization process following the midcycle gonadotropin surge, resulting in a failure in corpus luteum formation. Alternatively, it is possible that the complete loss of ovarian *NR5A2* will only interfere with corpus luteum survival in response to mating-induced prolactin stimulation, leading to corpus luteum regression.

Although the generation of a conditional *NR5A2* ovarian knockout mouse will certainly help in further defining the implication of NR5A2 in ovarian function, the exact mechanisms regulating and mediating NR5A2 luteal function will remain elusive. Since *NR5A2* expression was shown to be positively regulated by prolactin, which is essential for corpus luteum rescue and maintenance in rodents, it would be interesting to determine if *NR5A2* is expressed in prolactin receptor deficient mice. A null mutation of the prolactin receptor gene leads to female infertility due to a complete implantation failure resulting from impaired ovarian progesterone production (Ormandy et al., 1997). It is therefore tempting to speculate that NR5A2 may be involved in prolactin-regulated progesterone production in the corpus luteum. Another potential regulator of *NR5A2* expression and/or activity in the corpus luteum is LH, which is essential for

corpus luteum formation and maintenance. LH agonist-induced signaling was shown to modulate *NR5A2* expression, raising the possibility that NR5A2 might play a role in LH-regulated progesterone production (Liu et al., 2003). It is also possible that LH could modulate NR5A2 activity by promoting its phosphorylation.

In this thesis, we have provided evidence that one mechanism by which NR5A2 regulates progesterone synthesis is through modulation of StAR expression. Although StAR plays a central role in progesterone production (Stocco, 2001), many other factors are required for proper luteal progesterone biosynthesis to occur. For instance, the presence of the two enzymes involved in progesterone synthesis, CYP11A1 and 3β–HSD, and the LHR are crucial for luteal progesterone production. Interestingly, NR5A2 was shown to be an activator of *CYP11A1* and *3β-HSD* expression, raising the possibility that factors other than StAR may also be involved in NR5A2-mediated progesterone synthesis (Sirianni et al., 2002; Kim et al., 2005; Peng et al., 2003). Another requirement for luteal steroidogenesis to take place is the availability of the cholesterol precursor. Interestingly, the expression of *SR-B1*, which is responsible for cholesterol uptake in luteal cells, was shown to be positively regulated by NR5A2, suggesting that SR-B1 might be another factor mediating NR5A2 luteal function (Schoonjans et al., 2002). In order to identify downstream targets of NR5A2 in the corpus luteum, it would be informative to perform microarray analyses to examine the differential gene expression pattern in

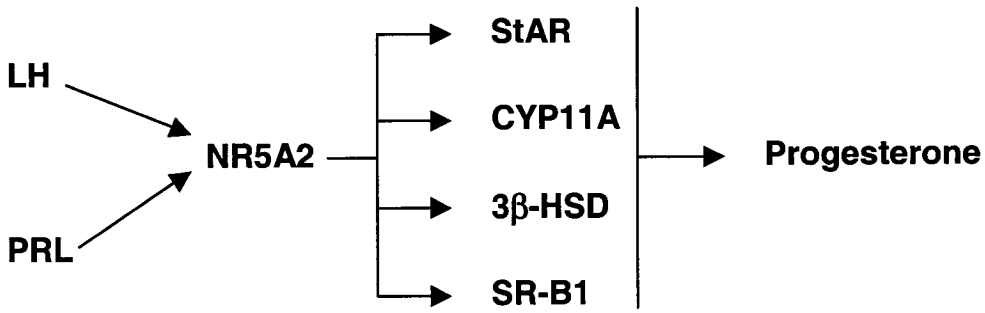
corpora lutea obtained from wild-type and *NR5A2* +/- females in response to hormonal treatment and during natural pregnancy. Figure 1 depicts a model for the role of NR5A2 in luteal function.

Infertility is a major problem worldwide and more than ¼ of clinical infertility cases are idiopathic in nature, owing to the poor understanding of the basic mechanisms regulating reproductive functions (Matzuk and Lamb, 2002). Since reproductive development and physiology are evolutionarily conserved across eutherian mammalian species, the development of genetic mouse models of infertility will certainly be beneficial for our understanding of human fertility. In this thesis, we used a mouse model haploinsufficient for *NR5A2* to demonstrate that this transcription factor is an important regulator of luteal function. NR5A2 is also expressed in human ovaries and was shown to regulate the expression of human genes involved in progesterone synthesis (Sirianni et al., 2002). It is therefore possible that impaired NR5A2 expression or function may be part of the molecular mechanisms underlying some clinical cases of female infertility. A better understanding of the role, regulation, and downstream targets of NR5A2 in the ovary will likely help deciphering the etiology of clinical reproductive dysfunctions and will potentially lead to the development of therapeutic tools for assisted reproductive technologies.

Figure 1. Model for the role of NR5A2 in the corpus luteum

This figure illustrates the potential regulators and mediators of NR5A2-induced progesterone production in the corpus luteum. All the factors present in this diagram have been shown to regulate or to be activated by NR5A2 *in vitro* or *in vivo*.

Figure 1. Model for the role of NR5A2 in the corpus lutem



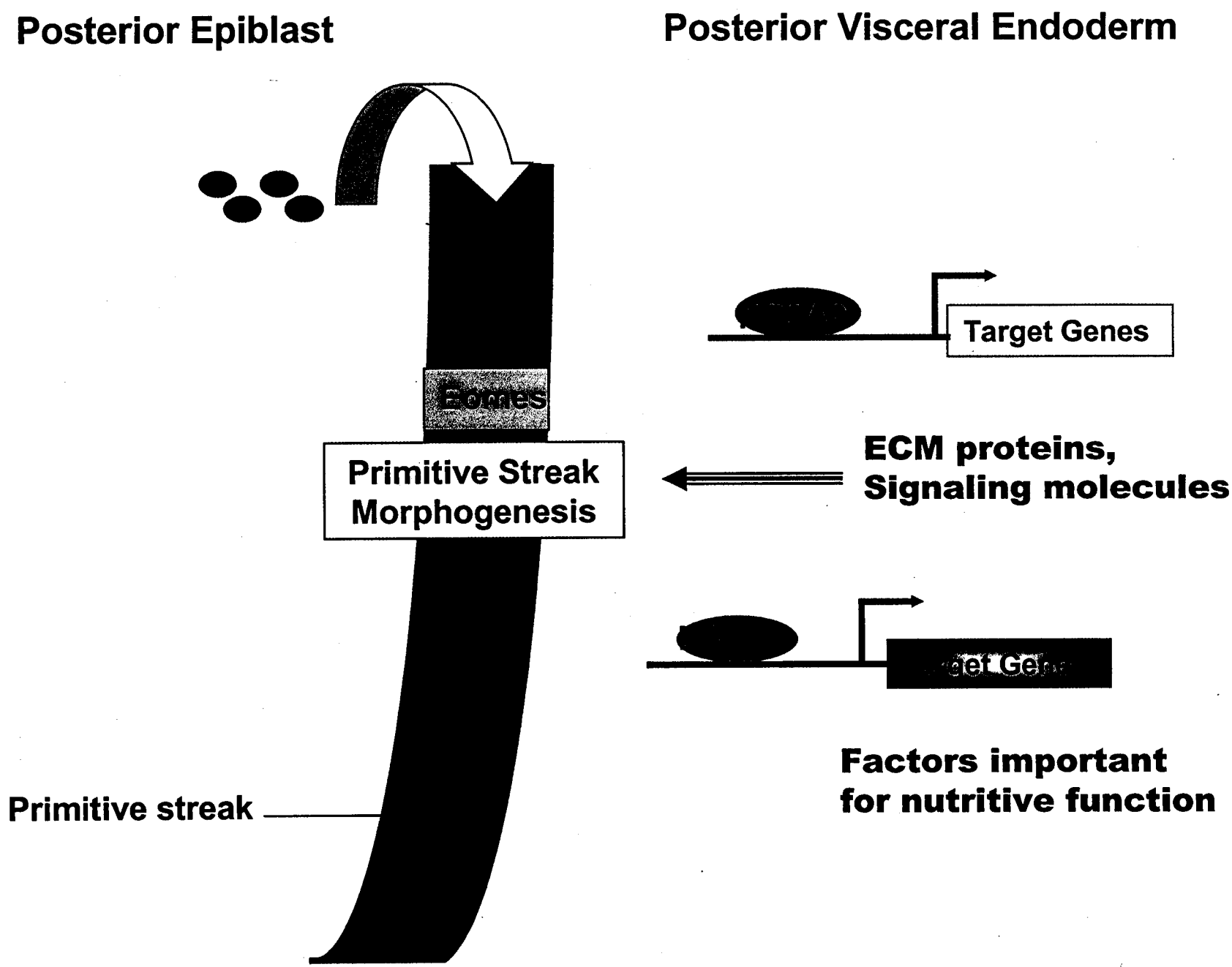
5.2 The role of NR5A2 in early mouse embryogenesis, perspectives and implications.

In this thesis, we performed a marker gene analysis on *NR5A2* -/- embryos to better characterize the role of NR5A2 in early embryogenesis. We have shown that NR5A2 is specifically required for proper development of the primitive streak, which marks the prospective posterior side of the embryo, and subsequent mesoderm formation. Using a tetraploid chimeric approach we also demonstrated a requirement for NR5A2 function in the VE, an extra-embryonic tissue, during the gastrulation period. Although the role of the VE in anterior patterning of the epiblast is well established, much less is known about its role in posterior patterning. In this study, we provide evidence supporting a role for the VE in posterior patterning of the epiblast since the loss of NR5A2 function in this tissue leads to posterior, but not anterior, patterning defects. Of particular interest, NR5A2 function in the VE appears to be of critical importance for the expression of the early mesodermal marker *Eomes* in the posterior epiblast. Further supporting a role for NR5A2 in the induction of *Eomes* expression in the epiblast, *NR5A2* -/- embryos and embryos lacking epiblastic *Eomes* display a similar phenotype consisting of an abnormal posterior thickening with morphological signs of EMT, impaired primitive streak morphogenesis, and no emergence of mesoderm (Russ et al., 2000). The identity of the NR5A2 targets promoting *Eomes* expression and proper primitive streak morphogenesis in the underlying epiblast are

unknown. Potential downstream NR5A2 targets are likely to encode extracellular signaling molecules, cell surface molecules and/or extracellular matrix proteins. For instance, embryos bearing mutations in the FGF or Wnt signaling pathways display specific posterior defects leading to early embryonic lethality. It is therefore tempting to speculate that NR5A2 may influence FGF and/or Wnt signaling. Another possibility is that NR5A2 might regulate the expression of extracellular matrix molecules or cell surface molecules present on visceral endodermal cells to promote cell migration and morphogenesis of the primitive streak.

In addition to its involvement in VE patterning activity, our results have also implicated NR5A2 in mediating the VE nutritive function during early mouse embryogenesis. During early embryonic development, the VE fulfills endodermal functions and is responsible for metabolism and nutrient uptake and transport (Bielinska et al., 1999). We have shown that, in addition to the defects in primitive streak formation, the expression of two genes involved in VE nutritive function, *HNF4 and GATA4*, is significantly reduced in *NR5A2* -/- embryos. This led us to propose a model in which NR5A2 plays a dual role in the VE during gastrulation, being involved in both the nutritive and patterning functions of this tissue (see Figure 2). Since the VE recapitulates the gene expression profile and function of endoderm-derived organs, the implication of NR5A2 in regulating endodermal gene expression in this tissue *in vivo* also supports the idea that NR5A2 acts as a key player in the development of

Figure 2. Model for the role of NR5A2 during gastrulation



endoderm-derived organs (Rausa et al., 1999; Paré et al., 2001; Annicotte et al., 2003).

As mentioned in the third chapter of this thesis, the tissue requirement for NR5A2 function during gastrulation was evaluated using a specific tetraploid aggregation approach. Usually, tetraploid chimeric analysis involves the aggregation of embryonic stem (ES) cells of a given genotype with tetraploid embryo of another genotype, taking advantage of the developmental bias of both ES and tetraploid cells. Whereas ES cells preferentially colonize the epiblast and its derivative (Beddington and Robertson, 1989), tetraploid cells predominantly populate extra-embryonic tissues (Nagy et al., 1990; Nagy et al., 1993). In our case, we were unable to generate *NR5A2* -/- ES cells. We have re-targeted *NR5A2* +/- ES cells, which are resistant to G418, using a similar *NR5A2* targeting vector containing a hygromycin-resistant cassette but were unable to establish G418/hygromycin double resistant ES cell lines. We therefore had to aggregate wild-type tetraploid embryos with diploid embryos derived from *NR5A2* +/- crosses to demonstrate that the presence of wild-type extra-embryonic tissues can rescue the early embryonic lethality of *NR5A2* -/- embryos (Appendix I). Unfortunately, this tetraploid aggregation approach is much more exhaustive than the usual ES cell- tetraploid embryo aggregation strategy and does not allow the production of a large number of embryos that would unable us to examine the requirement of NR5A2 during later developmental processes.

The inability to establish *NR5A2* -/- ES cell lines by re-targeting *NR5A2* +/- ES cells could result from a requirement for NR5A2 in ES cell proliferation, maintenance or survival in culture. NR5A2 is highly expressed in ES cells (Galarneau et al., 1996; Paré et al., 2004; Gu et al., 2005) and was shown to be involved in the regulation of proliferation of different cell types *in vitro* and *in vivo* (Botrugno et al., 2004; Wang et al. 2005; Annicotte et al., 2005; Schoonjans et al., 2005). It is possible that a single *NR5A2* allele is sufficient for ES cell proliferation and maintenance in culture but that the complete absence of NR5A2 leads to a blockade in ES cell proliferation, thereby preventing their maintenance in culture. This would explain why we were able to isolate ES cell clones resistant to both G418 and hygromycin, and therefore presumably *NR5A2* -/-, but were unsuccessful in maintaining them in culture. To further investigate the role of NR5A2 in ES cell proliferation, it would be interesting to determine if the expression of genes coding for factors involved in the regulation of the cell cycle are reduced in *NR5A2* +/- ES cells compared to their wild-type counterparts.

5.3 Expression of NR5A2 during craniofacial and neural development, perspectives and implications.

The inability to produce a large number of *NR5A2* -/- chimeric embryos rescued from the early embryonic lethality prevented further assessment of later developmental roles for NR5A2. The LacZ knock-in

approach used to inactivate the *NR5A2* gene, however, allowed us to better define the expression pattern of NR5A2 during mouse embryogenesis and to infer on its potential function post-gastrulation. By following the NR5A2-LacZ reporter activity, we demonstrated that NR5A2 is expressed during craniofacial and neural development. Interestingly, NR5A2 was shown to be expressed in neural crest cells prior to neural tube closure (Rausa et al., 1999). The results presented in this thesis indicate that NR5A2 is also expressed in cranial and trunk neural crest cell derivatives, notably in the trigeminal ganglia, the developing jaw, and the DRGs. In addition, we showed that NR5A2 is expressed in different types of spinal motor neurons from the time they form in the ventral spinal cord. This suggests that NR5A2 may be important for the establishment of pan motor neuron identity. The fact that NR5A2 expression in the developing head and spinal cord is evolutionarily conserved further supports a role for this nuclear receptor in craniofacial and neural development (Lin et al. 2000; Kuo et al., 2005).

Homeodomain transcription factors are of central importance for the specification and diversification of motor neuron identity in the ventral spinal cord (Jessell, 2000; Price and Briscoe, 2004). Interestingly, nuclear receptors of the NR5A subfamily are known to interact with and to be regulated by homeodomain proteins (Yu et al., 1997; Guichet et al., 1997; Liu et al., 2003; Annicotte et al., 2003), raising the possibility that NR5A2 expression and/or activity may be regulated by homeodomain proteins to

dictate spinal motor neuron cell fate. To examine this idea, it would be interesting to determine if NR5A2 can physically interact with homeodomain proteins implicated in specification or diversification of spinal motor neurons. For instance, NR5A2 expression co-localizes with that of islet-1 and lim-1 homeodomain transcription factors in motor neurons, suggesting that they might interact together to modulate gene expression and to influence motor neuron identity. On the other hand, it is possible that NR5A2 acts downstream or upstream of these homeodomain proteins to specify cell fate.

A recent study has established that hedgehog signaling positively regulates NR5A2 expression during skeletal muscle organization in zebrafish (Sheela et al., 2005). Since Shh signaling is essential for the specification of ventral cell fate in the spinal cord, it is tempting to speculate that shh signaling could also activate NR5A2 expression in the neural tube to specify ventral motor neural cell fate. It would be interesting to examine if NR5A2 expression is present in the spinal cord of *shh* -/- embryos and if shh can alter *NR5A2* expression *in vitro*.

Our results clearly demonstrate that NR5A2 is highly expressed in the DRGs. It is not clear, however, if NR5A2 is expressed in all or subsets of sensory neurons. It would be informative to evaluate if NR5A2 co-localizes with markers specific for proprioceptive and nociceptive sensory neurons present in the DRGs. Moreover, since NR5A2 expression is found in neural crest cells, it would be interesting to determine if *NR5A2*

expression is modulated by BMP signaling given that BMP signaling emanating from the epidermal ectoderm is required to induce the formation of neural crest cells.

The expression of NR5A2 in both motor and sensory neurons suggests the involvement of NR5A2 in the development of the sensory-motor nervous system. The early lethality resulting from *NR5A2* inactivation, however, precludes further assessment of NR5A2 function in neural development. The generation of a *NR5A2* conditional knock out mouse could help circumvent this problem. If NR5A2 is involved in the development and/or function of the sensory-motor nervous system, it is possible that *NR5A2* +/- mice may have mildly altered neurological functions. It would be interesting to address this possibility by performing neuro-behavioral studies aimed at identifying sensory and /or motor deficits.

5.4 Final remarks

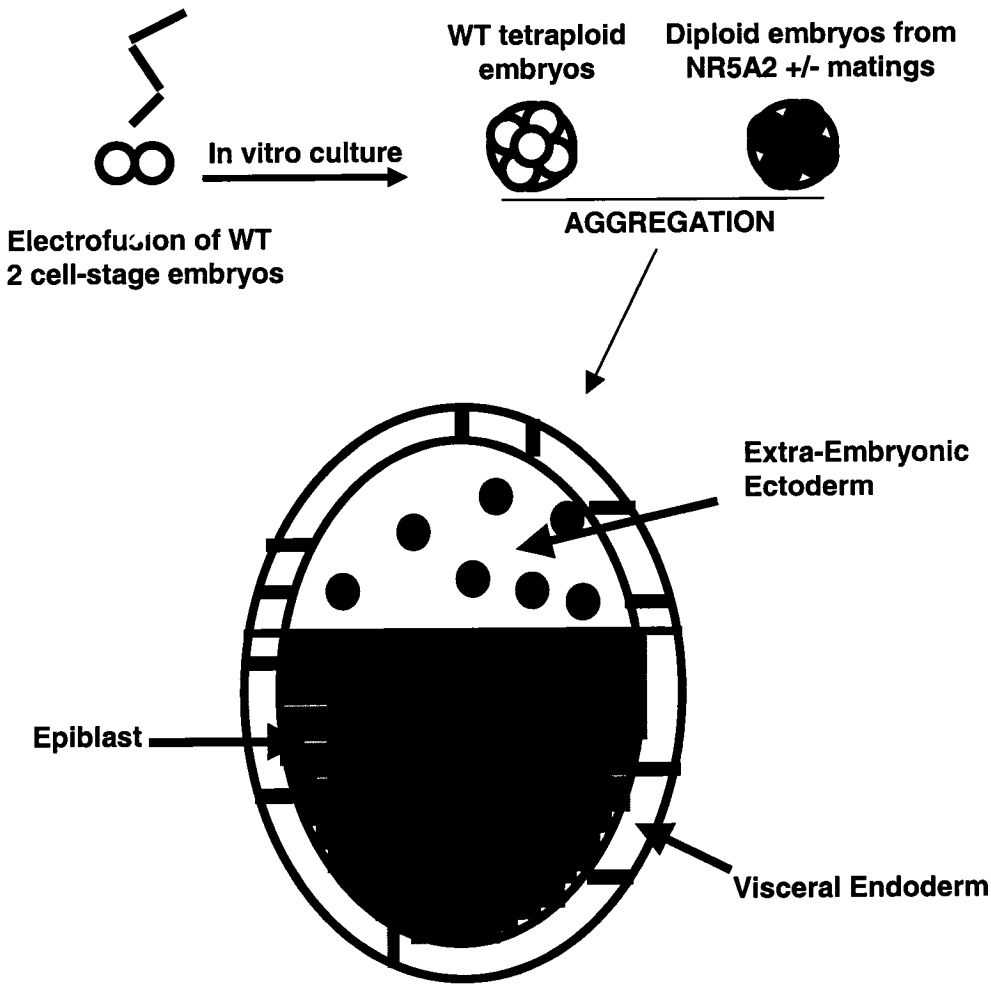
The nuclear receptor NR5A2 has been shown to play important roles in a variety of biological processes including cholesterol homeostasis, development, cell proliferation and tumorigenesis. Collectively, the studies presented in this thesis led to the identification of additional roles for NR5A2 and underscore the importance of this transcription factor in various developmental and reproductive processes in the mouse. The involvement of NR5A2 in this wide spectrum of

physiological functions makes this nuclear receptor an appealing therapeutic target. A better understanding of the roles, mechanisms of action and regulation of this nuclear receptor has potential clinical implications, for instance in assisted reproductive technology and cancer.

Appendix I: Generation of tetraploid chimeras

To evaluate the tissue requirement for NR5A2, a tetraploid aggregation analysis was used. Wild-type tetraploid embryos were generated by electrofusion of 2 cell-stage embryos and allowed to develop *in vitro* until they reached the morulae stage. They were then aggregated with morulae stage embryos obtained from *NR5A2* +/- matings. Aggregated chimeric embryos were then cultured *in vitro* until they reached the blastocyst stage and then transferred in the uterus of pseudopregnant females. In the resulting chimeric embryos the epiblast and its derivatives will be mainly derived from diploid cells derived from embryos obtained from *NR5A2* +/- crosses, whereas both diploid and wild-type tetraploid cells will compose the extra-emrbyonic tissues.

Appendix I: Generation of tetraploid aggregation chimeras



CHAPTER 6: REFERENCES

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