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ROLE OF THE ORPHAN NUCLEAR RECEPTOR NR5A2 IN OVARIAN FUNCTION

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Résumé

Le récepteur nucléaire orphelin NR5A2, également connu sous le nom de liver receptor homolog 1 (LRH-1), est exprimé dans l'ovaire, spécifiquement au niveau des cellules de granulosa. L'activation et le développement des follicules ovariens sont caractérisés par la multiplication des cellules de granulosa, des follicules primordiaux et leur unique rang de cellules de prégranulosa, aux follicules préovulatoires avec de nombreuses couches de cellules de granulosa cuboïdales. NR5A2 est un acteur bien connu de la régulation de la prolifération cellulaire dans des organes comme l'intestin et des pathologies comme le cancer du sein. L'hypothèse explorée est que NR5A2 régule le développement folliculaire en agissant sur la prolifération des cellules de granulosa et la régulation de l'activation des follicules primordiaux de la réserve ovarienne. Pour étudier ce facteur de transcription, un modèle de souris transgéniques caractérisées par la déplétion conditionnelle de NR5A2 dans les cellules de granulosa de tous les follicules (génotype *Nr5a2*$^{f/f}$; *Amhr2*$^{Cre+}$) a été développé. Les souris femelles mutantes, désignées comme cKO (pour *conditional knock-out*) sont infertiles suite à l'absence d'ovulation.

La première partie de l'étude a mis en évidence, et ce aussi bien *in vivo* qu'*in vitro*, une déplétion significative du nombre de cellules en phase S, phase du cycle cellulaire au cours de laquelle a lieu la réplication de l'ADN. Cette diminution du nombre de cellules en division s'accompagne d'une déplétion significative de l'expression de gènes essentiels pour la prolifération des cellules de granulosa, parmi lesquels les cyclines D et E. De plus, démonstration a été faite que la régulation de la multiplication cellulaire passait en partie par l'interaction de NR5A2 avec β-caténine et que l'impact négatif de la déplétion de NR5A2 sur

la prolifération des cellules de granulosa ne pouvait être secouru par l'adjonction d'estrogènes, un puissant mitogène des cellules ovariennes. Pour ce qui est du mécanisme, il a été déterminé que la régulation négative de CDKN1A, un inhibiteur du cycle cellulaire, est interropue en cas de déplétion de NR5A2. Ce gène a de ce fait été identifié comme un des principaux médiateurs de l'abrogation de la prolifération des cellules de granulosa gonadotropine-dépendante observée dans les souris cKO.

La seconde partie de l'étude s'intéresse à l'impact de la déplétion de NR5A2 sur la population de follicules primordiaux. Dans ce même modèle murin, la déplétion de NR5A2 engendre la multiplication des follicules primordiaux et coincide avec la surexpression de marqueurs liés à leur formation et à la survie cellulaire. Ces changements s'accompagnent également de la diminution de la quantité de transcrits codant pour des gènes favorisant l'activation folliculaire. Dans des souris de type sauvage, l'analyse de la localisation de NR5A2 par immunocytochimie a confirmé l'existence de deux sous-populations de follicules primordiaux, l'une exprimant NR5A2 dans les cellules de granulosa est considérée comme prête à s'activer, l'autre caractérisée par l'absence de ce même facteur de transcription est constituée de follicules primordiaux quiescents. Chez les souris cKO une augmentation significative dans l'abondance de la quantité de transcrits de gènes favorisant la quiescence a aussi été établie, ce qui plaide en faveur du concept des deux populations de follicules primordiaux. En conclusion, NR5A2 joue un rôle dans la formation des follicules primordiaux, leur survie et leur activation.

Pris dans leur ensemble, ces résultats démontrent que le récepteur nucléaire orphelin NR5A2 est un des régulateurs clé des évènements pré et peri-ovulatoires en agissant en particulier sur l'activation des follicules primordiaux et la prolifération des cellules de granulosa.

Mots-clés : NR5A2, cellules de granulosa, prolifération, follicules primordiaux, réserve ovarienne, souris transgéniques, déplétion conditionnelle.

Abstract

The orphan nuclear receptor NR5A2, also known as liver receptor homolog 1 (LRH1), is expressed in the ovary, specifically in granulosa cells. Activation and development of ovarian follicles are characterized by the multiplication of granulosa cells, from primordial with only one layer of pregranulosa cells, to pre-ovulatory stage with multiples layers of cuboidal granulosa cells. NR5A2 is a well known actor in regulation of cell proliferation in organs such as the intestine and in conditions such as breast cancer. The hypothesis that NR5A2 regulates follicular development by acting on granulosa cells proliferation and the regulation of the primordial follicle pool activation was tested. To study this transcription factor, a knock-out mouse model characterized by the conditional depletion of NR5A2 in granulosa cells of all follicles (genotype *Nr5a2*$^{f/f}$, *Amrh2*$^{Cre/+}$) was developed. These female mutant mice, designated as cKO (conditional knock-out), were infertile due to the absence of ovulation.

The first part of the study highlighted, *in vivo* and *in vitro*, a significant decrease of the number of cells reaching the S phase, where the DNA is replicated. This decrease in the dividing cells was accompanied by significant depletion of genes essential for granulosa cell proliferation, the cyclins D and E among others. Moreover, an effect of the depletion of NR5A2 on granulosa cell proliferation, via its interaction with β-catenin, was demonstrated and proliferation could not be rescued by the ovarian mitogen, estrogen. In terms of the mechanism, it was shown that the expected negative regulation of CDKN1A, a cell cycle inhibitor, did not occur following the depletion of NR5A2. This gene was therefore identified as the main mediator of the abrogation of gonadotropin dependent granulosa cell proliferation observed in the cKO mice.

The impact of NR5A2 depletion on primordial follicles population was next studied. In this mouse genotype, there was a several fold increase in the number of primordial follicles, with concomitant upregulation of markers linked to their formation and cell survival. These changes were accompanied by a decrease in the transcripts coding for genes linked to follicle activation. In wild type mice, quantitative analysis by immunocytochemistry confirmed that there are two subpopulations of primordial follicles, one where NR5A2 is expressed in granulosa cells and one where it is absent, the former was judged to be poised for activation, while the latter was quiescent. The cKO mice are characterized by a significant increase in transcript abundance of genes promoting quiescence, support for this concept. We conclude that Nr5a2 plays a role in primordial follicle formation, survival and activation.

Taken together these results demonstrate that the orphan nuclear receptor NR5A2 plays a key role in pre and peri-ovulatory events, acting particularly on primordial follicles activation and granulosa cells proliferation

Keywords : NR5A2, granulosa cells, proliferation, primordial follicle, ovarian reserve, conditional knockout mouse

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List of abbreviations

3βHSD	3 β Steroid Dehydrogenase
3Cl-AHPC	Adamantyl Hydroxyphenol Chlorocinnamic Acid
ABCA	ATP Binding Cassette
ABCG2	ATP Binding Cassette Subfamily G Member 2
ABCG5	ATP Binding Cassette Subfamily G Member 5
ABCG7	ATP Binding Cassette Subfamily G Member 7
ABCG8	ATP Binding Cassette Subfamily G Member 8
ACTH	Adenocorticotrophic Hormone
ADAMST1	ADAM Metallopeptidase With Thrombospondin Type 1 Motif 1
AF-2	Activation Function-2 Motif
AGT	Angiotensinogen
AHR	Aryl Hydrocarbon Receptor
AKR1C18	Aldo-Keto Reductase Family 1 Member C18
ALAS1	5-Aminolevulinic Acid Synthase 1
αGSU	α Glycoprotein Subunit
AMH	Anti-Mullerian Hormone
AMHR2	Anti-Mullerian Hormone Receptor Type 2
ANGPT1	Angiopoietin 1
AP-1	Activator Protein 1
APOA1	Apolipoprotein A1
AREG	Amphiregulin

ASPM	Abnormal Spindle Microtubule Assembly
ATP	Adenosine Triphosphate
BAD	BCL2 Associated Agonist Of Cell Death
BAX	BCL2 Associated X, Apoptosis Regulator
BCL2	B-cell Lymphoma 2
BCL2L1	Bcl-2-Like 1
BMP2	Bone Morphogenetic Protein 2
BMPR1B	Bone Morphogenetic Protein Receptor type-1B
BRCA2	Breast Cancer 2
BrdU	5-Bromo-2´-Deoxyuridine
BSA	Bovine Serum Albumin
BTC	Betacellulin
C57BL/6	C57 Black 6
CAK	CDK-Activating Kinase
cAMP	Cyclic Adenosine Monophosphate
CASC5	KNL1, Kinetochore scaffold 1
CBP	CREB Binding Protein
CBX2	Chromobox homolog 2
CCND1	Cyclin D1
CCND2	Cyclin D2
CCNE1	Cyclin E1
CCNE2	Cyclin E2
CD28	Cluster of Differentiation 28

CD7	Cluster of Differentiation 7
CDH2	Cadherin-2
CDK2	Cyclin dependent kinase 2
CDK7	Cyclin dependent kinase 7
CDKN1A	Cyclin Dependent Kinase Inhibitor 1A
CDKN1B	Cyclin Dependent Kinase Inhibitor 1B
CH25H	Cholesterol 25-Hydroxylase
ChIP	Chromatin Immunoprecipitation
cKO	Conditional knock-out
CL	Corpus Luteum
cMyc	MYC Proto-Oncogene, BHLH Transcription Factor
CNS	Central Nervous System
CON	Control
COUP-TF	Chicken-ovalbumin upstream-transcription factor
CREB	cAMP response element-binding protein
CREB1	cAMP Responsive Element Binding Protein 1
CRH	Corticotropin releasing hormone
CXCL1	C-X-C Motif Chemokine Ligand 1
CXCL10	C-X-C Motif Chemokine Ligand 10
CYCH	Cyclin H
CYP11A1	Cytochrome P450 Family 11 Subfamily A Member 1
CYP11B1	Cytochrome P450 Family 11 Subfamily B Member 1
CYP17	Cytochrome P450 Family 17

CYP17A1	Cytochrome P450 Family 17 Subfamily A Member 1
CYP19A1	Cytochrome P450 Family 19 Subfamily A Member 1
CYP51	Cytochrome P450 Family 51
CYB5R3	Cytochrome B5 Reductase 3
DAPI	4',6-Diamidino-2-Phenylindole
DAX1	Dosage-sensitive sex reversal, Adrenal hypoplasia critical region, on chromosome X, gene 1
DAZL	Deleted In Azoospermia Like
DBD - C domain	DNA Binding Domain
DDX4	DEAD-Box Helicase 4
DHCR24	24-Dehydrocholesterol Reductase
DHCR7	7-Dehydrocholesterol Reductase
DHH	Desert Hedgehog
DLPC	1,2-Dilauroyl-SN-glycero-3-phosphocholine
DMRT1	Doublesex And Mab-3 Related Transcription Factor 1
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribose Nucleic Acid
DNMT3A	DNA Methyltransferase 3 α
DPC	Day post coitum
E	Embryonic day
E2F1	E2F Transcription Factor 1
E2F2	E2F Transcription Factor 2
E2F4	E2F Transcription Factor 4

E2F5	E2F Transcription Factor 5
EBP	Emopamil binding protein
eCG	Equine chorionic gonadotropin
EDN2	Endothelin 2
EDN8	Endothelin 8
EGR1	Early Growth Response 1
ERα	Estrogen receptor α
EREG	Epiregulin
ERK	Extracellular signal-regulated kinases
ES	Embryonic stem cells
ESR1	Estrogen Receptor 1
ESR2	Estrogen Receptor 2
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum
FBXO5	F-Box Protein 5
FDFT1	Farnesyl-Diphosphate Farnesyltransferase 1
FDPS	Farnesyl Diphosphate Synthase
FDR	False discovery rate
FDX1	Ferredoxin 1
FIGLA	Factor in the Germline α
FMN2	Formin 2
FOS	Fos Proto-Oncogene, AP-1 Transcription Factor Subunit
FOXD3	Forkhead Box D3

FOXA1	Forkhead Box A1
FOXL2	Forkhead Box L2
FOXO3	Forkhead Box O3
FSH	Follicle Stimulating Hormone
FSHβ	Follicle Stimulating Hormone β subunit
FTZ	Fushi Tarazu
FTZ-F1	Fushi Tarazu Factor 1
FXR	Farnesoid X receptor
FZD4	Frizzled Class Receptor 4
GATA4	GATA Binding Protein 4
GCNF	Germ Cell Nuclear Factor
GDF9	Growth Differentiation Factor 9
GJA1	Gap Junction Protein α 1
GnRH	Gonadotropin-Releasing Hormone
GSTM1	Glutathione S-Transferase Mu 1
H12	Helix 12
H2	Helix 2
HBEGF	Heparin Binding EGF Like Growth Factor
HDAC3	Histone Deacetylase 3
HDL	High Density Lipoprotein
HEXB	Hexosaminidase Subunit β
HLH	Helix Loop Helix
HMG-CoA	3-Hydroxy-3-Methyl-Glutaryl-Coenzyme A reductase

HNF	Hepatocyte Nuclear Factor
HOXA10	Homeobox A10
HSC	Hematopoietic Stem Cell
HSD17B1	Hydroxysteroid 17-β Dehydrogenase 1
HSD17B11	Hydroxysteroid 17- β Dehydrogenase 11
HSD17 B12	Hydroxysteroid 17- β Dehydrogenase 12
HSD17B7	Hydroxysteroid 17- β Dehydrogenase 7
HSD3 B1	Hydroxy-δ-5-Steroid Dehydrogenase, 3 β - And Steroid δ -Isomerase 1
HSD3B2	Hydroxy- δ -5-Steroid Dehydrogenase, 3 β - And Steroid δ -Isomerase 2
iCRT3	2-[[[2-(4-ethylphenyl)-5-methyl-4-oxazolyl]methyl]thio]-N-(2-phenylethyl)acetamide
IDI1	Isopentenyl-Diphosphate δ Isomerase 1
IGF1	Insulin Like Growth Factor 1
IGFR1	Insulin Like Growth Factor 1 Receptor
IGFs	Insulin Like Growth Factor
IgG	Immunoglobulin G
IHH	Indian Hedgehog
IMMP2L	Inner Mitochondrial Membrane Peptidase Subunit 2
INHA	Inhibin Subunit α
INHBA	Inhibin Subunit β A
INHBB	Inhibin Subunit β B
IU	International Unit
KDR	Kinase Insert Domain Receptor

KEGG	Kyoto Encyclopedia of Genes and Genomes
KGN	KGN ovarian granulosa-like tumor cell line
KI67	Marker Of Proliferation Ki-67
KISS1	Kisspeptin 1
KITL	Proto-Oncogene Receptor Tyrosine Kinase (KIT) ligand
LBD - E domain	Ligand-Binding Domain
LDL	Low Density Lipoprotein
LDLR	Low Density Lipoprotein Receptor
LH	Luteinizing Hormone
LH β	Luteinizing Hormone β subunit
LHCGR	Luteinizing Hormone Choriogonadotropin Receptor
LHX9	Lim Homeobox protein
LIPE	Lipase E
LRH-1	Liver Receptor Homolog 1
LRP8	LDL Receptor Related Protein 8
LSS	Lanosterol Synthase
MAD2L2	Mitotic Arrest Deficient 2 Like 2
MAPK	Mitogen-Activated Protein Kinase
MAT1	Ménage à trois 1
MBF1	Multiprotein-Bridging Factor 1
MCL1	Induced Myeloid Leukemia Cell Differentiation protein
MDC1	Mediator Of DNA Damage Checkpoint 1
MECP2	Methyl-CpG Binding Protein 2

mIR-027b-3p	MicroRNA 027b-3p
miR1275	MicroRNA 1275
miR134	MicroRNA 134
miR30d	MicroRNA 30d
miR320	MicroRNA 320
miR383	MicroRNA 383
MIS	Mullerian Inhibiting Substance
MMP2	Matrix Metallopeptidase 2
MMP9	Matrix Metallopeptidase 9
mRNA	Messenger RNA
MVD	Mevalonate Diphosphate Decarboxylase
MVK	Mevalonate Kinase
NCOR	Nuclear Receptor Corepressor
NCOR1	Nuclear Receptor Corepressor 1
NCOR2	Nuclear Receptor Corepressor 2
NCS1	Neuronal Calcium Sensor 1
NFY	Nuclear transcription factor Y
NKX	NKX-homeodomain factors
nNOS	Neuronal Nitric Oxide Synthase
NOS3	Nitric Oxide Synthase 3
NOTCH2	Neurogenic locus Notch homolog protein 2
NPC1	NPC Intracellular Cholesterol Transporter 1
NPC1L1	NPC1 Like Intracellular Cholesterol Transporter 1

NR0B1	Nuclear Receptor Subfamily 0 Group B member 1 (also known as DAX1)
NR0B2	Nuclear Receptor Subfamily 0 Group B member 2 (also known as SHP)
NR1H4	Nuclear Receptor Subfamily 1 Group H member 4 (also known as FXR)
NR5A1	Nuclear Receptor Subfamily 5 Group A Member 1 (also known as SF1)
NR5A2	Nuclear Receptor Subfamily 5 Group A Member 2 (also known as LRH-1)
NR6A1	Nuclear Receptor Subfamily 6 Group A Member 1 (also known as GCNF)
NRIP1	Nuclear Receptor Interacting Protein 1
NSDHL	NAD(P) Dependent Steroid Dehydrogenase-Like
OCT4	Octamer-binding Transcription factor 4
P21	Cyclin-dependent kinase inhibitor 1 or CDK-interacting protein
PAF	Paraformaldehyde
PAQR8	Progestin and AdipoQ Receptor family member 8
PBS	Phosphate-Buffered Saline
PBST	Phosphate-Buffered Saline Tween
PBX1	PBX Homeobox 1
PCNA	Proliferating Cell Nuclear Antigen
PDGFRA	Platelet Derived Growth Factor Receptor α
PDX1	Pancreatic And Duodenal Homeobox 1
PGC1	Peroxisome proliferator-activated receptor-γ (PPAR-g)-Coactivator-1
PGC1α	Peroxisome proliferator-activated receptor γ Coactivator 1-α
PGC1β	Peroxisome proliferator-activated receptor γ Coactivator 1-β
PGE2	Prostaglandin E2
PGR	Progesterone Receptor

PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PKA	Protein Kinase A
PLA2G4A	Phospholipase A2 Group IVA
PLAGL1	PLAG1 Like Zinc Finger 1
PND	Postnatal Day
POD1	TCF21, Transcription Factor 21
POU5F1	POU Class 5 Homeobox 1
PPARD	Peroxisome Proliferator-Activated Receptor δ
PPAR γ	Peroxisome Proliferator-Activated Receptor γ
PPP2R5A	Protein Phosphatase 2 Regulatory Subunit 5 α
PRKAA2	Protein Kinase AMP-Activated Catalytic Subunit α 2
PRKAR2B	Protein Kinase CAMP-Dependent Type II Regulatory Subunit β
PROX1	Prospero-Related Homeobox Transcription Factor 1
PTEN	Phosphatase and TENsin homolog
PTGS2	Prostaglandin-Endoperoxide Synthase 2
PTS	6-Pyruvoyltetrahydropterin Synthase
qPCR	Quantitative Polymerase Chain Reaction
RBL1	RB Transcriptional Corepressor Like 1
REC8	REC8 Meiotic Recombination Protein
RNA	Ribonucleic acid
RPS6KA2	Ribosomal Protein S6 Kinase A2
RXR	Retinoid X Receptor
S203	Serine 203

S238	Serine 238
S243	Serine 243
SC5D	Sterol-C5-Desaturase
SCARB1	Scavenger Receptor Class B Member 1
SCP2	Sterol Carrier Protein 2
SE	Standard Error
SF1	Steroidogenic Factor 1
SHB	SH2 Domain Containing Adaptor Protein B
SHP	Small Heterodimer Partner
SHRNA	Short Hairpin RNA
SIGMAR1	Sigma Non-Opioid Intracellular Receptor 1
siRNA	Small interfering RNA
SIRT1	Sirtuin 1
SMAD3	Mothers against decapentaplegic homolog 3
SMRT	Silencing Mediator for Retinoic acid and Thyroid hormone receptor
SNAI2	Snail Family Transcriptional Repressor 2
SOAT	sterol O acyltransferase
SOAT1	sterol O acyltransferase 1
SOX2	SRY-Box 2
SOX9	SRY-Box 9
SP1	Specificity Protein 1
SP3	Specificity Protein 3
SRC1	Steroid Receptor Co-activator 1

SRC2	Steroid Receptor Co-activator 2
SRC3	Steroid Receptor Co-activator 3
SREBP	Sterol Regulatory Element-Binding Protein
SREBP1	Sterol Regulatory Element-Binding Protein 1
SREBP1A	Sterol Regulatory Element-Binding Protein 1A
SREBP1C	Sterol Regulatory Element-Binding Protein 1C
SREBP2	Sterol Regulatory Element-Binding Protein 2
STAR	Steroidogenic Acute Regulatory protein
STAT5A	Signal Transducer And Activator Of Transcription 5A
STAT5B	Signal Transducer And Activator Of Transcription 5B
SUMO	Small Ubiquitin-like Modifier
TCF4	Transcription Factor 4
TET	Tet Methylcytosine Dioxygenase
TGFβ3	Transforming Growth Factor β 3
TM7SF2	Transmembrane 7 Superfamily Member 2
TNFAIP6	Tumor Necrosis Factor stimulated gene-6
TSPO	Translocator Protein
USF1	Upstream Transcription Factor 1
USF2	Upstream Transcription Factor 2
VEGFA	Vascular Endothelial Growth Factor A
VMH	Ventromedial nucleus of the Hypothalamus
WNT1	Wnt Family Member 1
WNT4	Wnt Family Member 4

WNT5	Wnt Family Member 5
WT	Wild type
ZFP36	Zinc Finger Protein 36

To Bruce D. Murphy, for all the (nice) things I did not say.

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I always wondered why everyone is so cheesy and melodramatic in this part of the thesis, and as one of my friends told me « There aren't that many synonyms for « Thank you », specially if you write it in English, so keep it brief ». I am going to make my life easier and thank none other than myself so no one will be upset for having been forgotten[1]. In the end, this is my work[2], my results[3], that I obtained and analyzed by myself with my little neurons and my little hands[4]. I must say that I am quite happy with the result[5,6].

---

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[5] Is it really my role to judge that? Probably not. So I would like to thank the members of my committee Dr André Tremblay, Dr Christopher Price and Dr Nicolas Gévry and the members of the jury that accepted to read, correct and judge this thesis Dr Lawrence Smith, Dr Gustavo Zamberlam, Dr Jay Baltz, and Dr Greg Fitzharris.

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Introduction

Ovulation, or the expulsion of an oocyte by the ovary of the female mammals, is a complex phenomenon, regulated by time-specific signals from the oocyte, ovarian somatic cells and extra-ovarian endocrine sources (2). The mammalian female gonads are composed of follicles at different stages of development that evolve from the pool of primordial follicles, considered as the cornerstone of female fertility as it determines the longevity and the quality of female reproduction. Despite the emergence of key markers such as AMH, the processes by which this ovarian reserve is established and primordial follicles irreversibly enter the growing pool remain mainly unknown (1, 6). The primordial follicles are characterized by a single layer of a dozen or so flattened pre-granulosa cells. Following activation, the granulosa cells undergo multiple rounds of replication with a consequent follicular population of $4.0x10^7$ cells in the preovulatory bovine follicle (7, 8) making proliferation of the somatic component encased in the follicle one of the characteristics of follicular development. It is well known that regulation of granulosa cell proliferation from secondary follicles forward is dependent on the follicle stimulating hormone (FSH), that FSH targets proliferation related genes such as proliferating cell nuclear antigen (PCNA) and also up-regulates NR5A2 (3).

NR5A2 is a nuclear receptor, an intracellular protein, acting as a transcription factor. Although it possesses the classic nuclear receptor domain structure, it lacks identified signaling ligands and so is considered an orphan nuclear receptor (9). Constitutively active, it plays a role in major biological processes including development, cell growth, homeostasis, reproduction and embryogenesis. It is expressed in tissues of endodermal origin such as the liver and the intestine but also the gonads, in both the testis and the ovaries (5). In the female gonad, it is

specifically expressed in granulosa cells of all stages of developing follicles, as well as corpora lutea (4). Mutant mouse models have been widely used in research to understand the role of specific genes in diverse tissues, and, based on our previous findings with *Nr5a2*$^{f/f}$; *Amhr2*$^{Cre+}$ mice, we know that NR5A2 is a key regulator of the ovulatory process. Indeed, *Nr5a2*$^{f/f}$; *Amhr2*$^{Cre+}$ conditional knock-out mice, characterized by the depletion of NR5A2 in granulosa cells of all follicles, are infertile due to failure of ovulation from multiple causes (4). Because NR5A2 is expressed in the ovary of several species, it is a potential gene of interest in the study of the fertility of both animals and humans.

The overall hypothesis addressed in this thesis is that NR5A2 is an indispensable regulator of the granulosa cell growth and differentiation that characterize the development of the ovarian follicle. The first objective of this study was to show that NR5A2 is a major regulator of follicular development by acting on follicular growth via granulosa cell proliferation. The second objective aimed at demonstrating that NR5A2 regulates the ovarian reserve through primordial follicle formation, survival and activation.

CHAPTER 1: Literature review

THE ORPHAN NUCLEAR RECEPTORS STEROIDOGENIC FACTOR-1 AND LIVER RECEPTOR HOMOLOG-1: STRUCTURE, REGULATION AND ESSENTIAL ROLES IN MAMMALIAN REPRODUCTION

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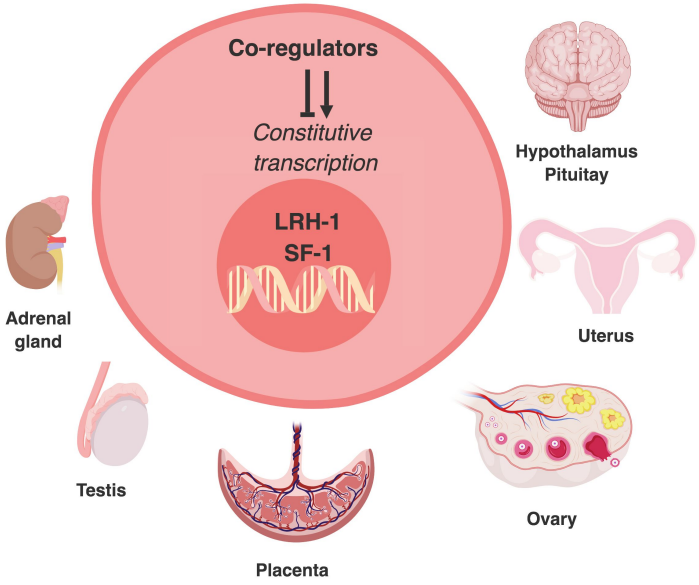
Abstract

Nuclear receptors are intracellular proteins that act as transcription factors. Proteins with classic nuclear receptor domain structure lacking identified signaling ligands are designated orphan nuclear receptors. Two of these, steroidogenic factor-1 (NR5A1 aka SF-1) and liver receptor homolog-1 (NR5A2: aka LRH-1), bind to the same DNA sequences, with different and non-overlapping effects on targets. Endogenous regulation of both is achieved predominantly by cofactor interactions. SF-1 is expressed primarily in steroidogenic tissues, LRH-1 in tissues of endodermal origin and the gonads. Both receptors modulate cholesterol homeostasis, steroidogenesis, tissue-specific cell proliferation and stem cell pluripotency. LRH-1 is essential for development beyond gastrulation, SF-1 for genesis of the adrenal, sexual differentiation and Leydig cell function. Ovary-specific depletion of SF-1 disrupts follicle development, while LRH-1 depletion prevents ovulation, cumulus expansion and luteinization. Uterine depletion of LRH-1 compromises decidualization and pregnancy. In humans, SF-1 is present in endometriotic tissue, where it regulates estrogen synthesis. SF-1 is underexpressed in ovarian cancer cells and overexpressed in Leydig cell tumors. In breast cancer cells, proliferation, migration and invasion, and chemotherapy resistance are regulated by LRH-1. In conclusion, the NR5A orphan nuclear receptors are non-redundant factors that are crucial regulators of a panoply of biological processes, across multiple reproductive tissues

Graphical abstract



Clinical relevance

The literature relevant to two orphan nuclear receptors, LRH-1 and SF-1, has been reviewed with focus on the effects of these two factors on reproductive processes in mammals. The absence of LRH-1 results in early embryo death, while the absence of SF-1 causes adrenal agenesis. SF-1 mutations in male humans result in adrenal dysgenesis and phenotypic female genitalia due to regression the Wolffian ducts and persistence of the Müllerian anlage. SF-1 expression is found in endometriotic tissue, where it plays a role in ectopic estrogen synthesis. Misregulation of both SF-1 and LRH-1 occurs in ovarian cancers, and LRH-1 is implicated in human pancreatic cancers. LRH-1 also promotes proliferation, metastasis and invasion of breast cancer cells. LRH-1 antagonists may therefore serve as therapeutic modalities for cancer treatment.

1. Introduction

A fascinating narrative of the last century describes discovery of the many mechanisms of cell signaling. From these investigations, we have learned much about how extra- and intracellular messages are received, transduced and translated within their target cells. A commonality of cell signaling is the presence of protein receptors that can be found integrated into the plasma membrane, in the cytoplasm, or within the nucleus. The signals that provoke cellular responses have been shown to display a wide diversity in structure, but most transduction systems fall into a few broadly defined categories. Protein signals usually interact with membrane receptors to induce cascades of intracellular modifications that drive cell functions. In contrast, lipid signaling differs from protein messaging, in that the messenger is believed to diffuse freely through membranes. Among lipid signals are the steroids, a class of derived lipids, that have evolved with signaling pathways that employ a category of proteins displaying structural similarity, known as the nuclear receptor superfamily (Figure 1-1). In mammals, this extensive superfamily is composed of approximately 50 functional members, with 48 genes identified in the human genome, 49 in mice and 47 in rats (322). In addition to steroids, members of the superfamily transduce signals as diverse as the thyroid hormones, retinoic acid, vitamin D and bile acids. The unique characteristic of nuclear receptors, relative to other signaling modalities, is their capacity to bind directly to DNA, and thereby regulate transcriptional events, evoking a wide diversity of physiological actions. For this reason, nuclear receptors have been defined as a group of gene-specific transcription factors. The superfamily is subdivided into seven subfamilies (N0– N6), with three classes (I-III) based on the multiple similarities and differences that exist in their structure and DNA-binding characteristics (76).

Historically, the discovery of new hormones was achieved by analysis of their effects on physiological or developmental processes, and the purified hormone was subsequently used to identify its cognate receptor (255). By this means, the steroid receptor family (class I) was discovered, including the progesterone, estrogen, glucocorticoid, androgen and mineralocorticoid receptors; as was thyroid/retinoid family (class II), including the thyroid receptor and vitamin D receptor. Proteins that recapitulated the nuclear receptor domain structure, but for which no ligand was known, were designated as orphan nuclear receptors, and they compose the third family of nuclear receptors (class III). Recognition of their existence introduced a new era in endocrinology, in which the process of ligand-receptor discovery was inverted (71), where the orphan receptors were cloned and were then used to search for previously unknown ligands (255). By this means the ligand for the orphan RXR receptor, 9-cis-retinoic acid, was discovered, thereby allowing its new classification as an adopted nuclear receptor (71) (Figure 1-1).

Figure 1- 1 Classification of the 48 human nuclear receptors

Based on their discovery due to a known ligand, orphan receptors for which a ligand has been identified (adopted receptors) and true orphan receptors for which the ligand, if present, remains unknown. SF-1 and LRH-1, the subject of this review, are highlighted in red. (Compiled from (12, 14, 64, 74, 84, 104, 105, 140, 193, 237, 288))

Many of the members of the nuclear receptor superfamily, when liganded, exert regulatory effects on mammalian reproductive processes. Some are clearly direct, best exemplified by the estrogen and progesterone receptors, while the effects of others, such as the thyroid (63) and retinoic acid receptors (92) on reproduction are both direct (91) and indirect (8).

The two orphan receptors treated herein are found in the NR5A family: NR5A1 (common name: steroidogenic factor-1 or SF-1) and NR5A2 (common name: liver receptor homolog-1 or LRH-1). They have been shown to be essential and significant regulators of reproductive processes. In spite of their closely related structures, these two nuclear receptors display differing and often non-overlapping effects, in particular, on reproductive target tissues. This is remarkable, in that they bind to the same, or highly similar response element in the genes they regulate (Figure 1-2) (52), and both are often expressed in the same cells and tissues (Figure 1-3).

This review is an attempt to shed light on the multiple similarities and differences between these two receptors, in structure, in signaling and in their roles in the mammalian reproductive system. We refer to these two receptors as SF-1 and LRH-1 throughout this treatise.

Figure 1- 2 Consensus DNA sequence to which both SF-1 and LRH-1 have been shown to bind on target genes.

The image is derived from the JASPAR open access database of non-redundant transcription factors, release 7 (http://jaspar.genereg.net).

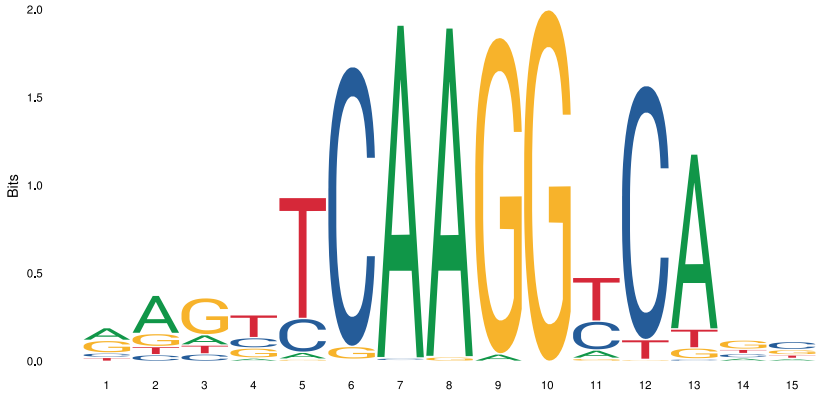
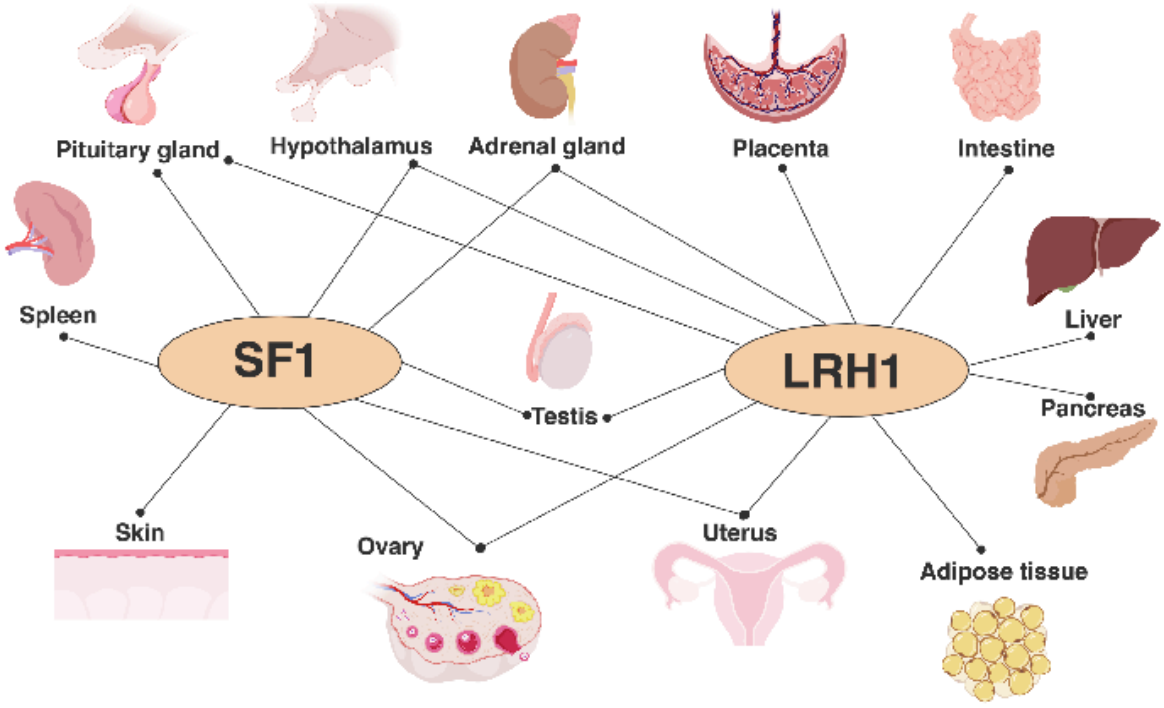


Figure 1- 3 Tissue distribution of the NR5A receptors in mammals

LRH-1 expression exclusively in the digestive tract and glands, SF-1 expression specific to the spleen and skin and overlap of the expression of the two receptors that occurs, principally in endocrine and neural tissue.



2. Discovery

During the last decade of the 20th century, the initial evidence of a gene responsible for the transcriptional activation necessary for the proper expression of the fushi tarazu (*ftz*) gene was described in *Drosophila* (278). *Ftz* is a member of the pair-rule class of genes governing *Drosophila* embryo segmentation, and the purified sequence-specific DNA-binding factor was then called Ftz-f1 (108). The embryonic long terminal repeat-binding protein in the mouse, now known as SF-1, proved to be a mammalian homolog of Ftz-f1 (151).

Some twenty years ago, the late Keith Parker reviewed the literature relative to SF-1, therein identifying it as a key determinant of the endocrine function at various levels within the hypothalamic-pituitary-gonadal axis (211). He noted that this transcription factor was detected in tissues known to express the cytochrome P450 steroid hydroxylases, genes regulating steroid hormone biosynthesis, hence its name. New *in vivo* data for SF-1 were becoming available at that time, showing that it is highly expressed in steroidogenic tissues and that it plays an essential role in activating the expression of various steroidogenic enzymes, thereby regulating adrenal and gonadal formation, as well as sex determination and differentiation (176, 211, 234). Early studies also showed that SF-1 functioned as a factor in the development of hypothalamic control of pituitary function (116). Due to its constitutive activity, and the *in silico* predictions of secondary structure suggesting ligand-independent active conformation, SF-1 was initially identified as an orphan nuclear factor (62).

The homology of Ftz-f1 with LRH-1 was recognized when the sequence of the latter was determined (234, 252). LRH-1 plays a major role in multiple processes, and was identified early as a regulator of intracellular cholesterol homeostasis (reviewed in (76)). Its roles in

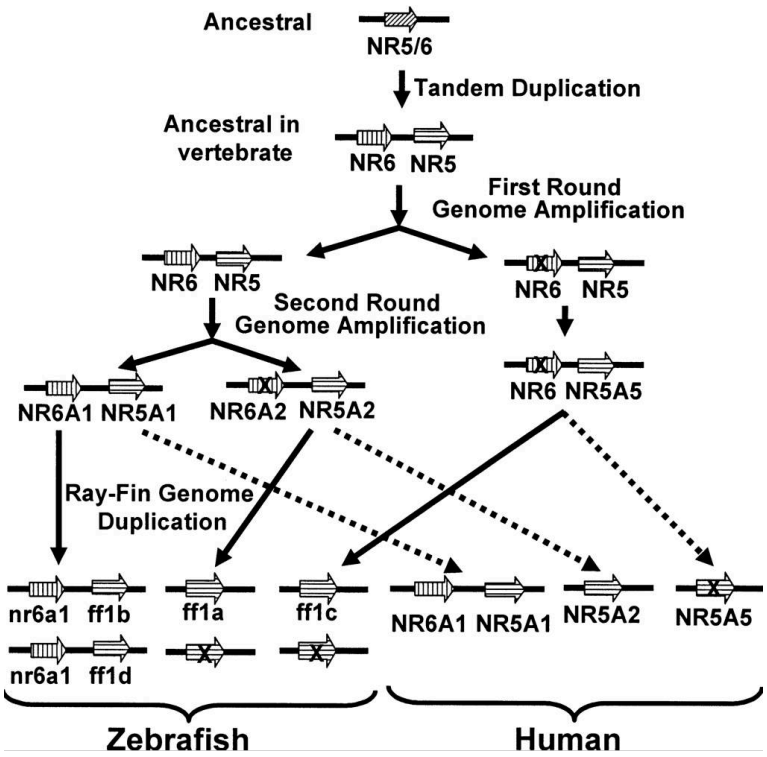
steroidogenesis, embryogenesis and reproductive function are amplified in subsequent sections of this review.

The evolution of the NR5A family was explored by Kuo et al. (147), who concluded that the mammalian versions of NR5A genes are orthologues of vertebrate forms that arose from a common ancestor, by means of a gene duplication event (Figure 1-4). The ancestral form gave rise to a third NR5A gene, NR5A5, present in teleost fish, but that has been lost in higher forms (55, 147).

Figure 1- 4 Evolution of NR5A genes

A**.** Schema to demonstrate the putative evolution of modern forms of the *NR5A* genes in vertebrates from an ancestral form containing one ancestral *NR5* gene that, after tandem duplication became the precursor of two forms, *NR5A5* and *NR5A6*. After two rounds of genome amplification the *NR5A1* and *NR5A2* genes emerged. Another product, *NR5A5* persists in fish, but has been lost in mammals. From ref 147 (reprinted with permission). B**.** Phylogenetic tree demonstrating the proposed origin and evolutionary relationship between LRH-1 and SF-1 in vertebrate species and in *Drosophila*. The evolutionary sequence and history was derived with the Neighbor-Joining method, and evolutionary distances from the Maximum Composite Likelihood method. Figure compiled using Molecular Evolutionary Genetics Analysis, version 7.0 for NCBI. The marker 0.2 represents a 20% sequence difference.

A


Ancestral
NR5/6
Tandem Duplication
Ancestral in vertebrate
NR6 NR5
First Round Genome Amplification
NR6 NR5
NR6 NR5
Second Round Genome Amplification
NR6A1 NR5A1
NR6A2 NR5A2
NR6 NR5A5
Ray-Fin Genome Duplication
nr6a1 ff1b
ff1a
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NR6A1 NR5A1
NR5A2
NR5A5
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Zebrafish
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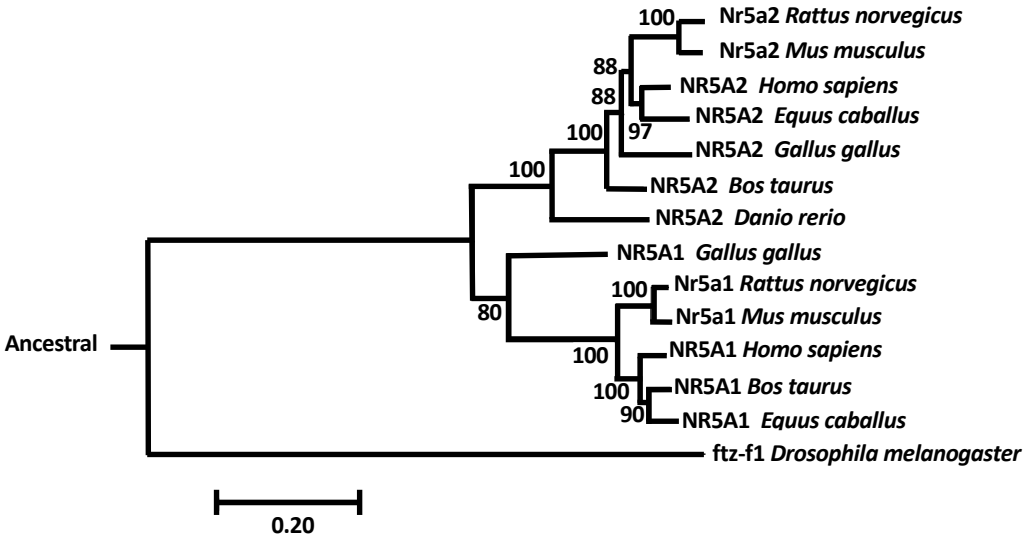

Nr5a2 Rattus norvegicus
Nr5a2 Mus musculus
NR5A2 Homo sapiens
NR5A2 Equus caballus
NR5A2 Gallus gallus
NR5A2 Bos taurus
NR5A2 Danio rerio
NR5A1 Gallus gallus
Nr5a1 Rattus norvegicus
Nr5a1 Mus musculus
NR5A1 Homo sapiens
NR5A1 Bos taurus
NR5A1 Equus caballus
ftz-f1 Drosophila melanogaster
Ancestral
100
88
88
97
100
100
80
100
100
90
0.20

3. Structure

LRH-1 is located on human and mouse chromosome 1 (83), whereas SF-1 is located on human chromosome 9 and mouse chromosome 2 (168). As noted above, both are classified as nuclear receptors because both display the typical structure of this family, albeit, with some peculiarities. As nuclear receptors, they are imported from the cytoplasm via the nuclear pore complex, and therefore have two functional nuclear localization signals responsible for shepherding the receptors into the nucleus (162, 305). The domain structure of these two closely related nuclear receptors (Figure 1-5) is comprised of:

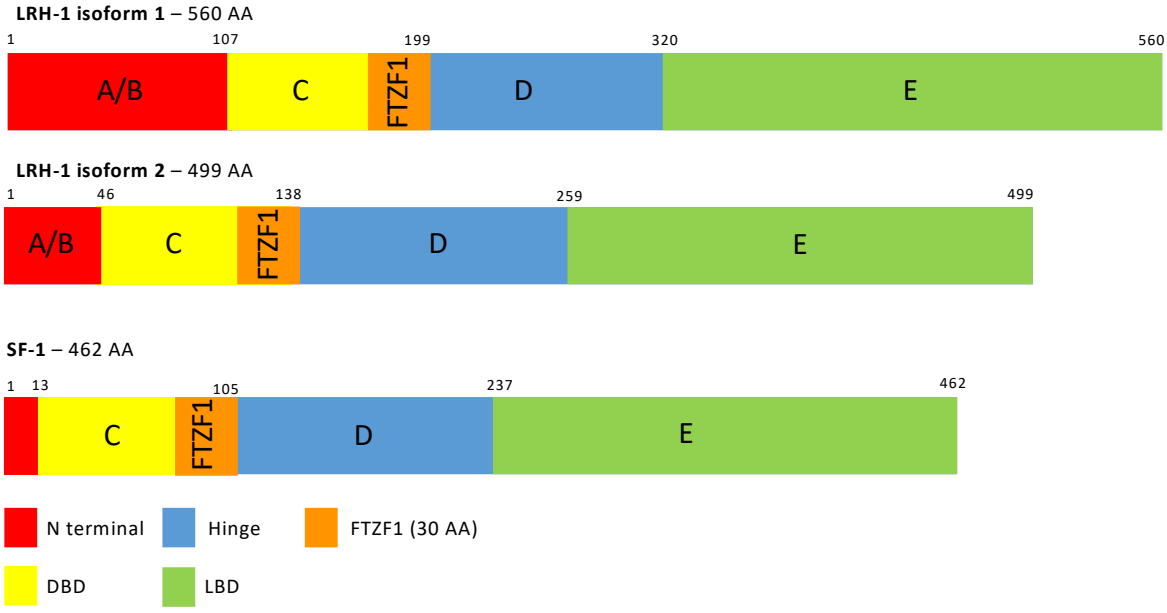
- a modulatory N-terminal A/B domain; that is, in contrast to other nuclear receptors, devoid of the ligand-independent activation function-1 domain (AF-1) at their N-terminals (76, 162);
- the highly conserved DNA-binding domain (DBD or C domain), responsible for targeting the receptor to specific DNA sequences, termed hormone response elements (213);
- the ligand-binding domain (LBD or E domain), which contains a conserved ligand-dependent activation function-2 (AF-2) motif that mediates co-activator interaction (213);
- the D domain serving as hinge between DBD and LBD (76, 211, 305), recently shown to be more than a flexible connector, as it is required to regulate the transcriptional activity of LRH-1 in humans (298) and has proven to be important for effective *in vitro* phosphorylation of LRH-1 (158).

Members of the NR5A subfamily further contain a hallmark feature, an additional 30-amino acid carboxyl-terminal extension, designated FTZ-F1 box or A box, located adjacent to the second zinc finger motif at the C terminus of the DBD (211, 270). The cooperativity between the P box in the first zinc finger motif of the DBD and the FTZ-F1 box allows for stable, high

affinity binding of NR5A factors to their target genes, thereby permitting their function as monomers (250, 269, 291). This was first shown in *Drosophila* Ftz-f1 by Ueda et al. (270), and subsequently confirmed in the mammalian homologs (reviewed in (174)). As mentioned above, the NR5A receptors are constitutive, in that they have the capacity to adopt an active conformation without requiring a ligand or other modifications. The un-liganded activity of the NR5A receptors evokes the two views of the evolution of nuclear receptors. Firstly, parent forms were un-liganded, and binding to specific ligands was a trait acquired independently by nuclear receptor lineages (70). Alternatively, the liganded forms were ancestral, a trait lost in the NR5A family (25). The presence of a ligand-binding domain and pocket in the NR5A genes argues for the latter scenario. In support of this view is the evidence that the active conformation is stabilized in SF-1 and LRH-1 when amino acid residues connect between helices, replacing connections usually found between the ligand and the receptor (25).

Figure 1- 5 Representative domain structure of the NR5A proteins using the mouse, where two isoforms of NR5A2 (LRH-1) are present.

One at 499 amino acids, that displays elevated homology with its mammalian homologs, and one with an extended A/B domain. The NR5A1 (SF-1) protein at 462 amino acids has similar domains, but the amino acid homology by BLAST is only 58 %. DBD, DNA binding domain, LBD, Ligand binding domain, FTZF1, FTZ-1 or A box specific to the *NR5A* genes.



4. Tissue distribution

SF-1 expression is most pronounced in steroidogenic organs such as the adrenal cortex and the gonads. It is present in the urogenital ridge of the mouse at embryonic day (E) 9.5, in fetal and adult adrenocortical cells, in the Leydig and Sertoli cells of the developing and mature testes, as well as in granulosa and theca cells of the ovary (117, 118, 120). While early studies reported that there is no expression in luteal cells (76), more recent studies suggest that it is, in fact, present in corpora lutea of the cow (190) as well as in the rat (72) and mouse (106). Studies have also located SF-1 expression in non-steroidogenic tissues, more specifically the ventromedial hypothalamus (VMH), where it plays an important role in hypothalamic regulation of pituitary gonadotroph organization and function (245), not to mention in endothelial cells of the sinus and pulp vein of the spleen and in the skin (195, 212).

In contrast (Figure 1-3), LRH-1 distribution is more widespread, as it is found in multiple tissues of endodermal origin, including the liver, pancreas and intestine (76). Elevated expression of LRH-1 was also found in the ovary, where it is restricted to the granulosa cell compartment (76). It is present at more modest levels in the hypothalamus and anterior pituitary gonadotrophs (80, 325), endometrium (318) and placenta, along with the adrenal gland and the testis (248).

5. Regulation

A. Potential ligands

As noted above, a stable and active monomeric NR5A LBD exists in the absence of ligand, co-activator peptide or homo- or heterodimeric receptor partner, indicating that ligands are

dispensable for SF-1 and LRH-1 basal activity (174, 291). Nevertheless, crystallography and mass spectrophotometry analyses revealed large and hydrophobic pockets in both SF-1 and LRH-1 LBD occupied by phospholipids, such as phosphatidyl ethanolamine and phosphatidyl glycerol (79). Experimental addition of bulky side chains into LRH-1 empty hydrophobic ligand-binding pocket results in equal or greater activity of the nuclear receptor, suggesting that it can accommodate potential ligands (232). Other phospholipids, such as phosphatidylcholine or second messenger phosphatidylinositol-phosphates, can modulate the two NR5A receptor interaction with co-activators (79, 144, 163, 205, 231). Variations in lipid environment and metabolism can modulate SF-1 function. For example, it has been shown that sphingosine can inhibit SF-1, and that cAMP can enhance SF-1 activity by inducing sphingosine catabolism (271). Introducing point mutations blocking phospholipid binding to these nuclear receptors generates mutant proteins, some of which are unable to be phosphorylated and then fail to recruit co-activators and induce transcription (161, 285).

While it is not clear whether there are active endogenous ligands for either of the NR5A receptors, a number of pharmacological ligands have been developed for SF-1 and LRH-1. Given that both receptors interact with the same gene sequences, the activity of these ligands is expected to be both gene target and cell context specific. Low-molecular weight compounds with cis-bicyclo [3.3.p] oct-2-ene core structure selectively increase SF-1 activity, while 4-alkuloxy-phenol derivatives have an inverse agonist effect and suppress the constitutive activity of the receptor ((60, 289), reviewed in (237)). LRH-1 can be activated by dilauryol-phosphatidyl-choline (DLPC), a ligand agonist that, through its regulation of bile acids and glucose homeostasis, has been shown to decrease the quantity of glucose in diabetic mice (157).

Inverse agonists of LRH-1 that inhibit its constitutive activity, such as ML179 and ML180, have been synthesized (32). They function by inducing the translocation of LRH-1 from the

nucleus (52) to the cytoplasm, thereby inactivating it. Another synthetic agonist molecule able to accommodate SF-1 and LRH-1 ligand binding pocket is GSK8470, developed by the GlaxoSmithKline company (289). This compound is described as capable of stimulating the transcription of downstream targets of LRH-1 in hepatic cells, as well as some downstream targets of the NR5A co-repressor short heterodimeric protein (SHP or NR0B2), an atypical orphan nuclear receptor that does not possess a DNA-binding domain (289). The retinol related molecule, adamantyl hydroxyphenol chlorocinnamic acid (3Cl-AHPC), is another example of anti-agonist molecule that represses LRH-1 by increasing its interaction with SHP (185). In addition to phosphatidylcholines, the signaling phospholipid, phosphatidylinositol 3,4,5-triphosphate (PIP3) binds to LRH-1 (182), although downstream effects of this interaction have yet to be determined. It also binds to SF-1, inducing slight modifications in the LBD structure, indicating that a dynamic exchange of potential ligands may regulate the activity of this receptor (20).

While molecular dynamic simulations showed no overall conformational changes in SF-1 and LRH-1 when bound to phospholipids, the ligands affect the recruitment and affinity with cofactor peptides, with consequences on transcriptional capacity (30, 98, 231). It has been suggested that the phospholipid molecule is not an endogenous ligand for LRH-1, and even that there might be no endogenous ligand for this receptor (320). Further, it has been postulated that the phospholipids that occupy the ligand pocket in LRH-1 and SF-1 serve not to stimulate, but rather to stabilize the molecule and to reduce inhibitory cofactor binding (161). Although it is clear that ligands exist, information is yet fragmentary, and their role, mechanism of action and function in cellular processes all require further investigation.

B. Co-regulators

Ligand-activated nuclear receptors are first used as adaptors between gene regulatory regions and the chromatin modifying enzyme complexes, and second as activators of ribonucleic acid (RNA) polymerase II, to suppress or enhance target gene expression (38). In turn, the nuclear receptors are modulated not only by ligand binding and post-translational modifications, but also by recruitment of co-regulators. Co-activators and co-repressors are positive and negative co-regulatory proteins (201) and their actions may well be the most important mode of functional regulation of orphan nuclear receptors (153).

Several tissue-specific co-activators and co-repressors are known to regulate the transcriptional activity of SF-1 and LRH-1 in a context-specific manner (Table 1.1). Importantly, these co-regulators bind to domains whose structure has been altered by binding of ligands and/or post-translational modifications (100). Whether the post-translational modifications are crucial to initiate, maintain, or simply facilitate the interaction is uncertain. It is, nonetheless, known that once the nuclear receptors are constitutively activated, they can interact with co-repressors that then further modulate their activity (76). This modification takes the form of alteration to the structure of the chromatin, leading either to a condensation that represses transcription or to a decondensation that facilitates the recruitment of the basal transcription machinery (24). In general, the structure that mediates the ligand-driven interaction with co-repressors and co-activators is the helix 12 (H12) of the receptor, which assumes an extended position in the absence of ligand, permitting the binding of co-repressors. Binding of agonist reorients H12 to a sequestered position that blocks the co-repressor binding site, while simultaneously forming a new docking surface for co-activators (222). The long H2 twists LRH-1 into an agonist-like conformation by affecting H12, even when LRH-1 ligand binding pocket

is empty (232), while H1 and H12 of SF-1 are packed against the α-helical bundle, demonstrating its LBD ligand-independent active conformation (62).

Table 1- 1 The principal mechanism for regulation of the transcriptional activity of the NR5A receptors is interaction with cofactors

This regulation can be either positive (co-activators) to enhance constitutive activity, or it can be negative (co-repressors) to inhibit induction of transcription of target genes. The mechanisms vary and some are unknown. LBD: ligand binding domain, DBD: DNA binding domain.

Co-regulator	Regulation	Interaction Site/Mode	Nuclear receptor	References
SHP (NR0B2)	Negative	Attaches C terminal domain and interacts with AF-2 domain	SF-1, LRH-1	(88, 159)
DAX1 (NR0B1)	Negative	AF-2 domain	SF-1, LRH-1	(6, 123, 262)
NCOR1, NCOR2	Negative	Recruited by DAX1	SF-1, LRH-1	(123, 252, 254)
ALIEN	Negative	Recruited by DAX1	SF-1, LRH-1	(123)
FXR	Negative	Co-enriched nuclear receptor half site	LRH-1	(48, 178, 320),(286)
PROX1	Negative	Interact with LBD and DBD	LRH-1	(224, 252)
SMRT	Negative	No direct interaction	LRH-1	(297)
MBF1	Positive	Bridging factor	LRH-1	(24)
SRC1, SRC2, SRC3	Positive	Binding to the LBD	LRH-1, SF-1	(159, 298)
PGC1	Positive	AF-2 domain	LRH-1	(311)
β-catenin	Positive	Distinct from the known interaction surfaces of LRH-1	SF-1, LRH-1	(23, 127, 317)

The two best known SF-1 and LRH-1 cofactors are SHP, mentioned above, and dosage-sensitive sex reversal-adrenal hypoplasia congenital region gene on the X chromosome, gene-1 (DAX1; NR0B1), both of which act as repressors of these NR5A orphan receptors. SHP attaches

to the nuclear receptor C terminal domain and represses SF-1 and LRH-1 activity by interacting with its AF-2 transactivation domain (159, 163). There appears to be a negative feedback loop by which SF-1 and LRH-1 bind to the SHP promoter region to induce its transcription, which in turn reduces their activation and consequent transcriptional activity (88, 160). Structural and biochemical probe analysis has shown that SHP interaction with LRH-1 is significantly stronger than with SF-1, due to differential binding events occurring between the core LXXLL motif of SHP and the SF-1 coactivator binding site (164).

DAX1 functions as a ligand-independent nuclear receptor, and its repressive mechanism indicates that it is a competitive transcriptional co-repressor (233). Like SHP, DAX1 lacks a DNA-binding domain and additionally has neither a modulatory domain nor a hinge region (123, 204). SF-1 and LRH-1 also interact with DAX1 through its LBD with the N-terminal LXXLL related motifs (262), binding with high affinity to the AF-2 domain, and repressing their transcriptional activity (6, 123, 262). There is also evidence that DAX1 recruits co-repressors, such as the nuclear receptor co-repressor (NCOR) and ALIEN, providing a further inhibitory mechanism of NR5A activity (53, 123). NCOR1 acts in the SUMOylation process of LRH-1, and one of the consequences of this association is the trans-repression of acute phase response proteins (252, 254). On the other hand, NCOR recruitment persists regardless of SF-1 SUMOylation state, as observed in un-sumoylatable SF-1 knock-in mice (156). The homolog of NCOR1, NCOR2 (aka silencing mediator for retinoic acid and thyroid hormone receptor; SMRT), also represses the transcriptional activity of SF-1 and LRH-1 (99, 254). The mechanism of interaction between SF-1, LRH-1 and the NCOR2 is not yet well understood, as no direct regulation has yet been demonstrated. It could be, as is the case for NCOR1, that a supplementary protein is needed to link NCOR2 and the two nuclear receptors, indicating that it could be an indirect cofactor (297).

Another co-activator of LRH-1 related to SHP activity is the farnesoid X receptor (FXR; NR1H4), principally expressed in the liver, kidney, adrenals and small intestine (178, 286). Studies with mice fed with FXR show that it is involved in cholesterol and bile acid metabolism, as well as in the regulation of glucose metabolism (178, 321). The interaction of FXR with LRH-1 allows for FXR mediated activation of SHP, retinol dehydrogenase 9, pyruvate carboxylase, and phosphatidylethanolamine N-methyltransferase (48). The prospero-related homeobox transcription factor, PROX1, is another recognized co-repressor that acts similarly to DAX1 and SHP by directly interacting with both LBD and DBD of LRH-1 (224, 252). The interaction between the nuclear receptors and PROX1 leads to the repression of several target genes of LRH-1, including the steroidogenic gene *CYP17a1* (224).

Other cofactors act as bridging factors to regulate SF-1 and LRH-1, such as the multiprotein bridging factor (MBF1), which does not possess histone-modifying activities, but rather enables interactions of the nuclear receptors with the transcription machinery (24, 129). The three homologs of the p160 family, steroid receptor co-activators (SRC1, SRC2 and SRC3) act as strong regulators of the two NR5A nuclear receptor transcriptional activity by binding to their ligand binding domains (159, 298). SRC-1 interacts directly with the LRH-1 LBD in helix 1 and AF-2 (298) while SRC3 potentiates the interaction between CREB and LRH-1 (96). The phosphorylating kinase A (PKA) downstream of cAMP, has been shown to stimulate SRC1 and SRC3 activation of SF-1 dependent transcription, but this signaling pathway also represses SRC2 co-activation of SF-1 by increasing ubiquitin-mediated degradation of SRC2 (22, 109), (111).

The co-activator peroxisome proliferator-activated receptor-γ (PPAR-γ)-coactivator-1 (PGC1) binds to the AF-2 domain of LRH-1 to promote differentiation of granulosa cells into progesterone-producing luteal cells (312). Interestingly, a novel isoform of LRH-1 in human

granulosa cells was shown to be coordinately regulated by SF-1 and PGC1 (134). Blocking PGC1 is one of the strategies by which SHP and DAX1 repress LRH-1 activity (153), and LRH-1 is also inhibited following the recruitment of PGC1 by the sterol regulatory element-binding proteins (SREBP2) (130).

In addition to this wide and growing list of co-regulators for NR5A receptors, it has been shown that β-catenin is important for stable interactions with SF-1 and LRH-1, acting synergistically with both orphan receptors. In terms of downstream effects, LRH-1 interacts with β-catenin to promote cell cycle gene expression and cell proliferation in the intestinal crypt and granulosa cells (23, 183). Likewise, SF-1 and β-catenin interact in the signaling pathway that produces testosterone, a collaboration that can be interrupted by WNT4 overexpression (127).

As with other members of the nuclear receptor family, the regulation of the activity of NR5A receptors is a complex, context-specific process that depends heavily on cofactors. These interactions are the major, if not the principal mechanisms of modulation of the multiple actions of SF-1 and LRH-1.

C. Transcriptional regulation

The SF-1 gene contains seven exons and is closely situated downstream of the germ cell nuclear factor (GCNF; *NR6A1*) gene, separated by approximately 13kb (122, 292). An intervening insulator element is present, composed of sites that are target regions for the CCCTC-binding factor, histone tail acetylation regulation, and other nuclear matrix interaction to induce insulator activities, such as the adrenal specific DNase hypersensitive sites (adHS1-3) (122). Transient transfection experiments and protein/DNA binding assays suggest that the basal promoter of the SF-1 gene interacts with the ubiquitous transcription factors, such as nuclear

transcription factor Y (NFY), stimulatory protein 1 and 3 (SP1/3) and upstream stimulatory helix loop helix (HLH) factors 1 and 2 (USF1/2), the latter of which enhances transcription through interactions with an E-box located on the basal promoter (293). Another HLH-factor, Pod-1/capsulin, has been shown to repress SF-1 expression via regulation of the E-box, and, while studies have failed to demonstrate direct interaction between this HLH-factor and the basal promoter, targeted deletion of Pod-1/capsulin leads to an increased SF-1 expression in the developing testis and in adrenocortical tumor cells (56, 57, 264).

Chromobox homologue 2 (CBX2) is another factor shown to bind the SF-1 promoter (133), and mice carrying CBX2 null mutation display reduced SF-1 and sex determining region Y (*Sry*) expression, leading to a sexually dimorphic phenotype, small adrenal glands and spleen malformation (132). Human CBX2 has been shown to bind directly to the SF-1 promoter and mutations in this gene also show XY sex reversal (18). Other factors involved in gonadal development have been shown to interact with the SF-1 basal promoter, such as Wilm's tumor suppressor (WT1), Lim homeobox protein (LHX9), SOX9 and GATA4 (268, 290). Additionally, multiple intronic enhancers are specific to gonads, the adrenal gland, ventromedial hypothalamus (VMH) or pituitary gland, indicating, not unexpectedly, that regulation of SF-1 transcription is tissue-specific (111).

DNA methylation also plays a role in the epigenetic regulation of the SF-1 gene. Studies in mouse tissues have shown that the SF-1 basal promoter is hypermethylated when SF-1 is not expressed, showing binding of DNA methyltransferase 3a (DNMT3A) and MECP2 factor to the SF-1 basal promoter (110, 302). In the hypo or unmethylated state, SF-1 is expressed, with recruitment of transcription factors such as USF2 and RNA polymerase II (110, 302). Interestingly, analysis of the SF-1 promoter region in endometriotic stromal cells, which show

aberrant increases in expression of SF-1, due to a high level of acetylation of associated histones, regulated by acetyltransferases (192).

From the above, it can be seen that SF-1 expression is modulated by a wide range of transcriptional regulatory processes, comprising transcription factors, intron enhancers and epigenetic elements. This multiple array of factors is expected to be responsible for the highly variable, developmental stage, tissue and even species-dependent expression of SF-1.

Much less is known about the factors regulating the transcription of LRH-1. Studies of the 5' upstream region of gene have been summarized by Fayard et al. (76), indicating multiple transcriptional activators in regulation of LRH-1. In the mouse, these include GATA, HNF and NKX motifs (76). Mouse *Pdx1* is co-expressed with LRH-1 and it has been shown that there are functional binding sites for this homeobox gene on the LRH-1 promoter (2). These findings have been interpreted to indicate that PDX1 exercises control over LRH-1 transcription during development.

D. Post-translational regulation

The expression of SF-1 and LRH-1 is also regulated by multiple modifications that occur following their translation. The intracellular second messenger, cAMP, acting via protein kinase A (PKA) can induce p300 to acetylate SF-1 (47). This post-translational change increases SF-1 DNA binding, induces its recruitment to nuclear clusters and increases its dynamic interaction with regulatory cofactors (47). PKA has also been shown to promote dissociation of DAX1 from SF-1, thereby activating or amplifying transcriptional activity of the latter (73). LRH-1 possesses a large hinge domain on which the serine residues S238 and S243 can be phosphorylated, an action brought on by activation of the protein kinase-C and the MAPK/ERK pathways (144, 158). The overall effect is an increase in the transcriptional activity of LRH-1.

SF-1, on the other hand, has only one phosphorylation site in the AF-1 domain of the hinge region, S203, and it can be phosphorylated by the MAPK/ERK signaling cascade, as well as by cyclin-dependent kinase 7 (CDK7). The latter will form the CDK-activating kinase (CAK) complex with cyclin H (CYCH) and ménage à trois 1 (MAT1), which then anchors SF-1 to the basal transcriptional machinery of many of its target genes (161).

Another post-translational modification of the NR5A receptors is the reversible covalent interaction engendered by conjugation to the small ubiquitin-like modifier (SUMO), at lysine residues (SUMOylation)(45, 306). Both NR5A receptors are direct substrates for the SUMO conjugation machinery, such that their activity is repressed by SUMOylation (40, 45, 308). The modification of SF-1 transcriptional activity by SUMOylation takes the form of reduced receptor binding to its cognate DNA sequences (35), or translocation of the transcription factor from the chromatin to inactive nuclear bodies (40, 45, 308). For example, the SUMO-E3 ligase RING finger protein 31 (RNF31) is involved in the ubiquitination (and stabilization) of DAX1, and present in the complex formed of DAX1, SMRT and HDAC to induce SF-1 inactivation on target gene promoter regions (69). SUMOylation has been identified as a factor in the interaction of corepressor PROX1 with LRH-1 to inhibit its role in cholesterol transport (253). Moreover, SUMO conjugation is implicated in the subnuclear localization of LRH-1, and the accumulation of LRH-1 in rat primary granulosa cells nuclear bodies can be suppressed by forskolin and cholera toxin treatment in rat primary granulosa cells, suggesting the cAMP pathway is also involved in regulating LRH-1 activity (306). While *in vitro* studies showed that overexpression of un-SUMOylatable SF-1 and LRH-1 increased cell-based reporter activity (40, 161), the permanent elimination *in vivo* of SF-1 SUMOylation in mice did not lead to increased SF-1 activity, rather it resulted in endocrine abnormality that reflected the inappropriate activation of specific SF-1 target genes (156). Indeed, loss of SF-1 SUMOylation elevates or induces ectopic

activation of sonic hedgehog (*Shh*) in mouse testis and adrenal glands to amplify hedgehog signaling, promoting steroidogenesis and resulting in abnormal endocrine tissue development (156). *In vivo* studies using unSUMOylatable LRH-1 mice lead to increased expression of genes involved in cholesterol transport and animals with diminished atherosclerosis development (253).

A further post-translational regulation process that affects NR5A family members is ubiquitination, essential for many cell processes including signaling cascades, regulation of the cell cycle, DNA repair and maintenance of protein integrity (44). It is generally inhibitory, and with respect to nuclear receptors, including SF-1, ubiquitination plays an important role in the reproductive and endocrine pathology. HDAC inhibitors such as trichostatin A and valproic acid are known to promote SF-1 ubiquitination, which then reduces steroidogenesis in adrenal tumor cells (46).

E. MicroRNAs

A wide range of micro-RNAs (miRNAs) is expressed in mammals and they have been found to participate in physiological and developmental processes. They aid in modulation of gene expression by mediating RNA transcript degradation or regulating translation rate. In mouse embryonic stem cells, miR134 has been shown to promote differentiation due to its translational attenuation of LRH-1 and NANOG, both known as positive direct regulators of OCT4/POU5F1 and stem cell proliferation (265). Micro-RNAs also play a role in several types of human malignancies such as cancer (249). For example, the overexpression of miR-30d induces cell-cycle arrest at G0/G1, decreases cell proliferation, migration, invasion and tumor growth while increasing cell apoptosis *in vitro* (304). Bioinformatic analysis and dual-luciferase reporter assay revealed that LRH-1 is a direct target of miR-30d in colorectal cancer cells. The same

study showed that LRH-1 overexpression could restore the inhibitory effect of miR-30d on these cells (304).

MicroRNAs are also involved in steroidogenic gene regulation and normal reproductive function. A recent report indicates that in the porcine ovary, miR-1275 is an endogenous regulator of LRH-1, reducing LRH-1 induction of CYP19A1 by means of effects on the 3'UTR of the LRH-1 gene (170). Studies have shown that various microRNAs interact with SF-1 to regulate its transcriptional activity. For example, miR-320 decelerates granulosa cell proliferation by decreasing SF-1 mRNA stability and impeding its transcriptional activity, while miR-764-3p has been shown to bind directly to SF-1 mRNA in mouse granulosa cells to inhibit aromatase transcription, resulting in reduced estrogen production (281, 314, 315). SF-1 has itself been shown to bind to the promoter region of miR-383 host gene, inducing its expression and allowing the miR-383-mediated estradiol release from granulosa cells (315).

F. Epigenetic regulation

As noted above, SF-1 and LRH-1 both function as direct regulators of transcription and as transcriptional enhancers. They may also function as pioneer or licensing transcription factors, the elements that can program the epigenome during cell differentiation, by modifying chromatin accessibility to other transcriptional constituents (66). Nuclear receptors, including the glucocorticoid receptor (97) and the ecdysone receptor (246), have been demonstrated to act as pioneer factors in differentiating tissues. The evidence for LRH-1 as a possible pioneer factor has been derived from breast cancer cell lines where it regulates proliferation (16). In that study, it was shown by chromatin immunoprecipitation analysis that LRH-1 cooperates with the well-known pioneer factor, forkhead box protein A1 (FOXA1), to bring about expression of cell cycle genes (16). Further, knockdown of LRH-1 altered FOXA1 binding and induced a second

epigenetic effect, the depletion of histone deacetylase 2 from the regulatory region of cell cycle proteins. The concept of NR5A receptors as epigenetic modulators is novel and requires further investigation in the multiple tissues where these proteins are expressed.

6. Cellular processes related to reproduction

A. Cholesterol homeostasis

Cholesterol plays a central role in diverse biological processes, including in the formation of cellular membranes, and serves as the parent molecule for steroid hormone synthesis. Improper transformation, transport, or storage of cholesterol is the basis for a number of diseases, thus homeostatic regulation is essential, not only for normal reproductive function, but also, in some cases, for survival. Overall, appropriate intracellular concentrations of cholesterol are maintained by four complementary mechanisms: *de novo* synthesis; importation as elements of high (HDL) or low-density lipoproteins (LDL); reverse cholesterol transport, primarily as HDL; and cholesterol esterification and liberation(197). NR5A receptors play a significant part in cholesterol homeostasis by direct effects on some key regulators involved in these mechanisms.

The *de novo* synthesis of cholesterol, which relies on the rate limiting enzyme HMG-CoA reductase (reviewed in (227)), does not appear to be directly influenced by SF-1. One reporter assay study showed that LRH-1 does not drive transactivation of this gene (130), while Datta et al. report binding of LRH-1 to the HMG-CoA promoter to induce specific activation of its transcription (58). Both of these studies are based solely on transient transfection assays, thus, further investigation is required to determine the biological significance of these contradicting findings.

In many species, the main source of supply of cholesterol for steroid synthesis is via its importation from circulation by the HDL receptor, scavenger receptor class B type 1 (SCARB1) (197). It has been shown that SF-1 transactivates the SCARB1 gene (37) and mediates the uptake of cholesterol via this receptor (171). Similarly, chromatin immunoprecipitation (ChIP) analysis revealed that LRH-1 binds to its response element in the *SCARB1* promoter to induce its expression in both mouse and human tissue (150, 238).

Reverse cholesterol transport, another homeostasis process completed principally by HDL via SCARB1, is regulated by LRH-1 activity (76). Intermediates that shuttle cholesterol to HDL for efflux include members of the ATP binding cassette (ABC) family (reviewed in (219)), and transient transfection assays have shown that LRH-1 activates transcription of isoforms ABCG5 and ABCG8 by binding to their promoter regions (82). In addition, hypomorphic expression of LRH-1 in the liver of heterozygote LRH-1 germline knockout mice leads to diminished expression of both *Abcg7* and *Abcg8* (300). One isoform of the ABC family, ABCG2, transports xenobiotics rather than cholesterol in reproductive tissues, and has been shown to be upregulated by SF-1 in mouse Sertoli cells (295). The role of SF-1 in the expression of factors involved in reverse cholesterol transport has yet to be elucidated.

Cholesterol storage and its liberation are also important factors in sterol homeostasis, particularly in steroidogenic cells. Free cholesterol is esterified by the enzymes of the sterol O-acyltransferase (SOAT) family (228). At least one of the members of the family, SOAT1, was identified as a target of SF-1 in adrenocortical cells, and overexpression of SF-1 in an adrenal cell line upregulates its expression (77). In contrast, promoter assays have suggested that SF-1 also liberates cholesterol from the esterified state by activating hormone sensitive lipase (LIPE), the principal lipase in steroidogenic tissues (112, 145). The latter concept is more in keeping of

the role of SF-1 as a factor in steroid synthesis induction. No information appears available at this time on the role of LRH-1 in regulation of either SOAT1 or LIPE.

The sterol regulatory binding proteins (SREBPs) are transcription factors that have been shown to be the dominant regulators of components in cholesterol metabolism processes (241). Lopez and McLean (171) reported a synergistic interaction between SF-1 and SREBP1a in induction of transcription of SCARB1. Synergistic transactivation has also been demonstrated in the context of another lipogenic gene, the Niemann-Pick C1(NPC1) -like 1 (155). Given that LRH-1 also induces SCARB1 expression in both mouse and human tissue (76), it is somewhat surprising that at least two studies suggest reciprocal antagonism between LRH-1 and SREBPs. Lee et al. reported that treatment with the LRH-1 agonist, dilauroyl phosphotidylcholine (DLPC) reduced the expression of SREBP1c (157). Others presented evidence that LRH-1 directly inactivates SREBP1 transactivation in promoter assays and they further showed that SREBP2 directly inhibits LRH-1 activity (130). It is proposed that this is achieved by binding of LRH-1 to SREBPs, which inhibits the recruitment of their co-activators CBP and PGC1β , potentiating SREBP transcriptional activity (167, 202). It is speculated that the inhibitory effects of SREBPs on LRH-1 activity have greater impact than the effects of LRH-1 on the SREBP functions because abundance of active SREBPs in the nucleus varies more strongly in response to metabolic alterations. The dissonance between multiple studies showing that LRH-1 stimulates SREBP-driven genes *in vivo* (13, 68) and *in vitro* (155) suggests that the antagonism between LRH-1 and SREBP may be gene-specific.

Cholesterol transport within the cell is necessary for multiple biological processes and since the aqueous intracellular milieu is hydrophobic, transport mechanisms are required. Among these are the cytosolic lipid transfer proteins including NPC1, sterol carrier protein-2 (SCP2) and the steroidogenic acute regulatory protein (STAR) (294). All of these factors are

essential for reproductive function (reviewed in (256)). Studies have shown that SF-1 regulates the transcription of the NPC1 gene in concert with cAMP (86). The promoter region of SCP2 contains an SF-1 (and presumably a LRH-1) response element, suggesting regulation by the NR5A receptors (172).

The STAR protein, first discovered by Clark and Stocco (50), transfers cholesterol from the outer mitochondrial membrane into the cristae, where it can initiate the enzymatic cascade that comprises steroid synthesis. There is a great deal of information implicating the NR5A receptors in modulating STAR expression, from early studies showing promoter transactivation by SF-1 in granulosa cells (229, 259) to more recent ChIP analysis of bovine thecal cells, demonstrating increased SF-1 binding to the bovine theca cell *STAR* promoter in response to luteinizing hormone (LH) stimulation (196). SF-1 induces STAR synthesis, not only through classic promoter binding, but also by acting as an enhancer at sites >3Kb upstream of the *STAR* transcription start site (189). A non-exhaustive list of tissues where SF-1 regulates STAR includes adrenal cell lines (296), Leydig cells (273), theca (196), granulosa (210) and endometrial cells (67). LRH-1 also is an essential factor for expression of STAR in steroidogenic tissues. An early investigation showed that LRH-1 induces *STAR* transcription in human adrenal, testis and ovarian tissues (217). Cre-loxP depletion of LRH-1 in the mouse ovary from primordial follicles forward (13, 14, 68) results in dramatic reduction in *Star* expression. Moreover, ChIP analysis by Duggavathi et al. (68) demonstrate that LRH-1 binds directly to the *STAR* promoter.

In summary, it is clear that there is remarkable scope in the regulation of cholesterol homeostatic and transfer mechanisms by the NR5A receptors. Virtually all of these processes are significant to mammalian reproductive function, as cholesterol is the parent molecule of the steroids that orchestrate folliculogenesis, spermatogenesis and gestation.

B. Steroidogenesis

As noted above, cholesterol is transported from the cytosol into the inner membrane of the mitochondrion of steroidogenic cells to allow steroid hormone biosynthesis (50). Cholesterol side chain cleavage enzyme (P450scc, CYP11A1), resident in the mitochondrion, converts cholesterol to pregnenolone and the diverse steroid products can subsequently be synthesized following transformations of pregnenolone by a variety of cytochrome P450 oxidases (CYPs) and steroid dehydrogenases (HSD) enzymes (86, 211) (reviewed in (187)). The promoter regions of several of these enzymes have been shown to contain the SF-1/LRH-1 consensus site (194, 226) (Table 1.2). These discoveries led to identification of the presence of SF-1 in most mouse steroidogenic tissues, including the corpus luteum (CL) (106) and LRH-1 was later shown to be present in the ovary and testis (248, 311) (Table 1.2). Under the control of gonadotropins (150), the NR5A receptors enhance the activity of steroidogenic genes, such as STAR, CYP11A1, CYP17A1, 3βHSD and the steroid 11β-hydroxylase (CYP11B1). Moreover, LRH-1 serves as a critical factor in the transcriptional regulation of the aromatase (*CYP19A1*) gene, the rate limiting enzyme in ovarian estrogen biosynthesis (184). In addition to regulating enzymes directly involved in cholesterol transport and steroid biosynthesis, SF-1 and LRH-1 control the expression of ferrodoxin 1 (*FDX1*), an iron-sulfur protein which functions as the electron donor for the catalytic activity of P450scc in ovarian granulosa cells (119). Activation of the *FDX1* promoter has been shown to occur following stimulation by cAMP (119).

Table 1- 2 Both LRH-1 and SF-1 regulate the process of steroid synthesis in reproductive target tissues, sometimes in the same cells.

STAR: steroidogenic acute regulatory protein; CYP11A1: P450 side-chain cleavage CYP17A1: P450 family 17; CYP 19: P450 aromatase; HSD3B1: 3ß-hydroxy-steroid dehydrogenase-1; HSD3B2: 3ß-hydroxy-steroid dehydrogenase-2.

SF-1		
Steroidogenic Gene	**Principal reproductive tissue localization**	**References**
STAR	Theca, Granulosa, Luteal, Leydig cells	(39, 49, 102, 125, 210)
CYP11A1	Theca, Granulosa, Luteal, Leydig cells	(27, 51, 102, 125, 210, 243, 244, 279)
CYP17A1	Theca, Granulosa, Luteal, Leydig cells	(27, 209, 210)
CYP19	Granulosa, Leydig, Sertoli cells	(177, 188, 280)
HSD17B1	Endometriotic tissue	(4)
HSD3B1	Leydig, Theca cells	(27, 188)
HSD3B2	Luteal, Leydig cells	(180, 272)

LRH-1		
Steroidogenic Gene	**Principal reproductive tissue localization**	**References**
STAR	Granulosa, Luteal, Leydig cells	(137, 181)
CYP11A1	Granulosa, Luteal, Leydig cells	(136, 244)
CYP17A1	Granulosa, Luteal, Leydig cells	(72, 181, 311)
CYP19	Granulosa, Leydig	(106, 218)
HSD3B1	Granulosa cells	(188)
HSD3B2	Luteal cells, Leydig cells	(180, 215)

C. Cell proliferation

We have recently shown that LRH-1 plays a key role in granulosa cell proliferation, as depletion of LRH-1 causes a significant decrease in the number of granulosa cells entering S-phase of the cell cycle, and in the abundance of transcripts of key genes such as the cyclins D and E and their downstream targets E2F1 and E2F2 (183). This effect on proliferation has been

shown to be mediated by LRH-1 interaction with β-catenin and CDKN1A, a cell cycle inhibitor, all of which are direct targets of LRH-1, as revealed by ChIP (183).

While there seems to be little information about the role of SF-1 in gonadal cell proliferation, several studies of SF-1 in mouse adrenal function have demonstrated direct correlation between adrenal size and SF-1 gene expression, and that SF-1 gene copy number has an impact on proliferative potential adrenocortical cells (15, 19, 133). SF-1 overexpression in human adrenocortical cells increases the proliferation rate *in vitro*, and transcript and microarray analysis showed increased expression of regulators of cell cycle progression and reduced expression of pro-apoptotic factors (65). An interesting study of the role of SF-1 on glycolytic gene transcription demonstrated that knocking down SF-1 *in vitro* led to reduced proliferation of adrenocortical Y-1 cells (5). In terms of the mechanism by which this effect occurs, it was postulated that the reduced expression of key glycolytic genes due to SF-1 absence leads to significantly lower levels of cellular ATP production, essential for the generation of daughter cells (274).

D. Stem cell differentiation

After discovery of SF-1 in the early 1990's, Milbrandt et al. demonstrated that it directed mouse embryonic stem (ES) cells towards a steroidogenic lineage, by inducing endogenous *Cyp11a1* expression via cAMP and retinoic acid activation (54). These differentiated cells were nonetheless unable to biosynthesize steroids, due to the lack of cholesterol transport proteins. This initial discovery led other groups to explore the role of both SF-1 and LRH-1 in stem cell differentiation, showing binding to the promoter region of the *OCT4* gene and the consequent activation of the transcription of this pluripotency factor, in both murine and human ES lines (94, 307). These authors showed that, as ES cells began to differentiate, both SF-1 and OCT4

expression decreased, indicating SF-1 plays a role in maintaining pluripotency (307). More recently, it has been demonstrated that both LRH-1 and SF-1 regulate and can replace OCT4 in stem cells (282). In fact, LRH-1 can take the place of OCT4 in the derivation of induced pluripotent stem cells from mouse somatic cells, with enhanced reprogramming efficiency, relative to the classic four factors required for induction of pluripotency (OCT4, SOX2, FOXD3 and NANOG) (7).

Following exit from the pluripotent state, cells respond differently to the NR5A receptors. LRH-1 is capable of inducing mesenchymal stem cell differentiation without SF-1 expression (311). SF-1 is also capable of inducing the adipose tissue mesenchymal stem cells (MSC) to differentiate into steroidogenic cells, as confirmed by the secretion of corticosterone (87). In contrast, the steroidogenic cells derived from bone marrow MSC secreted gonadal rather than adrenal steroids (87). Recently, it was reported that mouse ES cells could be differentiated into Leydig-like cells via SF-1 overexpression *in vitro,* and that these cells are able to rescue testosterone secretion when transplanted in the testes of rats where Leydig cells had previously been ablated (309). In terms of potential mechanisms, SF-1 directs stem cell differentiation by inducing of chromatin alterations, and by modifying the chromosomal conformation of genomic regions, via histone eviction and chromatin loop formation (189, 207).

In overview, there is substantial evidence to implicate the orphan nuclear receptors of the NR5A family in two prominent events in reproductive function, the regulation of stem cell pluripotency and differentiation.

7. Physiological processes related to reproduction

A. Embryogenesis

i. Early embryonic development

Both SF-1 and LRH-1 are expressed at multiple stages in embryonic and fetal life, and both are essential for normal embryonic development, demonstrated by their deletion via targeted mutagenesis in the mouse. LRH-1 is abundantly expressed in the morula and inner cell mass of the early embryo (94). At embryonic day E7.5 in the mouse, LRH-1 is detected in foregut endoderm, and is progressively expressed during the differentiation of the foregut into liver, intestine and pancreas (76). At day E17.5, LRH-1 exhibits its adult expression profile in the liver, exocrine pancreas, intestinal crypts and stomach epithelium (76). By day E11.5, bipotential gonads express LRH-1 and at E15.5, when testis and ovaries are anatomically distinct, and the signal for LRH-1 declines in gonads of both sexes (76). At day E17.5, LRH-1 exhibits its adult expression profile in the liver, exocrine pancreas, intestinal crypts and gastric epithelium (76).

Mice homozygous for a germline mutation in the gene encoding LRH-1 die between E6 and E7.5 (Table 1.3), a time that corresponds to gastrulation, indicating that LRH-1 plays a crucial role in the formation of the early embryo (208). Interestingly, development of the embryo to a multicellular stage occurs (148), indicating that proliferation can occur in the absence of LRH-1. Embryos heterozygous for the mutation display growth retardation, epiblast disorganization, and impaired primitive streak morphogenesis (148). Failure of gastrulation is believed to be secondary to defective visceral endoderm development (148). The mechanism is postulated to be related to the maintenance of pluripotency of the embryonic cells. As noted above, LRH-1 co-localizes with the pluripotency factors, OCT4 in the inner cell mass and in the embryonic

epiblast of the mouse, and LRH-1 is required to maintain OCT4 expression at the epiblast stage of embryonic development (94).

The ontology of SF-1 expression during development is less clear (Table 1.3). Its first appearance is in the adrenal/gonadal primordium region of the mesoderm that condenses to become the urogenital ridge on E9 in the mouse, and by E11 it is found in the separated gonadal and adrenal anlagen (117). SF-1 expression then continues in the developing steroidogenic portion of the adrenal gland and then in the outer cortical region (E11-12) (117). It is not indispensable, as mice with a germline mutation of SF-1 survive *in utero*, but succumb by the eighth postnatal day due to adrenal agenesis and consequent adrenocortical insufficiency (176).

Table 1- 3 Phenotypic characteristics of mouse models with germline deletion of LRH-1 or SF-1 or tissue-specific depletion of either gene in gonadal tissues showing the consequences of depletion on reproductive processes and consequent fertility

		LRH-1		SF-1		Reference
Germline deletion	Homozygous	Embryo lethality at gastrulation period (E6-7.5) Defective visceral endoderm development Impaired primitive streak morphogenesis		Postnatal lethality up to 8 days after birth Absence of adrenal gland and gonad regression at E11.5-12 Adrenocortical insufficiency		(176, 208) (148)
	Heterozygous	Reduced fertility in females due to impaired progesterone production		Fertile		(149, 176)
Cre-loxP conditional depletion	**Cre recombinase**	**Male**	**Female**	**Male**	**Female**	
	Amhr2	*Fertile*	Depletion in granulosa cells of all follicles including corpora lutea Infertile No ovulation No luteinization Decrease in granulosa cells proliferation No cumulus expansion	Depletion in Leydig cells of testes Infertile Arrested spermatogenesis Delayed fetal testes organization Hypoplastic, undescended testes Decrease in testes somatic cell proliferation	Depletion in granulosa cells of all follicles Infertile No ovulation Absence of CL Decrease in granulosa cells proliferation And reduced follicle number	(68) (214)
	Cyp19a1	*Fertile*	Depletion in granulosa cells from antral follicles forward Infertile Incomplete cumulus expansion No ovulation No luteinization	*Fertile*	*Fertile*	(14)
	Pgr	*Fertile*	Depletion in corpus luteum Infertile Corpus luteum unable to maintain pregnancy. Impaired dedicualization	*Fertile*	Depletion in corpus luteum Subfertile to infertile Impaired luteinization	(318) (203)

ii. Neural development

Tissue-specific depletion strategies indicate that SF-1 expression is required at multiple sites in the hypothalamic-pituitary-gonadal axis (125, 138). In the adult mouse brain, SF-1 is expressed exclusively in the VMH (40). In the hypothalamus, SF-1 is found in mouse VMH precursor cells from E11.5 onward (61, 251). Its expression is essential for correct formation of this structure, (116). In SF-1 knockout mice, the VMH is present at E17, but with decreased cellularity and abnormal organization that persists, at least until birth (116). Germline knockout studies are buttressed by results from a central nervous system (CNS)-specific SF-1 knockout mice model, where the animals show similar disruption of the VMH organization (139). Together these findings indicate that SF-1 is not involved in the early stages of VMH development, but that absence of this transcription factor results in incorrect architecture and premature regression of the structure.

SF-1 germline knockout animals can survive if supplemented with corticosteroids, and transplantation with wild-type adrenal glands restores hypothalamic-pituitary-adrenal function(179). These animals showed significant decreased locomotor activity and late-onset obesity (179). This suggests a role of SF-1 in energy homeostatic regulation, a concept supported by the occurrence of severe obesity in CNS specific SF-1 knockout mice (139).

iii. Pituitary development

SF-1 is found in gonadotrophs of the developing pituitary at E13.5, following α-glycoprotein subunit appearance (αGSU), but before the expression of the β-subunits of LH and follicle-stimulating hormone (FSH) (120). DNA methylation analysis has shown that SF-1 is silenced in progenitor αT1 gonadotrophic cell lines, but active in both immature (αT3-1) and mature (LβT2) gonadotrophic cell lines (152). This indicates that SF-1 does not direct precursor cells to the gonadotrophic cell fate, rather, it regulates the final differentiation steps.

iv. Gonadal development

SF-1 is strongly expressed in the genital ridge of both male and female mice, in the undifferentiated gonads from E9-E13, before the sex determining region Y (SRY) protein expression begins to induce sexual differentiation (117). Germline inactivation of SF-1 in mice does not interfere with normal early gonad development or with germ cell colonization, but differentiation is arrested around E11–11.5 and the cells degenerate via apoptosis (176). This suggests that SF-1 is not involved in the early stages of gonad identity specification, but required for differentiation, survival and growth of the somatic cells already present in the early indifferent gonad. SF-1 has been shown to participate in the transcription of *Sox9* by binding to its gonad specific enhancer region together with SRY (240), and SOX9 in turn activates Anti-Müllerian hormone (*Amh*) transcription together with SF-1 to induce male sexual development by inhibiting the formation of female gonads (59).

As gonad differentiation occurs, SF-1 continues to be expressed in a diffuse manner in the interstitial region, the testicular cords and seminiferous tubules of the testis (E12.5-15). In contrast, it disappears from the developing ovary between E13.5 and 16.5, only to reappear after birth in this organ (117). Sex-specific regulation is achieved by repression of SF-1 by the forkhead box L2 (FOXL2) transcription factor during ovarian development (263). As chronicled above, one of the principal mechanisms by which SF-1 regulates gonadal function is the induction of steroidogenic enzymes (117). Further it has been shown that SF-1 (and LRH-1) can transform both pluripotent and mesenchymal stem cells into steroidogenic cells *in vitro* (310). The combination of SF-1 with WT1, DMRT1, GATA4 and SOX9 transformed mouse fibroblasts into Sertoli-like cells (28). Together these observations demonstrate the pivotal roles for both SF-1 and LRH-1 in gonadal and reproductive tract development.

B. Gonadotropin synthesis and release

Neurons that are positive for SF-1 in the VMH express estrogen receptor α (ESR1) as well as leptin receptors, both known to be essential for appropriate gonadotropin secretion (299). This regulation has been attributed to impingement of VMH projections onto gonadotropin releasing hormone (GnRH) neurons (89), thus, it is to be expected that GnRH secretion would be impaired as the consequence of developmental disruption of the VMH in the absence of SF-1 (116). Support for this concept comes from CNS-specific SF-1 knockout mice that display diminished LH responses to exogenous GnRH relative to control animals (138). It was concluded that the absence of SF-1 in cells of the CNS and consequent abnormality has downstream effects in the form of reduced GnRH priming or synthetic capacity of pituitary gonadotrophs.

LRH-1 also plays a prominent role in the hypothalamus, particularly in the regulation of the female reproductive axis. LRH-1 expression in the CNS is localized to a limited area of the brain, the arcuate nucleus that includes kisspeptin (Kiss) neurons, a region well known for the regulation of GnRH secretion (3, 146). In mice, LRH-1 controls FSH levels, follicle maturation, and estrous cycle by binding directly to the *Kiss1* promoter and stimulating its transcription (3). The consequence of the depletion of LRH-1 is the reduction in the secretion of GnRH, which in turn reduces gonadotropin secretion (3).

The promoter regions of the common α-subunit and the subunits specific to the gonadotropic hormones, FSHß and LHß, display the SF-1/LRH-1 response element, indicating direct regulation by the NR5A receptors (81). Mutation of this site eliminated LHß promoter activity (135). As noted elsewhere, germline deletion of SF-1 has severe consequences on prenatal reproductive development and postnatal reproductive function. The synthesis and secretion of both LH and FSH are impaired, as is the expression of the GnRH receptor on

gonadotroph membranes (reviewed in (323)). Pituitary-specific knockout, targeting floxed SF-1 by means of Cre recombinase driving the αGSU common subunit, depleted FSH and LH content in the pituitary to the point of near absence, while all other pituitary hormones were unaffected (323). The expected abrogation of fertility followed.

LRH-1 has recently been detected in the anterior pituitary gland; but its functional significance *in vivo* is only partially understood. Indeed, LRH-1 mRNA and protein expression were also found in both primary pituitary cells and gonadotroph-derived cell lines (325). LRH-1 has been shown to regulate gonadotropin gene expression, activating the FSH and LH secretion from the rat anterior pituitary gland and in gonadotropic cell lines, *in vitro* (325). This notwithstanding, a recent study using a mouse model with a gonadotroph-specific deletion of LRH-1 demonstrated that these mice had normal pituitary FSH and LH expression and intact fertility, indicating its expression in the pituitary is dispensable *in vivo* (80).

C. Gonadal function

i. Testis

As noted above, it has been shown that SF-1 and LRH-1 are involved in the differentiation of mesenchymal stem cells into steroid hormone-producing cells, and in induction of the expression of the androgen-specific enzyme, CYP17A1 (313). They therefore play a pivotal role in steroid hormone production in human Leydig cells (311).

Given the demonstrated importance of both SF-1 and LRH-1 in regulation of steroidogenic enzymes and factors (76), various groups have explored their roles in testicular function. Interestingly, LRH-1 was undetectable in the Sertoli cells that regulate the development of germ cells and where SF-1 expression is high (218, 248). On the other hand, LRH-1 is expressed at appreciable levels, quantitatively greater than SF-1, in Leydig cells as well as in pachytene

spermatocytes and round spermatids (218, 248). Further, LRH-1 is expressed in several rat and mouse testicular cell types where it regulates aromatase expression (218).

Additionally, as discussed above, SF-1 plays a role in the development of testis. It stimulates the expression of AMH, inducing the regression of the Müllerian ducts in the developing fetus, and inhibiting female genitalia formation (242) and, in the absence of SF-1 in male germline, a sex reversal phenotype is observed demonstrating the essential role SF-1 in testis formation and function (120, 235, 245). In mature testes, SF-1 is expressed in the Leydig cells, where it regulates progenitor cell formation and survival, and in the Sertoli cells of the seminiferous tubules, where, as it is the case in the developing testes, it plays a role in AMH transcription (113, 131). Leydig-specific SF-1 knockout mice have been produced via the inactivation of SF-1 in cells expressing the AMH type 2 receptor (AMHr2) (Table 1.3)(125). Male mice in this model were infertile and also showed undescended, hypoplastic testes with abnormal structure. The lumina of the seminiferous tubules were closed, and spermatogonia failed to develop into sperm. This effect was attributed to androgen deficiency, and indeed, CYP11A1 and STAR expression, essential upstream proteins in testosterone production, were significantly reduced.

Precocious expression of LRH-1 in mice leads to precocious induction of androgen synthesis and early puberty (68) most likely due to effects on the hypothalamic-pituitary mechanisms regulating the onset of reproductive function. In contrast, mice heterozygous for LRH-1 mutation, i.e. where only a single functional allele is present, have circulating testosterone levels that are less than half of what is observed in their wild type littermates (277). Recent investigation showed by immunogold localization that LRH-1 is present in the head of human spermatozoa, with markedly reduced expression in the neck and across the tail, but also in different stages of testicular germ cells development (191). This study demonstrates that

LRH-1 plays a role in sperm motility, survival and cholesterol efflux, and appears to serve as a downstream target of estrogenic signaling.

ii. Ovary

In granulosa-specific SF-1 knockout mice, obtained via the depletion of SF-1 in the granulosa cells by means of Amhr2 Cre, females were infertile, showed hypoplastic ovaries with reduced numbers of oocytes and complete absence of luteal formation (214). This was an important indicator that SF-1, like LRH-1, is crucial for normal ovarian function. Indeed, as noted in the section on steroidogenesis, SF-1 has been shown to directly regulate theca cell transcription of CYP19A1, and also to bind CYP17 and STAR promoter regions in the ovary (196, 280). While studies have mainly reported low SF-1 expression in the mouse corpus luteum (106), bovine studies have shown that SF-1 inhibition leads to significant decrease in progesterone production in luteal cells (190). Analysis of NR5A receptor expression in the rat ovary indicated that SF-1 was expressed in ovarian cell types, i.e. granulosa, thecal and luteal cells at a higher level than LRH-1, which is restricted to the granulosa cells (72). In the macaque corpus luteum, SF-1 regulates the luteal secretion of inhibin-α (Inha), known to play a crucial role in suppression of FSH secretion (261). One of the important roles of SF-1 in granulosa cells, beyond biosynthesis of steroids, is its capacity to modulate the expression of AMH, a hormone involved in the ovarian reserve. In this context, SF-1 requires interaction with FOXL2 to bind the *AMH* promoter and induce its transcription (126). These interactions have not yet been well studied, but it has also been shown that FOXL2 is a suppressor of SF-1, leading to the inhibition of *Cyp17a1* transcription and interrupted follicle development (209).

SF-1 has other target genes in ovarian tissue. One of these in human steroidogenic cells is 5-aminolevulinic acid synthase 1 (*ALAS1*), a rate-limiting enzyme for heme biosynthesis in

mammals (128). This enzyme plays a role in progesterone production via the supply of heme as a prosthetic group of P450 steroid hormone-synthesizing enzymes (128). In another example, SF-1 combines with SMAD3 to activate TGFβ3-induced CYP19A1 expression and subsequent estradiol synthesis and secretion in mouse granulosa cells (166). The expression of another SF-1 target gene, *Cyp17a1*, is negatively regulated by the AP-1 family member FOS, a proto-oncogene that can reduce SF-1 activity by blocking both its transcription and binding to its LBD hinge region (247).

LRH-1 is highly expressed in the mouse ovary, and is specific to the steroidogenic granulosa and luteal cells, and distinctly absent in theca cells and ovarian stroma (106). It has also been identified in equine (21), rat (72), rabbit (1), bovine (75) and human (248) ovaries. During folliculogenesis, LRH-1 is expressed in the pre-granulosa cells of primordial follicles, in the granulosa cells of primary follicles and at all later stages of follicular development (76, 183). LRH-1 plays a major, but not indispensable role in granulosa cell proliferation (68, 183), and is also induced significantly in the CL during pregnancy (107). In rodents, the expression of LRH-1 is increased by FSH in granulosa cells, and by prolactin in luteal cells (72).

The essential role of LRH-1 in reproductive function and steroidogenesis *in vivo* is evidenced in mice heterozygous for a null mutation of LRH-1, where females are infertile due to a dysregulated luteal function (149). Additionally, granulosa-specific LRH-1 knockout females are also infertile, due to the failure in both cumulus expansion and ovulation, effects that cannot be redressed by gonadotropin stimulation (68, 125). The normal expression of a number of genes is disrupted in this mouse model, including the steroidogenic genes *Cyp11a1, Cyp19a1* and *Star*; as well as the rate-limiting gene in prostaglandin synthesis, the prostaglandin-endoperoxide synthase 2 (*Ptgs2*); and genes associated with cholesterol transfer such as *Scarb1* (68).

Another transgenic mouse model with the effect of depletion of LRH-1 in granulosa cells of antral follicles produced infertile females due to incomplete cumulus expansion, as well as a lack of ovulation (14). Key genes involved in the process of cumulus expansion and ovulation such as epiregulin (*Ereg*), amphiregulin (*Areg*), betacellulin (*Btc*) and tumor necrosis factor stimulated gene-6 (*Tnfaip6*) are significantly downregulated while connexin 43 (*Gja1*) is drastically upregulated in this knockout mouse model. Interestingly, the non-ovulated oocytes can be fertilized by intra-cytoplasmic sperm injection (ICSI), indicating that their viability is not affected, thus confirming that the absence of LRH-1 in granulosa cells and failure of ovulation is the genesis of their infertile condition (14). To further study the role of LRH-1 in ovulation, a CL-specific LRH-1 knockout mouse model was created and produced females capable of breeding, where ovulation and fertilization occurred, but the animals were infertile. In this mouse model, luteal size is reduced, and luteal function compromised, as evidenced by reduced circulating levels of progesterone. Ovarian expression of steroidogenic factors, including STAR and CYP11A1 is dramatically reduced, demonstrating that LRH-1 is required for luteal function (318).

These ovarian results are largely recapitulated in another transgenic mouse model generated by means of an inducible shRNA under the influence of a TET promoter, where LRH-1 depletion is actuated at will *in vivo* (85). The TET-treated females present a consistent reproductive phenotype that mimics the LRH-1 granulosa-specific knockout. Interestingly, the infertility is fully reversible after the cessation of LRH-1 knockdown, with no signs of permanent changes due to the transient reduction of LRH-1 expression.

These various examples demonstrated that SF-1 and LRH-1 are essential modulators of ovarian function in mammals, and that they are promising targets for novel fertility and

contraceptive treatments.

D. Gestation

Years of fundamental and applied research have shown that progesterone, produced principally by the corpus luteum during early gestation, is crucial for the initiation of pregnancy, for embryo implantation, for maintenance of pregnancy, and for suppression of the LH secretion that induces ovulation in mammals. Progesterone further drives decidualization, a process by which stromal cells differentiate into decidual cells. Decidualization of the maternal stroma is key to a successful implantation and appropriate placental formation, and it confers maternal immunotolerance to the fetus (reviewed (221)). Studies have reported that SF-1 expression is absent from the fetal components of the placenta, including trophoblast cells in humans and in rodents (258, 303). SF-1 expression does not appear to be a regulatory factor in the healthy uterine tissues; although, its continued expression in the CL of pregnant bovine and porcine ovaries indicates that it plays an essential role in maintaining gestation (190, 223). The cofactors DAX1 and WT1 have been shown to inhibit the cAMP-dependent transcriptional activity of SF-1 on CYP19A1 expression in cultured human endometrial cells (95). In mouse models where SF-1 expression is depleted specifically in granulosa cells, uterine development is significantly reduced in the epithelial, myometrial and stromal layers, resulting in absent or fewer complex endometrial glands (214). The effect was not interpreted to be direct, rather, absence of SF-1 in ovarian granulosa cells leading to impaired steroidogenic gene expression, and the consequent reduction in ovarian estradiol production, is insufficient to stimulate normal uterine differentiation. In a novel mouse model, induction of overexpression of SF-1 in cells expressing progesterone receptor (PGR) was characterized by abnormal uterine morphology, with enhanced endometrial gland and cyst development, and consequent infertility in females (275).

Endometrial cells of the SF-1 overexpressing mice did not express PGR and were unable to decidualize in response to hormone stimulation, suggesting that SF-1 silencing is essential for normal uterine function.

Zhang et al (318) demonstrated that LRH-1, expressed in the mouse and human endometrium, is necessary for endometrial decidualization, placenta formation and, ultimately, successful pregnancy. This was shown in a CL-specific LRH-1 knockout mouse that displayed luteal insufficiency, and where gestational failure cannot be mitigated by progesterone supplementation (318). Progesterone treatment induce successful implantation, but embryo development fails due to defects in placentation. These effects transpire at different times among animals, some soon following implantation, some as late as day 16 of pregnancy, and some at varying intervals. Uterine genes essential for gestation that are deregulated when LRH-1 is depleted from mouse CL and endometrium are *Hoxa10*, *Wnt4, Wnt5, Bmp2, Ppard* and *Hbegr*. In humans, siRNA reduction of LRH-1 impaired decidualization of the endometrium (318), and therefore the development of the placenta by affecting the invasion of extravillous trophoblastic cells into the uterine decidua (319). Thus, LRH-1 is essential for appropriate establishment of the maternal-fetal connection, and insufficient expression may be a factor in human gestational pathology, including pre-eclampsia (319).

E. Reproductive behavior

As mentioned above, SF-1 is strongly expressed in the VMH nucleus and is essential for the normal function of neurons of this region, with impact on mammalian behavior including physical activity, anxiety and aggressiveness (90, 179, 324). The VMH also regulates reproductive behavior, and the female sex hormones estrogen and progesterone secreted at different stages of the ovarian cycle leads to modifications in VMH neuron morphology (90).

CNS-specific SF-1 knockout mice show impaired female reproductive function, with abnormal sexual behavior, irregular estrous cycles and subfertility (138). The marked reduction in lordosis and receptivity observed in females of this mouse model may be caused directly by altered VMH organization, preventing neuron projection to the medial central gray and peri-aqueductal gray regions where lordosis is controlled (36), or indirectly, where ventromedial neurons of the VMH are unable to transmit excitatory signals to GnRH neurons and with effects on steroid synthesis and subsequent reproductive behavior (316). Although studies have shown that some components of male sexual behavior and copulatory performance are induced by androgen activation in the VMH, in addition to the essential androgen receptor activity in the medial preoptic area (MPOA), CNS-specific SF-1 knockout male mice do not show impaired reproductive behavior (101, 138). To our knowledge, no studies have been published on the effect of LRH-1 on reproductive behavior.

8. Pathological processes related to reproduction

A. Genetic abnormalities

Although no complete deletions of the SF-1 gene appear to have been observed in humans to date, non-silent single nucleotide polymorphisms, frameshifts and partial deletions have been described as causal to disorders in the heterozygous state (reviewed in (260)). Due to its essential role in steroidogenesis, the SF-1 mutations in humans were initially linked to adrenal insufficiency and gonadal dysgenesis, where 46XY patients presented external female genitalia, uterine and upper vagina structures and primary adrenal failure (46,XY DSD) (77). Heterozygous mutations in SF-1 are the cause of up to 20% of 46,XY DSD cases, without affecting adrenal function, and reported to account for approximatively 4% of infertile men with

severe spermatogenic failure that do not have chromosomal anomalies (9, 141). Single nucleotide variations and missense mutations in the human SF-1 gene have been associated with low testosterone levels, elevated gonadotropin secretion, azoospermia, oligozoospermia and hypospadias (9, 142). Women that carry SF-1 heterozygous mutations show a wide range of clinical phenotypes, some with no impact whatsoever, while others show 46,XX gonadal dysgenesis or agenesis, accompanied by conditions such as primary or secondary amenorrhea, precocious depletion of the ovarian reserve and consequent premature ovarian failure (34, 173). Recent clinical evaluations have demonstrated that presence of SF-1 mutations in women with primary ovarian insufficiency (POI) to be rare, excluding it as a prevalent genetic factor for this condition (124, 276). Most clinical accounts of SF-1 anomalies have been associated with loss of function, but there have been reports of SF-1 overexpression and overactivity as well. This has been attributed, in some instances, to copy number variation of SF-1 through genomic duplication at its chromosome locus or, alternatively, upregulation of SF-1 through decreased promoter methylation, leading to tumor development (78, 220). There appears to be no published information on human genetic abnormalities attributable in LRH-1.

B. Endometriosis

Endometriosis is characterized by presence of endometrial glands and stroma in ectopic locations, usually the peritoneal cavity, causing abnormal growth that can lead to persistent pelvic pain and infertility (31). In addition to some genetic predisposition, estrogen-dependency, progesterone resistance and inflammation are clear molecular indicators of the disease, mediated by growth factors, metalloproteinases, prostaglandins and cytokines (26, 31, 230). Bulun et al.(200) showed that, while normal endometrial tissues do not express steroidogenic genes, uterine tissues from women with endometriosis showed detectable expression of CYP19A1 and

estrogen production. Further studies showed that inflammation and estrogen production in endometriosis are connected by positive feedback that promotes expression of factors including STAR, CYP19A1, and PTGS2, all of which have been shown to be aberrantly overexpressed in endometriotic lesions (29, 206). The regulation of these steroidogenic genes strongly suggests that SF-1 and LRH-1 play a role in endometriosis. Indeed, studies have shown that while in normal endometrial cells, chicken-ovalbumin upstream-transcription factor (COUP-TF) and WT1 bind to the promoter region of aromatase to inhibit its expression, in endometriotic stromal cells, the presence of SF-1 expression competes with these two transcription factors to induce steroidogenic gene transcription (4, 95). Experiments in which prostaglandin E2 (PGE2) was overexpressed *in vitro* demonstrated that *de novo* steroidogenesis in endometriotic tissues is regulated by the PGE2-cAMP-SF-1 pathway, with driving estradiol production (4). Moreover, Tian et al. showed that the abundance of SF-1 and its target gene, STAR to be correlated with the severity of endometriosis (267).

In terms of mechanisms, it has been shown that the SF-1 gene is differentially methylated in the endometrium of women with endometriosis, compared to those not afflicted with the disease (114). The hypothesis that SF-1 is a determinant factor in endometriosis has been supported by the fact that, in non-pathological conditions, the SF-1 promoter and exon I region of endometrial stromal cells shows a dense methylation pattern, epigenetically silencing SF-1 expression (301). When aberrant demethylation of the SF-1 promoter occurs, as it is observed in endometriosis, expression of SF-1 is upregulated (301). In turn, this overexpression of SF-1 causes an increase in steroidogenesis, favoring inflammation and growth of ectopic endometrial tissue. The mouse model described above with conditional uterine overexpression of SF-1, where mice showed enlarged, aberrant endometrial glands and activated immune response, provides experimental support for a role of this transcription factor in endometriosis (275).

C. Cancer

Accumulating evidence indicates that LRH-1 participates in the pathogenesis of tumors of multiple sorts (reviewed in (198)) including pancreatic (11, 216), breast (266), gastric (284), and colon cancer (239). Suppression of LRH-1 in colon cancer (10) or osteosarcoma cells (165), inhibits, but does not eliminate proliferation. Recent studies showed that, in some pancreatic cancer cell lines, higher LRH-1 mRNA levels were present compared to normal pancreatic ductal epithelium cells (11). Overexpression of LRH-1 in these pancreatic cancer cell lines is characterized by a phenotype of increased cell proliferation, via upregulation of genes, including cyclins D1/E that regulate cell cycle, and cMyc, a proto-oncogene that controls generation of self-renewing metastatic cancer cells (121). Also upregulated are the metalloproteinases, MMP2 and MMP9, implicated in metastasis, and known to facilitate tumor growth, cell migration and tumor invasion (93). Moreover, LRH-1 can promote pancreatic cancer metastasis (11, 169) and promotes intestinal tumor proliferation in gastrointestinal tumors by activating the Wnt/β-catenin pathway (10, 284). LRH-1 also contributes to intestinal tumor formation via its interaction with β-catenin/TCF4, known to induce cyclins D1/E1, which in turn regulate the cell entry in the G1/S phase of the cell-cycle and their subsequent proliferation (23). LRH-1 has been shown to drive colon cancer cell growth by repressing the expression of CDKN1A in a p53 dependent manner (143), the same mechanism that is involved in LRH-1 regulation of breast cancer (see below). Overall, LRH-1 acts in the initiation of intestinal tumor formation through effects on the cell cycle and through impact on inflammatory pathways.

Estrogens have been shown to promote proliferation of malignant ovarian cancer cell lines, while progesterone inhibits proliferation and promotes apoptosis of these cells (175). Ovarian cancer researchers have identified SF-1 as a potential repressor of cancer cell proliferation, due to its crucial role in progesterone biosynthesis. Studies have demonstrated that

transient expression of SF-1 in certain human ovarian cancer cell lines inhibits estrogen-induced proliferation and promotes apoptosis (225). While SF-1 gene expression is observed in human epithelial and metastatic tumors of the ovary, these adenoma and carcinoma cells do not present SF-1 immunoreactivity (236). Conversely, clinical studies have suggested that ovarian tumors with functioning stroma secrete estradiol via the regulation of aromatase, due to overexpression of SF-1 (103). A recent extensive meta-analysis of a wide range of ovarian cancer clinical studies was aimed at determining whether SF-1 is associated with ovarian tumor progression (115). The results demonstrated that SF-1 expression level is significantly lower in ovarian cancer than in normal ovarian tissues, perhaps due to epigenetic silencing via increased methylation of the SF-1 gene (186). A further conclusion was that SF-1 expression pattern could serve as a marker to differentiate ovarian sex cord stromal tumors, where SF-1 is higher, from ovarian cancer, where it is markedly lower. A similar observation was made in granulosa cell tumors, where both SF-1 and LRH-1 expression were increased when compared to normal ovarian tissues as well as, in the case of SF-1 only, cells from other types of ovarian cancer (mucinous and serous) (42). This study also demonstrated that in the granulosa cell tumor-like *in vitro* cell line (KGN), SF-1 binds preferentially to the aromatase promoter II region relative to LRH-1, indicating that SF-1 is driving aromatase expression in this type of ovarian cancer (42). LRH-1 action in this context remains unknown, possibly functioning to activate proliferation. Similar results were observed in both human and rat testicular Leydig cell tumors, where elevated levels of aromatase mRNA were linked to increased SF-1 expression (257).

These clinical and cellular cancer studies demonstrate that both SF-1 and LRH-1 play different roles in ovarian cancer, depending on the type of tumor, or even the cell type of origin of the tumor.

Finally, there is considerable evidence implicating LRH-1 involvement in breast cancer (84). In the human breast, LRH-1 is expressed in the stromal compartment and in undifferentiated adipose tissue where it regulates aromatase expression and promotes estrogen biosynthesis (84). Its expression promotes proliferation, migration and invasion of breast cancer cells *in vitro* (154). LRH-1 expression is regulated by estrogen and its mRNA transcript levels have been proven to be higher in ESR1-positive but more stable in ERα-negative cells (154). In ERα-positive breast cancer cells, LRH-1 promotes cell proliferation by increasing estrogen biosynthesis by regulating aromatase expression (266) and by ESR1-mediated transcription of target genes such as *GREB1*(43). Estrogen-dependent breast cancer is often treated with aromatase inhibitors or estrogen receptor agonists such as tamoxifen. Altered expression and functions of microRNAs have been reportedly associated with tamoxifen resistance (326). The microRNA, miR-027b-3p directly targets and inhibits the expression of LRH-1 and CREB1, and its levels were found to be significantly negatively correlated with LRH-1 and CREB1 levels in breast cancer tissues (326). As further evidence of a role for LRH-1 in mammary carcinogenesis, LRH-1 levels have been shown to be particularly elevated in chemoresistant breast cancer tissues from patients after recurrent chemotherapy (283). LRH-1 promotes breast cancer cell resistance to chemotherapy by upregulating the checkpoint protein, MDC1, to enhance DNA damage repair (283). Further, independent of its interactions with estrogen, LRH-1 overexpression was observed to promote remodeling of the actin cytoskeleton and E-cadherin cleavage, contributing to increase migration and invasion of the cancerous cells (41). Analysis of breast cancer samples by Bianco et al. (17) also revealed that LRH-1 regulates cell proliferation by inhibiting CDKN1A expression, thus removing an endogenous brake on proliferation. As above, this mechanism occurs independent of the proliferation involving ESR1 or p53. High levels of LRH-1 are also associated with poor breast cancer prognosis (16, 17).

Given its important role in the progression of both estrogen positive and negative cancers, and its involvement in regulating hormone-independent pathways such as CDKN1A, it is clear that inhibition of LRH-1 could provide a powerful new approach for the treatment of endocrine-resistant breast cancer (16, 17). To our knowledge, no studies have shown SF-1 deregulation or involvement in breast cancer.

9. LRH-1/SF-1 interactions

Given the structural similarities of SF-1 and LRH-1, along with their demonstrated ability to bind to the same DNA sequences, one would expect that they could reciprocally compensate for one another. In a number of tissues, particularly those in the digestive system, compensation does not occur because of the lack of significant overlapping expression (199). Other factors may be in play, due to differences in structure between the two nuclear receptors that have been shown to exist, including variation in helix length (144), which could result in differential responses to endogenous agonists. There are species differences as well, particularly in the size and primary structure of the ligand-binding pocket, such that the mouse appears to have undergone a radical reduction in the capacity to bind ligands by both SF-1 and LRH-1 (144). In addition, there is some evidence that the two nuclear receptors are differentially sensitive to the inhibitory effects of DAX1, and this cofactor is more potent in repressing the transactivation of steroid synthetic enzymes by LRH-1 than by SF-1 (33).

LRH-1 and SF-1 are co-expressed in the ovary at all stages of the estrous cycle (106). The ovary-specific models of depletion targeting the follicular granulosa cells by means of the Amhr2 Cre driver argue that compensation of LRH-1 for SF-1 depletion, or conversely, SF-1 for LRH-1, does not occur (68). Indeed, there are only modest increases in mRNA for LRH-1

in the ovaries of the Amhr2/SF-1 granulosa specific knockout mouse, in both the gonadotropin stimulated and untreated conditions (214). As noted above, the Amhr2/SF-1 female mouse is infertile due to disruption of the follicle development trajectory (214) while in the Amhr2/LRH-1 ovary, large antral follicles that appear structurally normal are present, but these fail to ovulate (13, 68). These differences occur even though the two receptors regulate many of the same genes, particularly those associated with steroidogenesis, and in the same cells.

Both SF-1 and LRH-1 are present and active in the murine corpus luteum (106), but, as above, SF-1 cannot compensate for LRH-1 deficiency in this tissue (318). This may be due to the differential amounts that are recruited to promoters of the genes of luteal function in response to luteotropic stimuli, as reported by Weck et al.(287). By means of a chromatin immunoprecipitation technique, these authors showed that SF-1 binding to the *Inha* subunit promoter decreases and LRH-1 binding increases following activation of the cAMP signal that induces granulosa cell differentiation to luteal cells. One potential mechanism for this change is the relative expression (LRH-1 up, SF-1 down) of the two nuclear receptors following stimulation of the ovarian cells with gonadotropins (287). A further possibility is that stimulatory ligands may differentially regulate LRH-1 vs. SF-1 transactivation by acting through different intracellular signaling mechanisms (287). The mechanisms of selective action clearly merit further investigation.

10. Summary, conclusions and future perspectives

The NR5A receptors are nuclear receptor proteins that act as transcription factors. They are evolutionarily conserved, as orthologues and paralogues are found in metazoans, from roundworms to mammals. The two mammalian forms, known as LRH-1 and SF-1, are common

to reproductive tissues, but are also differentially expressed across a wide range of organs. While they interact with the same or highly similar DNA sequences, they have multiple, often non-overlapping actions, and cannot compensate for each other. While a good deal is known about SF-1 and LRH-1, the interaction, synergy or antagonism of the multiplex array of regulators, from cofactors to miRNA to epigenetic mechanisms, remains far from completely explained. Regulation by the two receptors appears to be cell and context specific, and the extent of commonality is unclear.

Much new information has emerged in recent years with respect to their differential roles in the regulation of fertility, derived primarily from conditional, cell-specific mutations in mice (Table 1-3 and Figure 1-6). Again, this information demonstrates that SF-1 depletion in granulosa cells disrupts the follicle development process, while folliculogenesis proceeds to the large antral stage in LRH-1 depleted follicles. Most models of depletion of either of the nuclear receptors in granulosa cells are anovulatory, with the exception of the knockdown at the late peri-ovulatory state in the PgrCre/LRH-1 mouse. LRH-1 is an essential regulator of endometrial decidualization, while SF-1 appears to be present in endometriotic tissue. Both LRH-1 and SF-1 regulate steroid synthesis, but often in different tissues, LRH-1 in granulosa cells, SF-1 in theca and Leydig cells. In some tissues, such as the corpus luteum, LRH-1 and SF-1 are co-expressed and the extent of overlap in their targets is yet unknown.

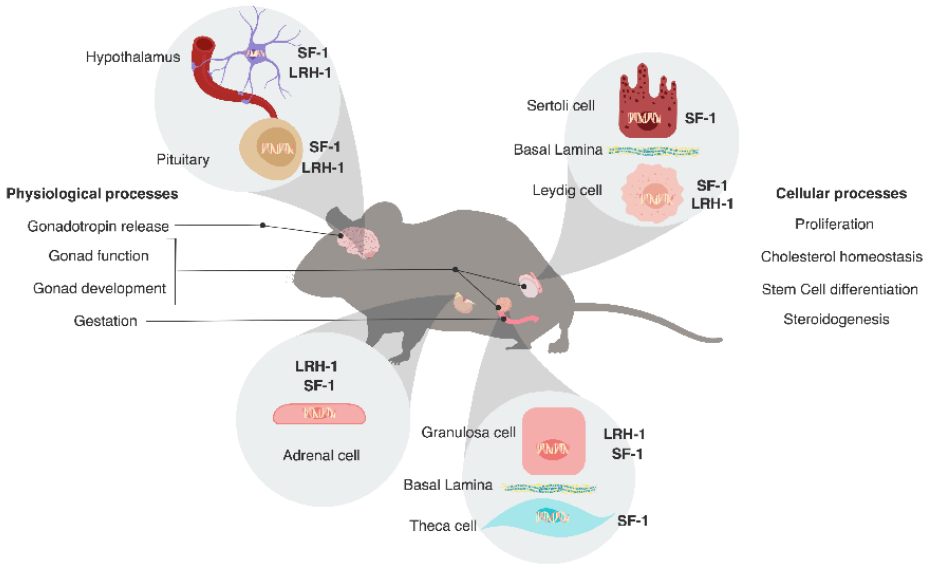
It is enigmatic that two receptors that bind to the same DNA sequence in the same tissue can have disparate effects. Are there yet undiscovered endogenous ligands that selectively modulates their activity? Are the differential effects related to the dose of the receptors present in the cell? Both receptors are implicated in stem cell pluripotency, but in other circumstances, such as decidual conversion of endometrial stromal cells, LRH-1 is essential for terminal differentiation. LRH-1 appears to be a potent impetus for cell proliferation, but is not an absolute

requirement, as embryos develop to a multicellular stage in germline deleted mice, and depletion in the ovary only partially compromises granulosa cell multiplication. These are among the many questions that need to be addressed in future research.

11. Disclosures

The authors have no conflicts of interest, financial or otherwise to disclose.

Figure 1- 6 Summary of the essential cellular and physiological effects of the NR5A receptors LRH-1 and SF-1 in reproductive tissues using the mouse as a model.



CHAPTER 2: Hypothesis and objectives

Hypothesis

Based on the previous published work demonstrating that NR5A2 is specifically expressed in granulosa cells of all follicles and its essential role in the ovulatory process, cumulus expansion and luteinization, we hypothesize that NR5A2 modulates follicular development leading to ovulation.

The purpose of this investigation was to determine the role of NR5A2 in the ovarian function, specially in the regulation of follicular activation and growth.

Objectives

1. To examine the role of NR5A2 in granulosa cell proliferation *in vivo* using a mice conditional knock-out model *Nr5a2*$^{f/f}$, *Amhr2*$^{Cre+}$ and *in vitro*. The corresponding results are described in the Chapter 3.

2. To determine what is the role of NR5A2 in the regulation of the ovarian reserve using the same mouse model that in objective 1. The results corresponding to this objective are discussed in Chapter 4.

CHAPTER 3 : First manuscript

The Orphan Nuclear Receptor Liver Receptor Homolog-1 (NR5A2) Regulates Ovarian Granulosa Cell Proliferation

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Précis: The nuclear receptor, NR5A2, regulates gonadotropin-induced proliferation in mouse granulosa cells by regulating the expression of cell cycle kinases and their inhibitors.

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Abstract

In mouse ovaries, liver receptor homolog-1 (NR5A2) expression is restricted to granulosa cells. Mice with NR5A2 depletion in this cell population fail to ovulate. To determine whether NR5A2 is essential for granulosa cell proliferation during follicular maturation, we generated granulosa-specific knockout mice (genotype *Nr5a2*$^{f/f}$*Amhr2*$^{Cre+}$, hereafter cKO) with NR5A2 depletion from primary follicles forward. Proliferation in cKO granulosa cells was substantially reduced relative to control counterparts (CON), as assessed by bromodeoxyuridine incorporation (BrdU), proliferative cell nuclear antigen (PCNA) expression and fluorescent-activated cell sorting (FACS). Microarray analysis revealed more than 2000 differentially regulated transcripts between cKO and CON granulosa cells. Major gene ontology pathways disrupted were proliferation, steroid biosynthesis, female gamete formation and ovulatory cycle. Transcripts for key cell cycle genes, including *Ccnd1, Ccnd2, Ccne1, Ccne2, E2f1,* and *E2f2* were in reduced abundance. Transcripts from other cell cycle related factors, including *Cdh2, Plagl1, Cdkn1a, Prkar2b, Gstm1, Cdk7,* and *Pts* were overexpressed. Although the follicle-stimulating hormone and estrogen receptors were overexpressed in the cKO animals, *in vivo* treatment with estradiol-17β failed to rescue decreased proliferation. *In vitro* inactivation of NR5A2 using the ML180 reverse agonist similarly decreased cell-cycle related gene transcripts and downstream targets as in cKO mice. Pharmacological inhibition of β-catenin, a NR5A2 cofactor, decreased cyclin gene transcripts and downstream targets. TUNEL immunofluorescence and qPCR of pro/anti-apoptotic and autophagic markers showed no differences between cKO and CON granulosa cells. Thus, NR5A2 is essential for granulosa cell proliferation, but its depletion does not alter the frequency of apoptosis nor autophagy.

Keywords: Granulosa cells, orphan nuclear receptor, proliferation, cell cycle.

1. Introduction

In mammals, the process of follicle development serves to provide the structure and mechanisms to bring about maturation of the ovum and its subsequent expulsion during ovulation (1). It begins prior to birth with the formation of the primordial follicles. In its earliest postnatal stage, the primordial follicle is surrounded by a dozen or so granulosa cells (2). Its activation, by processes not yet completely understood, initiates a long developmental process in which most follicles that are activated are lost to atresia. In those that survive to the preovulatory stage, the granulosa cells undergo multiple rounds of replication with a consequent follicular population of 4.0×10^7 cells in the bovine model (2). Associated with granulosa cell replication is the formation of the follicular antrum, beginning following approximately ten rounds of proliferation (2). Gene deletion studies in mice have shown that regulation of proliferation is stage-dependent, in primordial and primary follicles, paracrine stimuli from the oocyte dominate, while growth factors are significant to preantral development (3). Antrum formation coincides with the acquisition of receptors for follicle-stimulating hormone (FSH) and the downstream synthesis of estrogens serves as a formidable proliferative stimulus to granulosa cells in antral follicles (4,5).

Recent studies have demonstrated that, in addition to estrogens, signals from orphan nuclear receptors play a role in granulosa proliferation and, thus, follicle growth. Those in the Nr5a family, steroidogenic factor-1 (SF-1, NR5A1) (6) and liver receptor homolog 1 (LRH-1; NR5A2) (7) are expressed in the ovary. NR5A2 and NR5A1 are closely related, classic zinc finger transcription factors that are believed to interact with the same or similar DNA sequences (8,9). While NR5A1 is expressed in both follicular theca and granulosa

cells, NR5A2 is restricted to granulosa cells of primary and all subsequent follicles in the ovary (8). Its expression in human ovarian follicles, as measured by transcript abundance, increases as follicles proceed through the developmental trajectory (10). Conditional ablation studies of these orphan nuclear receptors has shown that both are essential for successful development of the mouse follicle and consequent ovulation (6,7). Both are known play important roles as regulators of cell proliferation in tumor tissues. NR5A1 supports proliferation of prostate cancer cells indirectly, by promoting the steroid hormone synthesis that stimulates replication (11). NR5A2, on the other hand, is a mitogen in that it has been shown to directly induce proliferation in cancer cells from the liver (12), mammary gland (13), colon (14) and bone (15). Its effects on proliferation *in vivo* in granulosa cells, as well as the extent to which conditional depletion of NR5A2 in these cells contributes to infertility in mouse models have been little explored.

NR5A2 is a constitutively active transcription factor reported to be a direct regulator of proliferation in intestinal crypt cells, by acting to promote transition from the G0/G1 phase to the S phase of the cell cycle (16). In that study, it was shown that it directly promotes expression of cell cycle genes, cyclin D1 and E1. It was further concluded that the intracellular signaling molecule, β-catenin, is a potent coactivator of NR5A2 in the induction of proliferation (16). The synergistic effect of these two factors has been extended to include several cancer cell lines (17), but there is no current evidence for this interaction *in vivo* or in ovarian cells.

The objectives of the present study were to explore the role of NR5A2 in ovarian function, with focus on its contribution to granulosa cell proliferation. We used a mutant mouse model where NR5A2 has been depleted from the granulosa cells of follicles at all stages from the primary follicle forward.

2. Material and Methods

A. Animals and colony maintenance

Animal experiments were approved by the University of Montreal Animal Care Committee and were conducted according to the guidelines of the Canadian Council on Animal Care. All mutant and control mice were maintained on the C57BL/6 background, under a 14-hour light, 10-hour dark cycle and provided food and water *ad libitum*. Euthanasia was performed with isofluorane anesthesia followed by cervical dislocation, as previously described (18). *Nr5a2* floxed (*Nr5a2*$^{f/f}$) mice have been described previously (7,18). Granulosa-specific depletion of NR5A2 from primary follicle forward was generated by crossing these animals with mice expressing Cre-recombinase driven by the Anti-Müllerian type II receptor (*Amhr2*$^{Cre+}$) (7,18) to produce conditional knockout (cKO) mice (genotype *Nr5a2*$^{f/f}$*Amhr2*$^{Cre+}$). Following DNA extraction from tails, littermates were genotyped. Control (CON) mice in these trials were nonmutant, *Nr5a2*$^{f/f}$*Amhr2*$^{Cre-}$ females (19). Wild type mice were employed as controls in only in experiments where granulosa cells were isolated and treated with the NR5A2 inverse agonist ML180 (Cayman Chemical Co., Cedarlane, Burlington, ON, Canada).

B. Superstimulation protocol

Superstimulation (18) was achieved in 22 to 25 day-old mice by interperitoneal injection of 5 IU equine chorionic gonadotropin (eCG, Folligon; Intervet, Kirkland, QC, Canada) to stimulate follicular development. Animals were euthanized 44 to 48 hours later. Ovaries were collected, weighed, fixed in paraformaldehyde or formalin (Sigma-Aldrich, Oakville, ON, Canada) and embedded in paraffin. In other trials, granulosa cells were

isolated by ovarian puncture with 25 g needles in PBS 1x or culture medium and mechanically separated from the oocyte before filtration with 40-µm Nylon Falcon Cell Strainer (BD Inc, Mississauga, ON, Canada). As it has previously been shown that NR5A2 regulates the expression of cytochrome P450 19a1, (CYP19A1, aka aromatase), the rate limiting enzyme in estrogen synthesis, we addressed the possibility that the effects of NR5A2 depletion were solely due to disruption of estrogen synthesis. This was achieved by treatment of CON and cKO mice with a single injection of estradiol 17β, 1mg/animal according to our previous protocol (5). Ovaries were collected for analysis of proliferation (see below).

C. Bromodeoxyuridine (BrdU) incorporation and proliferating cell nuclear antigen (PCNA) and KI-67 expression

Immature cKO and CON mice were superstimulated with equine chorionic gonadotropin (eCG Folligon; Intervet, Kirkland, QC, Canada) as described above and injected with BrdU, Sigma-Aldrich, Oakville, ON, Canada), 30mg/kg, 24 hours before euthanasia. Ovaries were fixed, processed and sectioned. Slides were then rehydrated, incubated with trypsin (Invitrogen Inc, Burlington, ON, Canada) for 20 min at 37°C and 10 min at room temperature. DNA was denaturated by HCl 1N and 2N for 10min on ice and 10 min at room temperature/20min at 37°C respectively before being blocked 1h with 5% normal rabbit serum (NRS) (Jackson Immuno Research, West Grove, PA, USA) in PBS. Tissues were then incubated overnight with the first antibody sheep polyclonal against BrdU (Abcam, Toronto, ON, Canada) 1:100 in 5% NRS overnight. The following day, slides were incubated with the second antibody rabbit polyclonal to sheep IgG-H&L (FITC) (Abcam) in PBS 1x 1:400 for 1 hour and 4′,6-diamidino-2-phenylindole (DAPI)

(Sigma-Aldrich, Oakville, ON, Canada) 1 :1000 in PBS1x for 5min. Slides were mounted with Permafluor (Thermo Fisher Scientific, Mississauga, ON, Canada). Ovarian distribution of BrdU was observed by Axio imager M1 (Zeiss Microscopy, Toronto, ON, Canada) and dividing cells were counted using CellProfiler Software (Broad Institute, Cambridge, MA, USA) (4). Colored pictures were filtered to isolate the channel of interest (blue for DAPI and green for BrdU), then converted to grayscale. A sample of five follicles per section was filtered individually to obtain a significant average, then the total number of cells in the selected follicles were counted based on shape recognition in the DAPI image and the number of dividing cells that incorporated BrdU was determined based on intensity measurement in the BrdU image. The ratio of these values gave the percentage of proliferating cells. For PCNA immunofluorescence, the slides followed the same rehydration protocol before being boiled for 30 min in sodium citrate for antigen retrieval and blocked for 1 hour with 5% Bovine Serum Albumin (BSA) (Jackson Immuno Research, West Grove, PA, USA) in PBS at room temperature. We then treated with a rabbit polyclonal antibody against PCNA (Biomol SA194) first antibody 1:200 in BSA 5 %/PBS at 4C overnight and with cyanine-3 (Cy3)- conjugated goat antirabbit IgG second antibody (Jackson Immuno Research 111-165-144) diluted 1:400 in PBS 1× for 1 hour at room temperature. Finally, granulosa cells treated for KI-67 immunofluorescence were fixed with PAF 4% for 20min and blocked with 5% BSA/PBS before being treated with KI-67 goat polyclonal conjugated first antibody 1:500 (Abcam ab15580) and CY3 conjugated donkey anti-goat IgG second antibody (Jackson Immuno Research 705-165-003) 1:400 both for 1 hour at room temperature. In both cases DAPI was used for counterstaining. A similar paradigm to that employed for BrdU was used to measure the intensity signal following immunostaining for PCNA and KI-67.

D. Global analysis of gene expression by microarray

Mice were superstimulated as described above and ovaries collected at 40 h after eCG treatment. Granulosa cells were isolated from large antral follicles by laser microdissection, as previously described (7) and RNA extracted by with RNeasy Kits (Qiagen). Samples comprising pooled granulosa cells of large antral follicles from each mouse RNA samples (10 μg; 3 per genotype) with RNA integrity number (RIN) >7 were chosen for microarray analysis. Microarray experiments were performed using the Affymetrix mouse 430-2 chips to profile the gene expression levels with approximately 40,000 unique probes. Each RNA sample was converted to cRNA and hybridized on individual chip according to the manufacturer's instructions (Affymetrix and http://www-microarrays.u-strasbg.fr). Raw microarray data (.CEL files) were normalized using the = Gcrma R-pipeline. Background normalized data were then subjected to bioinformatics analysis using the web application, Network Analyst (20). Differential expression analysis was performed on quantile-normalized data using Limma algorithm on the Network Analyst platform. Probe densities (mean probe densities for genes with multiple probe sets) were compared between cKO and CON granulosa cells and differentially regulated genes were identified with parameters: false discovery rate (FDR) < 0.05 and fold change ≥ 2. The microarray data will be deposited in the NCBI Gene Expression Omnibus (http://ncbi.nlm.nih.gov/geo/). Subsequent gene ontology and pathway analyses were conducted using the Panther (http://pantherdb.org/) and Kegg (http://www.genome.jp/kegg/) databases.

E. RNA extraction and real time PCR

RNA from granulosa cells was extracted with a shredder followed by RNeasy Mini Kit (Qiagen, Toronto, ON, Canada) following the manufacturer's instructions. Reverse

transcription was performed using the SuperScript III Reverse Transcriptase (Invitrogen Inc, Burlington, ON, Canada). Real-time qPCR was performed using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories Inc, Hercules, CA, USA) with the CFX 96 Real-Time System, C1000 Touch Thermal Cycler (Bio-Rad). The transcripts were amplified following the same cycling program: 30 sec at 95 °C then 40 cycles of 15 sec at 95°C and 30 sec at 60°C followed by 5 sec steps of 0.5°C increase between 65 and 95°C. Primers employed can be found in Supplementary Table 2.1.

F. Pharmacological treatments

Granulosa cells from superstimulated immature CON mice were treated at 19 hours after plating with 3 µM of ML180. Treatments were dissolved in dimethylsulfoxide (DMSO, Sigma-Aldrich) and in both cases, a DMSO only control group was included. Treatment duration was 6 hours, after which cells were harvested for analysis of cell cycle genes by qPCR . To evaluate the effects of ML180 on granulosa cell proliferation, cells from CON mice were treated *in vitro* every 6 hours for 24 hours before quantifying proliferation by measuring incorporation of the cellular marker, KI-67. In addition, cKO cells were treated with the inhibitor to ß-catenin-responsive transcription (iCRT3, Sigma-Aldrich) at 20 µM respectively for 6 h and compared to DMSO-treated controls.

G. Cleaved caspase-3 immunofluorescence

Slides of formalin fixed paraffin embedded ovaries from superstimulated immature mice were treated as previously described (18), blocked one hour with 5% Normal Goat Serum(NGS) (Jackson Immuno Research, West Grove, PA, USA), incubated with cleaved caspase-3 antibody (Cell Signaling Technology Inc, Danvers, MA, USA) as first antibody

1 :150 in 5% NGS overnight, with cyanine-3 (Cy3)- conjugated goat antirabbit IgG second antibody (Jackson Immuno Research) diluted 1:400 in PBS 1X for 1h at room temperature and counterstained with DAPI, diluted 1:1000 in PBS 1X, for 5 min before being mounted with Permafluor. The same CellProfiler Software pipeline used for BrdU was used to count the number of atretic follicles and apoptotic cells marked with cleaved caspase-3 in ovaries of CON and cKO mice.

H. TUNEL evaluation of apoptosis

In situ Apoptosis Detection Kit (ab206386, Abcam) that allows the recognition of apoptotic nuclei, was used according to the manufacturer's instructions on slides from the same blocks as used for caspase-3 immunofluorescence. In summary, samples were rehydrated, permeabilized using Proteinase K 23 for 23min, endogenous peroxidases were inactivated with 3% H2O2 over 5 min, samples were labeled with TdT Enzyme for 2 h, blocked with blocking buffer for 10 min, incubated with conjugate for 35 min, incubated with DAB Solution for 20 min and counterstained with Methyl Green for 1 min, before being dehydrated and mounted with a coverslip.

I. Flow cytometry

Fluorescence-activated cell sorting (FACS) was performed on granulosa cells isolated by follicle punctured and pooled from both ovaries of superstimulated immature CON and cKO mice using BD Accuri C6 Cytometer (BD Inc,) according to the manufacturer's instructions. After calibrating the cytometer using beads provided by the manufacturer, the samples were diluted in Krishan buffer to a concentration allowing the

machine to count 30000 events. Results were analyzed using ModFit LT Software (Verity Software House, Topsham, ME, USA).

J. Statistical analyses

All data were analyzed using JMP (version 9.0; SAS Institute Inc, Cary, NC, USA) statistical software. Differences between mutant and control mice were determined by Student's t-test. All numerical data are represented as mean ± SEM. Significant difference was recognized at $P < 0.05$.

3. Results

A. Conditional depletion of NR5A2 in granulosa cells decreases cell proliferation

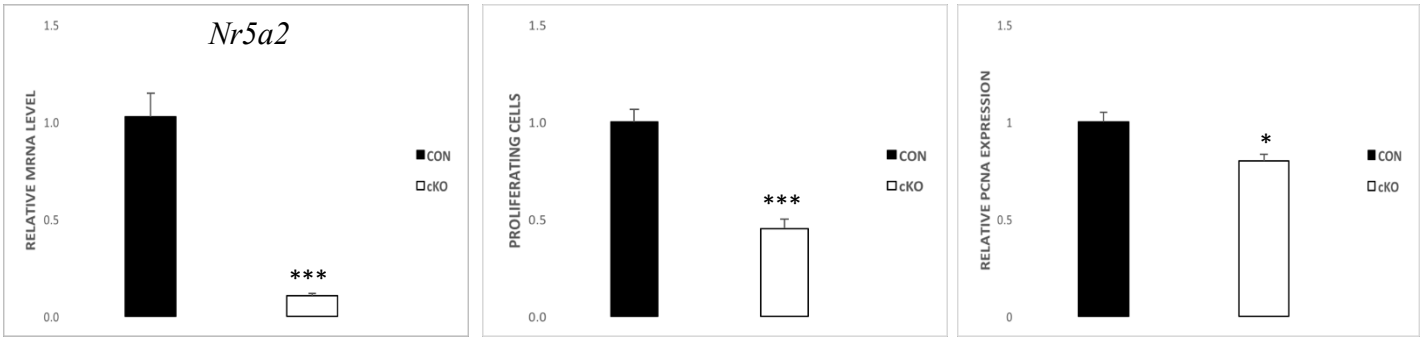
We have previously shown that the cKO mouse model, obtained by crossing *Nr5a2* floxed mice with mice expressing Cre-recombinase, driven by the *Amhr2* promoter, are infertile due to multiple factors including failure to ovulate (7). Analysis by qPCR of the *Nr5a2* transcript from granulosa cells at 44 h after eCG treatment confirmed depletion of the *Nr5a2* gene by > 90 % in the cKO relative to the CON animals (Figure 3-1A). At 24h after eCG treatment, there was no difference in mean ovarian mass (CON, 6.3 ± 0.6; cKO, 7.3 ± 1.1 μg) indicating that the cKO ovaries were not hypomorphic. Figure 3-1B depicts ovarian sections displaying nuclear incorporation of BrdU in cKO and CON mice and Figure 3-1C, PCNA. The evident reductions in abundance of incorporation of BrdU and the intensity of the signal for PCNA were quantified (Figure 3-1A). Both markers demonstrated that the number of proliferating granulosa cells in cKO mice relative to their

CON counterparts was reduced by more than 50 % in BrdU incorporation (Figure 3-1A). FACS analysis (Figure 3-2) of granulosa cells revealed that there were significantly more cells in the G0/G1 phase of the cell cycle in the cKO mice relative to the CON counterparts, but fewer cells in the G1/S and G2/M phases of the cell cycle. Together, these results indicate that proliferation is substantially impaired in granulosa cells in cKO mice relative to their wild type counterparts.

Figure 3- 1 Proliferation of mouse granulosa cells is compromised in NR5A2-depleted follicle

(A, left) Amhr2Cre+ depletion of Nr5a2 transcripts in granulosa cells from mouse antral follicles (n = 5). (A,middle and right) BrdU and PCNA expression in CON and cKO granulosa cells at 44 h after gonadotropin treatment, as evaluated by quantitative image analysis. Data are means ± standard error of the mean (SEM); *P <0.05, ***P <0.001. (B) Representative images showing the expression of BrdU in CON and cKO ovaries at 44 hours after gonadotropin stimulation *in vivo* (n = 10/n = 6). (C) Representative images for PCNA at 44 hours after gonadotropin stimulation (n = 4). In this and subsequent figures, CON signifies control animals, cKO the granulosa-specific mouse knockout line (genotype Nr5a2$^{f/f}$; Amhr2Cre+). Original scale bars, 50µm.

A



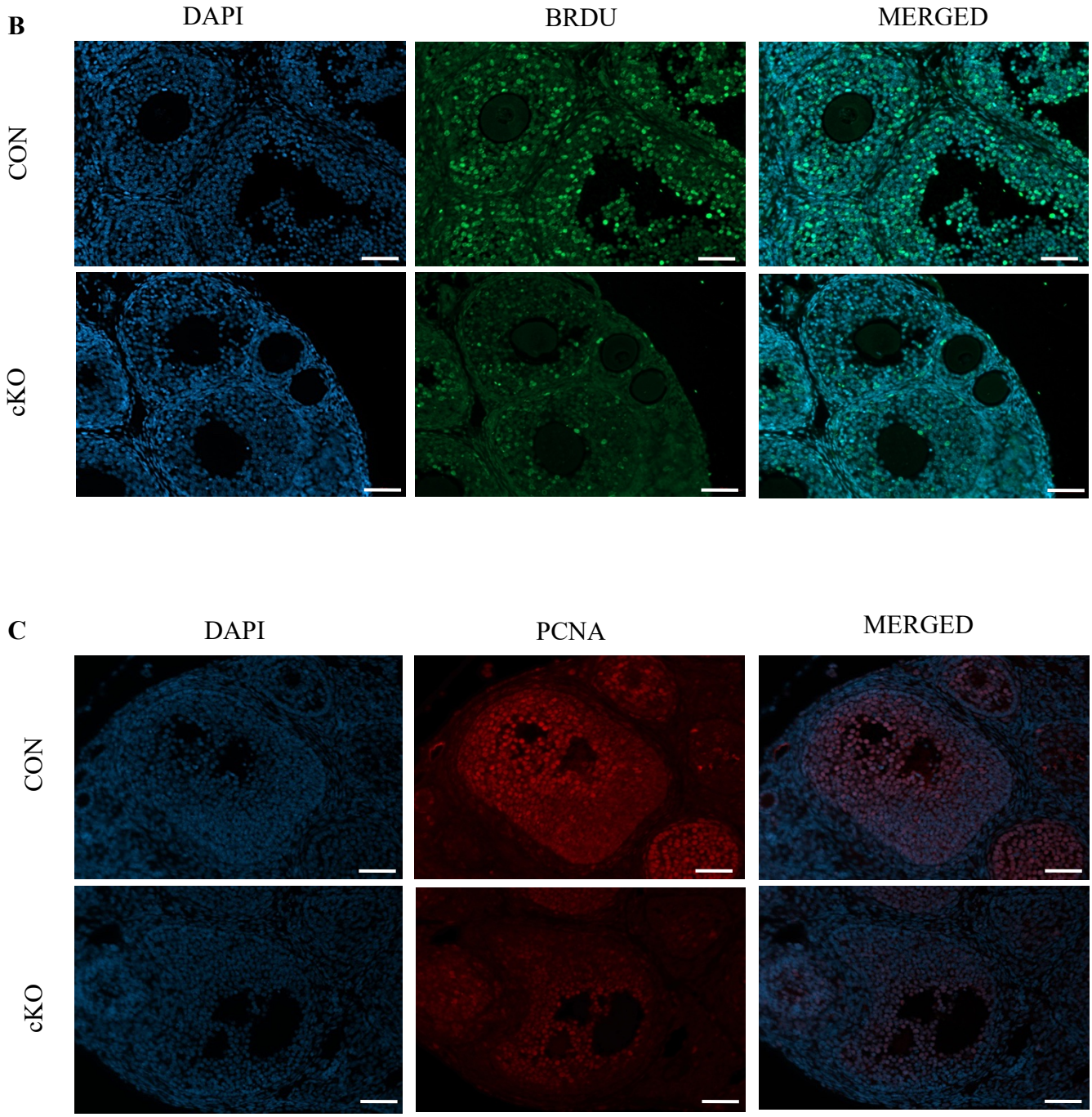
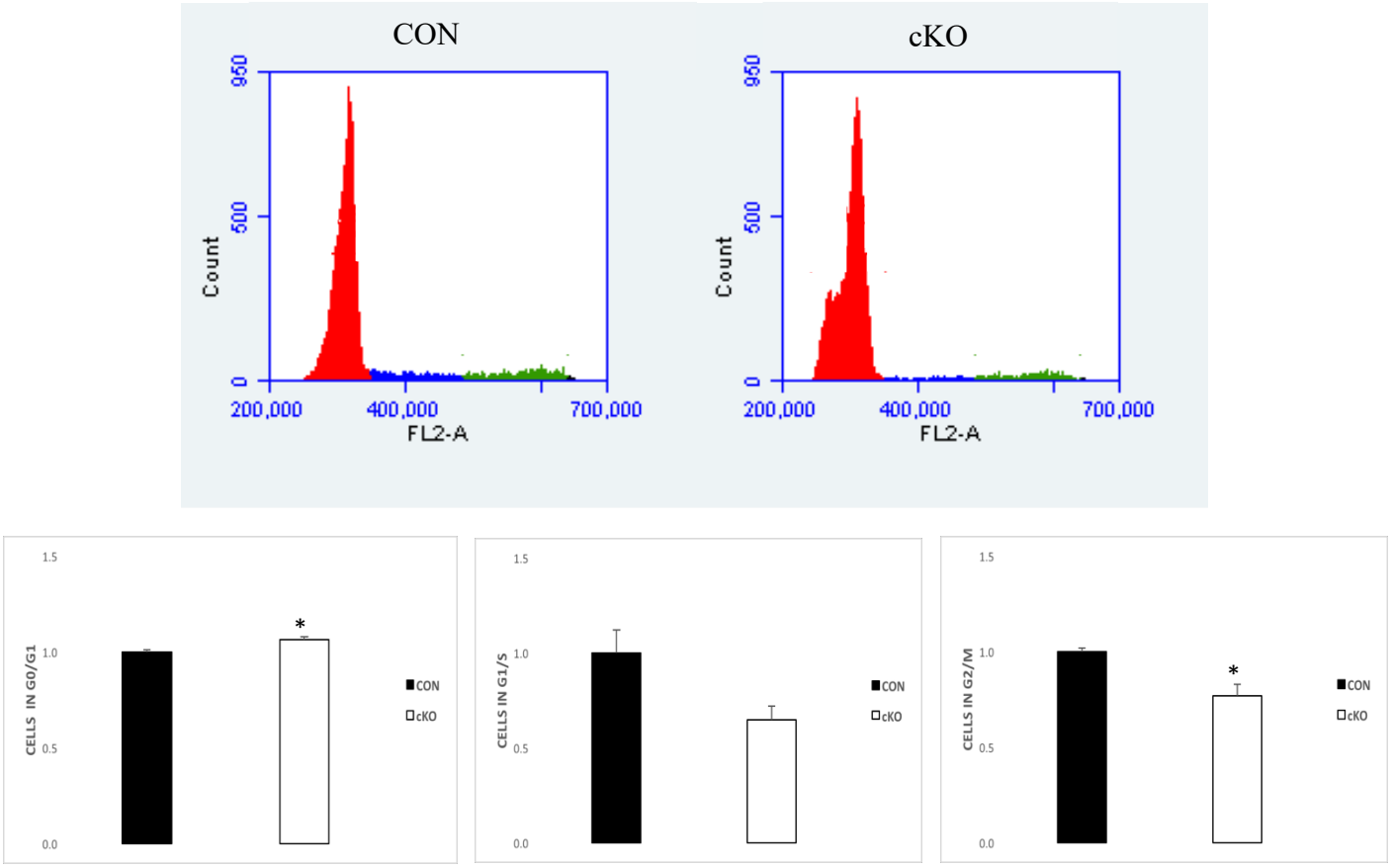

B
DAPI
BRDU
MERGED
CON
cKO
C
DAPI
PCNA
MERGED
CON
cKO

Figure 3- 2 FACS of granulosa cells from gonadotropin-stimulated CON and cKO mice.

(Upper) Representative scan data. (Lower) Means ± SEM of cell numbers in G0/G1 (red), G1/S (blue), and G2/M (green) phases of the cell cycle (n = 3). *P <0.05. FL-2, fluorescence 2.



B. <u>Substantial modification of the granulosa cell transcriptome accompanies NR5A2 depletion</u>.

Microarray data analysis using the Network Analyst platform yielded 2136 differentially regulated genes at the statistical parameters of FDR = 0.05 and and a two-fold or greater change between cKO and CON mice at 48h post-eCG. Of these, 1089 genes were upregulated and 1047 genes were downregulated. Lists of the 100 genes with the greatest definable variation relative to CON granulosa cells are presented in Supplemental

Tables 3-2 (upregulated genes) and 3-3 (downregulated genes). Tables 3-1 and 3-2 represent gene ontology analysis by Panther and KEGG, respectively, demonstrating pathways and gene clusters that differed substantially between the cKO and CON samples. Pathway analysis revealed, as expected, that proliferation and mitotic cell cycle processes were major cellular functions disrupted by NR5A2 depletion, as shown in the heat map in Figure 3-3A. We have previously demonstrated, using this (7) and other depletion paradigms (21,22), that genes associated with steroidogenesis are, with few exceptions, reduced as reflected in the heat map (Figure 3-3B). The gene clusters associated with the ovulation cycle and female gamete generation, not surprisingly, showed inappropriate up and down regulation of multiple genes (Figures 3-4A & B).

Table 3- 1 Top nine gene ontology pathway (GO: Biological Processes)

Top nine gene ontology pathway enriched among all differentially regulated genes in the granulosa cells of mice with depletion of NR5A2 in granulosa cells (cKO mice) relative to control (CON) mice, as revealed by microarray and Panther bioinformatic analysis.

Pathway	Total	Expected	Hits	P.Value	FDR
Cell division	509	54.6	95	3.83E-08	3.14E-05
Mitotic cell cycle	683	73.2	118	7.90E-08	3.24E-05
Regulation of lipid metabolic process	232	24.9	52	1.67E-07	4.57E-05
Steroid metabolic process	342	36.7	68	3.13E-07	5.47E-05
Steroid biosynthetic process	164	17.6	40	4.39E-07	5.47E-05
Mitosis	366	39.2	71	4.60E-07	5.47E-05
Lipid biosynthetic process	587	62.9	102	4.67E-07	5.47E-05
Female gamete generation	94	10.1	27	1.13E-06	0.000108
M phase of mitotic cell cycle	382	40.9	72	1.18E-06	0.000108

Table 3- 2 Top ten KEGG pathways

Top ten KEGG pathways enriched among all differentially regulated genes in the granulosa cells of mice with depletion of NR5A2 in granulosa cells (cKO mice) relative to control (CON) mice, as revealed by microarray and bioinformatic analysis as enriched among all differentially regulated genes.

Pathway	Total	Expected	Hits	P.Value	FDR
Cell cycle	127	14.1	33	1.81E-06	0.000386
Prostate cancer	87	9.68	24	1.59E-05	0.00141
Pathways in cancer	306	34.1	58	2.24E-05	0.00141
Steroid biosynthesis	17	1.89	9	2.64E-05	0.00141
HTLV-I infection	223	24.8	45	4.03E-05	0.00152
Fat digestion and absorption	8	0.891	6	4.27E-05	0.00152
Pancreatic cancer	70	7.79	19	0.000153	0.00467
p53 signaling pathway	69	7.68	18	0.000389	0.00981
Progesterone-mediated oocyte maturation	81	9.02	20	0.000415	0.00981
Focal adhesion	199	22.2	38	0.000516	0.011

C. Differential regulation of cell cycle genes and downstream targets in granulosa cells depleted of Nr5a2

By realtime PCR it was found that the abundance of transcripts of numerous genes involved in the cell cycle, including cyclin D1 and cyclin E1 are significantly decreased in granulosa cells depleted of NR5A2 (Figure 3-5A). The same effects were observed on their paralogs, cyclin D2 and cyclin E2. It is known that downstream targets of these cyclins that play key roles in execution of the S phase by acting on the transcription of genes responsible for DNA replication (23). These include the E2f activators, *E2f1*, *E2f2*, that are also downregulated in granulosa cells from cKO mice (Figure 3-5A).

Figure 3- 3 Heat map derived from gene ontology analysis of microarray data from CON and cKO mice following gonadotropin stimulation *in vivo*. Represented are two of the top nine enriched pathways for (A) cell-cycle and (B) steroidogenesis-related genes, demonstrating twofold or greater variation in expression in granulosa cells.

As noted in the color bar, genes in the cKO column are underexpressed relative to CON values, whereas genes in red are overexpressed. Probability cutoff was $P < 0.05$.

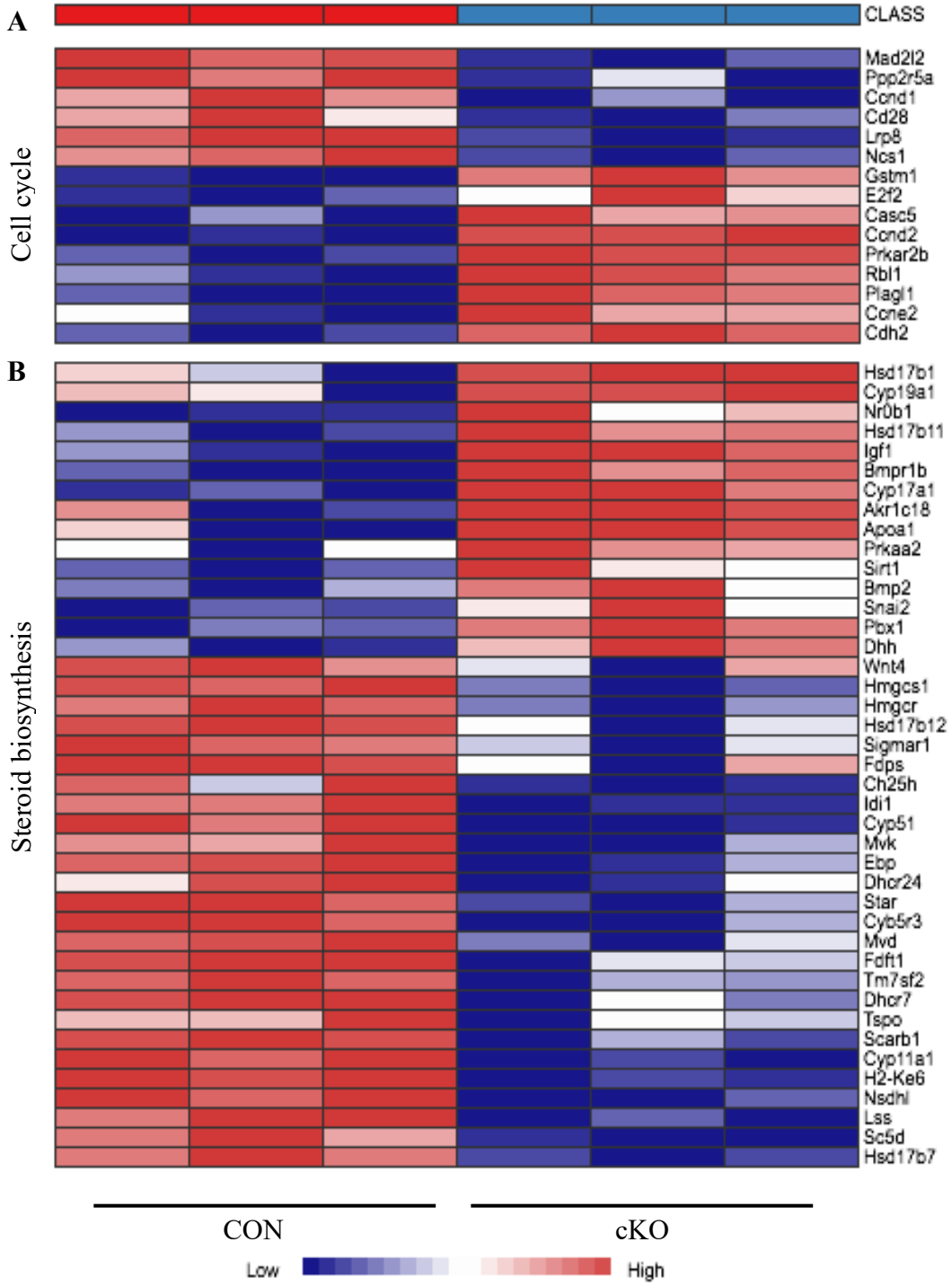
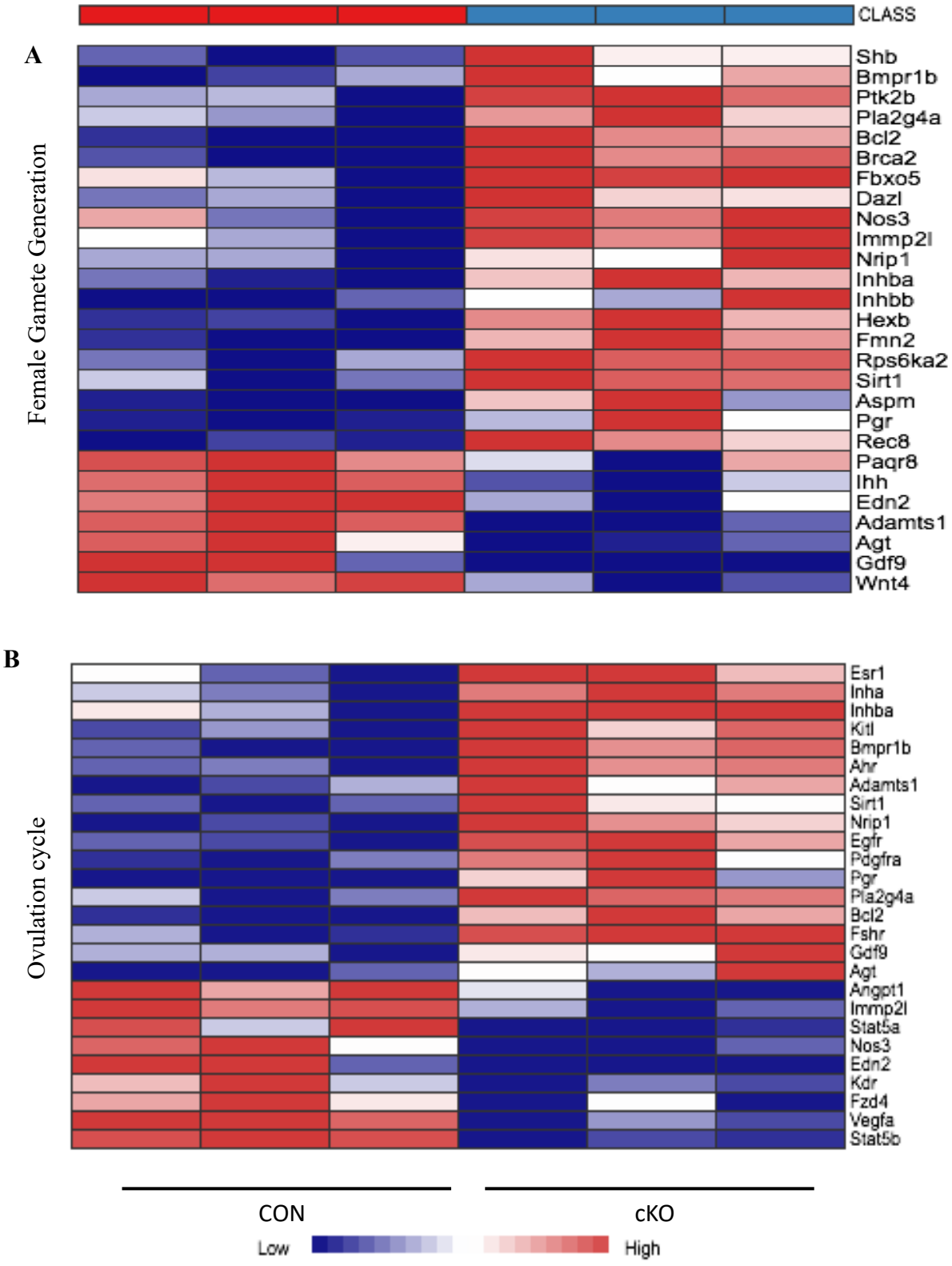


Figure 3- 4 Heat map derived from gene ontology analysis of microarray data from CON and cKO mice following gonadotropin stimulation *in vivo*. Represented are two of the top 10 enriched pathways, in this case for (A) female gamete generation and (B) ovulation cycle



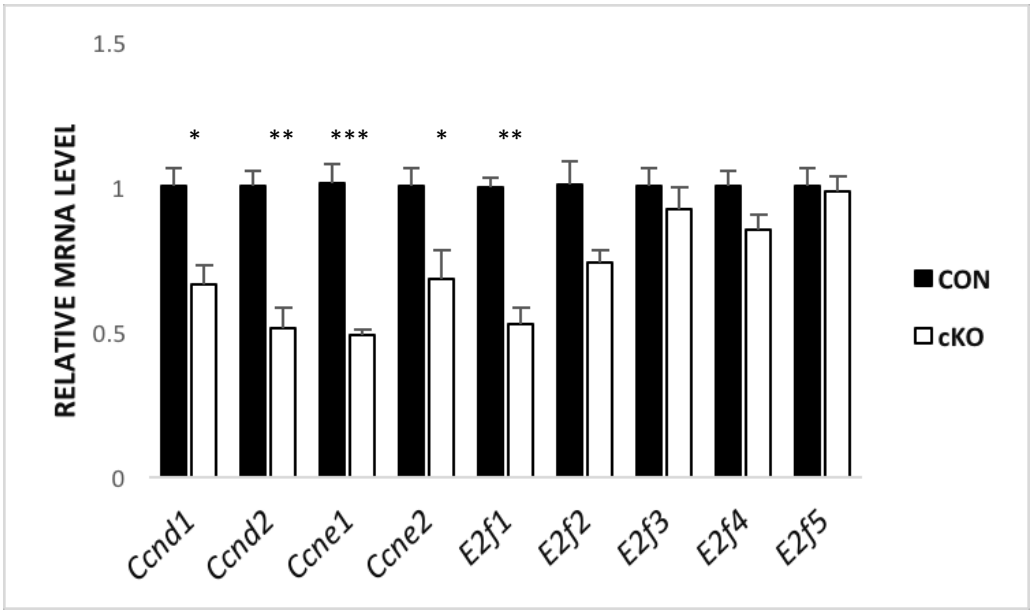
Cyclin dependent kinases associated with cyclins D and E; *Cdk4*, *Cdk6* and *Cdk2* respectively; and transcripts for the E2f repressors, *E2f4* and *E2f5* did not show any significant difference in their abundance between the cKO and CON animals.

PCR validation of the global transcriptome also demonstrated that number of genes associated with the cell cycle were upregulated in the granulosa cells of cKO animals relative to the CON counterparts, including *Cdh2*, *Plagl1*, *Igfr1*, *Prkar2b*, *Gstm1*, *Cd7*, *Cdk7* and *Pts* (Figure 3-5B). We further examined two cell cycle inhibitors, *Cdkn1a* (*p21*) and *Cdkn1b*, and discovered the former to be substantially upregulated, while the latter showed no difference between CON and cKO granulosa cells following gonadotropin stimulation.

Figure 3- 5 Real-time PCR analysis of differentially expressed genes in mouse granulosa cells harvested following gonadotropin stimulation.

Data are expressed as means ± SEM for genes (A) underexpressed in cKO mice relative to CON and (B) overexpressed in cKO mice relative to CON mice (n = 5 to 9 animals/group). *P <0.05, **P < 0.01, ***P < 0.001.

A



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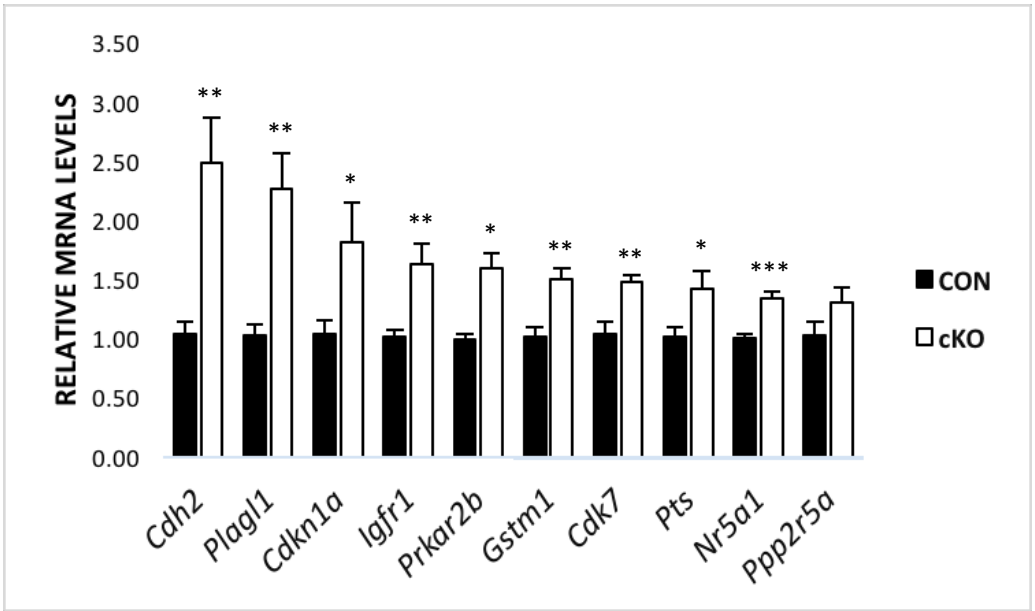


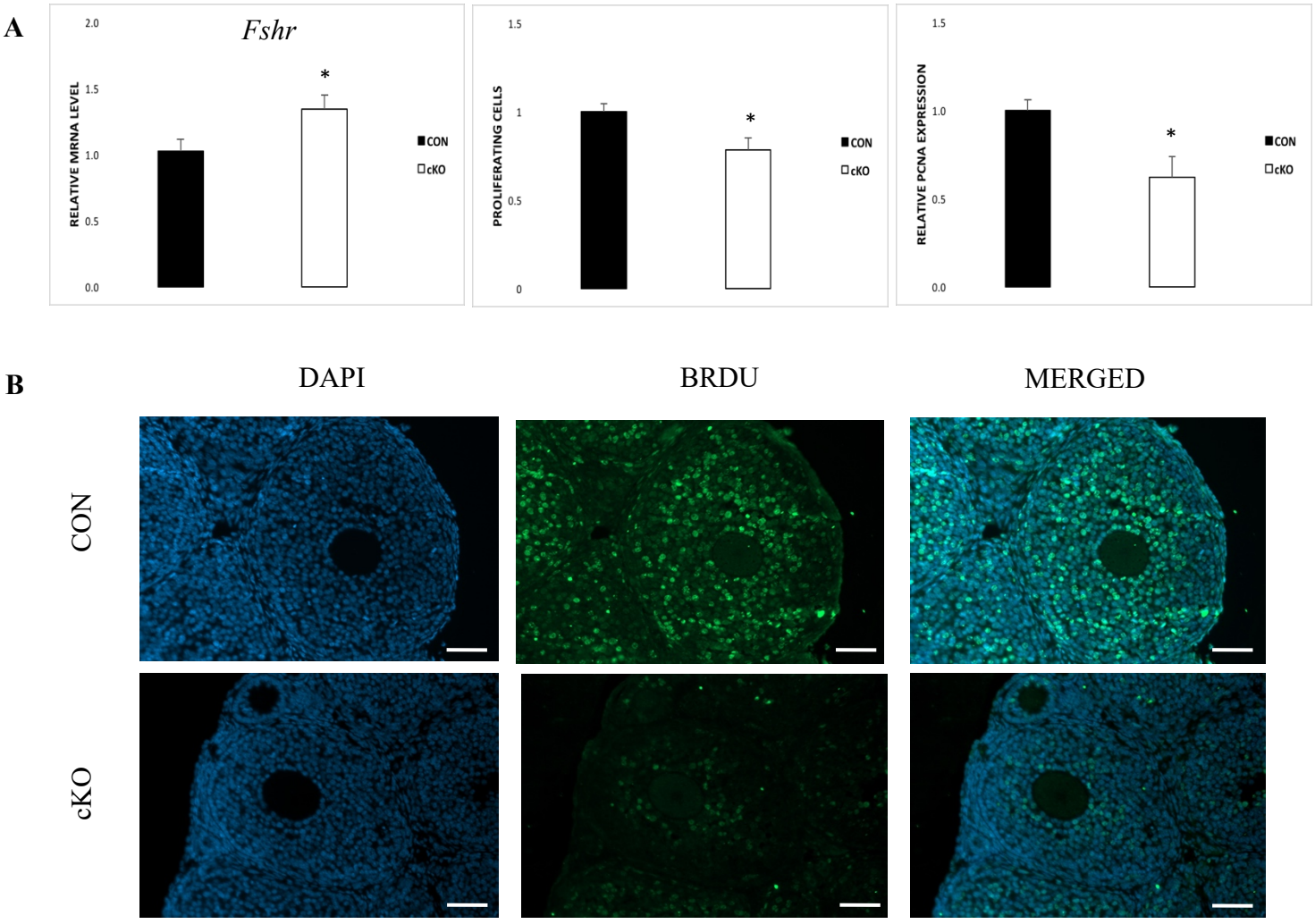
D. NR5A2 depletion affects gonadotropin receptor transcripts and estrogen cannot rescue proliferation

Another transcript that was substantially upregulated in the microarray analysis was the receptor for follicle-stimulating hormone (FSHr), while the receptor for luteinizing hormone (LHCGr) was less affected (Figure 3-6A). In this model, in particular, there is overexpression of CYP19A1 (7), a target of FSH and the rate-limiting enzyme in estrogen production. Further, we have shown that estradiol17β concentrations are more than double in the follicles of cKO mice following superstimulation (7). Estrogens are potent mitogens for granulosa cells (5), and microarray analysis indicated that both estrogen receptors, ESR1 and ESR2 are overexpressed by approximately three-fold in cKO granulosa cells. We therefore treated 24-day old immature CON and cKO mice with estradiol 17β, followed by euthanasia 24h later, according to our earlier protocol (5). Ovarian sections were probed for BrdU and PCNA expression by quantification of immunofluorescence to establish

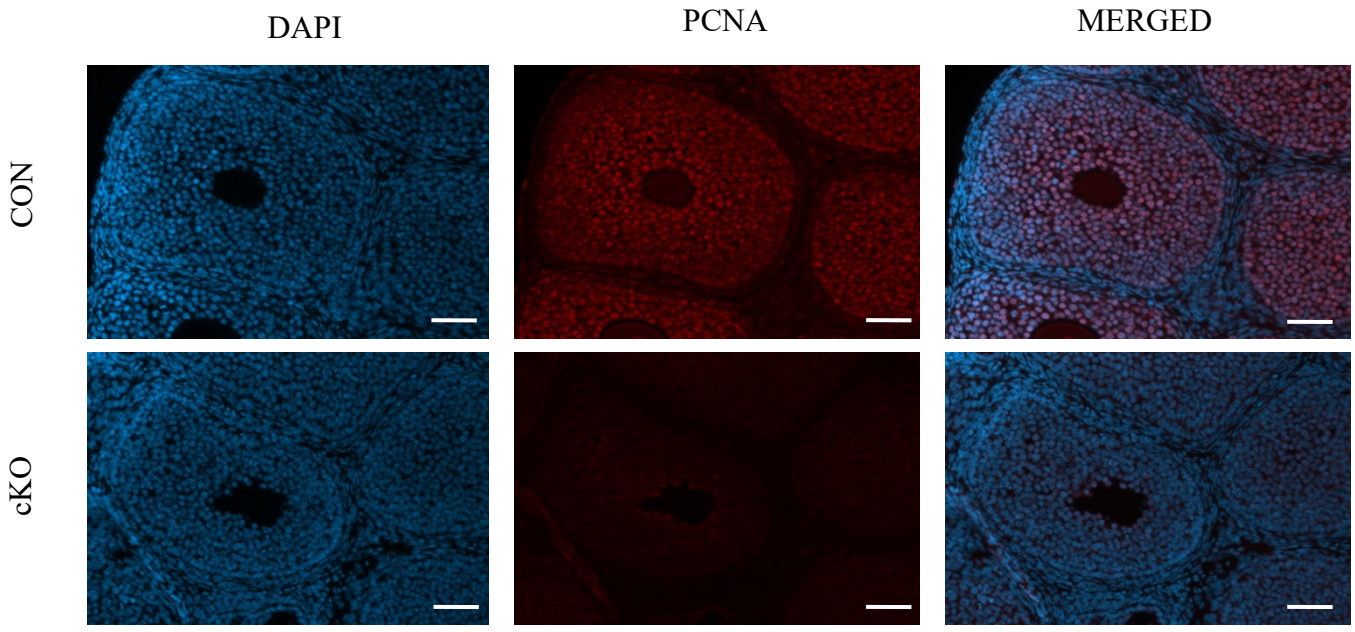
whether the reduction in proliferation in cKO mouse granulosa cells could be reversed by estrogen treatment. The results (Figure 3-6A & B) indicate that, in spite of treatment with pharmacological doses of estrogen, the rate of proliferation remained well below that

Figure 3- 6 FSHr is elevated in cKO granulosa cells, and exogenous estrogen cannot rescue proliferation relative to CON animals.

(A, left) Abundance of *Fshr* transcripts in CON and cKO mice following gonadotropin stimulation (n = 5). (A, middle) The number of proliferating cells following 1 mg estrogen administration *in vivo*, as determined by quantitative imaging of BrdU expression. (A, right) The quantitative analysis of PCNA expression between granulosa cells from CON and cKO animals. Data are expressed as means ±SEM of n = 4 animals/group. *P< 0.05. (B) Representative photomicrographs of BrdU incorporation in ovaries from estrogen-treated CON and cKO mice. (C) Representative photomicrographs showing PCNA expression in the ovaries of estrogen-treated CON and cKO mice. Original scale bars, 20μm.



C



E. Pharmacological inhibition of NR5A2 and its cofactor β-catenin (CTTN1B) interferes with *in vitro* expression of cell cycle genes in granulosa cells of CON and cKO mice

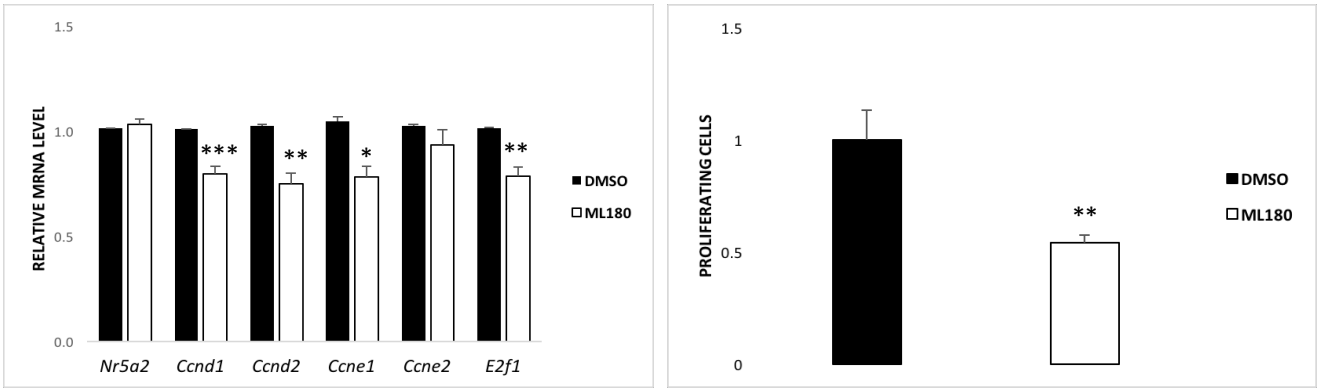
Incubation of CON granulosa cells with the inverse Nr5a2 agonist, ML180, for 6 h caused no net difference in the abundance of *Nr5a2* transcripts relative to DMSO controls. There was a demonstrable reduction in transcripts for *Ccnd1*, *Ccnd2*, *Ccne1*, and *E2f1* further recapitulated the reduction in these genes in cKO granulosa cells at 6h of treatment (Figure 3-7A). At 24h of treatment, proliferation was suppressed by half (Figures 2.7A and B), consistent with *in vivo* results. NR5A2 is a constitutively active transcription factor, and previous studies have shown that β-catenin functions as its coactivator (16). To explore its potential role in the ovary, we treated cKO granulosa cell cultures with the oxazole compound, iCRT3, that interferes with β -catenin action, by inhibition of its translocation to the nucleus and its interaction with the transcription factor, TCF4 (24). TCF4, as measured by microarray, was two-fold greater in cKO granulosa cells, indicating no

impairment of this element of the β -catenin signaling mechanism (data not shown). Nonetheless, analysis at 6h indicated that there was depletion of *Nr5a2*, its targets the cyclins D and E and other downstream genes such as the E2fs activators, *E2f1* (Figure 3-7C).

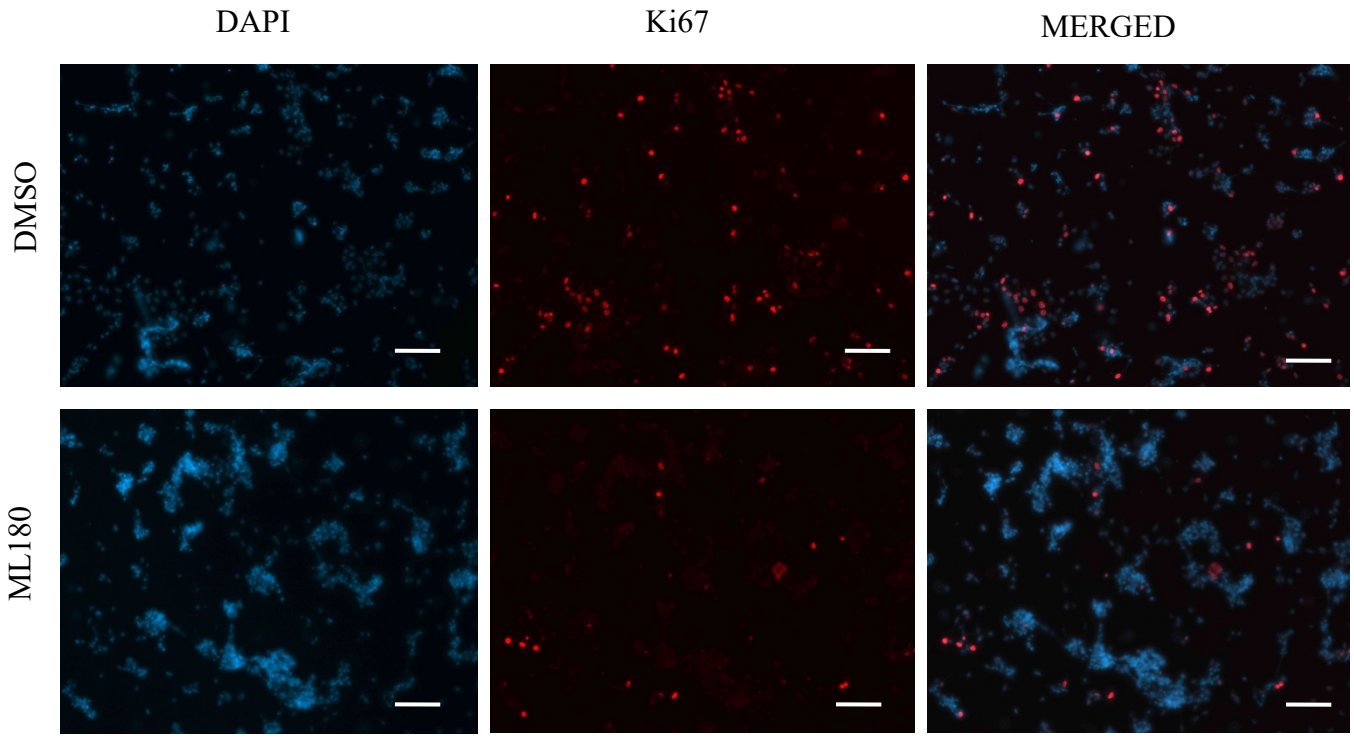
Figure 3- 7 Effects of the Nr5a2 reverse agonist, ML180

(A, left) Effects on genes associated with cell proliferation. (A, right) Mean reduction in proliferation, as indicated by the reduced expression of the marker KI-67 by quantitative image analysis. (B) Representative image demonstrating reduction in KI-67 expression. Original scale bars, 100 μm. (C) Effects of the β-catenin activity inhibitor iCRT3 on cell-cycle genes in granulosa cells *in vitro* harvested from gonadotropin-stimulated cKO mice. Means ± SEM from cultures of granulosa cells from five mice per group. *P <0.05, **P <0.01, ***P <0.001.

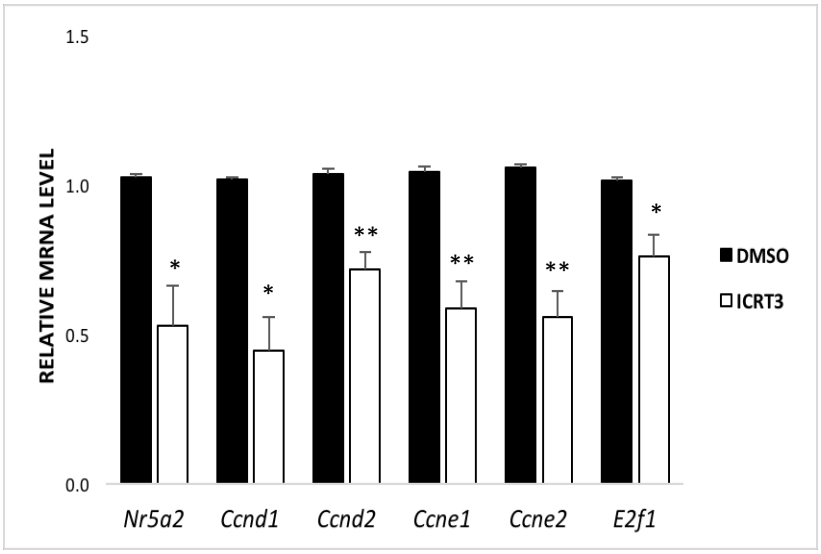
A



B


DAPI
Ki67
MERGED
DMSO
ML180

C

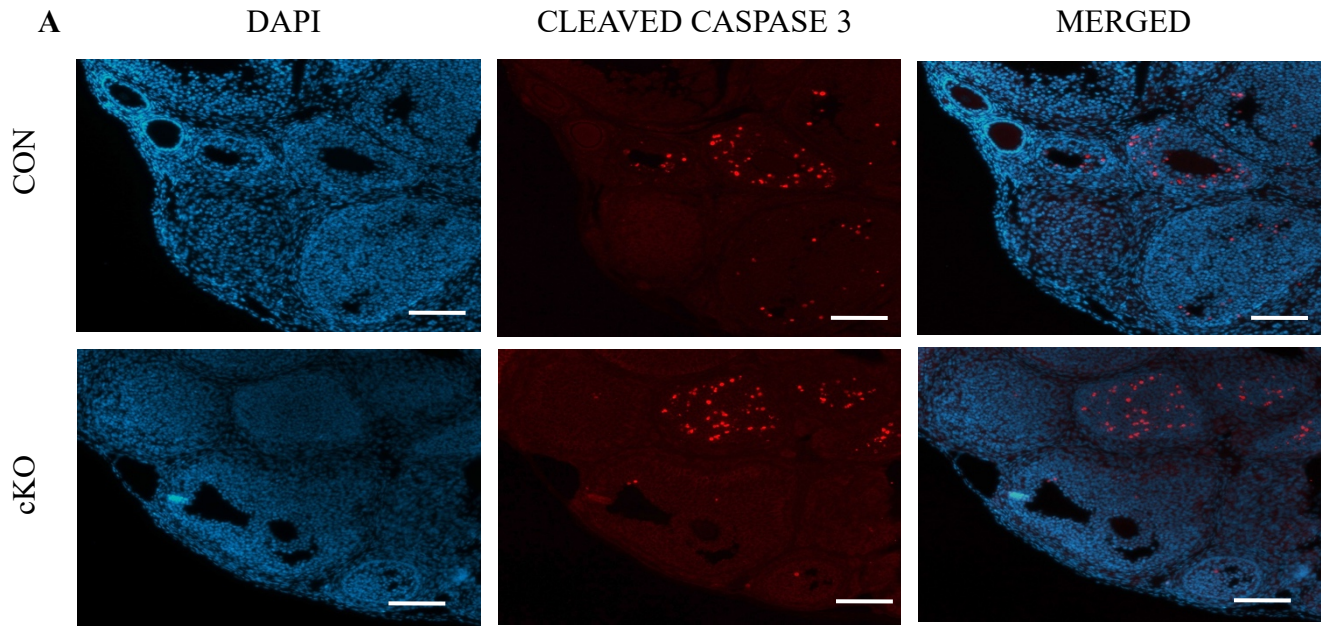

1.5
1.0
0.5
0.0
RELATIVE MRNA LEVEL
*
*
**
**
**
*
DMSO
ICRT3
Nr5a2
Ccnd1
Ccnd2
Ccne1
Ccne2
E2f1

F. NR5A2 depletion does not affect apoptosis or autophagy in ovarian granulosa cells

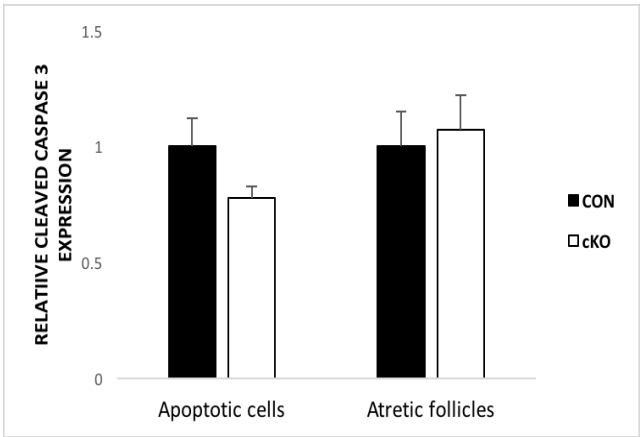
We next determined whether the reduced proliferation in cKO mouse granulosa cells *in vivo* could be attributed to programmed cell death or autophagy. In this context, cleaved caspase 3 immunofluorescence results were evaluated using CellProfiler software (Figure 3-8A & B). There was no difference in the number of atretic follicles, nor in the number of atretic cells in these follicles between the control and the cKO mice (n=5/genotype). TUNEL analysis (Supplemental Figure 3-1) confirmed this observation. Evaluation of the pattern of gene expression of anti-apoptotic *Bcl2*, *Bcl2l1*, *Mcl1* and pro-apoptotic *Bax*, and *Bad* (n=5/genotype) further indicated that there is no difference in patterns of programmed cell death genes (Fig 2.8B). While it is known that granulosa cell depletion can also be the result of autophagy (25), qPCR analysis indicated no difference in transcript abundance for a panel of genes associated with autophagy and signaling of autophagy beyond a moderate increase in the abundance of Beclin (Figure 3-8C). The results indicate that NR5A2 depletion does not increase the frequency of autophagy.

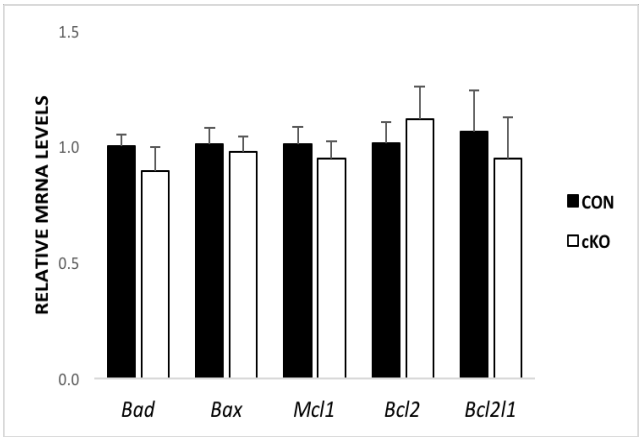
Figure 3- 8 Depletion of Nr5a2 does not increase apoptosis nor autophagy in mouse granulosa cells

(A) Immunolocalization of cleaved caspase-3, a marker of apoptosis in the ovaries of gonadotropin-stimulated CON and cKO mice. Original scale bars, 50μm. (B, left) Counts of the total number of follicles with apoptotic nuclei by cleaved caspase-3 immunofluorescence analysis from ovaries of CON and cKO mice. (B, right) Means ± SEM of apoptotic marker genes. (C) Means ± SEM of the effects of NR5A2 depletion *in vivo* on expression of autophagy marker genes in gonadotropin-stimulated mice (n = 5/group). *P , 0.05.

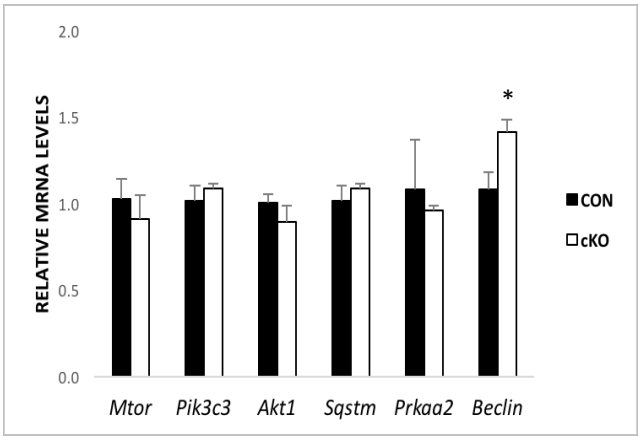


B





C



4. Discussion

We have previously shown that depletion of NR5A2 from mouse granulosa cells, either prior to (7,18), or following the ovulatory gonadotropin signal (21) results in infertility. Short term depletion of the *Nr5a2* transcript by shRNA results in the infertility with the same anovulatory phenotype (26). Herein, we provide clear evidence that, when depletion is initiated from the primary follicle forward, gonadotropin induced proliferation of the granulosa cell population in antral follicles is compromised. The role of NR5A2 in proliferation has been studied primarily in embryonic tissue and cancer cells, *in vitro*. Although germline deletion of NR5A2 is lethal during early embryogenesis, proliferation of the embryo to the gastrulation stage occurs (27), indicating proliferation can occur in the absence of this nuclear receptor. Likewise, suppression of NR5A2 in colon cancer (28) or osteosarcoma cells (15), inhibited, but did not eliminate proliferation, consistent with the present findings.

In concordance with studies of NR5A2 depletion in cancer cell lines (13,28), FACS analysis revealed that greater proportion of the granulosa cells were in the G0/G1 phase, while there were fewer in both the S and G2/M phases. The decrease we report in granulosa

cells following NR5A2 depletion is of lower magnitude than that seen in cancer cells (7 % vs 12-15 %), while the abundance of the cell population in the G2/M phase was greater by 23 % in cKO mice vs. 10- 18 % in cancer cell lines (13,28). Thus, these, the first *in vivo* results of disruption of the cell cycle by NR5A2 depletion, indicate the ubiquity of the role of NR5A2 in proliferation.

We employed microarray and gene abundance evaluation in search of the potential explanations for the reduced proliferation in granulosa cells. The number of dysregulated transcripts (>2000) far exceeded the number (637) observed in the ovary in a study employing an 80 % shRNA depletion *in vivo* in a similar model at 40 after eCG stimulation (26). The present results further show that depletion of NR5A2 reduced abundance of cell cycle transcripts such as *Ccnd1* and *Ccne1*, both *in vivo* and *in vitro*, consistent with results of depletion studies in cancer cell lines (28). Another remarkable similarity between our *in vivo* data and cancer cell line results is the increase in expression of *Cdkn1a*, or *p21*, the cell cycle inhibitor that was identified by both microarray and PCR validation. Suppression of expression of this gene has previously been implicated as a major mechanism by which NR5A2 influences proliferation in breast (13) and colon (14) cancer cell lines. In addition, CDKN1A has been shown by chromatin immunoprecipitation assays to be a direct target of NR5A2 in breast cancer cells (13). Promoter assays revealed that Nr5a2 recruitment to the *Cdkn1a* promoter region in colon cancer cell lines activates transcription (14). Male mice with germline deletions of CDKN1A are fertile, but displayed a 48 % increase in the number of Sertoli cells, the male analog of the granulosa cell (29). Global deletion of *Cdkn1a* appears to have little effect on mouse granulosa cell proliferation in the periovulatory follicle after the ovulatory signal, while double knockout of CDKN1A and CDKN1B dramatically increases proliferation in this context (30). Based on our findings

and the information in the literature, we propose that the failure of Cdkn1a downregulation in the absence of Nr5a2 is the principal mediator of the abrogation of gonadotropin-induced granulosa cell proliferation in the present study.

Follicle progression to the antral stages occurs in this cKO model (7), an event that could not occur without granulosa cell proliferation (2). It is well known that the early phases of follicle development are gonadotropin independent, driven primarily by elements of the transforming growth factor-β family (3). The experimental paradigm of the present study was comparison of granulosa cell proliferation in cKO and CON mice following treatment with eCG, a potent stimulator of follicle development from the early antral stage forward. As noted above, our data demonstrate that the gonadotropin induction of proliferation is substantially impaired by depletion of NR5A2. Both the LH and FSH receptors are present the granulosa cells of the cKO mouse, and the latter are overexpressed.

Insulin like growth factors (IGF) act in synergy with FSH in induction of antral follicle formation, and deletion of Igfr1 from granulosa cells abrogates this process completely (31). In the present study, the abundance of both *Fshr* and *Igf1r* transcripts was elevated in cKO granulosa cells relative to the CON model. In ruminant follicles, FSH induces the expression of Igfr1 (32,33). It is therefore possible that superstimulation by eCG, in the presence of elevated Fshr is responsible for the observed increase *Igf1r* transcript abundance.

One of the principal roles of FSH in the ovarian follicle is the induction of CYP19A1 and consequent production of estradiol (34). Neither of these events appear impaired in this study, indeed, in microarray data and in our previous reports (7) indicate that CYP19A1 is overexpressed. Not only are both isoforms of the estrogen receptor (ESR1

and ESR2) overexpressed, but also, intrafollicular concentrations of estradiol are more than two-fold greater in this cKO mouse model (7). Further, proliferation could not be rescued by injection of cKO mice with pharmacological doses of estradiol, a treatment that we previously showed to induce granulosa cell mitosis *in vivo* (5). The estrogen signaling pathway appears intact, as microarray data show a robust expression of its iconic target mediator of proliferation, GREB1 (35). We conclude that the mitogenic actions NR5A2 and estrogen are achieved by separate mechanisms.

The canonical intracellular signal in the Wnt/β -catenin pathway, β -catenin, has been shown to bind stably and interact with NR5A2 via the ligand binding pocket of the latter (36). This interaction has significant consequences, in that induction of cyclin-mediated proliferation of pancreatic and hepatic cell lines by NR5A2 is potentiated by β -catenin (16). The β -catenin signal also functions as a NR5A2 co-activator in the ovary (37). Thus, it was no surprise that in the present study, inhibition of nuclear ß-catenin activity in cKO granulosa cells drastically depleted the abundance of transcripts for the same cell cycle genes that were affected by Nr5a2 depletion alone. The β -catenin inhibitor we employed, iCRT3, abrogates activation of transcription via TCF4. The inhibitor, iCRT3, has been shown to have modest effects on other signaling pathways, including STAT and NOTCH (24) . Thus, the reduction in transcription of NR5A2 engendered by this treatment indicates that NR5A2 may be downstream of β -catenin.

5. Conclusion

Herein we provide the first evidence that NR5A2 is a critical regulator of proliferation in ovarian granulosa cells. Follicle development, driven by granulosa cell proliferation, occurs in the cKO mouse model employed, indicating that NR5A2 is not sine qua non for mitosis. Nonetheless, severe impairment of the cell cycle is engendered in cKO animals, interfering with the normal proliferative response to gonadotropins.

6. Acknowledgments

The authors are grateful to Vickie Roussel for technical support. This study was funded by OGP12596 from the Canadian Institutes of Health Research to BDM.

7. Supplementary Data

Supplementary table 3- 1 Primers sequences employed for PCR analysis

	Gene	Forward	Reverse
Genotyping	*Lrh1*	GCTATAGGGAGTCAGGATACCATGG	GTTCGTACCACTTTCATCTCCTCACG
	Amhr2gCre	GAACCTGATGGACATGTTCAGG	AGTGCGTTCGAACGCTAGAGCCTGT
Housekeepings	*Rpl19*	CTGAAGGTCAAAGGGAATGTG	GGACAGAGTCTTGATGATCTC
	B2m	GACCGGCCTGTATGCTATCC	TTTCAATGTGAGGCGGGTGG
	Hprt	ATGATCAGTCAACGGGGGAC	GAGAGGTCCTTTTCACCAGCA
Proliferation	*Nr5a2*	TCATGCTGCCCAAAGTGGAGA	TGGTTTTGGACAGTTCGCTT
	Ccnd1	GAGCCATCCAAACTGAGGAA	AGCCAGAGGTGTGCAAATTA
	Ccnd2	AGTTCCGTCAAGAGCAGCAT	GACGAACACGCCTCTCTCTT
	Ccne1	CTGGACTCTTCACACAGATGAC	GCCTATCAACAGCAACCTACA
	Ccne2	GAAAGCTTCAGGTTTGGAATGG	GCTTCACTGGACTCACACTT
	E2f1	GGTGATACCTTAAGTCCCTGTTC	CCCTCTCCCTTTCCCAATAAAT
	E2f2	CATGTTTCCCTGGGAGGATTAT	ATCTGACCTTGGAAGTGAAGTG
	E2f3	GAGTGGGTGAGTTTGGACTAAG	TCCAGTTTGGGACACCATTC
	E2f4	GGAGGGTTGGTACCTAAGAATG	TCTCTGCAATGGCTCTAAATGA
	E2f5	ACACCAGCTGCAGAAGTATC	GGGTAGGAGAAAGCCGTAAA
	Fshr	AGAAAGCAGGTGGATGGATAAA	GAAGACCCTGTTAGAGCAATGA
	Cdh2	CTCCAACGGGCATCTTCATTA	TCAAGTGAAACCGGGCTATC
	Plagl1	CAGGAGCTGATGCAAGAGAATA	GAAAGGAGGCATGGGATCAA
	Cdkn1a	CCAGCTAGGATGACAGTGAAG	GAGTCGGGATATTACGGTTGAG
	Prkar2b	CCCTGTCTTCTTCCTCAGTTT	AGAACAGAGCTGGCATTCATAG
	Gstm1	GCCCACGTTTCTCTAGTAGTC	GACGTTCCAGTATCCCAGTATC
	Cdk7	GTTGGTCTAGGCTGGCTATAAA	CAACTGCTCACTCACCACTAA
	Pts	CTCACACGGGAAGCATAGAC	CTCTCCAGCCCAAGATGAAATA
	Ppp2r5a	CCGAAAGACAGAGAGAGAAGAAG	CACTGGTACTGCTGCGAATA
Apoptosis	*Bad*	CAACATTCATCAGCAGGGAC	TCCATCCCTTCATCCTCCTC
	Bax	GCTGATGGCAACTTCAACTG	ATCAGCTCGGGCACTTTAG
	Mcl1	TCGGACTTCAGAGCACTTTATG	GGAAGTCAGGCTCCTAGTAAAC

	Bcl2	GAGCAGGTGCCTACAAGAAA	CTTTGTCCTCTGACTGGGTATG
	Bcl2l1	CTGTCGCCGGAGATAGATTTG	CTGGGTCTGCTCTGTGTTTAG
Autophagy	*Mtor*	CGGGACTACAGAGAGAAGAAGA	CATCAACGTCAGGTGGTCATAG
	Pik3c3	AGAGAGGGTGTCCAGAAGTTA	CTCTGTCTGCAGCAGCTTTA
	Akt1	ACGACGTAGCCATTGTGAAG	GCCGTTCCTTGTAGCCAATA
	Sqstm	AACAGATGGAGTCGGGAAAC	AGACTGGAGTTCACCTGTAGA
	Prkaa2	AGACTTAACAGCCTGGAACATAC	CTACGGGCAGTCTCACATTTAG
	Beclin	GTGTGCAGCAGTTCAAAGAAG	TGCCTCCAGTGTCTTCAATC

Supplementary table 3- 2 The 100 genes most differentially overexpressed in the Nr5a2 depleted mouse granulosa cells from immature mice at 44 h after equine chorionic gonadotropin stimulation.

Listed in the order of overexpression. logFC=log fold change, AveExpr=average expression, t=student's t-test value, P.Value=probability, adg.P.Val=adjusted probablility value.

Symbols	logFC	AveExpr	t	P.Value	adj.P.Val
Rgs13	7.3545	7.406	7.3805	8.75E-05	0.0026378
5830474E16Rik	6.3627	6.1282	9.0622	2.05E-05	0.0011805
Gm2044	6.029	7.1863	6.4957	0.00020932	0.0042506
Elovl2	5.9864	8.1539	8.3501	3.68E-05	0.0016213
Car14	5.948	9.209	6.0137	0.00034929	0.0057499
Lect1	5.9462	10.712	4.9491	0.0012023	0.012035
5730433N10Rik	5.8126	6.7977	13.698	9.68E-07	0.00039753
Mro	5.8088	8.4099	6.8976	0.00013943	0.0034321
Otor	5.6808	5.3713	11.998	2.61E-06	0.00055423
Slc38a5	5.6257	8.4001	5.3904	0.00070703	0.0086611
Aicda	5.5569	6.4551	10.93	5.22E-06	0.0007058
Zbtb8b	5.5467	6.5552	6.9201	0.00013636	0.0034007
Cyp19a1	5.4223	10.594	3.431	0.0092663	0.043788
Clec12b	5.3895	5.2463	18.609	9.42E-08	0.00013547
Fam171b	5.3603	7.6709	6.9402	0.0001337	0.0033563
Ptger2	5.3431	8.1901	16.736	2.12E-07	0.00017098
Fshr	5.2948	8.1717	10.956	5.13E-06	0.0007058

Prrt4	5.2064	7.2269	7.6052	7.11E-05	0.0023416
Slfn4	5.1629	7.7685	5.2817	0.00080374	0.0094273
Grem2	5.1396	10.362	5.0597	0.0010498	0.011174
Dnajb13	5.1318	5.1549	18.105	1.16E-07	0.00013547
Fhl1	5.1059	8.8477	9.9257	1.06E-05	0.00088323
C030009J22Rik	5.0808	5.1446	7.7569	6.19E-05	0.002178
Syt7	5.0297	8.8813	8.9342	2.27E-05	0.0012205
Slc26a7	4.9974	9.2134	8.8415	2.45E-05	0.0012825
Mfsd2a	4.9966	8.3992	6.8681	0.00014357	0.0034625
Lrrtm3	4.9699	5.0534	20.35	4.75E-08	8.29E-05
6430537K16Rik	4.913	6.3985	10.382	7.62E-06	0.0007912
5430401H09Rik	4.8931	6.1848	16.992	1.89E-07	0.00017098
Hpgd	4.88	6.9927	9.7661	1.19E-05	0.00094091
2610007B07Rik	4.8374	6.7564	7.7774	6.08E-05	0.00216
Plxnc1	4.8272	7.4394	16.861	2.00E-07	0.00017098
1110032F04Rik	4.7736	7.7039	5.5266	0.00060343	0.0078349
Mamdc2	4.7219	8.6166	4.16	0.003332	0.023093
Slc18a2	4.721	5.8614	5.2709	0.00081419	0.0094968
Arg1	4.6573	4.7399	4.9848	0.0011506	0.011817
Efna5	4.61	8.8858	7.4464	8.23E-05	0.0025417
Apoa4	4.5573	7.1788	6.9014	0.00013891	0.0034321
Chst8	4.555	7.5398	4.6253	0.0018066	0.015682
Nppc	4.5382	5.4757	8.4111	3.49E-05	0.0015739
1110006E14Rik	4.5145	5.6599	7.9954	5.00E-05	0.0019522
Kcnh2	4.4974	6.9555	7.5811	7.27E-05	0.0023588
Neb	4.4959	6.4615	5.3283	0.00076063	0.0090865
Omd	4.4957	6.9545	5.9324	0.00038186	0.0060395
Gsta2	4.4561	9.5705	11.104	4.64E-06	0.00067611
Cbs	4.4367	5.7424	9.7507	1.21E-05	0.00094091
Tac1	4.3951	6.2723	10.689	6.15E-06	0.00072691
Inhba	4.3613	12.919	5.2459	0.00083872	0.009689
Scg5	4.3423	5.6581	13.9	8.67E-07	0.00039753

6330415B21Rik	4.3408	4.4056	5.1531	0.00093746	0.010416
Kctd14	4.3188	9.8341	8.809	2.51E-05	0.0013014
D630039A03Rik	4.2666	5.1419	15.602	3.62E-07	0.00022302
Drd4	4.266	4.7308	6.0298	0.0003432	0.005686
Irs1	4.2527	10.767	10.245	8.40E-06	0.00081531
Car12	4.2474	7.2559	9.25	1.77E-05	0.0010976
Slc25a35	4.2	7.7386	8.7511	2.63E-05	0.0013275
Grem1	4.1986	7.3558	4.1215	0.0035101	0.023762
Sytl4	4.192	10.361	13.64	1.00E-06	0.00039753
Gprasp2	4.1871	6.4711	8.753	2.63E-05	0.0013275
Hey2	4.1696	9.3719	7.608	7.09E-05	0.0023416
2810039B14Rik	4.1585	5.185	12.566	1.85E-06	0.00053821
Asb4	4.1582	6.4769	11.893	2.79E-06	0.00055423
Map3k5	4.108	7.9812	8.8504	2.43E-05	0.0012797
Bcl2l10	4.0855	7.1712	4.2139	0.0030988	0.022104
Gm4926	4.0792	5.4019	8.0109	4.94E-05	0.0019457
Srgap3	4.0639	8.1412	11.769	3.02E-06	0.00055423
Sybu	4.0631	5.0119	12.227	2.27E-06	0.00055423
Fam13a	4.0599	8.0766	10.66	6.27E-06	0.00072691
Mamld1	4.0532	5.6362	11.06	4.78E-06	0.00068706
Myocd	4.0467	5.662	6.8918	0.00014024	0.0034358
Hsd17b1	4.0417	12.578	4.6544	0.0017406	0.015352
Rbm20	4.0379	5.7779	10.329	7.91E-06	0.00080842
Hsd11b2	4.0328	9.6875	8.1849	4.24E-05	0.0017413
Fam81a	4.0181	8.6325	9.5615	1.39E-05	0.0010194
Phex	4.0133	6.0272	5.9168	0.00038846	0.0060979
Epha5	4.0078	4.6131	9.0058	2.14E-05	0.0012019
Grik3	4.0022	4.4151	9.6636	1.29E-05	0.00098496
D12Ertd673e	3.9929	5.0168	5.3541	0.00073785	0.0089343
Rai2	3.9664	5.1103	7.5356	7.58E-05	0.0024372
Rps6ka5	3.9572	5.1384	16.437	2.43E-07	0.00018215
Scara3	3.946	6.7931	14.158	7.55E-07	0.00039753

Obox2	3.9213	5.9985	5.5736	0.00057165	0.0075683
Mfsd7c	3.918	5.7047	10.115	9.22E-06	0.00083355
Tmem45a	3.8882	7.4154	5.4067	0.00069363	0.0085771
Kcnt2	3.8749	8.1511	6.6933	0.00017103	0.0037536
Foxo1	3.8413	10.631	11.969	2.66E-06	0.00055423
Gpr56	3.8296	8.2089	6.0996	0.00031816	0.0054779
Pcdh8	3.8008	7.2749	7.3754	8.80E-05	0.0026378
Bmpr1b	3.8005	7.7849	11.243	4.24E-06	0.00065205
Rian	3.7996	4.9405	10.514	6.94E-06	0.00074299
Cyp2s1	3.7758	8.2092	13.024	1.41E-06	0.00045403
C530014P21Rik	3.7656	5.9503	6.0268	0.00034433	0.005686
Mycn	3.7652	7.9038	6.6445	0.00017972	0.0038748
Cxxc4	3.7614	4.951	6.3179	0.00025202	0.0047702
Crim1	3.7352	8.7714	11.29	4.11E-06	0.00064934
Tmtc2	3.7334	7.2455	8.9661	2.21E-05	0.0012145
Nr0b1	3.729	4.3173	5.4689	0.00064514	0.0081603
Nxf3	3.7172	6.3459	4.1573	0.0033445	0.023133
Inhbb	3.7168	10.714	5.1538	0.00093666	0.010416
Apoc3	3.7078	6.3388	7.0215	0.00012345	0.0032041
Mctp1	3.7059	7.4122	10.387	7.59E-06	0.0007912
Cyp17a1	3.702	10.221	11.624	3.31E-06	0.00056857
Robo2	3.691	5.0331	7.3508	9.00E-05	0.0026443
Zdhhc23	3.672	6.397	13.428	1.12E-06	0.00042111

Supplementary table 3- 3 The 100 genes most differentially underexpressed in the Nr5a2 depleted mouse granulosa cells from immature mice at 44 h after equine chorionic gonadotropin stimulation.

Listed in the order of underexpression. logFC=log fold change, AveExpr=average expression, t=student's t-test value, P.Value=probability, adg.P.Val=adjusted probablility value.

Symbols	logFC	AveExpr	t	P.Value	adj.P.Val
Bhmt	-8.0511	10.139	-8.3906	3.56E-05	0.0015798
Slc12a8	-7.1192	6.5436	-23.884	1.38E-08	7.24E-05
Mmel1	-6.8498	7.72	-7.2662	9.75E-05	0.0027715
Unc13d	-6.7761	6.7075	-21.355	3.27E-08	8.29E-05
Pik3c2g	-6.5806	5.7261	-29.548	2.66E-09	2.79E-05
Parvb	-6.4546	6.4038	-21.25	3.40E-08	8.29E-05
Tmem184a	-5.9611	5.6401	-10.57	6.68E-06	0.00072691
Rdh9	-5.9574	6.5823	-15.654	3.53E-07	0.00022302
Pla2g5	-5.9431	6.9025	-13.975	8.32E-07	0.00039753
Plin5	-5.834	8.6377	-6.0597	0.00033222	0.0056063
Cd177	-5.7657	5.1161	-11.72	3.11E-06	0.00055423
5330417C22Rik	-5.5899	6.0507	-11.223	4.29E-06	0.00065205
Atp1a3	-5.5496	6.6767	-7.7172	6.42E-05	0.0021917
Saa1	-5.4704	6.3688	-5.3941	0.00070397	0.0086466
Hp	-5.4448	6.7498	-8.874	2.38E-05	0.0012619
Hal	-5.394	5.0077	-20.597	4.32E-08	8.29E-05
Plch2	-5.3824	6.8901	-9.9943	1.01E-05	0.00087442
Emb	-5.2653	7.3621	-7.0222	0.00012336	0.0032041
Cdh15	-5.1421	5.0204	-11.759	3.04E-06	0.00055423
Cd83	-5.1312	6.6169	-5.9773	0.00036346	0.0058725
Mapkapk3	-5.0479	10.66	-18.352	1.05E-07	0.00013547
Clic3	-5.0363	5.4877	-12.858	1.56E-06	0.00048028
Ydjc	-5.0039	5.6235	-11.935	2.72E-06	0.00055423
Slc47a1	-5.0011	5.7447	-10.993	5.00E-06	0.0007058
Dmbt1	-4.9462	4.6657	-5.4163	0.00068591	0.0085017
Rhod	-4.8995	6.8155	-7.3891	8.68E-05	0.0026243

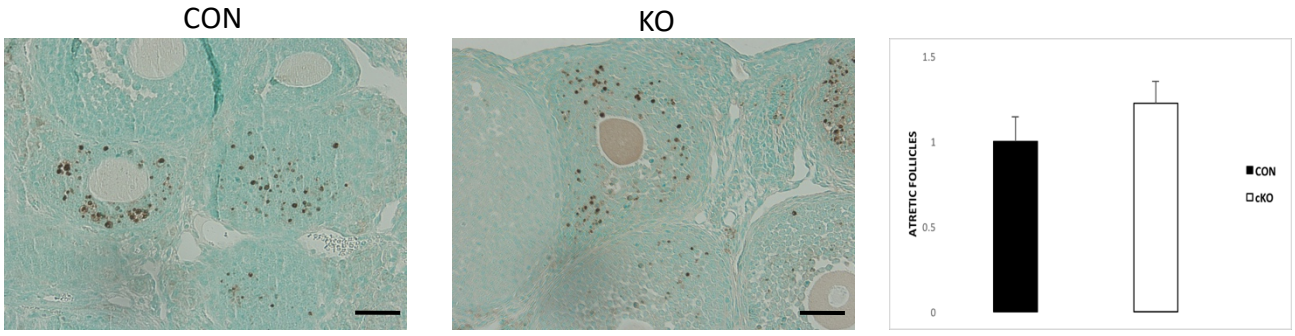
Cited1	-4.8538	5.3043	-9.9759	1.02E-05	0.00087442
Pdlim2	-4.7723	7.6958	-9.176	1.87E-05	0.0011181
Bace2	-4.7646	6.4558	-10.31	8.02E-06	0.00080842
Tinagl1	-4.6845	8.525	-7.914	5.38E-05	0.0020355
Dpysl4	-4.6797	6.966	-16.807	2.05E-07	0.00017098
Sv2c	-4.6411	4.6653	-10.05	9.67E-06	0.00086631
Ltf	-4.5959	4.7599	-10.57	6.68E-06	0.00072691
Star	-4.5298	11.382	-10.232	8.48E-06	0.00081595
Chst1	-4.4852	9.1379	-11.876	2.82E-06	0.00055423
Wisp2	-4.4686	7.7075	-4.2093	0.003118	0.022181
Saa3	-4.4661	10.276	-7.4659	8.08E-05	0.0025417
Fbxl16	-4.4421	7.1566	-14.378	6.71E-07	0.00039113
Rgs7	-4.4225	4.4341	-13.72	9.57E-07	0.00039753
Rims4	-4.3381	6.9038	-16.184	2.74E-07	0.00019129
Apol6	-4.2951	6.5368	-13.597	1.02E-06	0.00039753
Ntf5	-4.2601	6.4629	-7.4589	8.14E-05	0.0025417
Dusp1	-4.2373	10.455	-7.6377	6.90E-05	0.0023183
Adamtsl4	-4.1987	5.6512	-9.4556	1.51E-05	0.0010683
Hsd17b7	-4.1635	9.5667	-12.082	2.48E-06	0.00055423
Shb	-4.1621	4.817	-12.28	2.20E-06	0.00055423
Apof	-4.1334	4.9383	-9.963	1.03E-05	0.00087442
Homer2	-4.1158	5.9569	-10.659	6.28E-06	0.00072691
Kcne3	-4.0929	5.9438	-8.1898	4.22E-05	0.0017413
Rora	-4.074	7.1455	-9.4317	1.53E-05	0.0010683
Slc29a4	-4.0574	4.6902	-7.4336	8.33E-05	0.0025417
Mcam	-4.0538	6.3826	-5.8058	0.00043945	0.0065178
Angpt1	-4.0183	6.5808	-6.2235	0.00027857	0.0050114
Avpi1	-4.0109	10.444	-9.4108	1.56E-05	0.0010691
Etos1	-4.0046	4.7175	-7.793	5.99E-05	0.00216
Adm	-3.9975	6.0691	-8.5359	3.15E-05	0.0014861
Cited4	-3.9621	7.2443	-5.4405	0.0006668	0.0083638
Ppp1r14a	-3.9423	10.37	-6.8812	0.00014171	0.0034399

Igfbp3	-3.9363	7.1613	-4.7082	0.0016254	0.014714
Cish	-3.9308	9.1465	-13.106	1.35E-06	0.00045403
Slc27a3	-3.9152	5.5084	-7.5955	7.17E-05	0.0023495
Acsl4	-3.9107	9.7112	-6.3912	0.00023335	0.0045345
Retsat	-3.8147	8.0835	-9.5637	1.39E-05	0.0010194
Dsc2	-3.8073	7.8125	-11.274	4.15E-06	0.00064934
Gsg1l	-3.7968	8.683	-6.4308	0.0002239	0.0044233
Rgs10	-3.7636	8.9274	-8.2448	4.03E-05	0.0017095
Sbsn	-3.7506	9.98	-7.0442	0.00012075	0.0031696
Mvd	-3.7492	8.4939	-6.8209	0.00015046	0.0035342
Hck	-3.7417	4.6463	-5.5979	0.00055592	0.0074362
Thrsp	-3.7366	5.343	-10.819	5.63E-06	0.00072691
Parm1	-3.7199	9.0723	-6.8604	0.00014467	0.0034714
Mapk13	-3.6994	4.7291	-4.6681	0.0017105	0.015213
Kifc3	-3.6568	5.9827	-6.1969	0.00028658	0.0050934
Apln	-3.6549	8.0636	-9.393	1.58E-05	0.0010699
Mfsd4	-3.6547	5.8751	-10.165	8.89E-06	0.00081809
Prodh	-3.6403	7.002	-9.1969	1.84E-05	0.0011101
Mbp	-3.634	6.2666	-5.6863	0.00050269	0.0070377
Gamt	-3.633	8.7859	-5.3781	0.0007173	0.0087563
Synm	-3.5989	8.3449	-4.7297	0.0015818	0.014473
Hddc3	-3.5951	7.0121	-4.7283	0.0015846	0.014486
Esrp2	-3.5763	5.8128	-6.8382	0.00014789	0.0035165
Slc16a2	-3.5732	9.1828	-8.2939	3.86E-05	0.0016591
Rab27a	-3.5723	5.4108	-11.769	3.02E-06	0.00055423
Cmbl	-3.5682	7.9619	-4.6613	0.0017253	0.015296
Fads6	-3.5578	8.9706	-5.9296	0.00038302	0.0060396
Tnfrsf12a	-3.5423	8.705	-3.9018	0.0047451	0.028811
Tspan5	-3.5329	6.9241	-8.8269	2.48E-05	0.0012914
A2m	-3.5222	6.0946	-3.931	0.004557	0.02801
Hs6st1	-3.5125	8.7941	-9.6507	1.30E-05	0.00098731
Rnaseh2a	-3.5075	9.9248	-7.9944	5.01E-05	0.0019522

Wnt4	-3.5025	10.71	-3.3287	0.010759	0.04809
Lama5	-3.4682	5.3834	-6.1745	0.00029353	0.0051747
Npl	-3.4588	6.7231	-5.2038	0.00088201	0.010068
Lrrn3	-3.455	6.8454	-6.3109	0.0002539	0.0047886
Myc	-3.4514	8.4461	-6.6573	0.00017739	0.0038512
D630023F18Rik	-3.4484	8.6207	-5.904	0.00039398	0.0061569
Saa2	-3.4454	5.8663	-5.7815	0.00045154	0.006648
Spns2	-3.4114	9.1044	-7.5315	7.61E-05	0.0024389
Nr1d1	-3.379	4.8809	-9.0823	2.02E-05	0.0011734
Tspan15	-3.3776	5.3131	-9.8106	1.15E-05	0.00094091
Fabp4	-3.3756	5.4228	-4.2558	0.0029301	0.021411

Supplementary figure 3- 1 TUNEL analysis showing no difference in the frequency of apoptosis in ovaries of immature, gonadotropin stimulated CON and cKO mice.

Ovaries were taken at 44h after treatment with eCG. Original scale bars 20µm. Data are expressed as means ±SEM



CHAPTER 4 : Second manuscript

The Orphan Nuclear Receptor Liver Receptor Homolog-1 (LRH-1, NR5A2) Regulates The Ovarian Reserve

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Précis: The nuclear receptor, Nr5a2, regulates ovarian reserve by regulating primordial follicle formation, activation and survival.

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Abstract

In mouse ovaries, Liver Receptor Homolog-1 (LRH-1, NR5A2) is restricted to granulosa cells of all follicles and corpora lutea. Mice with NR5A2 depletion in this cell population are anovulatory and infertile. To determine whether NR5A2 is essential for regulation of the ovarian reserve, we generated granulosa-specific knockout mice (genotype *Nr5a2* $^{f/f}$*Amhr2*$^{Cre+}$, hereafter cKO) with NR5A2 depletion from primordial follicles forward. Cre recombinase was expressed in all follicles, as demonstrated by immunolocalization of Amrh2 and the *Nr5a2* mRNA level was significantly depleted at at postnatal day 4 (PND4) and postnatal day 13 (PND13). The number of primordial follicules in cKO mice relative to control counterparts (CON) was substantially larger at PND4, the end of the germ cell cyst breakdown, as well as at (PND13). Ten day *in vitro* culture of PND4 ovaries confirmed the increased population of primordial follicles in cKO mice compared to their CON littermates, and indicated that, while CON mice developed primary follicles in the ovarian medulla the cKO animals failed to do so. Immunocytochemistry and *in situ* hypbridization of NR5A2 demonstrated the existence of two subsets of primordial follicles one which expressed NR5A2, and the second which did not. Neither expressed the mitotic marker, KI-67. There was a dysregulation of activation-related genes such as *Foxl2*, *Foxo3*, *Pten*, and the increase of expression of quiescence markers like *Cxcl1*, *Cxcl10*, *Egr1, Lin28a* in cKO mice ovaries indicated that recruitment of primordial follicles into the growing pool is impaired by NR5A2 depletion. Moreover, primordial follicle formation related genes *Figla, Notch2* were upregulated and *Amh* was downregulated in the cKO ovary, indicating that NR5A2 plays a role in formation of primordial follicle pool. Finally the role of NR5A2 in the survival of primordial follicles

was assessed and it was demonstrated that the depletion of NR5A2 upregulates pro-survival factors such as *Mcl1* and downregulates pro-apoptotic genes such as cleaved caspase 3 suggesting a third mechanism to explain the differences in primordial follicle population between cKO and CON mice. It is concluded that the presence or absence of NR5A2 defines two populations of primordial follicles and is essential for primordial follicle formation, activation and survival.

Keywords: Ovarian reserve, formation, activation, survival, NR5A2, conditional knock out, primordial follicles.

1. Introduction

The development of the mouse ovary begins with sexual differentiation on embryonic day 10.5 (45), characterized by mitotic replication of oogonia. Most of the incipient oocytes initiate meiosis between embryonic day 13.5 to day 15.5 (32). Those that persist can be found densely packed in structures known as germ cell cysts (51). Beginning on approximately day 19.5 of gestation, these germ cell cysts undergo a programmed breakdown, when the pregranulosa cells invade and surround each oocyte, forming the primordial follicles (33). This process continues through the first four days of postnatal life, and oocytes that fail to be encapsulated succumb to apoptosis (31). In the mouse, approximately 33% of the oocytes survive to form the primordial follicles (33). The consensus view is that this process of primordial follicle formation establishes the ovarian reserve that will provide oocytes throughout reproductive life (56).

Although enormous progress has been made in understanding the events and regulation of the later stages of ovarian follicular development, the early stages of development remain, to a large extent, a mystery (11). The majority of the follicles in the reserve are dormant, and it has been shown that the maintenance of primordial follicles in this quiescent state is not a passive process, but that the resting pool of primordial follicles is under constant inhibitory influence (49, 50). Nonetheless, once activated, continuation of development is an irreversible process, and follicles that have initiated growth undergo atresia if not selected for subsequent stages of maturation (38). Activation of primordial follicles occurs via a gonadotropin-independent process, whereby they are gradually selected from the quiescent reserve into the growing follicle pool (26, 44). Extensive bidirectional signaling takes place between oocytes and granulosa cells to ensure follicle

development from primordial stage onwards (4, 36). As oocytes begin to increase in size, the granulosa cells change shape from flattened to cuboidal, increase their proliferation and form multiple layers (9). Multiple activator and repressor signaling pathways are involved in the control of primordial follicle activation as indicated by the oocyte markers such as GDF9, FOXO3 and PTEN or the granulosa cell specific AMH, and FOXL2 (22).

Recent studies have shown that the orphan nuclear receptor, liver receptor homolog 1 (LRH-1, NR5A2), is essential for follicular development, due to its effects on multiple processes, including granulosa cell proliferation (27). NR5A2 expression is restricted to granulosa cells of primordial and all subsequent follicles in the ovary, as well as to luteal cells (57). Germline deletion of NR5A2 results in early embryo lethality (23), thus, a conditional depletion strategy is necessary for exploration of its role in the ovary. By means of ovary-specific depletion, beginning in either primordial or antral follicles, or restricted to the corpus luteum, we have previously shown that NR5A2 is required, not only for successful follicle development and subsequent ovulation, but also for pregnancy (2, 3, 10, 55). To date, very little is known about the developmental dynamics of primordial follicles under physiological conditions, and no information has emerged on the role of NR5A2 in either primordial follicle activation or maintenance of the quiescence of the follicle reserve.

The objectives of the current study were to explore the role of NR5A2 in ovarian function, with focus on its contribution to primordial follicle activation. We used a mutant mouse model in which NR5A2 was depleted from the granulosa cells of follicles at all stages, from primordial follicles forward. Our results demonstrate a role for NR5A2 in the regulation of primordial follicle activation.

2. Material and Methods

A. Animals and colony maintenance

Animal experiments were approved by the University of Montreal Animal Care Committee and were conducted according to the guidelines of the Canadian Council on Animal Care. All mutant and control (CON) mice were maintained on the C57BL/6 background, under a 14-hour light, 10-hour dark cycle and provided food and water *ad libitum*. Euthanasia was performed with isoflurane anesthesia, followed by cervical dislocation, as previously described (2). *Nr5a2* floxed (*Nr5a2*$^{f/f}$) mice have been described previously (2, 10). Granulosa- specific depletion of NR5A2 was generated by crossing these animals with mice expressing Cre-recombinase driven by the anti-Mullerian type II receptor (*Amhr2*$^{Cre+}$) (2, 10) to produce conditional knockout (cKO) mice (genotype *Nr5a2*$^{f/f}$; *Amhr2*$^{Cre+}$). Following DNA extraction from tails, littermates were genotyped. Control mice (CON) mice in these trials were nonmutant, *Nr5a2*$^{f/f}$; *Amhr2*$^{Cre-}$ females (42).

B. RNA extraction and Real-Time PCR

RNA was extracted from postnatal day 4 (PND4) and postnatal day 13 (PND13) ovaries with PureLink RNA mini extraction kit according to the manufacturer's instructions (Invitrogen #12102385). Reverse transcription was performed using the SuperScript III reverse transcription (Invitrogen #18080093). Real-time quantitative polymerase chain reaction (qPCR) was performed using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories #1725274) with the CFX 96 Real-Time System, C1000 Touch Thermal Cycler (Bio-Rad Laboratories). The transcripts were amplified following the same

cycling program: 30 seconds at 95°C and then 40 cycles of 15 seconds at 95°C and 30 seconds at 60°C, followed by 5-second step of a 0.5°C increase between 65°C and 95°C. Primers employed can be found in Supplemental Table 1.

C. RNA *in situ* hybridization (RNAScope)

Tissues were fixed in 10% formalin overnight, paraffin embedded and cut in 5 μm sections. RNA in situ hybridization was performed with RNAscope® 2.5 HD Detection Kit (RED) (ACD Bio, # 322350) following manufacturer's instructions and as previously described (52). The tissue sections were hybridized with probes spanning mouse *Amhr2* and *Nr5a2* mRNA (ACD-bio #543579 for Nr5a2, #489821 for Amhr2) in the HybEZ hybridization oven (ACD) for 2 hours at 40°C, following a series of pretreatment steps. The slides were then processed for standard signal amplification steps per manufacturer's instructions. Red chromogen development was performed following the RNAscope 2.5 HD detection protocol. The slides were then counterstained in 50% hemotoxyin for 2 minutes, air-dryed and coverslipped with EcoMount.

D. Histology and follicle counting

Tissue processing and embedding were performed using standard techniques. Ovaries were serially sectioned at 5μM and stained with hematoxylin and eosin (Thermo Fisher Scientific #72511, Thermo Fisher Scientific #71311). For follicle counting, 4-9 ovaries were assessed per genotype for each age group and follicles were classified as follows. Primordial follicles displayed a single layer of squamous pregranulosa cells around the oocyte. Primary follicles were those with one layer of cuboidal granulosa cells, and secondary follicles with more than one layer of cuboidal granulosa cells surrounding the

oocyte. Data were expressed as ratio of the number of follicles of each class per section in the cKO ovary to the number counted in the control sections. At PND13 a total of 62 tissues were assessed for the cKO and 45 for the CON, at PND4 89 for the CON and 96 cKO.

E. Fluorescent immunocytochemistry

Slides of formalin or paraformaldehyde-fixed paraffin-embedded PND13 and PND4 ovaries from control and cKO mice were rehydrated as previously described (2) and blocked 1 hour with 5% bovine serum albumin (Jackson ImmunoResearch Laboratories) in PBST 0.1%. Slides treated for NR5A2 immunofluorescence were blocked for a further hour with MOM blocking reagent (Vectorlabs #FMK2201). Slides were then incubated overnight at 4°C with antibodies against FOXL2 (1:8000 in BSA5% / PBST 0.1% - generous gift from Dr. Dan Bernard), NR5A2 (1:200 in MOM kit dilution reagent, Vectorlabs #FMK2201), AMHR2 (1:200 in BSA5% / PBST 0.1%, R&D Systems #AF4749), cleaved caspase 3 (1:250 in BSA5%/PBST 0.1%, Cell Signaling #9661), Pten (1:200 in BSA5% / PBST 0.1%, Cell Signaling #138G6), DDX4 (1:200 in BSA5% / PBST 0.1%, Invitrogen #PA5-23378) or KI-67 (1:200 in BSA5% / PBST 0.1%, Abcam #Ab15580). In case of double staining, NR5A2/ KI-67 both antibodies where diluted in MOM kit dilution reagent. CY3 conjugated anti-mouse (Jackson ImmunoResearch #115-165-146) diluted 1:400 in BSA 5%/PBST 0.1% was used for NR5A2 and AMHR2 immunofluorescence. CY3 conjugated goat anti-rabbit IgG (Jackson ImmunoResearch #111-165-144 or Invitrogen #A11008), was used as second antibody for PTEN, FOXL2, cleaved caspase 3 and KI-67 immunolocalization, at the same concentration. Finally, slides were counterstained with DAPI (Thermo Fisher Scientific #D1306), 1:1000 in PBS for 5 minutes before being washed three times in PBS and mounted with Permafluor (Thermo

Fisher Scientific #TA006FM). CellProfiler Software (5) was used to quantify signal intensity.

F. Ovary culture

Postnatal day 4 (PND4) mice ovaries from CON and cKO animals were dissected and placed in 50µl drops of media and cultured for 10 days on 0.4-µm floating filters (Millipore Corp., #PICM01250) at 37°C in a chamber containing 5% CO_2 (21, 47). Filters were placed in 4-well culture plate (Nunc plate, Fisher Scientific #176740) on 0.5 ml DMEM-F12 media supplemented with 0.1% Albumax (Thermo Fisher Scientific #11021029), penicillin-streptomycin (Invitrogen #15140148), 0.1% FBS (Thermo Fisher Scientific #SH30071.03), 27.5 µg/ml transferrin (Sigma #T1147), and 0.05 mg/ml L-ascorbic acid (Sigma #A4403) (21, 47). Culture medium was changed every second day. After 10 days of culture, ovaries were fixed for 4 h in 4% PAF, washed with PBS and mounted in blocks before being stained with Ddx4 to facilitate oocyte/follicle counting (see previous section).

G. Statistical analyses

All values are expressed as mean ± SEM. To determine significance between means in all experiments, comparisons were made using Student's t-test analysis with Welch's correction on Graphpad Prism V7.0 (Graphpad Software). $P < 0.05$ was considered significant.

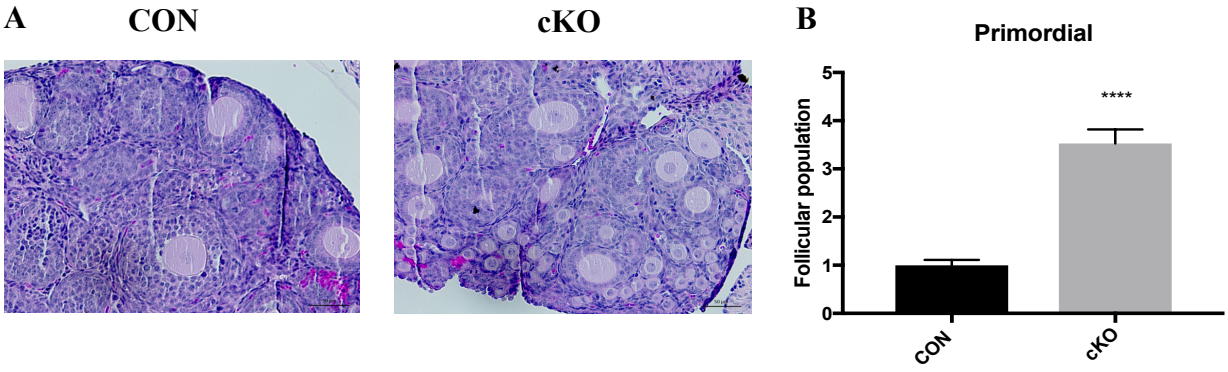
3. Results

A. Phenotypic differences between the cKO and CON ovaries at Day 13

To study the role of NR5A2 in preantral folliculogenesis, we collected postnatal day 13 (PND13) ovaries from CON and cKO mice, as the follicle population is limited to primordial, primary and secondary follicles at this time (20). Histological evaluation of ovarian sections, confirmed the presence of these three follicle subtypes in both CON and cKO mice (Figure 4-1A&B). The total number of primordial follicles in the cKO, compared to the CON ovary population of these follicles was more than three times greater in the cKO compare to the CON (Figure 4-1B). This increased number of primordial follicles was found predominantly in the ovarian cortices of cKO mice (Figure 4-1A).

Figure 4- 1 NR5A2 depletion increases the number of primordial follicles at PND13

(A) Hematoxylin/Eosin staining analysis showed that the number of primordial follicles was increased in cKO mice ovary at PND13 compared to the CON (n=5/n=9). The primordial follicles are mainly located in the cortices of the ovaries. Scale bar 50 µm. (B) The population of primordial follicles was quantified. ****P<0.001 Data are mean ± standard error of the mean (SEM)



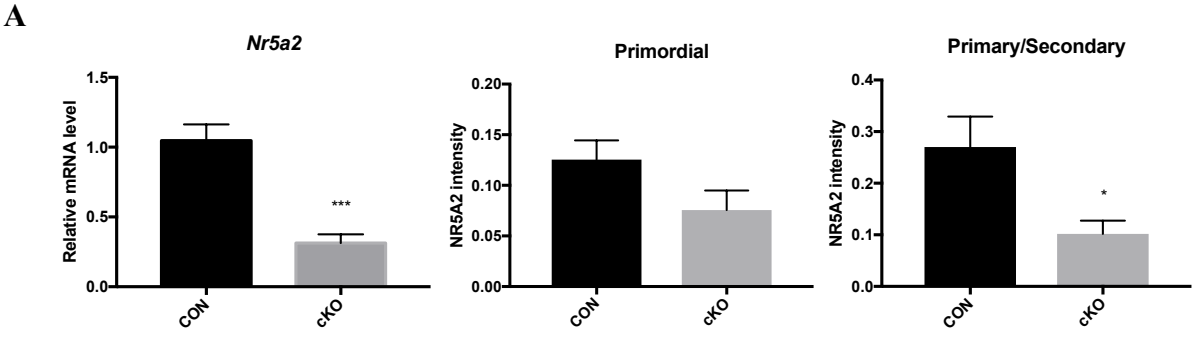
B. NR5A2 mRNA and proteins are depleted in cKO mice at Day 13

We then sought to confirm the depletion of NR5A2 in the ovaries of cKO mice by qPCR and quantitative immunocytochemistry at PND13 (Figure 4-2). Whole ovary RNA

analysis revealed a 70 % decrease ($p<0.001$) in *Nr5a2* mRNA abundance in the cKO mice (Figure 4-2A). Evaluation of NR5A2 protein by immunofluorescence demonstrated the expected nuclear localization of NR5A2 in CON ovaries (Figure 4-2B). Visual analysis indicated that only a small subset of the primordial follicles in the CON mouse expressed NR5A2. In the cKO ovaries, little signal was observable in primordial follicles, confirmed by a trend toward reduction detectable by CellProfiler software ($p=0.11$, Figure 4-2B). In primary and secondary follicles, the immunofluorescent signal was reduced by greater than 60 % ($p<0.05$, Figure 4-2B) in keeping with the reduction seen by qPCR.

Figure 4- 2 NR5A2 is significantly depleted in cKO ovaries at PND13

(A left) Abundance of *Nr5a2* transcripts in CON and cKO mice ovaries at PND13 (n = 13/n=7 animals per genotype). (A Center and right) Quantitative analysis of NR5A2 expression in CON and cKO granulosa cells at PND13 in primordial (center) and primary/secondary (right) follicles ***P<0.001, *P<0.05. Data are expressed as means ±SEM (B) Immunolocalization of NR5A2 in the ovaries of PND13 CON and cKO mice. Original scale bars 20μm.



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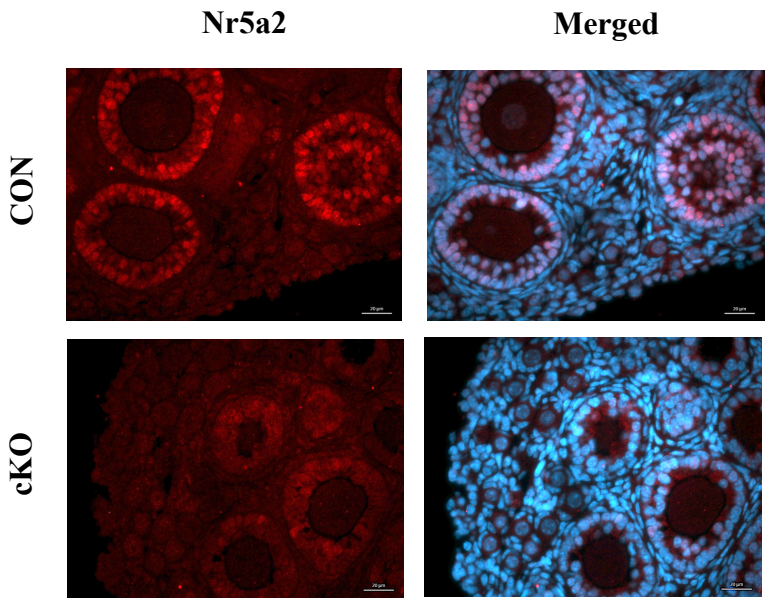
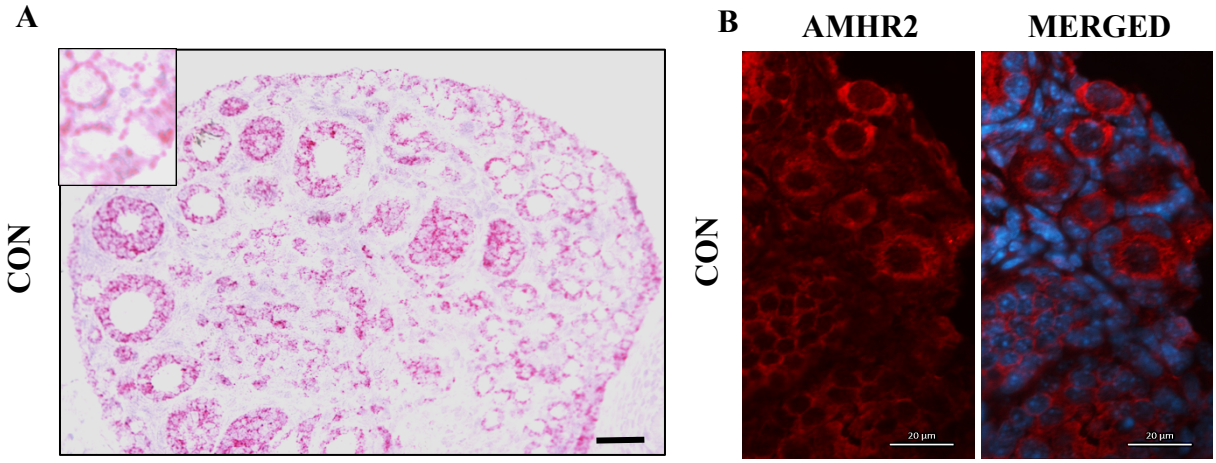


C. AMHR2 is expressed in the primordial follicles

Given the demonstrable variability in follicle populations and in expression of NR5A2 in preantral follicles, we employed a combination of *in situ* hybridization (RNAScope) and immunocytochemistry to determine where AMHR2 was expressed in the immature ovaries of the CON mice, and thus where *Amhr2*^{Cre} recombined with the *Nr5a2* floxed sites in the immature ovaries of cKO mice. As can be seen in Figure 4-3A, mRNA for *Amhr2* localized to the granulosa cells of primordial, primary and secondary follicles in the immature ovary, as well as to the surface epithelium. Immunohistochemistry revealed that AMHR2 protein is present in primordial, as well as primary and secondary follicles (Figure 4-3B). Together these findings provide evidence for Cre/lox recombination and consequent depletion at least as early as the primordial follicle stage of the follicle development trajectory.

Figure 4- 3 AMHR2 is expressed in granulosa cells of all follicles

(A) RNA *in situ* hybridization of *Amhr2* in CON ovaries at PND6. (B) Immunolocalization of AMHR2 in the ovaries of PND13 CON mice. Original scale bars 50µm (A) 20µm (B).



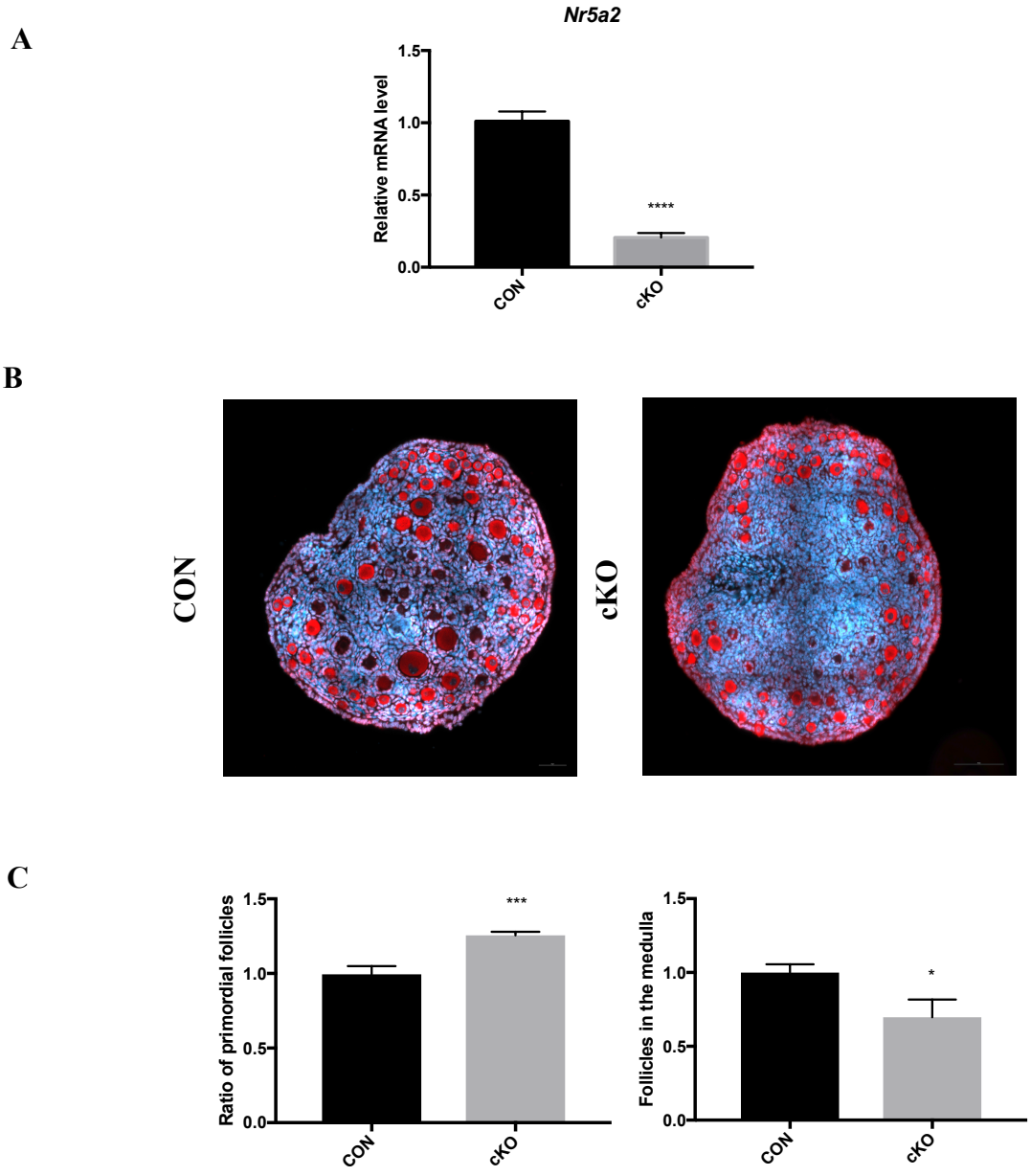
D. Ovaries in culture show more primordial and fewer developing follicles in the medulla of the ovary of the Nr5a2 cKO mouse

To further study the role of Nr5a2 in follicular development, we developed an organ culture model of postnatal day 4 (PND4) ovaries incubated over 10 days. The abundance of *Nr5a2* mRNA was markedly lower in the cKO ($p<0.0001$) compared to the CON ovaries by qPCR at PND4 (Figure 4-4A). It is known that in organ culture of neonatal mice ovaries, the growing follicles are principally concentrated in the central part of the ovary (12, 28, 39). To facilitate oocyte identification, DDX4 immunofluorescence was performed. In our CON mice we observed primordial follicles in the cortex and developing primary follicles, mainly located in the medulla, consistent with previously published data (39). In the cKO mice ovaries we showed a significant increase in the ratio of primordial to primary and secondary follicles (Figure 4-4B&C), in keeping with result *in vivo* at PND13 (Figure 4-

1). Moreover, we show a significant decrease in the number of developing follicles in the medullary zone of the ovary of the cKO mice (Figure 4-4B, C).

Figure 4- 4 Depletion of Nr5a2 impacts follicle development *in vitro*

(A) Abundance of *Nr5a2* transcripts in CON and cKO mice ovaries at PND4 (n=5/n=10 per genotype). (B) CON and cKO mice ovaries cultured for 10 days *in vitro*. Oocytes stained with DDX4. (C left) Quantification of the primordial follicles population. (C right) Quantification of the follicles in the medulla (n=13/n=8 animals per group). ****P<0.001, ***P<0.005, *P<0.05. Data are expressed as means ±SEM. Original scale bars 200µM.



E. NR5A2 mRNA and proteins are depleted at PND4, the time of cyst breakdown and follicular formation

The process of cyst breakdown and primordial follicle formation typically begins at 17.5 days post coitum (DPC) and is completed by PND 5 (17). As NR5A2 appeared to be depleted at PND4 (Figures 3.4A, 5A&B), we collected PND4 CON and cKO mice ovaries for histological examination, to assess the role of NR5A2 in follicular formation. At PND4, we observed an increased number of primordial follicles in the cKO compared to control mice. Moreover, the cKO mouse ovaries were characterized by a decrease of primary follicles (Figure 4-6A&B), which led us to hypothesize that NR5A2 is involved in primordial follicle activation.

Figure 4- 5 Depletion of NR5A2 in PND4 cKO ovaries

(A) Immunolocalization of NR5A2 in PND4 CON and cKO mice ovaries. Original scale bars 20μm (B) Quantitative analysis of NR5A2 expression between CON and cKO PND4 mice ovaries (n= 6/n=3 animals per genotype). *P<0.05. Data are expressed as means ±SEM.

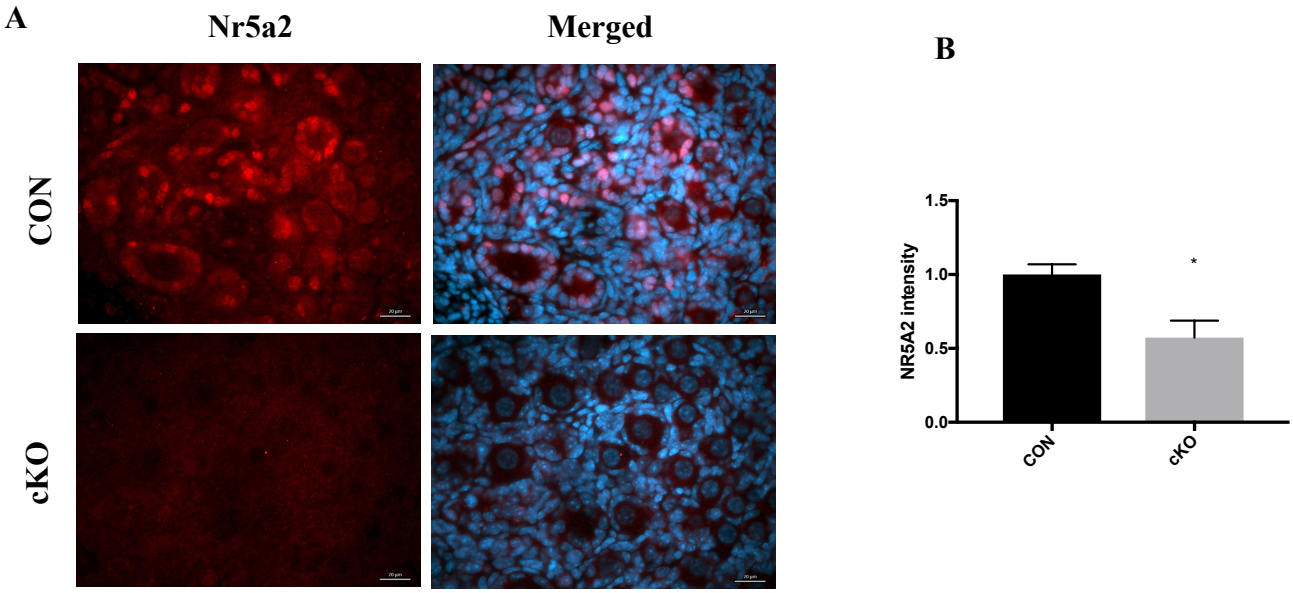
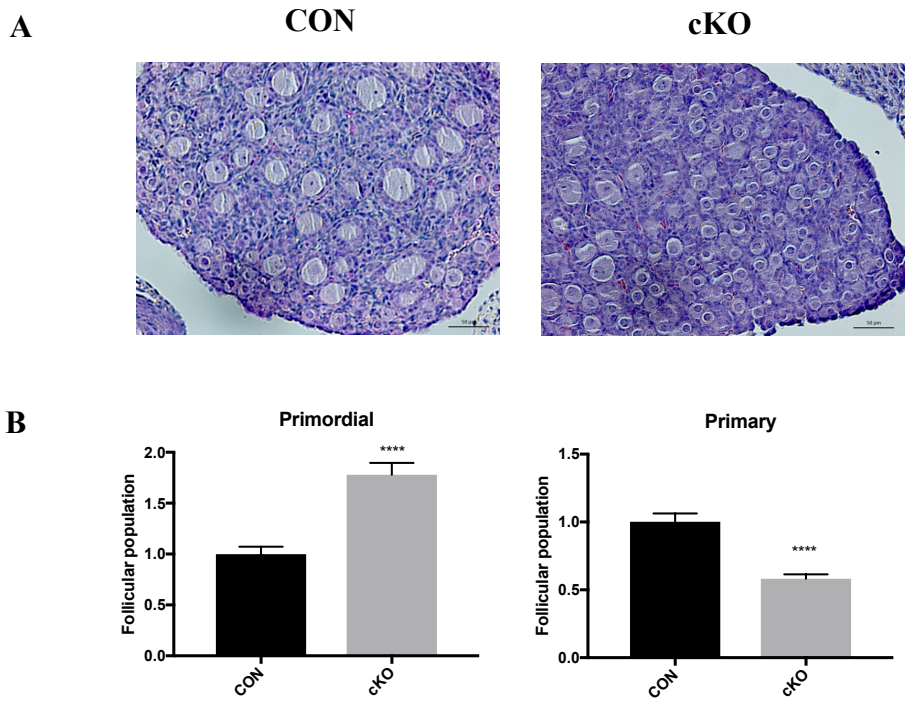


Figure 4- 6 NR5A2 depletion increases the number of primordial follicles at PND4

(A) Hematoxylin staining analysis showed that the number of primordial follicles was increased and the number of primary follicles decreased in cKO mice ovary at PND4 compared to the CON. Scale bar 50 µm. (B) The populations of primordial and primary follicles were quantified. (n= 5/n= 7 animals per genotype). ****P<0.001. Data are expressed as means ± SEM.



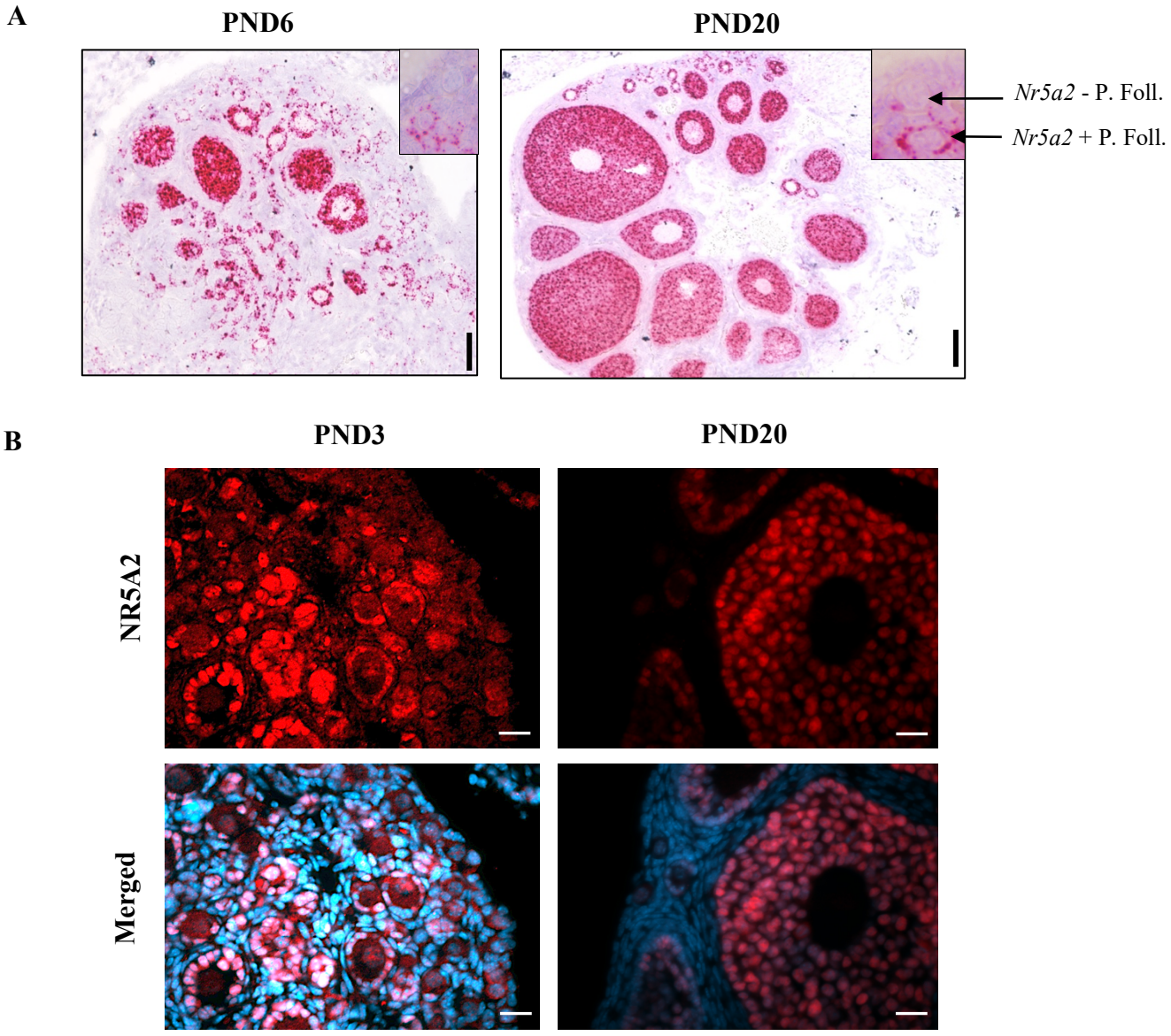
F. NR5A2 defines two subsets of primordial follicles

To demonstrate the implication of NR5A2 in primordial to primary follicle transition, we performed RNA *in situ* hybridization (RNAScope) and fluorescent immunocytochemistry in ovaries of wild type mice at PND3 and PND6 as well as PND20. As expected, the signal was detected in all primary and secondary follicles, but only in a subset of primordial follicles (Figure 4-7). In fact, we observed the existence of two populations of primordial follicles, one expressing NR5A2 and a second that appears devoid of the signal. To confirm that the follicles expressing NR5A2 have not yet been activated, we performed a double immunofluorescence to detect NR5A2 and KI-67, a

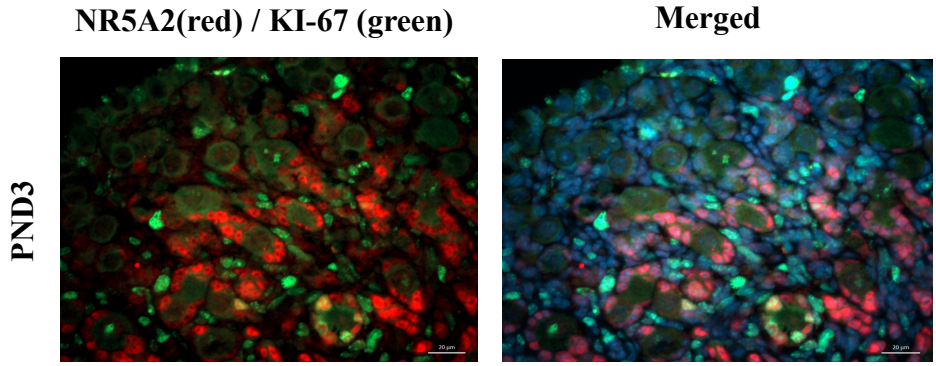
cellular proliferation marker (Figure 4-7C). As expected, we found that activated primary follicles expressed NR5A2 and KI-67. One subpopulation of primordial follicles expressed neither NR5A2 nor KI-67, and there was a demonstrable subset of primordial follicles that expressed only NR5A2 but not KI-67, and thus was not activated (Figure 4-7C).

Figure 4- 7 NR5A2 defines subsets of primordial follicles

(A) RNA *in situ* hybridization of *Nr5a2* in CON ovaries at PND6 and PND20. (B) Immunolocalization of NR5A2 in the ovaries of PND3 and PND20 CON mice. (C) Co-immunolocalization of NR5A2 (red) and KI-67 (green) in the ovaries of PND3 CON mice. Original scale bars 50µm (A) 20µm (B, C).



C

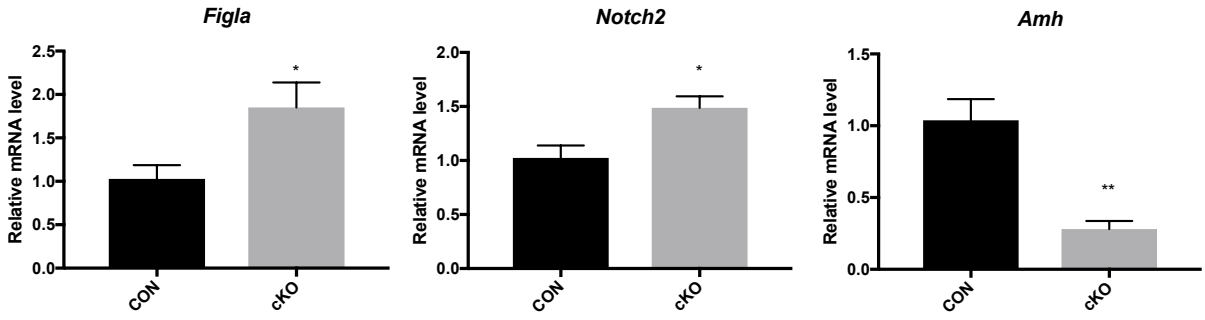


G. NR5A2 is involved in primordial follicle formation, survival and activation

To further explore the role of NR5A2 in the regulation of the ovarian reserve, we studied the impact of its depletion on primordial follicle formation (Figure 4-8), survival (Figure 4-9) and activation (Figure 4-10) by investigating known marker genes for these processes. Following depletion of NR5A2, mRNA level for the gene, Factor in the germline alpha (*Figla*), was significantly upregulated (Figure 4-8). NOTCH2 is a granulosa cell expressed factor that also plays a role in regulating breakdown of germ-cell nests and formation of primordial follicles (53). As with the *Figla, Notch2* transcripts were significantly increased in cKO mice at PND4 (Figure 4-8), suggesting an increase in the formation of the primordial follicles. AMH is an important regulator of primordial follicle assembly, as it mediates stromal-epithelial interaction in the developing ovary to inhibit follicle assembly (29). We observed a significant decrease in *Amh* mRNA level in the cKO mice (Figure 4-8) that accompanied the increase of *Figla* and *Notch2* (Figure 4-8).

Figure 4- 8 NR5A2 depletion impacts follicle formation related genes

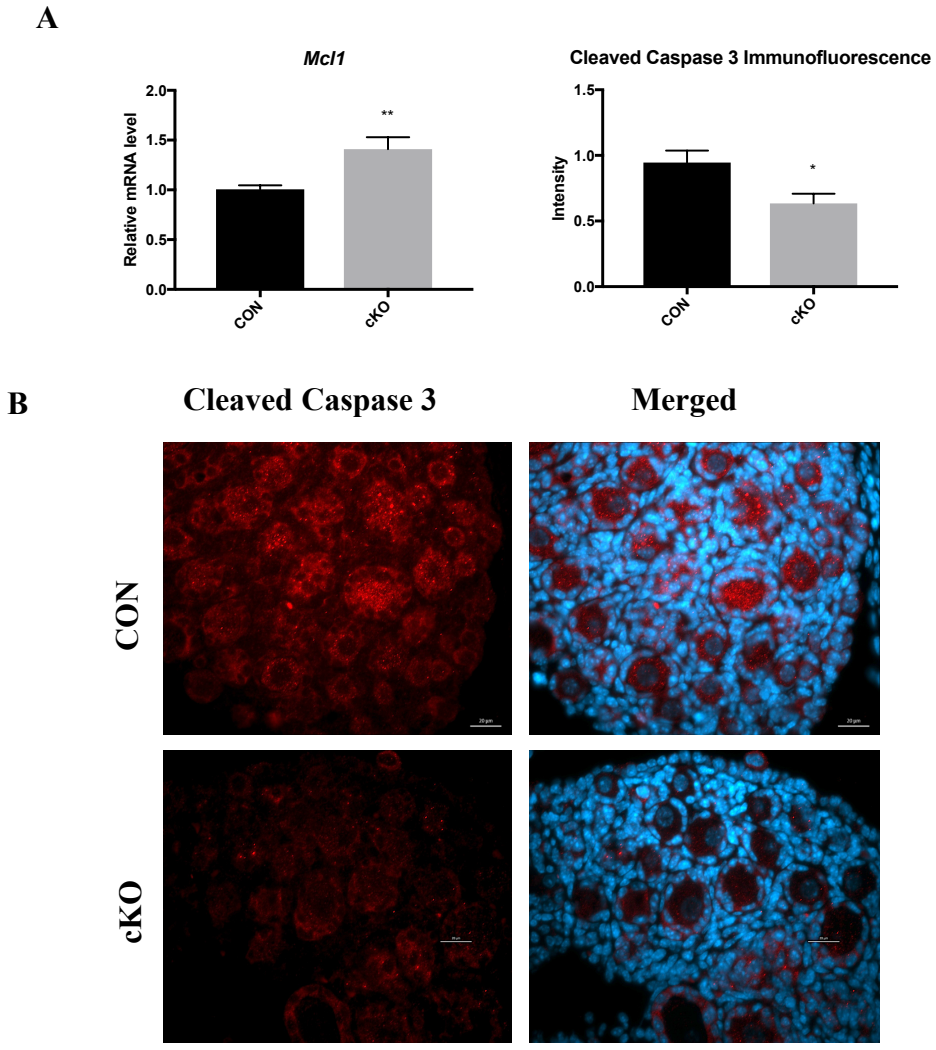
Abundance of *Figla*, *Notch2* and *Amh* transcripts in CON and cKO mice ovaries at PND4 (n=5 / n=10 animals per genotype). *P<0.05, **P<0.001. Data are expressed as means ±SEM



The oocytes that fail to be surrounded by granulosa cells to form follicles perish by apoptosis (30). To study this as a possible mechanism to explain the effects of NR5A2 on the primordial follicle population, we quantified the anti-apoptotic B-cell lymphoma 2 (BCL-2) family member myeloid cell leukemia-1 (MCL-1) mRNA and the pro-apoptotic cleaved caspase 3 protein levels (Figure 4-9). MCL-1 localized to both oocytes and somatic cells in primordial follicles of PND4 cKO mice, *Mcl1* was significantly upregulated in the ovaries of Nr5a2 cKO animals relative to their CON counterparts (Figure 4-9A). To further explore involvement of the apoptosis pathway in the phenotype observed in the postnatal cKO ovary, we measured the pro-apoptotic cleaved caspase 3 proteins level by immunofluorescence. The result was a significant decrease of the signal detected in the cKO mice (Figure 4-9A&B).

Figure 4- 9 NR5A2 depletion affects apoptosis related genes

(A left) Abundance of *Mcl1* transcripts in CON and cKO mice ovaries at PND4 (n=10/ n=5 animals per genotype). (A right) Quantitative analysis of cleaved caspase 3 expression between CON and cKO PND4 mice ovaries (n=9 / n=5 animals per genotype) (right). **P<0.01, *P<0.05. Data are expressed as means ±SEM (B) Immunolocalization of cleaved caspase 3 in CON and cKO mice ovaries at PND4 (n = 9 / n=5 animals/genotype). Original scale bar 20µm.

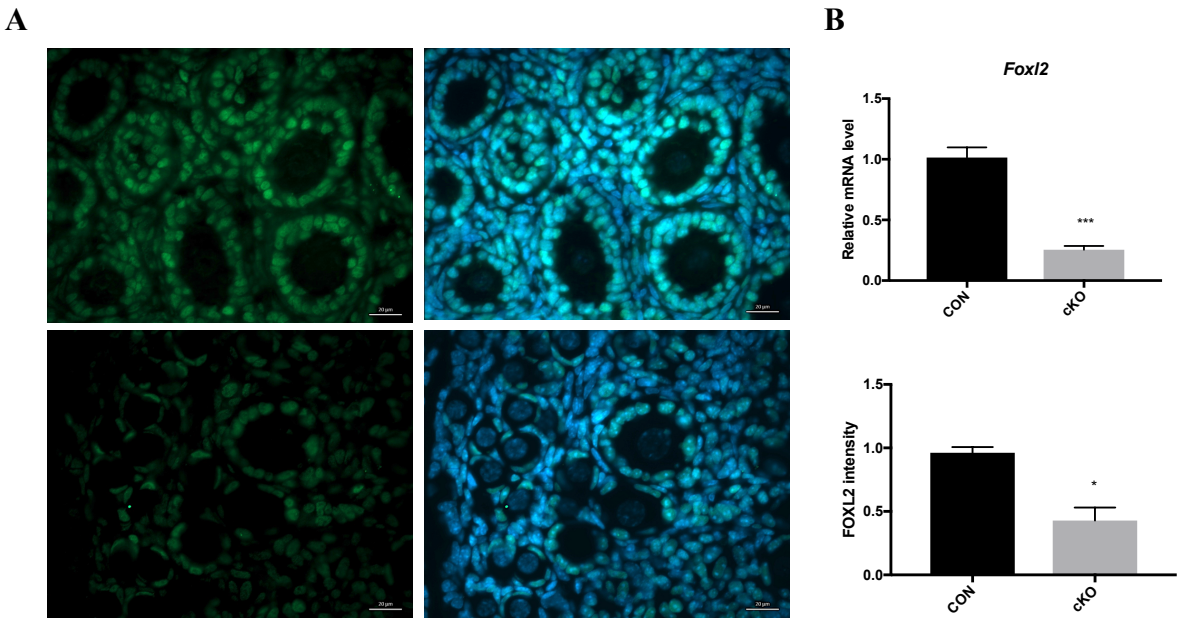


We then investigated the possibility that NR5A2 plays a role in primordial follicle quiescence and activation (Figures 3.10 & 3.11). In the cKO model we observe a drastic decrease in mRNA levels for the female follicle specific gene, *Foxl2* (Figure 10) and quantification of FOXL2 protein by immunofluorescence confirmed the significant decrease of FOXL2 presence in cKO mice ovaries (Figure 4-10A&B). Germline knockout

studies have identified the tumor suppressor, PTEN, as the principal constraint on the activation of primordial follicles (13). Here we show a significant increase in the abundance of PTEN protein and the mRNA of its downstream target, *Foxo3* in NR5A2 cKO PND4 ovaries (Figure 4-10C&D). These data argue that NR5A2 depletion maintains primordial follicular dormancy.

Figure 4- 10 NR5A2 depletion affects follicle activation related genes

(A) Immunolocalization of FOXL2 in CON and cKO mice ovaries at PND4 (B up) Abundance of *Foxl2* transcripts in CON and cKO mice ovaries at PND4 (n=5 /n=10 animals per genotype). Original scale bars 20μm (B down) Quantitative analysis of FOXL2 expression between CON and cKO PND4 mice ovaries (n =5/n=4 animals/genotype) (C) Immunolocalization of Pten in CON and cKO mice ovaries at PND4 (D up) Quantitative analysis of PTEN expression between CON and cKO PND4 mice ovarian cortices (n=4/n=6 animals per genotype). (D down) Quantitative analysis of *Foxo3* expression between CON and cKO PND4 mice ovaries (n=5/n=10 animals per genotype). ***P<0.005, *P<0.05. Data are expressed as means ±SE



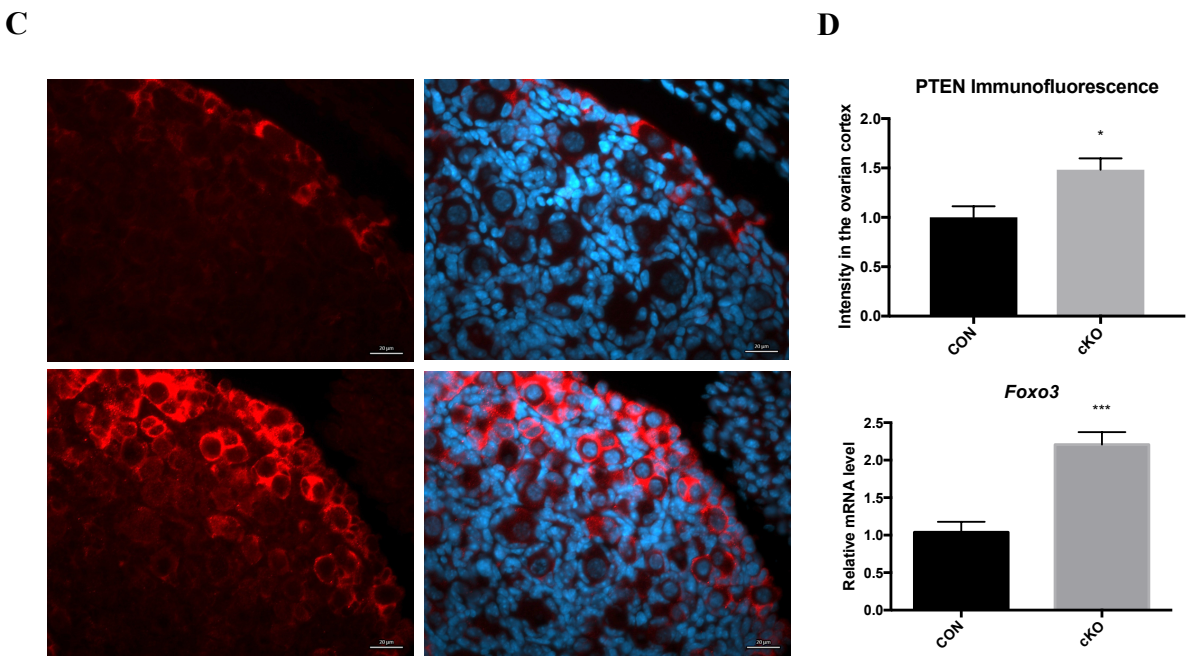
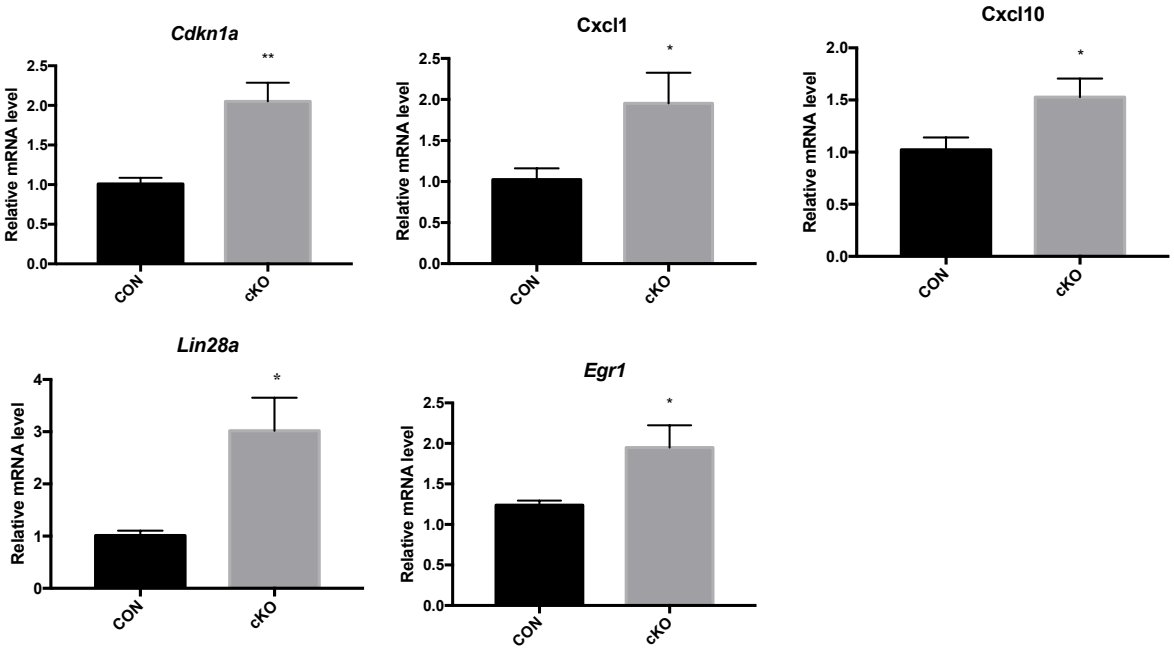


Figure 4- 11 Quiescence markers are upregulated in the absence of NR5A2

Abundance of *Cdkn1a*, *Cxcl1*, *Cxcl10*, *Lin28a* and *Egr1* transcripts in CON and cKO mice ovaries at PND4 (n=5 /n=10 animals per genotype). **P<0.01, *P<0. 05. Data are expressed as means ±SEM



To further investigate the role of NR5A2 in primordial follicle quiescence, we quantified transcripts of several genes involved in establishment or maintenance of the quiescent state in the PND4 ovaries of cKO mice (Figure 4-11). These included *Cxcl1* and *Cxcl10*, two chemokine ligands that are upregulated in human quiescent hematopoietic stem cells (HSC) (41). *Egr1* gene has also been reported to be downregulated in quiescent stem cells by induction of cell division and migration (54). The mRNA level for this gene was elevated in the cKO animals by qPCR validation. *Lin28a*, reported to be expressed in primordial germ cells and non activated primordial oocytes (8), was also significantly upregulated in the cKO (Figure 4-11).

4. Discussion

In the mammalian ovary, the progressive activation of the finite reserve of primordial follicles serves as the source of fertilizable ova during reproductive life. While progress has been made in recognition of the events in primordial follicle formation (24), factors regulating their activation under physiological conditions remain less well understood. Here we showed that NR5A2, an orphan nuclear receptor, well known to play essential roles in expansion of the cumulus oöphorus (3), ovulation (10) and granulosa cell proliferation (27), as well as in luteinization (55) is expressed in granulosa cells as early as in the primordial follicle stage of follicle development. Based on this observation, we investigated the role of NR5A2 in early folliculogenesis in mouse ovaries at PND4, recognized as the time of termination of the formation of the primordial follicle pool (17), and PND13, when the follicular population is limited to primordial to secondary stage follicles, but does not include any antral follicles. Herein, we present evidence to indicate

that there are two populations of primordial follicles in the mouse ovary, one quiescent where NR5A2 is absent, and a second, with NR5A2 expressed that is poised to activate and join the growing pool.

In aid of confirming the follicle population in which NR5A2 is depleted, we showed by immunofluorescence and RNA *in situ* hybridization that AMHR2 is expressed PND4 and PND13. This concurs with earlier studies in primordial follicles (19). It has been shown that the *Amhr2*-Cre allele is expressed even earlier, by E11.5 in both male and female urogenital ridges (16). By E12.5, expression is localized to somatic cells of the gonads and mesenchyme cells of the Müllerian ducts, and at E17.5, *Amhr2*-Cre expression is present in almost all somatic cells of the ovary (18). As the *Amhr2* driven Cre recombinase has been employed in a knock-out mice model to deplete NOTCH (53), and we had previously shown effective recombination and depletion in the ovary (10), we chose this model to explore the effects of NR5A2 on the early follicle population ($Nr5a2^{f/f}$; $Amhr2^{Cre+}$). Herein we demonstrated substantial (>80%) depletion of *Nr5a2* at both PND4 when only primordial follicles are present; and at PND13 when primordial, primary and secondary follicles populate the ovary.

The consequence of this NR5A2 depletion was a significantly greater number of primordial follicles in both PND4 and PND13 in the ovaries of the cKO mice. At PND4 we also observed a significant decrease in the population of activated primary follicles, as indicated by the absence of expression of proliferation markers in the somatic cell component of the follicle. We hypothesized that the increased number of primordial follicles observed in our NR5A2 cKO mice ovaries could result from one, or some combination of three mechanisms of action: a decreased rate of recruitment from a constant

follicular pool, an increase in the initial population, and/or a decreased rate of apoptosis-related loss.

In support of the hypothesis that depletion of NR5A2 affects the early follicle population by reducing recruitment, we showed PND4 ovaries in organ culture displayed a larger population of primordial follicles and a reduced number of primary and secondary follicles. To address potential mechanisms, we examined the expression of members of the PI3K pathway, PTEN and FOXO3, factors known to regulate follicle recruitment rate (reviewed in (1)). We found that both are substantially upregulated in the cKO follicle population at PND4. These findings are consistent with the role of PTEN and its downstream target, FOXO3 as the principal inhibitors of primordial follicle activation. Indeed, deletion of PTEN from oocytes of primordial follicles resulted in premature activation of the entire primordial follicle pool (35). Similarly, studies have shown that *Foxo3*$^{-}$ovaries were enlarged by PND14 with greater numbers of early-growing follicles, and the primordial follicle reserve was totally depleted by that time (6, 25). Thus, one consequence of depletion of NR5A2 in primordial follicles is abnormal maintenance of the constraint on follicle activation normally exercised by the depletion of PTEN and its downstream target FOXO3.

In ovarian somatic cells, the transcription factor, FOXL2, plays an important role in controlling the activation, and formation, of primordial follicles. It acts on differentiation of pre-granulosa into granulosa cells during follicular activation (40, 48). In the present study, we show substantially reduced FOXL2 transcript abundance in the NR5A2 cKO mouse. This finding is consistent with the germline depleting mutation of the *Foxl2* gene, with the consequence of abrogation of the squamous to cuboidal transition of the primordial follicle granulosa cells following follicular activation (40). Our unpublished ChIPseq data

in adult mouse granulosa cells demonstrate peaks of NR5A2 binding associated with the *Foxl2* gene, suggesting that FOXL2 may be downstream of NR5A2 in follicular activation.

Additional support for a reduced rate of activation of primordial follicles comes from evaluation of quiescence markers. For example, the abundance of the transcript for *Cdkn1a*, the cyclin dependent kinase inhibitor, is increased in PND4 cKO mice in the present study. This protein is known to induce and maintain cell cycle arrest under conditions such a senescence or quiescence (14, 34). Further, it is a direct target of NR5A2 (27). The chemokine ligands CXCL1, CXCL10 as well as EGR1 and LIN28A are also associated with mitotic quiescence (41, 54). All three were significantly upregulated at the mRNA level in NR5A2 cKO ovaries at PND4. Together the gene expression patterns support the hypothesis that NR5A2 depletion from primordial follicles diminishes the rate of recruitment of primordial follicles into the growing follicle pool.

Our second hypothesis was that depletion of NR5A2 from the follicle population increases the complement of primordial follicles in the ovarian reserve. To explore this supposition, we evaluated *Amh, Notch2* and *Figla* mRNA levels. AMH is well known for its role in the preservation of the ovarian reserve (19), and has recently been implicated it in the formation of primordial follicles, inhibiting primordial follicle assembly, thereby affecting the initial primordial follicle pool size in a rat ovarian organ culture (29). In the present study, we report significant downregulation of *Amh* in the cKO mouse ovary at PND4, suggesting its inhibitory effect is diminished. We have also shown that depletion of NR5A2 in primordial follicles results in elevated expression of the transmembrane protein, NOTCH2. Signaling via NOTCH2 has been shown to be essential for regulating breakdown of germ-cell nests and, thus, in formation of primordial follicles (7). Female mice with conditional deletion of the *Notch2* gene in somatic granulosa cells of the ovary

exhibited reduced fertility, accompanied by the formation of multi-oocyte follicles that resulted from defects in dissolution of the primordial germ-cell nests (7). The third element, the germ cell-specific factor, FIGLA, is expressed in the mouse ovary concurrent with the formation of the primordial follicles (15) and is essential for this formation to occur (43). We found that transcripts for *Figla* were increased by twofold at PND4 in ovaries from cKO mice. Together these results suggest that the NR5A2 cKO ovary is invested with a greater complement of primordial follicles is the WT counterpart, accounting, in part, for the increased ovarian reserve at PND4 and PND13.

Germ cell cyst breakdown and invasion by pre-granulosa cells to form the primordial follicles is accompanied by a significant loss of oocytes. As many as two thirds of the oocytes are lost through apoptosis (46). Genetic modification of anti-apoptotic genes in mice, such as MCL-1, showed that its depletion engenders premature exhaustion of the ovarian reserve, characterized by early primordial follicle loss (30) and increased apoptosis of primordial germ cells in embryonic oocytes (37). Moreover, oocyte numbers in the NOTCH2 conditional mutants were increased as a result of decreased oocyte apoptosis (53), showing that programmed cell death influences the ovarian reserve. To evaluate the effects of the depletion of NR5A2 in apoptosis, we quantified the relative mRNA levels of *Mcl1* and found them to be upregulated in the cKO ovaries at PND4. We also quantified cleaved caspase 3 protein, a crucial mediator of programmed cell death, by immunofluorescence in PND4 CON and cKO ovaries. The significant decrease in the level of expression of cleaved caspase 3 proteins in the cKO ovary confirmed that the increased number of primordial follicles in ovaries that characterized depletion of NR5A2 was attributable, at least in part, to reduced apoptosis in primordial follicles.

A key finding in the present study is that there is variation in the primordial follicle population with respect to the expression of *Nr5a2,* shown by *in situ* hybridization. By means of immunocytochemistry, we demonstrated two populations of primordial follicles, those in which NR5A2 was expressed in the pre-granulosa cells and those where it was not present. Neither of these populations expressed the mitotic marker KI-67, confirming their quiescence. The concept of more than one subset of primodial follicles is not new, previous studies of the developmental chronology of follicle development identified two distinct populations of primordial follicles (58, 59). A first wave of simultaneously activated follicles after birth is located in the medulla of the ovaries. They exist in the ovaries for around three months and contribute to the onset of puberty. The second wave of primordial follicles originate from the ovarian cortex and gradually replace the first wave of follicles to provide fertility until the end of reproductive life. In the present study, the ovaries of CON mice collected at PND4 and cultured for ten days showed extensive activation of the medullary follicle population, and that was was nearly absent in the cKO mice. Thus, the differential expression of NR5A2 in primordial follicles may be related in part to their location in the postnatal ovary. Nonetheless, *in situ* and immunocytochemical analyses identified populations of primordial follicles in the cortex of more mature ovaries that either express or are devoid of NR5A2. Given the multiple roles of NR5A2, including induction of cell proliferation, we speculate that the follicles where NR5A2 is absent are quiescent, and those expressing NR5A2 are in line for activation.

In summary, we have shown that depletion of NR5A2 from primordial follicles results a larger ovarian reserve. Three different, but perhaps interdependent mechanisms have been invoked: formation of a larger population of primordial follicles pre- and perinatally, reduced loss of follicle by apoptosis; and a decreased rate of recruitment into

the growing follicle pool. We further show that NR5A2 is differentially expressed in populations of primordial follicles, suggesting that this orphan nuclear receptor is an elemental generator of primordial follicle activation.

5. Acknowledgements

The authors are grateful to Vickie Roussel, Fanny Morin and Olivia Eilers-Smith for technical support. This study was funded by OGP12596 from the Canadian Institutes of Health Research to BDM.

6. Supplementary data

Supplementary table 4- 1 Primers sequences for PCR analysis

		Forward	Reverse
Genotyping	*Lrh1*	GCTATAGGGAGTCAGGATACCATGG	GTTCGTACCACTTTCATCTCCTCACG
	Amhr2gCre	GAACCTGATGGACATGTTCAGG	AGTGCGTTCGAACGCTAGAGCCTGT
qPCR primers	*Rpl19*	CTGAAGGTCAAAGGGAATGTG	GGACAGAGTCTTGATGATCTC
	B2m	GACCGGCCTGTATGCTATCC	TTTCAATGTGAGGCGGGTGG
	Hprt	ATGATCAGTCAACGGGGGAC	GAGAGGTCCTTTTCACCAGCA
	Nr5a2	TCATGCTGCCCAAAGTGGAGA	TGGTTTTGGACAGTTCGCTT
	Amh	ACAGAACCTCTGCCCTACT	TGCTTGGTTGAAGGGTTAAGA
	Cdkn1a	CCAGCTAGGATGACAGTGAAG	GAGTCGGGATATTACGGTTGAG
	Foxl2	CATAGCCAAGTTCCCGTTCT	TGATGAAGCACTCGTTGAGG
	Foxo3	CTCAAACTGACGGAAGACCTAC	GGGAGTCTCAAAGGTGTCAAG
	Lin28a	GGACTCAACTCCTTAGCCTTTC	GCTATCAACCATCACGCTACA
	Ckit	TCATCGAGTGTGATGGGAAA	GGTGACTTGTTTCAGGCACA
	Cxcl1	GGATTCACCTCAAGAACATCCAG	ATCTTTTGGACAATTTTCTGAACC
	Cxcl10	GGGCCATAGGGAAGCTTGAA	AGAGGCTCTCTGCTGTCCAT
	Egr1	GGAGAGGCAGGAAAGACATAAA	GCTCTGAGATCTTCCATCTGAC
	Figla	GTTCTGGAAGAAGCGAAGGT	CTGGTAGGTTGGGTAGCATTT
	Notch2	TGGAGGTCTCAGTGGCTATAA	ATTCTGGCATGGGTTAGAAAGA
	Mcl1	TCGGACTTCAGAGCACTTTATG	GGAAGTCAGGCTCCTAGTAAAC

CHAPTER 5: General discussion

1. Introduction

Infertility affects 12-15% of the couples in reproductive age in developed countries and these numbers reach up to 25% of the population across the developing world, a total of 186 million people worldwide (46). Ovarian dysfunction accounts for approximately 40% of reproductive disorders (50), and is the major cause of female infertility. Modern times have brought about a global trend of women delivering their first birth at later ages, even though a decline in fertility begins around 25 years of age (46)). This decline is due to either reduced oocyte quality, luteal insufficiency or, most commonly, a depletion of the ovarian reserve (46). The premature reduction of the primordial follicle pool engenders a loss of normal function in the ovary and premature ovarian failure (or premature ovarian insufficiency) (22). This condition stems from various causes that include the low number of follicles at birth, and side effects of invasive treatments such as chemotherapy (49). Thus, maintenance and preservation of the ovarian reserve is key for female fertility preservation. Other infertility causes come from subsequent events that follow initial primordial follicle recruitment such as ovulation and luteinization (13, 41)) that depend on the successful development of follicles from primordial to preovulatory stage, with cross-talk signals coming from the oocytes and granulosa cells in a space and time-dependent manner. Extensive progress has been made in the field of understanding ovulation (11), but the processes involved in the rapid and terminal differentiation of somatic cells in the peri-ovulatory follicle and the events that induce extrusion of the oocyte remain unclear (37).

Two orphan nuclear receptors are indispensable regulators of follicle development, ovulation and luteinization: steroidogenic factor-1 (SF-1, NR5A1), and liver homolog receptor-1 (LRH-1, NR5A2) (29). This thesis focuses on NR5A2, whose expression is

restricted to granulosa cells of all follicles, from primordial to antral stage, and to corpora lutea. It regulates a wide array of genes involved in growth, differentiation and metabolism, as well as many processes essential to the function of ovarian somatic cells (29). Germline deletion of NR5A2 in mice disrupts early embryogenesis, and is lethal prior to gastrulation at embryonic day 7.5, before gonadal differentiation (11). This lethality required a different approach to gene disruption.

2. Conditional knock-out mouse model

Advancements in transgenic mouse models have been vital to identify critical genes and pathways for ovarian development. Over the past 30 years, the use of tissue and cell-specific genetic recombination, such as the Cre/loxp system, permitted time and space specific patterns of deletion. Cre recombinase expression has been associated with investigation of conserved developmental proteins, such as the transforming growth factor beta (TGFβ), wingless-type MMTV integration site (WNT), phosphatidylinosito-4,5-bisphosphate 3-kinase (PI3K), phosphatase and tensin homolog (PTEN), thymoma viral proto-oncogene (AKT), and Notch. These have been studied due to their function in key events: primordial germ cell (PGC) specification, follicular growth, meiosis and ovulation. Our laboratory has developed several models to study the role of NR5A2 in female reproductive function. Based on the pattern of expression of the orphan nuclear receptor and the target cells of the promoters driving Cre recombinase, we developed conditional knock-out animals by crossing *Nr5a2*$^{f/f}$ mice crossed with *Amhr2*$^{Cre+}$, *Cyp19*$^{Cre+}$ and *Pgr*$^{Cre+}$ animals. Amhr2Cre and Cyp19Cre expression are reported in granulosa cells as early as E11.5 (23, 51) and PgrCre is detected starting at PND30 in the corpora lutea (43). Previous

studies from our laboratory using these transgenic mice models showed that NR5A2 is essential for ovulation (12), cumulus oophorus expansion (2) and luteinization (53). As the present work focuses on the role of NR5A2 on follicular growth, we could have used Cyp19Cre and Amhr2Cre mice, but not PgrCre, as recombination in this model begins later, after the gonadotropin signal that provokes ovulation (53). We previously demonstrated that depletion of NR5A2 driven by Cyp19Cre suppresses ovulation as a consequence of the absence of efficient cumulus expansion (53). The potential for earlier effects appear not to have been explored to date, in spite of reports that aromatase is expressed in fetal ovaries. This expression is at extremely low levels, such that neither the deletion (20) nor the mutation (6) of the aromatase protein affects ovarian formation. Aromatase (Cyp19) is also expressed at PND5 and PND8 in the granulosa cell layer of growing follicles (30) (reviewed in (44)). Although we detected no disruption of follicle development in previous studies of this model, it would be of interest to assess the effects of NR5A2 depletion under Cyp19Cre recombinase on the development of primary and secondary follicles that can be found in the ovaries at PND5 and 8. Later stages may be less interesting, as it has been reported that, in 21 day-old rats, aromatase expression disappears from growing follicles and is then limited to healthy large antral follicles.

For the above reasons, we chose to employ *Nr5a2*$^{f/f}$; *Amhr2*$^{Cre+}$ mice (conditional knock-out mice, cKO) and their control littermates *Nr5a2*$^{f/f}$ (CON). Amhr2Cre has been used with loxp animals to study the impact of the depletion of several genes on the female reproductive tract. For example, granulosa cell-specific ablation tuberous sclerosis complex 1 (TSC1) in the female reproductive tract by means of Amhr2Cre results in sterility in the form of premature follicular depletion, impaired oocyte maturation, embryo retention due to oviductal occlusion and embryo implantation defects (45). Although there

were few reports of Amhr2 expression in primordial follicles, the present work demonstrates, both by *in situ* hybridization and immunocytochemistry, that this receptor is present in the primordial population. Thus, *Nr5a2*$^{f/f}$; *Amhr2*$^{Cre+}$ conditional knockout animals are characterized by the depletion of NR5A2 in granulosa cells from primordial follicles forward. Although follicular development progresses until the antral stage in this model, no ovulation occurs due, at least in part, to the absence of cumulus expansion, rendering these transgenic mice infertile (12).

3. Role of NR5A2 in granulosa cell proliferation

Based on previous *in vitro* studies, NR5A2 has been shown to play a role in cell proliferation in the intestine (18) and in breast cancer (3). As granulosa cell proliferation is essential for follicle activation and development, the first objective of this thesis was to study the role of NR5A2 on replication in this essential follicular somatic cell (Chapter 3). The implication of NR5A2 in the regulation of granulosa cell proliferation was first confirmed by global microarray analysis of gene expression performed with granulosa cells from large antral follicles of CON and cKO mice. Gene ontology analysis of the differentially expressed genes revealed by microarray analysis using KEGG and Panther databases showed that the most profoundly dysregulated pathways were cell cycle, cell division and mitotic cell cycle, all related to cell proliferation. Interestingly, among the other most dysregulated pathways, we found cancer related gene clusters, and one of the characteristics of cancer cells is that in contrast to normal cells, which only divide a finite number of times, cancer cells persist in proliferation. Microarray data were therefore used to establish a list of markers to be validated by qPCR and, in case of discrepancy, the

specificity of qPCR detection and quantification was always trusted over microarray global analysis. These global findings sparked our interest in assessing the number of proliferating granulosa cells via *in vivo* BrdU incorporation. We then demonstrated that there was a significant decrease in the number of granulosa cells reaching the S phase the ovaries of the cKO animals. This decrease was then shown to be the result of the loss of NR5A2 regulation of cell cycle related genes and downstream effectors such as cyclins D, E, and E2F activators, as well as interaction with the co-activator, ß-catenin, and by repression of the transcription of the cell cycle inhibitor, p21 (*Cdkn1a*). Moreover, fluorescence activated cell sorting (FACS) on granulosa cells from superstimulated immature mice demonstrated that the decrease in the number of cells in G1/S seen in the BrdU incorporation/immunofluorescence experiments was accompanied by the increase of G0/G1 quiescent cells and a decrease in the number of cells reaching G2/M transition. This dysregulation could not be totally reversed by estradiol injections despite the fact that estradiol is a potent mitogen in granulosa cells (39). The decrease in proliferating granulosa cells was 40% in the superstimulated cKO mice compared to control animals. Although this was reduced to 20% in estradiol injected cKO mice compared to their CON counterparts, and there remained significantly fewer proliferating cells in cKO animals. This apparent gain of function in the cKO mice induced by estrogen treatment could not be confirmed by PCNA detection, where the ratio was actually reversed, with fewer proliferating cells in the E2 injected cKO mice than in the superstimulated animals.

Our *in vitro* experiments relying on the ML180 antagonist to inactivate NR5A2 in WT mouse granulosa cells by translocating it from its active state in the nucleus to the cytoplasm, confirmed the negative effects of NR5A2 depletion on granulosa cell proliferation described *in vivo*. In was then attempted to elucidate the mechanisms involved

in the regulation of granulosa cell proliferation by NR5A2 based on published data on its interaction with ß-catenin to regulate cell proliferation in the intestinal crypt (4). To address this issue, an *in vitro* primary granulosa cell culture system was developed and cells were treated with ICRT3, an inhibitor of

ß-catenin activation. The consequent findings demonstrated that the decrease in granulosa cell proliferation observed *in vivo* in our cKO mice model was not due primarily to the interruption or the dysregulation of the crosstalk between NR5A2 and ß-catenin. Indeed, by inactivating ß-catenin, we showed that positive regulators of cell cycle in cKO granulosa cells were even more depleted.

Based on the role of FSHR in the proliferation and differentiation of granulosa cells, both being essential processes necessary for follicular development and preovulatory follicle formation, *Fshr* and its target *Igf1r* mRNA levels were also quantified. Both were significantly upregulated, which correlated with previous results obtained in the same mouse model showing upregulation of CYP19 and the estrogen receptors downstream of FSHR through its action on IGF1R (1). As granulosa cell proliferation was drastically downregulated in our cKO mice model despite the significant increase of FSHR and its downstream targets we speculate that this is a compensatory mechanism set in action by the effects of the depletion of NR5A2 on other pathways regulating granulosa cell proliferation.

Given that neither ß-catenin nor FSH appear to be dominant regulator of NR5A2 effects on proliferation. we examined other factors. We came to the conclusion that the principal mode of action of NR5A2 on granulosa cell proliferation is via its effects on p21 (aka CDKN1A), a factor previously shown to be a direct target of NR5A2 (3).

Our previous studies (2, 12, 53) have aptly demonstrated the impact of the depletion of NR5A2 on the differentiation process in granulosa cells. As noted above, cumulus expansion is absent or modified, ovulation does not occur if depletion precedes the LH surge, and luteinization is impaired if the depletion follows the LH signal. These findings demonstrate the NR5A2 is unequivocally essential for programming terminal differentiation of granulosa cells (38). No dysregulation of LHR was observed, leading to the hypothesis that, even if NR5A2 plays a role in the differentiation process,it does not act through LH receptor but via other mechanisms (53).

The study presented in Chapter 3 is the first to demonstrate a role for NR5A2 in granulosa cell proliferation *in vivo* and it adds another dimension to the remarkably vast array of effects of NR5A2 on the granulosa cell and, consequently, on the ovarian follicle. One of the interesting aspects of the study was that mitotic arrest was not complete in the *Nr5a2* floxed mouse. This may be due to the fact that the mutation strategy with the Cre/lox system depletes (>90%), rather than deletes the expression of the target gene, *Nr5a2*. Thus, there may be sufficient expression of NR5A2 to support continued proliferation. The alternative hypothesis, perhaps more valid, is that there are multiple, independent mechanisms controlling granulosa cell proliferation during the development from the early antral to preovulatory follicle. This course of events engenders extensive estrogen synthesis (9), and estrogen is mitogenic in granulosa cells (39). Among other potential factors that could act in concert with NR5A2 to maximize granulosa cell proliferation include members of the TGFβ family, in particular TGFβ itself and the bone morphogenic proteins (BMP) (19).

4. Role of NR5A2 in regulation of ovarian reserve

One of the tenets of ovarian biology is that the granulosa cells of the primordial follicle population are mitotically quiescent. The effects of conditional knockout of NR5A2 on G0/G/1 cells in the antral follicle implied a role for NR5A2 in the regulation of cells in the dormant primordial state. Thus, we focused the second part of this thesis on the study of the impact of the depletion of NR5A2 on primordial follicles (Chapter 4). In this study, we provide the first known demonstration that NR5A2 is a major regulator of primordial follicle activation. Indeed, the depletion of NR5A2 results in a significant increase in the number of primordial follicles at postnatal day 4 (PND4), the time of termination of primordial follicle formation, and PND13, just prior to the detection of the first antral follicles, when follicular growth becomes gonadotrophin dependent. The significant increase in the quantity of transcripts of quiescent markers such as *Cxcl10*, *Cxcl1*, *Egr1,* as well as the dysregulation of primordial follicle formation and activation-related genes such *Figla*, *Foxl2*, confirmed that the depletion of NR5A2 has an impact on the ovarian reserve by maintaining the primordial follicle population.

In Chapter 3, we addresses the role of NR5A2 on follicular growth through granulosa cell proliferation in antral follicles. Transcript analysis by qPCR was performed using granulosa cells punctured from superstimulated immature mice ovaries, as antral follicles with attendant gonadotropin receptors are the only targets of the stimulating hormones. In studies at PND4 and PND13, we employed whole ovaries. The major rationale for this is, as we have previously shown, NR5A2 has but a single cellular site of expression in the mouse ovary, the nucleus of the granulosa cell, thus, at PND4 and PND 13, no non-follicular contaminating cells were present. In addition, there are practical

reasons for using whole ovaries, because of their size, needle based granulosa cell puncture technique previously used was excluded as it would not have allowed to collect a significant quantity of cells without pooling individuals. We could have pooled cells but, as the degree of Cre recombination varies between animals, specially at PND4, pooling would introduce further variation into the analysis. Alternatively, separation of cells and removal the oocytes either by FACS or by filtration, was considered but this approach was expected to result in degraded RNA quality. In summary, based on the compisition of the cKO mice model used, Amhr2Cre being expressed in granulosa cells of all follicles, and the fact that NR5A2 expression is restricted to granulosa cells, it was concluded that with RNA extracted from whole ovaries, any up or downregulated gene expression would come directly or indirectly from the effects of the granulosa cell-specific depletion of NR5A2.

Immunofluorescence was chosen over Western Blots because protein levels of NR5A2 in granulosa cells are not abundant, requiring pooling of samples to provide sufficient material for immunoblot analysis with attendant caveats. The semi-automated pipeline developed using CellProfiler Software, described in the methods of Chapters 3 and 4, permitted quantification on immunofluorescence images in individual ovaries, with independent quantification of the signal from follicles at different stages of development. It further allowed us to differentially quantify the signal in the ovarian cortex and the medulla and precise quantification of the number of positive cells. The potential weakness of this technique is that it required sampling of histological sections of the ovary, with the presumption that the sampling paradigm resulted in an adequate representation of the granulosa cell population.

A major finding of this part of the thesis was the existence of two populations of primordial follicles, highlighted by the localization of NR5A2 by *in situ* hybridization and

immunofluorescence. Based on previous publications reporting the existence of two waves of activation of primordial follicles (54), and the increase in the quantity of transcripts related to quiescence caused by the depletion of NR5A2, it was hypothesized that the primordial follicles that do not express NR5A2 are quiescent, and those that express NR5A2 may be poised for activation. The presence of the two primordial follicle populations was first identified by visual assessment, then confirmed by double immunofluorescence with NR5A2/KI67. The latter test addressed whether primordial follicles could activate in absence of NR5A2. No activated (i.e. KI67 positive) follicles were NR5A2 negative. We interpreted this finding to indicate that NR5A2 is required for primordial follicle activation. Further, two populations of primordial follicles, demonstrably quiescent as they did not express KI67, were evident. One was clearly positive for NR5A2 and, in the second, the signal could not be detected.

It has been shown that postnatal follicle development in the mouse begins with a wave focused in the medulla of the immature ovary beginning shortly after birth, followed by initiation of cortical activation for the remainder of the reproductive lifespan (7). In order to study the effects of the depletion of NR5A2 on the medullary wave of primordial follicle activation, an *in vitro* culture model of whole ovaries was established. This culture system, described in the literature for the PND4 rat ovary (10, 34) allows for incubation for up to 15 days. We maintained it for 10 days to compare the morphology of the cultured ovaries to PND13 ovaries with the histological findings in which we showed a significant increase in the population of primordial follicles in the cKO ovaries. The choice of the time point was directed by the fact that ovaries from younger animals survive better in culture than those from older animals, and because ovaries from prepubertal rats, and by extension mice, are small enough to allow the diffusion of nutrients and oxygen into intact tissue (10,

16). After DDX4 staining to facilitate oocyte identification, it was obvious that, in addition to an increased population of primordial follicles in the cKO ovary, congruent with *in vivo* results, that the population of developing follicles located in the medulla of CON ovaries was absent in cKO ovaries. This absence of developing follicles from the ovarian medulla is consistent with the conclusion that NR5A2 regulates follicle activation. The effect may be the result of the paucity of the *Foxl2* gene expression, downstream to NR5A2, as we demonstrated by qPCR. This is consistent with published studies implying that the first developing wave of primordial follicles are located in the medulla and contain granulosa cells arising from precursor cells that express FOXL2 in the fetal ovaries (32). In contrast, the cortical follicles with granulosa cells are derived from leucine rich repeat containing G-protein coupled receptor 5 (LGR5) are located in the cortex and remain quiescent until later in life, again, under the control of FOXL2 that is required for all primordial follicle activation (32).

Interestingly the *in vivo* study of the PND13 mouse ovary also showed a significantly increased population of activated primary, secondary and tertiary follicles in the cKO vs WT animals. This increase did not seem to persist until adulthood, as follicular counts did not show any significant difference between cKO and WT, apart from an increase in the number of apoptotic follicles. Definitive differences could not be confirmed by qPCR, perhaps attributable to the difficulty to distinguish atretic follicles from hematoxolin/eosin overstained specimens. Yet, the disappearance of the phenotype of increased number of primordial follicles between PND13 and adulthood is not surprising ,as it has been shown in other transgenic mice models where there is an increased primordial follicle pool. As an example, cKit promoter-induced overexpression of the Bcl2

gene in the ovary increases the size of the primordial follicle pool at birth but the phenotype does not persist into adulthood (21), nor are their effects on the adult follicle population.

In an attempt to better understand the mechanisms involved in NR5A2 regulation of primordial follicle activation, the abundance of the TGFβ family member AMH and its receptor, AMHR2, were evaluated. Indeed, AMH produced by granulosa cells of growing follicles in the ovary is the only known paracrine factor capable of inhibiting activation of primordial follicles (15). Unexpectedly, AMH was significantly downregulated in the transgenic animals. This was a counterintuitive result, given that Amh-/- mice are characterized by overactivation of primordial follicles, an increase in the number of growing follicles and completely depleted ovaries by13 months of age (14). The disparate findings may be reconciled by recognition of the decrease in the number of small developing preantral follicles cKO mice. These follicles are the source of AMH (47). Moreover, AMH depletion could also impact the primordial follicle population by increasing follicle assembly, as shown in a study employing rat ovarian organ culture (33).

AMHR2 is a specific receptor for AMH (48) and, because this same receptor is driving Cre recombinase expression in the cKO mice model use through this entire thesis it was of interest to assess its level of transcription. Our qPCR analysis showed strong downregulation trend in the cKO ovaries that did not seem to affect recombination, as *Nr5a2* depletion is still >80% at PND4. This could be due to *Amh* downregulation but also it may have been due to the means by which conditional depletion was achieved, via a knock-in disabling one of *Amhr2* alleles.

Extracting RNA from whole ovaries at PND4 also allowed to study the impact of the depletion of NR5A2 on oocyte specific factors such as FIGLA (42) and genes expressed in germ and somatic cells, including PTEN (17, 24). The latter protein is downstream of

the highly conserved PI3K/PTEN/AKT signaling pathway that is involved in primordial follicle development through its members Kit ligand (KL) (40). Activated PI3K/AKT represses FOXO3a and FOXO3a deficient mice and transgenic mice with *Pten* deficient oocytes have an excessive primordial follicle activation phenotype. These mutant mice have in common enlarged ovaries containing oocytes increased in size and elevated numbers of primordial follicles, followed by oocytes atresia and follicular depletion, triggering premature ovarian insufficiency (5, 36). In our *Nr5a2*$^{f/f}$; *Amhr2*$^{Cre+}$ mice PTEN and FOXO3 expression are both upregulated, with PTEN being specifically upregulated in the primordial follicles located in the cortex. As the cortex is the site where the second wave of primordial follicles is located (54) implying that NR5A2 activates primordial follicles that begin developing after birth (the medullary wave). The data suggest that NR5A2 could also regulate and preserve the quiescent state of the primordial follicles that develop later in life.

5. Future directions

While in the first part of this project addressing granulosa cell proliferation, there was confirmation and corroboration with similar mechanisms known to be present in *in vitro* studies focusing on other cell types (3, 25, 28, 52), the role of NR5A2 in primordial follicle activation raises many unanswered questions and unexplored possibilities. Our current understanding is that the increased number of primordial follicles in cKO compared to WT ovaries can be explained by a difference in the activation rate of primordial follicles. Markers involved in primordial follicle formation and oocyte survival are dysregulated, and these may well be the most important pathways by which NR5A2 affects the pool of

primordial follicles. While it is believed that the current study provides multiple clues to the mechanisms of NR5A2 action on the primordial population, further investigation is required to provide an integrated view of this regulation. An important future direction will be to exploit emerging technology explore the transcriptome of pre-granulosa cells of CON and cKO mice. This could be achieved by single cell RNAseq across a temporal sequence that includes PND4, a time point that has been reported to be close to the termination of primordial follicle formation. At this time, most, if not all, of the follicles present in the ovary are in the primordial or primary stage of development. This experiment would allow the identification of dysregulated genes and, using gene ontology, establish the pathways upon which NR5A2 depletion has an impact. Chromatin immunoprecipitation, followed by next generation sequencing (ChIPSeq) in the wild type ovary at PND4 could follow, to identify the population of significantly up or downregulated genes that are direct targets of NR5A2. This series of experiments would also provide the opportunity to highlight new markers specifically expressed in primordial follicles and test the hypothesis presented herein of the existence of two subsets in the early ovary, the quiescent and the poised primordial follicles. These newly discovered markers as well as the novel NR5A2 targets could then be quantified by immunofluorescence, *in situ* hybridization, qPCR and western blot to assess expression levels in CON and cKO animals.

Another possible experiment would be to expand the study that has been developed *in vitro* and described in this thesis (Chapter 4) with whole ovaries from PND4 wild type mice treated with NR5A2 recombinant protein. As mouse-specific recombinant protein is not available yet, a well-known NR5A2 agonist such as dilauroylphosphatidylcholine (DLPC) could be used (27). The goal would be to establish whether the quiescent population of primordial follicles observed in cKO mice can be activated and the phenotype

rescued following overexpression of NR5A2. A further endpoint would be to determine whether the global rate of activation of primordial follicles can be increased in CON mice. The same overexpression experiment could be conducted *in vivo* by injecting DLPC in the presence of a mitotic marker such as BrdU to determine whether the ovarian reserve can be prematurely activated, or even depleted leading to premature ovarian failure.

A new mouse line characterized by an increase in the transcription of NR5A2, as has been developed, with other factors in female mouse mammary epithelium is a useful tool for further study (26). This approach employs the lox/stop/lox (LSL) cassette. In a mouse bearing an inducible Cre (tamoxifen or tetracycline driven), NR5A2 could be overexpressed at will. The alternate experiment, the depletion of NR5A2 *in vitro* with adenovirus Cre infection or using ML180, an antagonist for NR5A2, could be employed in *Nr5a2* $^{f/f}$ or wild type ovaries from the same time point as the overexpression of NR5A2, to confirm what we report *in vivo*.

Results of the present study further suggest that there is a larger initial population of primordial follicles in the cKO mouse ovary at birth. A different angle of development for this project would be to explore this phenomenon in terms of the process of germ cell nest breakdown and primordial follicle formation. The initial step would be to evaluate the level of expression as well as the localization of NR5A2 by *in situ* hybridization and immunofluorescence in germ cell nests of cKO and CON animals in the ovaries at different time points from prenatal day post-coitum 17 (DPC17), before the cyst breakdown starts. The follow up would take place right after birth, when the process of formation of primordial follicles has been reported to be around 50% complete (35). The same sequencing experiments as those designed for PND4 ovaries could be undertaken to provide new information regarding gene expression associated with the formation of

primordial follicles. In addition, the role of NR5A2 in the process of cyst breakdown could be addressed from the point of view of the aberrations observed in the cKO mice transcriptome. These results would provide the first insight into the temporal pattern of the gene expression associated with the formation of the follicles and the misregulation resulting from NR5A2 depletion could give us new information regarding the role of NR5A2 in this process.

Studying time points as early as the germ cell cyst breakdown could also provide the opportunity to confirm the involvement of NR5A2 in oocyte survival, as it is well known that an important wave of oocyte death occurs around birth, during the process of formation of primordial follicles. This follows from the present finding that some apoptotic and pro-survival markers such as cleaved caspase 3 or MCL1 were found to be respectively down and upregulated in our cKO mice model.

Based on the results obtained on the function of NR5A2 in primordial follicle activation, an exciting perspective would be to explore its capacity to preserve the ovarian reserve following chemotherapy. Indeed, young female cancer survivors frequently suffer from primary ovarian insufficiency. Chemotherapy damages the ovary by direct toxicity on follicles and also by indirect depletion of primordial follicle population due to over-recruitment (8, 31). It would be of great interest to explore if inhibiting primordial follicle activation through NR5A2 depletion could serve as a treatment to preserve fertility during chemotherapy. This new study could be approached by inducing premature ovarian failure in *Nr5a2*$^{f/f}$; *Amhr2*$^{Cre+}$ mice by treating them with chemotherapeutic agents or use WT mice treated with these same drugs and NR5A2 antagonist such as ML180.

Finally, recent preliminary unpublished data from our lab showed that women infertile from unknown causes present a significant downregulation of NR5A2 in granulosa

cells when compared to those from control (male infertility factor) women. This strongly suggests that this transcription factor plays a role in human fertility. Thus, its function could be studied using *in vitro* techniques based on culture of primary granulosa cells or immortalized human granulosa cell lines with specific depletion of Nr5a2 engendered by siRNA or CRISPR-Cas9 or overexpression induced by pharmacological treatments such as DLPC.

In the long-term perspective, NR5A2, as a major player in ovarian function will be a potential target for fertility preservation, via its actions on the ovarian reserve, and novel contraception options could be based on the infertility phenotype engendered by its depletion. I sincerely hope that this thesis will contribute to the field to solve the mystery of female infertility.

CHAPTER 6: Conclusion

To conclude, this study leads to a better understanding of the mechanisms involved in the ovarian function via the role of the orphan nuclear receptor NR5A2 in pre- and peri-ovulatory events (Figure 7-1). In the first part of this thesis, focused on the function of NR5A2 in granulosa cells proliferation, it has been shown that the depletion of this transcription factor decreases the number of dividing cells mainly through its interaction with β-catenin and the absence of negative regulation by CDKN1A. This work then addressed earlier stages of follicular development to determine the role of NR5A2 in the establishment and activation of the pool of primordial follicles. Here, it was demonstrated that NR5A2 is a key regulator of primordial follicle formation, survival and activation as primordial follicles persist in this cKO mouse model. Moreover, two subsets of primordial follicles, quiescent and poised, can be distinguished by immunolocalization of NR5A2 (Figure 7-2).

While this study successfully answers our two objectives, new questions arise from these newly found NR5A2 functions: Does NR5A2 play a role prior to primordial follicles formation, for instance in germ cell cyst breakdown? Would a transgenic mouse model with ovarian overexpression of NR5A2 develop ovarian cancer due to over-proliferation of granulosa cells? Could the decrease in fertility observed in older wild type female mice be delayed by the administration of NR5A2 antagonists? Can mouse data be linked to human ovarian dysfunction and apply these newly discovered functions of NR5A2 on primordial follicle activation to prevent premature ovarian failure?
These unanswered questions will certainly lead the scientific community to a variety of future lines of research to further elucidate the broad functions of NR5A2 in ovarian function and female reproduction.

Figure 6- 1 Effects of NR5A2 depletion on the ovarian function

Time and cell specific conditional knockout of NR5A2 based on Cre recombinase driven by *Amhr2*, *Cyp19*, or *Pgr* promoters engenders infertility due to the absence of cumulus expansion, ovulation and/or luteinization. In this thesis we showed that NR5A2 regulates granulosa cell proliferation and primordial follicle fate.

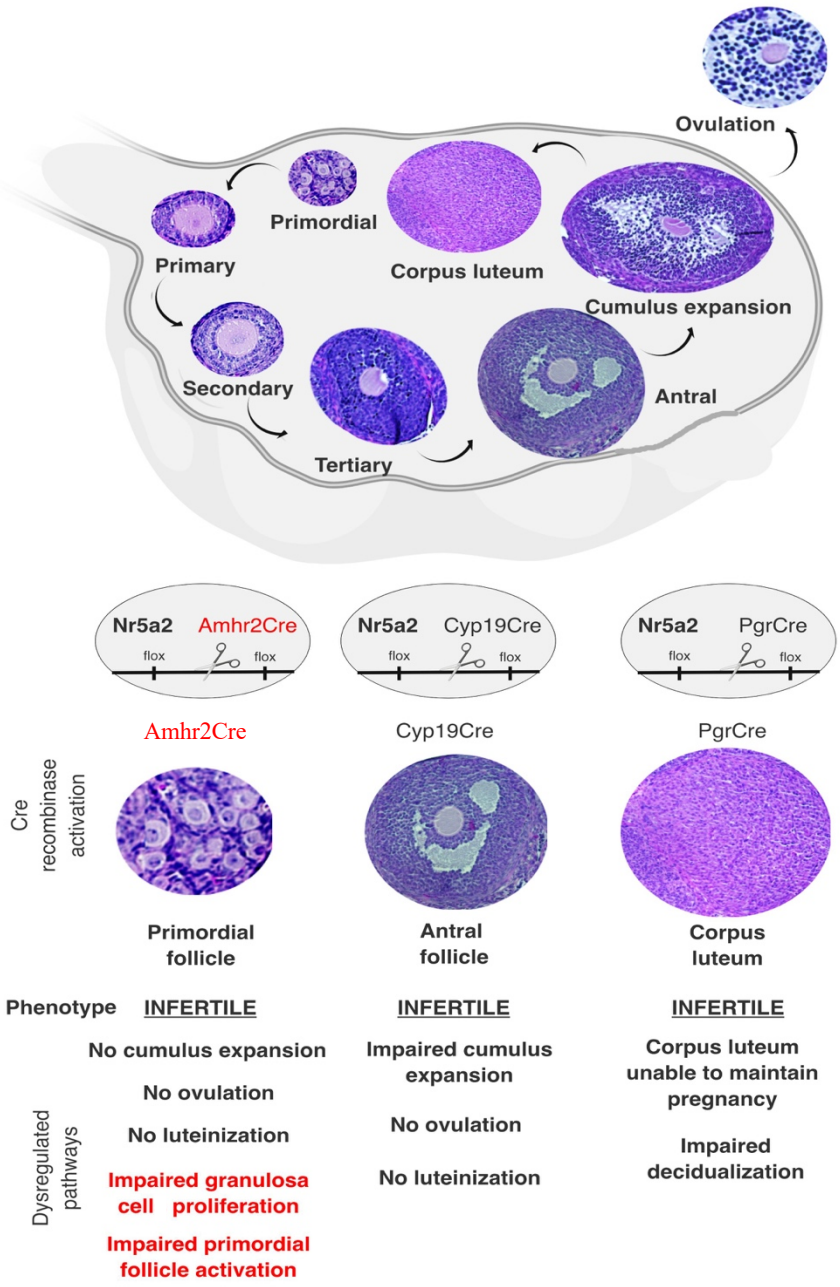


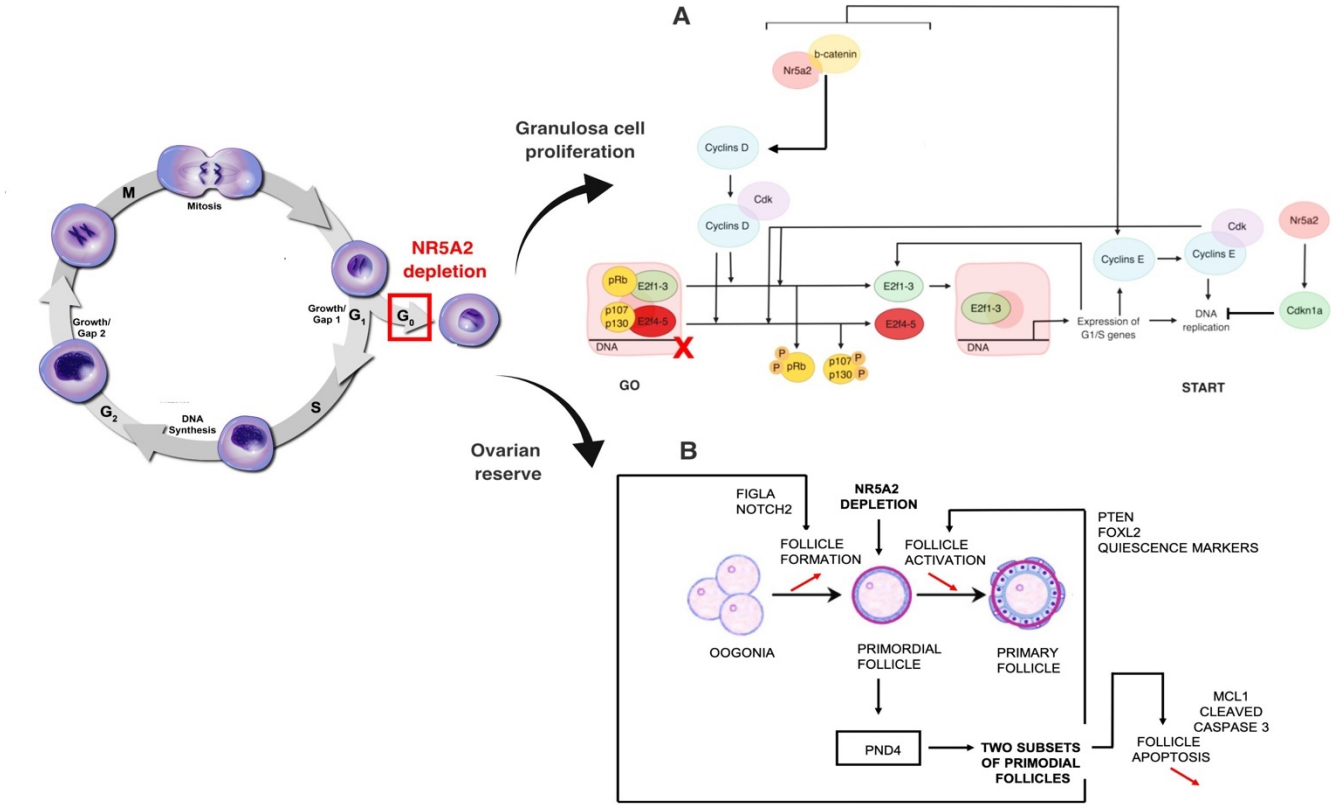
Figure 6- 2 NR5A2 regulates cell quiescence

Summary of the main findings of this thesis regarding the regulation of granulosa cells proliferation and primordial follicle activation by NR5A2.

A - In quiescent cells (G0/G1), the promoters of G1/S genes are repressed by the E2f repressor complexes and the members of the pRB family, P107 and P130. Weak concentrations of E2f activators are present, but inactivated by pRB. The NR5A2/β-CATENIN complex acts as a mitogen leading to Cyclin D/Cdk dependent phosphorylation of pRb and their dissociation from E2f activators. This increase in activity of E2f activators leads to the transcription of G1/S genes and DNA replication. NR5A2 also directly targets the cell cycle inhibitor, CDKN1A, to downregulate its activity and promote granulosa cell proliferation.

B – NR5A2 regulates primordial follicle activation by decreasing the expression of quiescence markers and PTEN and promoting transcription of factors essential for transition from the primordial to the primary follicle stage, such as FOXL2. NR5A2 depletion increases the formation of primordial follicles via NOTCH2 and FIGLA and decreases apoptosis by acting on pro-apoptotic and prosurvival factors including CASPASE 3 and MCL1.

Adapted from : MyCancerGenome.org and Araújo et al, Reprod Biol Endocrinol 2014



CHAPTER 7 : Bibliography

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CHAPTER 1

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CHAPTER 3

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CHAPTER 5

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