Université de Montréal

Role of the orphan nuclear receptor steroidogenic factor 1 in mouse reproductive function

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Thèse présentée à la Faculté de médecine vétérinaire

en vue de l'obtention du grade de *Philosophiae Doctor* (Ph. D)

en sciences vétérinaires, option reproduction

Avril 2021

Université de Montréal

Département de biomédecine, Faculté de médecine vétérinaire

Cette thèse intitulée

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Résumé

Le récepteur nucléaire orphelin facteur stéroïdogénique 1 (SF-1 ou NR5A1) est un modulateur indispensable du développement surrénal et gonadique et qui joue un rôle dans la détermination du sexe, le développent hypothalamique, la fonction hypophysaire et la stéroïdogénèse. Toutefois, les études sur SF-1 dans le milieu de la biologie de la reproduction portent majoritairement sur des modèles embryonnaires ou de mammifères immatures. L'objectif principal de cette thèse était de déterminer le rôle de SF-1 dans les évènements clés de la fonction reproductrice chez les mâles et femelles matures. Ce facteur de transcription est exprimé dans différents organes, principalement ceux de l'axe hypothalamo-hypophyso-gonadique, et dans divers types cellulaires des gonades. Nous avons donc généré 4 modèles de souris knockout conditionnels (cKO) en utilisant les allèles Cre-recombinase et flox de SF-1 ($SF\text{-}1^{f/f}$) afin d'identifier son rôle dans les différentes populations cellulaires des testicules et ovaires de souris matures.

Dans la première étude, nous avons présenté une analyse des souris femelles du modèle cKO du récepteur de la progestérone ($PR^{Cre/+}$;$Nr5a1^{f/f}$), où la suppression de SF-1 est spécifique aux cellules gonadotropes de l'hypophyse et aux cellules ovariennes de type granulosa suite au déclenchement du signal ovulatoire ainsi que les cellules lutéales du corps jaune. Cette étude a révélé de nouveaux rôles *in vivo* de SF-1 durant l'ovulation et la lutéinisation, tout en suggérant que SF-1 est un médiateur de la synthèse et sécrétion des gonadotrophines. Les femelles $PR^{Cre/+}$;$Nr5a1^{f/f}$ cKO étaient infertiles principalement en raison de l'importante réduction de FSH et LH sécrété dans la circulation, causé par le phénotype hypophysaire. Afin de contourner cette dysfonction hypophysaire, des traitements de gonadotrophines exogènes ainsi que des

transplantations d'ovaires nous ont permis de démontrer que SF-1 régule la transcription de gènes impliqués dans l'expansion du cumulus ainsi que la rupture de follicules pour induire l'ovulation. De plus, nous avons montré que l'absence de SF-1 dans les ovaires de souris matures peut mener à l'infertilité, indépendamment du phénotype hypophysaire. D'autre part, nos trouvailles indiquent que, malgré la basse expression de SF-1 dans le corpus luteum chez la souris, sa déplétion dans les cellules lutéales conditionnée par recombinase Cre inhibe la production de progestérone. Aucun phénotype reproductif a été observé chez les souris $PR^{Cre/+}$;$Nr5a1^{f/f}$ cKO mâles.

La deuxième étude a démontré le rôle essentiel de SF-1 dans la fonction du testicule mature. La souris mâle P450 17α-hydroxylase ($Cyp17^{Cre/+}$;$Nr5a1^{f/f}$) cKO, où la suppression de SF-1 est spécifique aux cellules de Leydig, était fertile malgré la taille réduite de ses testicules, la malformation de ses tubes séminifères, la perturbation de la spermiogénèse, ainsi que la réduction d'expression de gènes de la stéroïdogénèse. Bien que les mâles aromatase ($Cyp19^{Cre/+}$;$Nr5a1^{f/f}$) cKO, supprimant SF-1 dans les cellules de Sertoli, étaient fertiles et démontraient des capacités reproductives similaires aux mâles contrôle, les souris $Cyp17^{Cre/+}$+$Cyp19^{Cre/+}$; $Nr5a1^{f/f}$ cKO (dKO) étaient soit infertiles ou montrait une fertilité affaiblie. La dysgénésie sévère du cordon testiculaire ainsi que la spermatogénèse perturbée chez la souris dKO étaient causées par la déplétion simultanée de SF-1 chez les cellules de Leydig et Sertoli, suggérant que les cellules de Sertoli peuvent compenser pour l'absence de SF-1 dans les cellules de Leydig et vice versa. Ces données démontrent que SF-1 est requis pour une stéroïdogénèse testiculaire et une spermatogénèse ainsi qu'une fertilité normale, bien que savoir si la régulation de ces fonctions par SF-1 est directe ou indirecte reste à élucider. De façon intéressante, les femelles des trois lignées cKO étudié dans ce deuxième article étaient fertiles et la sous expression de SF-1 dans les cellules ovariennes de type granulosa ou de la thèque a produit des effets mineurs sur leur fonction reproductive.

En somme, la recherche présentée dans cette thèse contribue à l'avancement des connaissances sur SF-1 et son rôle dans la régulation d'événements reproductifs cruciaux dans l'hypophyse, l'ovaire et le testicule de souris matures. Les lignes de souris produites dans ce projet vont servir d'outil indispensable pour élucider les mécanismes de régulation de SF-1 sur la fonction gonadique and présenter de nouvelles avenues de recherches pour ce récepteur orphelin.

Mots clés : SF-1, Axe HPG, ovulation, lutéinisation, stéroïdogénèse, spermatogenèse, fertilité

Abstract

The orphan nuclear receptor steroidogenic factor-1 (SF-1 or NR5A1) is an indispensable modulator of adrenal and gonadal development, playing key roles in sex determination, hypothalamic development, pituitary function and steroidogenesis. Yet, studies to date of SF-1 in reproductive biology mostly focus on embryonic and immature mammalian models. The overall objective of this thesis was to determine the role of SF-1 in key events of mature male and female mouse reproductive function. This transcription factor is expressed in a variety of organs, mainly those of the hypothalamic-pituitary-gonadal axis, as well as in multiple cell types of the gonads. Therefore, we generated four conditional KO (cKO) mouse models employing Cre-recombinase and floxed alleles of SF-1 (SF-1$^{f/f}$) to identify its role in different cell types of the testes and ovaries of mature mice.

Our first study presents an analysis of female mice from the progesterone receptor (PR$^{Cre/+}$;*Nr5a1*$^{f/f}$) cKO model, where SF-1 depletion is specific to gonadotropes in the pituitary gland as well as granulosa cells of the peri-ovulatory follicle and luteal cells of the corpus luteum. This research highlighted new *in vivo* roles for SF-1 in ovulation and luteinization, and provided further evidence that SF-1 is a mediator of gonadotropin synthesis and secretion. PR$^{Cre/+}$;*Nr5a1*$^{f/f}$ cKO females were infertile, due in large part to the reduced secretion of FSH and LH, caused by the pituitary phenotype. Exogenous gonadotropin treatments and ovarian transplantation experiments allowed us to circumvent the pituitary dysfunction to demonstrate that SF-1 in granulosa cells regulates the transcription of cumulus expansion and follicle rupture genes to induce ovulation. In addition, we showed that the absence of SF-1 in ovaries of mature mice can

lead to female infertility, independent of the pituitary phenotype. Moreover, the data showed that, though SF-1 expression is reduced in mouse corpus luteum, its Cre-mediated depletion in luteal cells abrogates progesterone production. No reproductive phenotype was observed in $PR^{Cre/+}$;$Nr5a1^{f/f}$ cKO males.

Results from our second study demonstrated that SF-1 plays an essential role in mature testicular function. The P450 17α-hydroxylase ($Cyp17^{Cre/+}$;$Nr5a1^{f/f}$) cKO male mouse, where the SF-1 depletion is specific to Leydig cells, were fertile, though showed reduced testis size with disrupted seminiferous tubules and impaired spermiogenesis, in addition to reduced expression of steroidogenic genes. While the aromatase ($Cyp19^{Cre/+}$;$Nr5a1^{f/f}$) cKO males were fertile and showed reproductive capacities comparable to control males, the $Cyp17^{Cre/+}$+$Cyp19^{Cre/+}$; $Nr5a1^{f/f}$ cKO (dKO) model were either infertile or showed significantly impaired fertility. The dKO males displayed severe testis cord dysgenesis and impaired spermatogenesis caused by the depletion of SF-1 in both Leydig and Sertoli cells, suggesting that Sertoli cells can compensate for the absence of SF-1 in Leydig cells and vice versa. These data provide strong evidence that SF-1 is required for normal testicular steroidogenesis, spermatogenesis and male fertility, though whether the regulation of these functions is direct or indirect remains to be elucidated. Interestingly, the females of the three cKO mouse lines studied in this second article were fertile and the depletion of SF-1 in granulosa cells of antral follicles or in theca cells produced minor effects on their steroidogenic capacities.

Collectively, the research presented in this thesis contributes to advance our understanding of the role of SF-1 in the regulation of essential reproductive events in the pituitary, ovary and testis of mature mouse gonads. The mouse lines generated for this project will serve as valuable

tools to elucidate the mechanisms underlying SF-1 regulation of gonad function and present novel directions for the investigation of this nuclear receptor.

Keywords : SF-1, HPG axis, ovulation, luteinization, steroidogenesis, spermatogenesis, fertility.

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List of abbreviations

3βHSD	3 β Steroid Dehydrogenase
3βHSD1	Hydroxysteroid 3 β Dehydrogenase, Isomerase 1
3βHSD2	Hydroxysteroid 3 β Dehydrogenase, Isomerase 2
3Cl-AHPC	Adamantyl Hydroxyphenol Chlorocinnamic Acid
17βHSD1	Hydroxysteroid 17-β Dehydrogenase 1
Ab	Abnormal
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
ACAT1	Acetyl-coA Acetyltransferase 1
AF-2	Activation Function-2 Motif
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
AP-1	Activator Protein 1
AREG	Amphiregulin
ASPM	Abnormal Spindle Microtubule Assembly
ATP	Adenosine Triphosphate
AVPV	Anteroventral Periventricular Nucleus

AREG	Amphiregulin
BAD	BCL2 Associated Agonist of Cell Death
BAX	BCL2 Associated X, Apoptosis Regulator
BCL2	B-cell Lymphoma 2
BMP2	Bone Morphogenetic Protein 2
BSA	Bovine Serum Albumin
BTB	Blood Testis Barrier
BTC	Betacellulin
C/EBPβ	CCAAT/enhancer-binding protein β
C57BL/6	C57 Black 6
CAK	CDK-Activating Kinase
cAMP	Cyclic Adenosine Monophosphate
CBP	CREB Binding Protein
CBX2	Chromobox homolog 2
CD7	Cluster of Differentiation 7
CDK	Cyclin Dependent Kinase
CDK7	Cyclin Dependent Kinase 7
CDKN1A	Cyclin Dependent Kinase Inhibitor 1A
Cga	Chorionic Gonadotrophin Subunit Alpha
ChIC	Chromatin Immunocleavage
ChIP	Chromatin Immunoprecipitation
cKO	Conditional Knock-out
CL	Corpus Luteum
cMyc	MYC Proto-Oncogene, BHLH Transcription Factor
CNS	Central Nervous System
COC	Cumulus-Oophorus Complex

CON	Control
COUP-TF	Chicken-ovalbumin upstream-transcription factor
CREB	cAMP Response Element-Binding Protein
CREB1	cAMP Responsive Element Binding Protein 1
CRRF	Centre de Recherche en Reproduction et Fertilité
CX43	Connexin 43
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
DAPI	4',6-Diamidino-2-Phenylindole
DAX1	Dosage-sensitive sex reversal, Adrenal hypoplasia critical region, on chromosome X, gene 1
DBD	DNA Binding Domain
dKO	double cKO model
DLPC	1,2-Dilauroyl-SN-glycero-3-phosphocholine
DMRT1	Doublesex and Mab-3 Related Transcription Factor 1
DNA	Deoxyribonucleic Acid
DNMT3A	DNA Methyltransferase 3 α
DSD	Disorders of Sex Development
E	Embryonic Day
E2F1	E2F Transcription Factor 1
E2F2	E2F Transcription Factor 2
eCG	Equine Chorionic Gonadotropin

EGF	Epidermal Growth Factor
ER	Estrogen Receptors
EREG	Epiregulin
ERK	Extracellular signal-regulated Kinases
ES	Embryonic Stem Cells
ESR1	Estrogen Receptor α
FDX1	Ferredoxin 1
FOS	Fos Proto-Oncogene, AP-1 Transcription Factor Subunit
FOXD3	Forkhead Box D3
FOXA1	Forkhead Box A1
FOXL2	Forkhead Box L2
FSH	Follicle Stimulating Hormone
FSHβ	Follicle Stimulating Hormone β subunit
FTZ	Fushi Tarazu
FTZ-F1	Fushi Tarazu Factor 1
FXR	Farnesoid X receptor
GATA4	GATA Binding Protein 4
GCNF	Germ Cell Nuclear Factor
GJA1	Gap Junction Protein α 1
GnRH	Gonadotropin-Releasing Hormone
GnRHR	Gonadotropin-Releasing Hormone Receptor
GSE	Gonadotrope Specific Element
H12	Helix 12
H2	Helix 2
HAS2	Hyaluronan Synthase 2
HBEGF	Heparin Binding EGF-like Growth Factor

HCG	Human Chorionic Gonadotropin
HDAC	Histone Deacetylase
HDL	High Density Lipoprotein
HE	Hematoxylin and Eosin Staining
HLH	Helix Loop Helix
HMG-CoA	3-Hydroxy-3-Methyl-Glutaryl-Coenzyme A Reductase
HNF	Hepatocyte Nuclear Factor
HOXA10	Homeobox A10
HPG	Hypothalamus-Pituitary-Gonad
IgG	Immunoglobulin G
INHA	Inhibin Subunit α
INHBA	Inhibin Subunit β A
INHBB	Inhibin Subunit β B
INSL3	Insuline-like Polypeptide 3
IU	International Unit
KGN	Ovarian Granulosa-like Tumor Cell Line
KISS1	Kisspeptin 1
LBD	Ligand-Binding Domain
LDL	Low Density Lipoprotein
LDLR	Low Density Lipoprotein Receptor
LH	Luteinizing Hormone
LHβ	Luteinizing Hormone β subunit
LHR	Luteinizing Hormone Receptor
LIPE	Lipase E
LRH-1	Liver Receptor Homolog 1
MAPK	Mitogen-Activated Protein Kinase

MCL1	Induced Myeloid Leukemia Cell Differentiation Protein
MDM2	Mouse Double Minute 2 Homolog
mRNA	Messenger RNA
Mtor	Target of Rapamycin
N	Normal
NOTCH	Neurogenic Locus Notch Homolog
NOTCH2	Neurogenic Locus Notch Homolog Protein 2
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)
Ov^x	Ovariectomy
P21	Cyclin-dependent Kinase Inhibitor 1
P4	Progesterone
PAF	Paraformaldehyde
PAS	Periodic Acid Schiff
PBS	Phosphate-Buffered Saline
PBST	Phosphate-Buffered Saline Tween
PCNA	Proliferating Cell Nuclear Antigen
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

PR	Progesterone Receptor
PRKO	Progesterone Receptor Knockout Mouse Model
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PKA	Protein Kinase A
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
PROX1	Prospero-Related Homeobox Transcription Factor 1
PTEN	Phosphatase and TENsin Homolog
PTGS2	Prostaglandin-Endoperoxide Synthase 2
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
RXR	Retinoid X Receptor
S203	Serine 203
S238	Serine 238
S243	Serine 243
SCARB1	Scavenger Receptor Class B Member 1
SCP2	Sterol Carrier Protein 2
scRNA-seq	Single-cell RNA sequencing
SEM	Standard Error of the Mean
SF-1	Steroidogenic Factor 1
SHP	Small Heterodimer Partner
shRNA	Short Hairpin RNA

SIM	Single-minded gene
siRNA	Small Interfering RNA
SMAD3	Mothers Against Decapentaplegic Homolog 3
SMRT	Silencing Mediator for Retinoic Acid and Thyroid Hormone Receptor
SOAT	Sterol O Acyltransferase
SOAT1	Sterol O Acyltransferase 1
SOX2	SRY-Box 2
SOX8	SRY-Box 8
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
SUMO	Small Ubiquitin-like Modifier
TCF4	Transcription Factor 4
TET	Tet Methylcytosine Dioxygenase
TGFβ3	Transforming Growth Factor β 3
TNFAIP6	Tumor Necrosis Factor Stimulated Gene-6
TP53	Tumor Protein p53

USF1	Upstream Transcription Factor 1
USF2	Upstream Transcription Factor 2
VEGFA	Vascular Endothelial Growth Factor A
VMH	Ventromedial Nucleus of the Hypothalamus
WNT4	Wnt Family Member 4
WNT5	Wnt Family Member 5
WT	Wild Type
WT1	Wilms Tumor 1
ZFP36	Zinc Finger Protein 36

To Berinjela

Acknowledgements

To the members of my jury, Dr. Christopher Price, Dr. Gustavo Zamberlam and Dr. Stephanie Pangas, I thank you for taking the time to read and evaluate this thesis. To Dr. Sheila Laverty and Dr. Gustavo Zamberlam, the members of my committee, thank you for helping me see the bigger picture and providing words of encouragement.

A special thank you to Dr. Daniel Bernard for helping me decipher the pituitary phenotype in my model, and the members of his laboratory, in particular, Xiang Zhou, Dr. Luisina Ongaro and Dr. Gauthier Schang for their assistance in the hormonal dosage.

Merci à Dr. Alexandre Boyer et Dr. Adrien Levasseur de m'avoir donné plusieurs cours accélérés sur la fonction testiculaire des souris et de m'avoir aidé à interpréter mes données.

Au Dr. Nicolas Gévry et à la Dre. Stéphanie Bianco, merci pour votre contribution scientifique et votre chaleureux accueil lors de mes visites à votre laboratoire. En espérant continuer à collaborer avec vous dans de futurs projets.

Un grand merci à Geneviève Provost et Christine Blondin pour leur professionalisme et leur bonne humeur, et à Jacinthe Therrien, toujours prête à aider avec sa jovialité légendaire. Merci également au personnel de FANI pour le support technique et la bienveillance qu'ils ont portée aux soins des multiples souris utilisées aux fins de ma recherche.

To the members of the Murphy laboratory, past and present, thank you for making it a pleasure to come to the lab and for the stimulating scientific, existential and everyday life conversations. Un merci particulier à Fanny et à Vickie, toujours à l'écoute dans mes moments de

doute, pour votre aide dans les nombreuses expériences que j'ai entreprises durant ces quatre années dans votre labo. Vos sages conseils m'ont grandement aidé à traverser cette longue étape.

To Bruce, these years as your trainee have left me well equipped to face any scientific and bureaucratic challenge that lies ahead. Thank you for always keeping your office door open for questions and comedic relief. Et glorificatus sum ut sint vobis in novissimis doctorate discipulus.

Lauriane et Marie-Charlotte, mes complices du doc, merci pour votre soutien et réconfort moral, les longues conversations et les échanges d'idées lorsque les expériences n'aboutissaient pas, et les sorties à la crèmerie pour assouvir nos envies de sucre. J'adore notre trio en voyage, et j'espère qu'on pourra revivre l'expérience aux prochaines rencontres scientifiques!

To my CRRF buddies, Anthony, Elena, Emmanuelle, Esdras, Philip et Nour, I am so grateful for the friendship and positivity that came from our group gatherings. Los quiero mucho. I hope we get to dance together around a kitchen table again soon.

Para minha querida mamãe, que sempre soube me apoiar com o seu amor infinito e sua sabedoria, você foi uma parte integral do meu bem-estar durante estes últimos anos. Obrigada pelo carinho. Pops, you taught me to always question the world around me and to approach any problem in life with a level head and kind heart. Obrigada por ter visto em mim a capacidade de assumir este desafio. Jubis, my sunshine sister, thank you for your continuing encouragements every step of the way. Suave na nave. Michael John, hermano au grand coeur, obrigada por ser um exemplo de ambição e motivação. To Mik, my editor, motivator, problem solver, entertainer, personal chef, musical curator, thank you. We live, we learn, we forget.

And to all my friends who believed me every time I said I was almost done, thank you for playing along.

Introduction

The canonical understanding of mammalian reproductive function relies on the timely regulation and effective secretion of hormones that affect the organs of the hypothalamus-pituitary-gonad (HPG) axis. Gonadotropin-releasing hormone (GnRH) is secreted by the GnRH-secreting neurons of the hypothalamus into the portal blood circulation to stimulate Luteinizing Hormone (LH) and Follicle-Stimulating Hormone (FSH) synthesis in the gonadotrope cells of the anterior region of the pituitary gland. The secretion of these gonadotropins triggers cascades of reactions in the gonads of both sexes.

In the ovary, FSH stimulates follicle growth via granulosa cell proliferation and estradiol synthesis, a hormone that has a positive feedback effect on the pituitary gland to induce a surge of LH secretion. LH mediates oocyte maturation, cumulus expansion, ovulation and luteinization, the differentiation of theca cells and mural granulosa cells into luteal cells that synthesize progesterone. Progesterone secretion has a negative feedback effect on the hypothalamus and pituitary gland to reduce gonadotropin secretion. In the testis, LH induces steroid production in the Leydig cells and testosterone secretion, which in turn will have a negative feedback effect on the hypothalamus and pituitary. FSH in the male activates spermatogenesis by inducing the transcription of androgen-binding protein in Sertoli cells and ensuring high testosterone concentration within the testis. FSH is also involved in the formation of the blood-testis barrier in the seminiferous tubules.

Hormone production and action in the HPG axis is regulated by specific receptor binding and intracellular signaling cascades leading to the transcription of target genes that mediate reproductive functions. Nuclear receptors play essential roles as transcription factors in these

endocrine signaling pathways. Although they possess the classical nuclear receptor domain structure, Steroidogenic factor 1 (SF-1, Nr5a1) and Liver-receptor homologue 1 (LRH-1, Nr5a2) show constitutive activity and a ligand independent active conformation, which classifies them as orphan nuclear receptors. They act as transcription factors modulating cholesterol homeostasis and steroidogenesis. Recent studies have shown the essential role of LRH-1 in post-natal and adult mouse ovarian function, where it regulates primordial follicle formation, granulosa cell proliferation, ovulation and corpus luteum formation. On the other hand, many knockout mouse models have been generated to identify the role of SF-1 in gonadotropin secretion and steroidogenic organ development. These studies do not, however, provide a clear understanding of SF-1 regulation of mouse reproductive function after maturity. Indeed, much remains unknown on the role of this nuclear receptor in HPG axis organ function of the mature mouse, in particular sexually mature gonads.

This thesis is comprised of two studies that present original findings on the impact of SF-1 depletion in male and female reproductive function, through the use of conditional knockout mouse models targeting SF-1 in different gonadal cell types that allow a novel perspective. More specifically, these studies offer new insight on the role of SF-1 in gonadotropin secretion, ovulation, luteinization and spermatogenesis.

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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Running title: Orphan nuclear receptors in reproduction

Keywords: Orphan receptor, Steroidogenic factor-1, Liver receptor homolog-1, Cholesterol metabolism, Steroidogenesis, Testis, Ovary, Uterus,

Status: This manuscript was published in Physiological Review (Volume 99, Issue 2, pages 1249-1279) on Feb. 27 2019.

Author contributions: OS and MCM contributed equally to the review of the literature and writing of the review; OS on SF-1 principally, MCM on LRH-1. MCM designed the figures with help from OES and BDM. KB contributed to the review structure and writing of the review. BDM contributed to the design and editing of the review.

1.1 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.

1.2 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.

1.3 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). 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 (1). 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 (2).

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 (3). 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 (4), where the orphan receptors were cloned and were then used to search for previously unknown ligands (3). 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 (4) (Figure 1).

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 (5) and retinoic acid receptors (6) on reproduction are both direct (7) 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 2) (9), and both are often expressed in the same cells and tissues (Figure 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.

1.4 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* (10). *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 (11). The embryonic long terminal repeat-binding protein in the mouse, now known as SF-1, proved to be a mammalian homolog of Ftz-f1 (12).

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 (13). 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 (13-15). Early studies also showed that SF-1 functioned as a factor in the development of hypothalamic control of pituitary function (16). 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 (17).

The homology of Ftz-f1 with LRH-1 was recognized when the sequence of the latter was determined (15, 18). LRH-1 plays a major role in multiple processes, and was identified early as a regulator of intracellular cholesterol homeostasis (reviewed in (2)). 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. (19), 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 4). The ancestral form gave rise to a third NR5A gene, NR5A5, present in teleost fish, but that has been lost in higher forms (19, 20).

1.5 Structure

LRH-1 is located on human and mouse chromosome 1 (21), whereas SF-1 is located on human chromosome 9 and mouse chromosome 2 (22). 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 (23, 24). The domain structure of these two closely related nuclear receptors (Figure 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 (2, 24);

- the highly conserved DNA-binding domain (DBD or C domain), responsible for targeting the receptor to specific DNA sequences, termed hormone response elements (25);
- 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 (25);
- the D domain serving as hinge between DBD and LBD (2, 13, 23), recently shown to be more than a flexible connector, as it is required to regulate the transcriptional activity of LRH-1 in humans (26) and has proven to be important for effective *in vitro* phosphorylation of LRH-1 (27).

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 (13, 28). 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 (29-31). This was first shown in *Drosophila* Ftz-f1 by Ueda et al. (28), and subsequently confirmed in the mammalian homologs (reviewed in (32)). 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 (33). Alternatively, the liganded forms were ancestral, a trait lost in the NR5A family (34). 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 (34).

1.6 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 (35-37). While early studies reported that there is no expression in luteal cells (2), more recent studies suggest that it is, in fact, present in corpora lutea of the cow (38) as well as in the rat (39) and mouse (40). 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 (41), not to mention in endothelial cells of the sinus and pulp vein of the spleen and in the skin (42, 43).

In contrast (Figure 3), LRH-1 distribution is more widespread, as it is found in multiple tissues of endodermal origin, including the liver, pancreas and intestine (2). Elevated expression of LRH-1 was also found in the ovary, where it is restricted to the granulosa cell compartment (2). It is present at more modest levels in the hypothalamus and anterior pituitary gonadotrophs (44, 45), endometrium (46) and placenta, along with the adrenal gland and the testis (47).

1.7 Regulation

1.7.1 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 (30, 32). 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 (48). 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 (49). Other phospholipids, such as phosphatidylcholine or second messenger phosphatidylinositol-phosphates, can modulate the two NR5A receptor interaction with co-activators (48, 50-53). 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 (54). 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 (55, 56).

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 ((57, 58), reviewed in (59)). 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 (60).

Inverse agonists of LRH-1 that inhibit its constitutive activity, such as ML179 and ML180, have been synthesized (61). They function by inducing the translocation of LRH-1 from the nucleus (9) 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 (57). 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 (57). 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 (62). In addition to phosphatidylcholines, the signaling phospholipid, phosphatidylinositol 3,4,5-triphosphate (PIP3) binds to LRH-1 (63), 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 (64).

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 (51, 65, 66). 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 (67). 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 (56). 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.

1.7.2 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 (68). 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 (69) and their actions may well be the most important mode of functional regulation of orphan nuclear receptors (70).

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). Importantly, these co-regulators bind to domains whose structure has been altered by binding of ligands and/or post-translational modifications (71). 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 (2). 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 (72). 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 (73). The long H2 twists LRH-1 into an agonist-like conformation by affecting H12, even when LRH-1 ligand binding pocket is empty (49), while H1 and H12 of SF-1 are packed against the α-helical bundle, demonstrating its LBD ligand-independent active conformation (17).

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 (50, 74). 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 (75, 76). 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 (77).

DAX1 functions as a ligand-independent nuclear receptor, and its repressive mechanism indicates that it is a competitive transcriptional co-repressor (78). Like SHP, DAX1 lacks a DNA-binding domain and additionally has neither a modulatory domain nor a hinge region (79, 80). SF-1 and LRH-1 also interact with DAX1 through its LBD with the N-terminal LXXLL related motifs (81), binding with high affinity to the AF-2 domain, and repressing their transcriptional activity

(79, 81, 82). 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 (79, 83). 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 (18, 84). On the other hand, NCOR recruitment persists regardless of SF-1 SUMOylation state, as observed in un-sumoylatable SF-1 knock-in mice (85). 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 (84, 86). 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 (87).

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 (88, 89). 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 (88, 90). The interaction of FXR with LRH-1 allows for FXR mediated activation of SHP, retinol dehydrogenase 9, pyruvate carboxylase, and phosphatidylethanolamine N-methyltransferase (91). 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 (18, 92). The interaction between the nuclear receptors and PROX1 leads to the repression of several target genes of LRH-1, including the steroidogenic gene *CYP17a1* (92).

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 (72, 93). 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 (26, 74). SRC-1 interacts directly with the LRH-1 LBD in helix 1 and AF-2 (26) while SRC3 potentiates the interaction between CREB and LRH-1 (94). 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 (95, 96), (97).

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 (98). Interestingly, a novel isoform of LRH-1 in human granulosa cells was shown to be coordinately regulated by SF-1 and PGC1 (99). Blocking PGC1 is one of the strategies by which SHP and DAX1 repress LRH-1 activity (70), and LRH-1 is also inhibited following the recruitment of PGC1 by the sterol regulatory element-binding proteins (SREBP2) (100).

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 (101, 102). Likewise, SF-1 and β-catenin interact in the signaling pathway that produces testosterone, a collaboration that can be interrupted by WNT4 overexpression (103).

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.

1.7.3 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 (104, 105). 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) (105). 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 (106). 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 (107-109).

Chromobox homologue 2 (CBX2) is another factor shown to bind the SF-1 promoter (110), 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 (111). Human CBX2 has been shown to bind directly to the SF-1 promoter and mutations in this gene also show XY sex reversal (112). 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 (113, 114). 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 (97).

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 (115, 116). In the hypo or unmethylated state, SF-1 is expressed, with recruitment of transcription factors such as USF2 and RNA polymerase II (115, 116). 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 (117).

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. (2), indicating multiple transcriptional activators in regulation of LRH-1. In the mouse, these include GATA, HNF and NKX motifs (2). 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 (118). These findings have been interpreted to indicate that PDX1 exercises control over LRH-1 transcription during development.

1.7.4 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 (119). This post-translational change increases SF-1 DNA binding, induces its recruitment to nuclear clusters and increases its dynamic interaction with regulatory cofactors (119). PKA has also been shown to promote dissociation of DAX1 from SF-1, thereby activating or amplifying transcriptional activity of the latter (120). 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 (27, 52). 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 (56).

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)(121, 122). Both NR5A receptors are direct substrates for the SUMO conjugation machinery, such that their activity is repressed by SUMOylation (122-124). The modification of SF-1 transcriptional activity by SUMOylation takes the form of reduced receptor binding to its cognate DNA sequences (125), or translocation of the transcription factor from the chromatin to inactive nuclear bodies (122-124). 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 (126). SUMOylation has been identified as a factor in the interaction of corepressor PROX1 with LRH-1 to inhibit its role in cholesterol transport (127). 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 (121). While *in vitro* studies showed that overexpression of un-SUMOylatable SF-1 and LRH-1 increased cell-based reporter activity (56, 123), 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 (85). 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 (85). *In vivo* studies using unSUMOylatable LRH-1 mice lead to increased expression of genes involved in cholesterol transport and animals with diminished atherosclerosis development (127).

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 (128). 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 (129).

1.7.5 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 (130). Micro-RNAs also play a role in several types of human malignancies such as cancer (131). 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* (132). 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 (132).

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 (133). 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 (134-136). 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 (136).

1.7.6 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 (137). Nuclear receptors, including the glucocorticoid receptor (138) and the ecdysone receptor (139), 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 (140). 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 (140). 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.

1.8 Cellular processes related to reproduction

1.8.1 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(141). 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 (142)), 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 (100), while Datta et al. report binding of LRH-1 to the HMG-CoA promoter to induce specific activation of its transcription (143). 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) (141). It has been shown that SF-1 transactivates the SCARB1 gene (144) and mediates the uptake of cholesterol via this receptor (145). 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 (146, 147).

Reverse cholesterol transport, another homeostasis process completed principally by HDL via SCARB1, is regulated by LRH-1 activity (2). Intermediates that shuttle cholesterol to HDL for efflux include members of the ATP binding cassette (ABC) family (reviewed in (148)), and transient transfection assays have shown that LRH-1 activates transcription of isoforms ABCG5 and ABCG8 by binding to their promoter regions (149). 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* (150). 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 (151). 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 (152). 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 (153). 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 (154, 155). 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 (156). Lopez and McLean (145) 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 (157). Given that LRH-1 also induces SCARB1 expression in both mouse and human tissue (2), 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 (60). Others presented evidence that LRH-1 directly inactivates SREBP1 transactivation in promoter assays and they further showed that SREBP2 directly inhibits LRH-1 activity (100). 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 (158, 159). 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* (160, 161) and *in vitro* (157) 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) (162). All of these factors are essential for reproductive function (reviewed in (163)). Studies have shown that SF-1 regulates the transcription

of the NPC1 gene in concert with cAMP (164). The promoter region of SCP2 contains an SF-1 (and presumably a LRH-1) response element, suggesting regulation by the NR5A receptors (165).

The STAR protein, first discovered by Clark and Stocco (166), 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 (167, 168) 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 (169). 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 (170). A non-exhaustive list of tissues where SF-1 regulates STAR includes adrenal cell lines (171), Leydig cells (172), theca (169), granulosa (173) and endometrial cells (174). 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 (175). Cre-loxP depletion of LRH-1 in the mouse ovary from primordial follicles forward (160, 161, 176) results in dramatic reduction in *Star* expression. Moreover, ChIP analysis by Duggavathi et al. (161) 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.

1.8.2 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 (166). 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 (13, 164) (reviewed in (177)). The promoter regions of several of these enzymes have been shown to contain the SF-1/LRH-1 consensus site (178, 179) (Table 2). These discoveries led to identification of the presence of SF-1 in most mouse steroidogenic tissues, including the corpus luteum (CL) (40) and LRH-1 was later shown to be present in the ovary and testis (47, 180) (Table 2). Under the control of gonadotropins (146), 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 (181). 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 (182). Activation of the *FDX1* promoter has been shown to occur following stimulation by cAMP (182).

1.8.3 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 (102). 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 (102).

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 (110, 183, 184). 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 (185). 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 (186). 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 (187).

1.8.4 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 (188). 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 (189, 190). 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 (190). More recently, it has been demonstrated that both LRH-1 and SF-1 regulate and can replace OCT4 in stem cells (191). 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) (192).

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 (180). 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 (193). In contrast, the steroidogenic cells derived from bone marrow MSC secreted gonadal rather than adrenal steroids (193). 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 (194). 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 (170, 195).

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.

1.9 Physiological processes related to reproduction

1.9.1 Embryogenesis

1.9.1.1 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 (189). 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 (2). At day E17.5, LRH-1 exhibits its adult expression profile in the liver, exocrine pancreas, intestinal crypts and stomach epithelium (2). 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 (2). At day E17.5, LRH-1 exhibits its adult expression profile in the liver, exocrine pancreas, intestinal crypts and gastric epithelium (2).

Mice homozygous for a germline mutation in the gene encoding LRH-1 die between E6 and E7.5 (Table 3), a time that corresponds to gastrulation, indicating that LRH-1 plays a crucial role in the formation of the early embryo (196). Interestingly, development of the embryo to a multicellular stage occurs (197), 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 (197). Failure of gastrulation is believed to be secondary to defective visceral endoderm development (197). 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 (189).

The ontology of SF-1 expression during development is less clear (Table 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 (36). SF-1 expression then continues in the developing steroidogenic portion of the adrenal gland and then in the outer cortical region (E11-12) (36). 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 (14).

1.9.1.2 Neural development

Tissue-specific depletion strategies indicate that SF-1 expression is required at multiple sites in the hypothalamic-pituitary-gonadal axis (198, 199). In the adult mouse brain, SF-1 is expressed exclusively in the VMH (123). In the hypothalamus, SF-1 is found in mouse VMH precursor cells from E11.5 onward (200, 201). Its expression is essential for correct formation of this structure, (16). In SF-1 knockout mice, the VMH is present at E17, but with decreased cellularity and abnormal organization that persists, at least until birth (16). 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 (202). 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(203). These animals showed significant decreased locomotor activity and late-onset obesity (203). 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 (202).

1.9.1.3 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) (37). 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 (204). This indicates that SF-1 does not direct precursor cells to the gonadotrophic cell fate, rather, it regulates the final differentiation steps.

1.9.1.4 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(36). 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 (14). 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 (205), 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 (206).

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 (36). Sex-specific regulation is achieved by repression of SF-1 by the forkhead box L2 (FOXL2) transcription factor during ovarian development (207). As chronicled above, one of the principal mechanisms by which SF-1 regulates gonadal function is the induction of steroidogenic enzymes (36). Further it has been shown that SF-1 (and LRH-1) can transform both pluripotent and mesenchymal stem cells into steroidogenic cells *in vitro* (208). The combination of SF-1 with WT1, DMRT1, GATA4 and SOX9 transformed mouse fibroblasts into Sertoli-like cells (209). Together these observations demonstrate the pivotal roles for both SF-1 and LRH-1 in gonadal and reproductive tract development.

1.9.2 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 (210). This regulation has been attributed to impingement of VMH projections onto gonadotropin releasing hormone (GnRH) neurons (211), 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 (199). 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 (212, 213). In mice, LRH-1 controls FSH levels, follicle maturation, and estrous cycle by binding directly to the *Kiss1* promoter and stimulating its transcription (213). The consequence of the depletion of LRH-1 is the reduction in the secretion of GnRH, which in turn reduces gonadotropin secretion (213).

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 (214). Mutation of this site eliminated LHß promoter activity (215). 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 (216)). 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 (216). 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 (44). 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* (44). 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* (45).

1.9.3 Gonadal function

1.9.3.1 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 (217). They therefore play a pivotal role in steroid hormone production in human Leydig cells (180).

Given the demonstrated importance of both SF-1 and LRH-1 in regulation of steroidogenic enzymes and factors (2), 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 (47, 218). 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 (47, 218). 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 (219) 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 (37, 41, 220). 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 (221, 222). 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 3)(198). 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 (161) 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 (223). 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 (224). 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.

1.9.3.2 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 (225). 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 (169, 226). While studies have mainly reported low SF-1 expression in the mouse corpus luteum (40), bovine studies have shown that SF-1 inhibition leads to significant decrease in progesterone production in luteal cells (38). 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 (39). 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 (227). 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 (228). 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 (229).

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 (230). This enzyme plays a role in progesterone production via the supply of heme as a prosthetic group of P450 steroid hormone-synthesizing enzymes (230). In another example, SF-1 combines with SMAD3 to activate TGFβ3-induced CYP19A1 expression and subsequent estradiol synthesis and secretion in mouse granulosa cells (231). 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 (232).

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 (40). It has also been identified in equine (233), rat (39), rabbit (234), bovine (235) and human (47) 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 (2, 102). LRH-1 plays a major, but not indispensable role in granulosa cell proliferation (102, 161), and is also induced significantly in the CL during pregnancy (236). In rodents, the expression of LRH-1 is increased by FSH in granulosa cells, and by prolactin in luteal cells (39).

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 (237). 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 (161, 198). 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* (161).

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 (176). 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 (176). 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 (46).

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* (238). 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.

1.9.4 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 (239)). Studies have reported that SF-1 expression is absent from the fetal components of the placenta, including trophoblast cells in humans and in rodents (240, 241). 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 (38, 242). 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 (243). 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 (225). 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 (244). 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 (46) 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 (46). 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 (46), and therefore the development of the placenta by affecting the invasion of extravillous trophoblastic cells into the uterine decidua (245). 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 (245).

1.9.5 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 (203, 246, 247). 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 (199). 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 (248), 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 (249). 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 (199, 250). To our knowledge, no studies have been published on the effect of LRH-1 on reproductive behavior.

1.10 Pathological processes related to reproduction

1.10.1 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 (251)). 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) (153). 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 (252, 253). 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 (253, 254). 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 (255, 256). 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 (257, 258). 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 (259, 260). There appears to be no published information on human genetic abnormalities attributable in LRH-1.

1.10.2 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 (261). 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 (261-263). Bulun et al.(264) 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 (265, 266). 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 (243, 267). 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 (267). Moreover, Tian et al. showed that the abundance of SF-1 and its target gene, STAR to be correlated with the severity of endometriosis (268).

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 (269). 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 (270). When aberrant demethylation of the SF-1 promoter occurs, as it is observed in endometriosis, expression of SF-1 is upregulated (270). 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 (244).

1.10.3 Cancer

Accumulating evidence indicates that LRH-1 participates in the pathogenesis of tumors of multiple sorts (reviewed in (271)) including pancreatic (272, 273), breast (274), gastric (275), and colon cancer (276). Suppression of LRH-1 in colon cancer (277) or osteosarcoma cells (278), 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 (272). 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 (279). Also upregulated are the metalloproteinases, MMP2 and MMP9, implicated in metastasis, and known to facilitate tumor growth, cell migration and tumor invasion (280). Moreover, LRH-1 can promote pancreatic cancer metastasis (272, 281) and promotes intestinal tumor proliferation in gastrointestinal tumors by activating the Wnt/β-catenin pathway (275, 277). 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 (101). LRH-1 has been shown to drive colon cancer cell growth by repressing the expression of CDKN1A in a p53 dependent manner (282), 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 (283). 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 (284). 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 (285). Conversely, clinical studies have suggested that ovarian tumors with functioning stroma secrete estradiol via the regulation of aromatase, due to overexpression of SF-1 (286). 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 (287). 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 (288). 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) (289). 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 (289). 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 (290).

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 (291). 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 (291). Its expression promotes proliferation, migration and invasion of breast cancer cells *in vitro* (292). 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 (292). In ERα-positive breast cancer cells, LRH-1 promotes cell proliferation by increasing estrogen biosynthesis by regulating aromatase expression (274) and by ESR1-mediated transcription of target genes such as *GREB1*(293). 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 (294). 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 (294). 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 (295). LRH-1 promotes breast cancer cell resistance to chemotherapy by upregulating the checkpoint protein, MDC1, to enhance DNA damage repair (295). 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 (296). Analysis of breast cancer samples by Bianco et al. (297)

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 (140, 297). 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 (140, 297). To our knowledge, no studies have shown SF-1 deregulation or involvement in breast cancer.

1.11 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 (298). 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 (52), 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 (52). 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 (299).

LRH-1 and SF-1 are co-expressed in the ovary at all stages of the estrous cycle (40). 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 (161). 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 (225). As noted above, the Amhr2/SF-1 female mouse is infertile due to disruption of the follicle development trajectory (225) while in the Amhr2/LRH-1 ovary, large antral follicles that appear structurally normal are present, but these fail to ovulate (160, 161). 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 (40), but, as above, SF-1 cannot compensate for LRH-1 deficiency in this tissue (46). 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.(300). 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 (300). A further possibility is that stimulatory ligands may differentially regulate LRH-1 vs. SF-1 transactivation by acting through different intracellular signaling mechanisms (300). The mechanisms of selective action clearly merit further investigation.

1.12 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 3 and Figure 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.

1.13 Disclosures

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

1.14 Figures

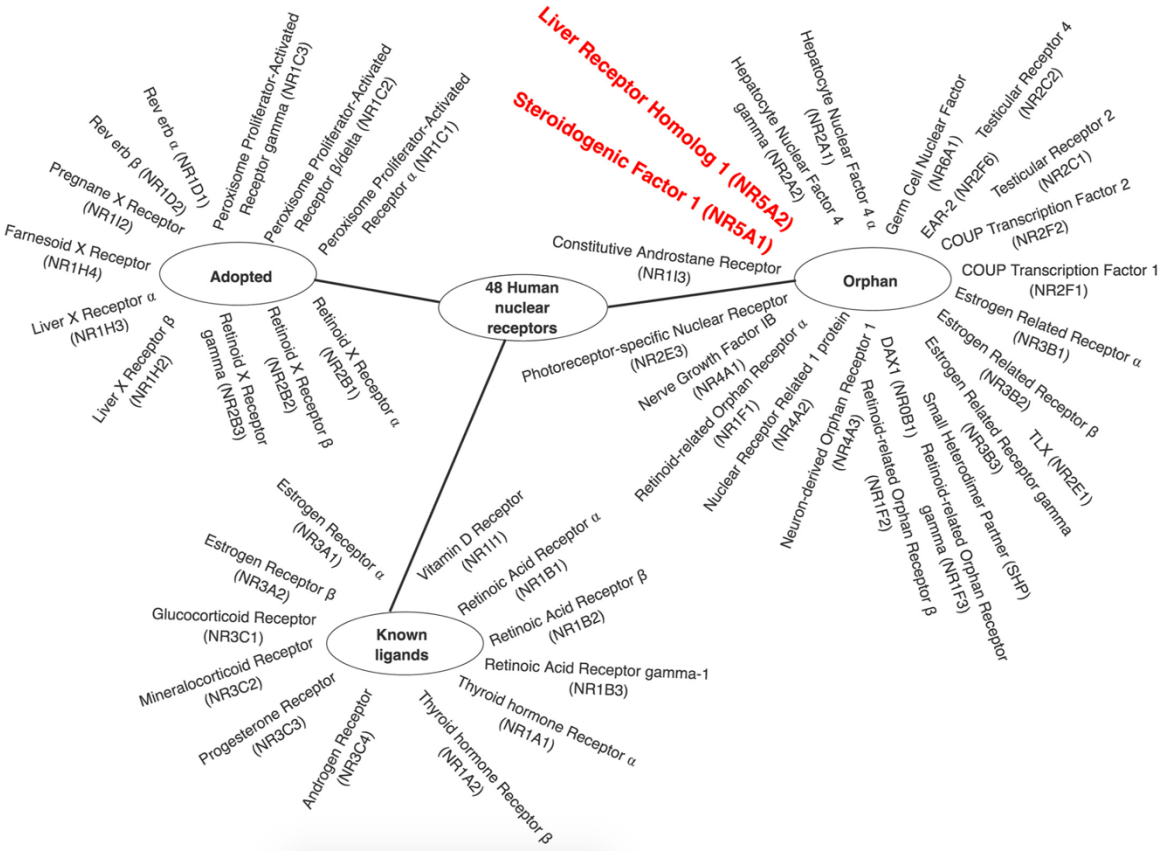

Figure 1.1

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 (59, 176, 291, 301-308))

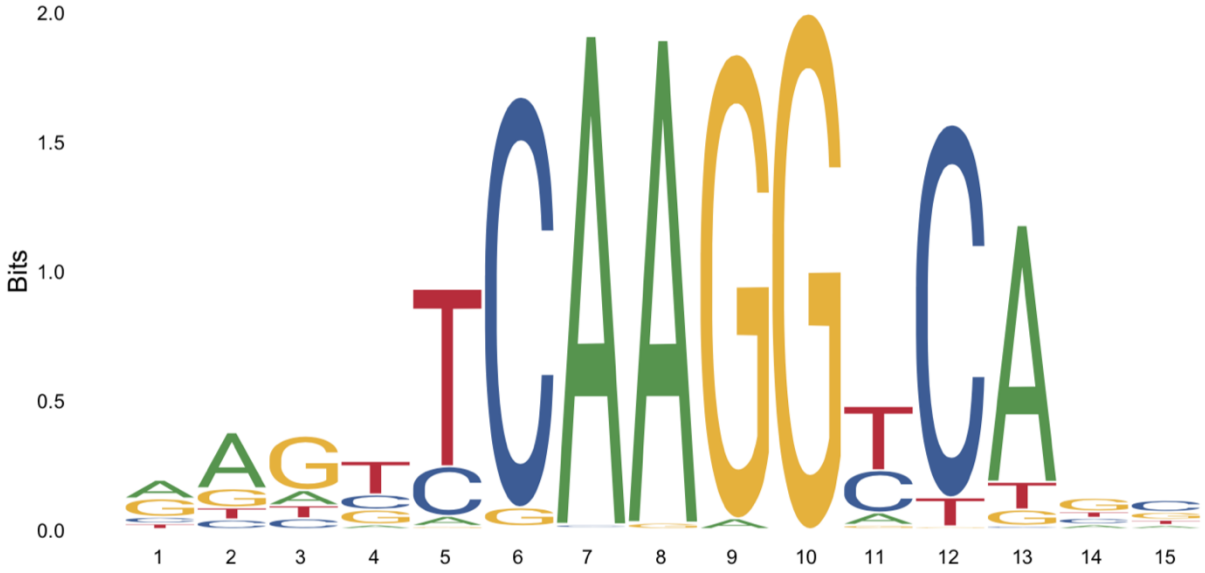

Figure 1.2

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).

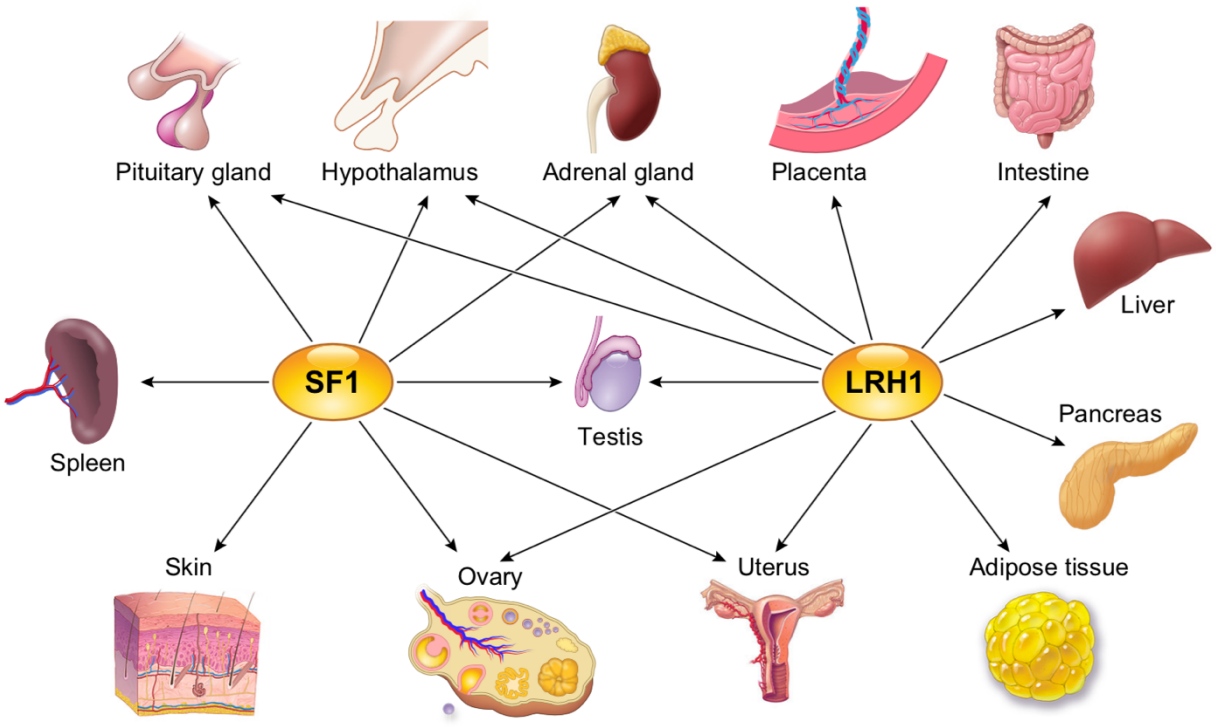

Figure 1.3

Tissue distribution of the NR5A receptors in mammals, showing 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.

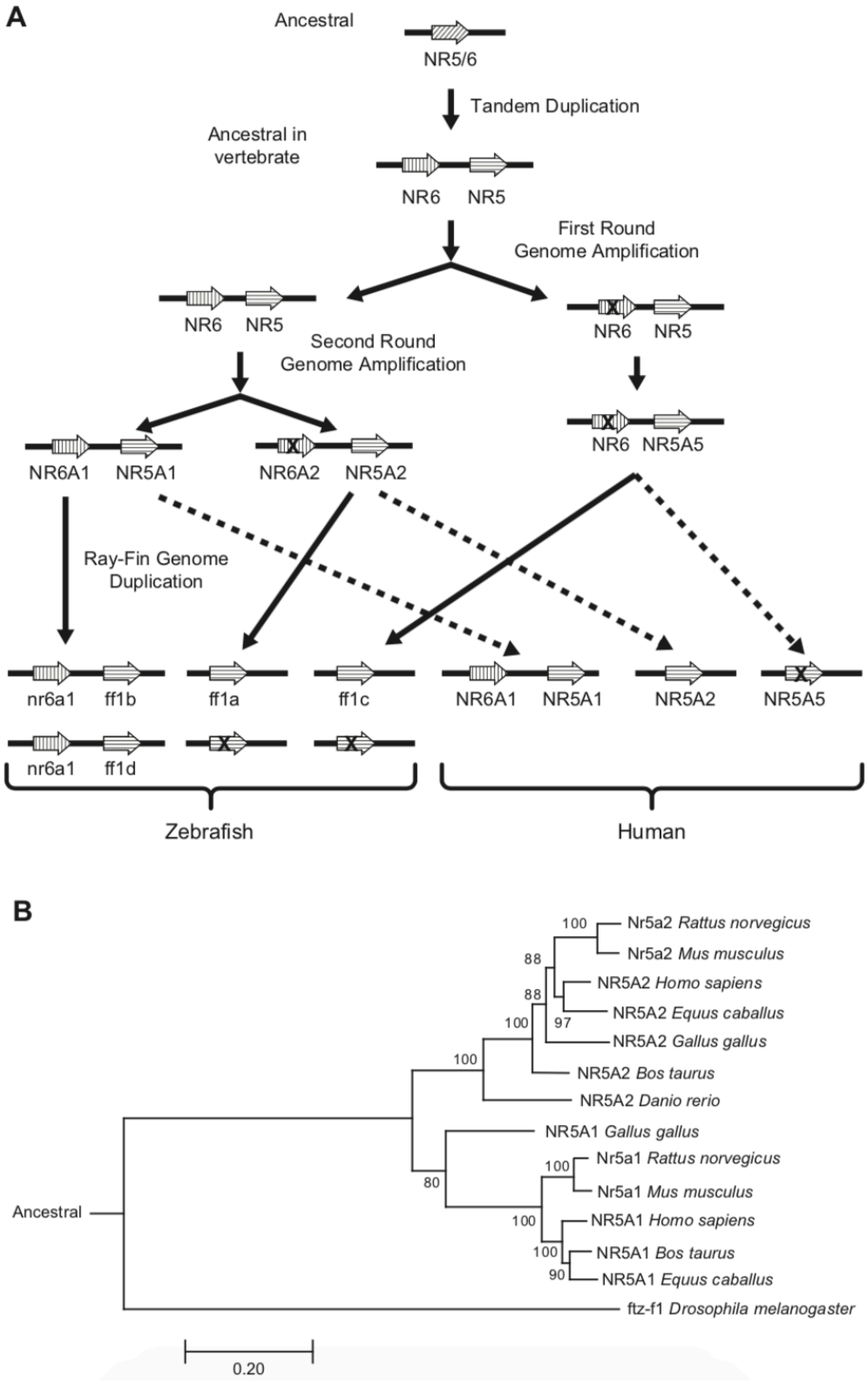
A
Ancestral
NR5/6
Tandem Duplication
Ancestral in vertebrate
NR6 NR5
First Round Genome Amplification
NR6 NR5
NR6 NR5
Second Round Genome Amplification
NR6 NR5A5
NR6A1 NR5A1
NR6A2 NR5A2
Ray-Fin Genome Duplication
nr6a1 ff1b
ff1a
ff1c
NR6A1 NR5A1
NR5A2
NR5A5
nr6a1 ff1d
Zebrafish
Human
B
Nr5a2 Rattus norvegicus
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
100
97
100
100
80
100
100
100
90
0.20

Figure 1.4

Evolution of the *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.

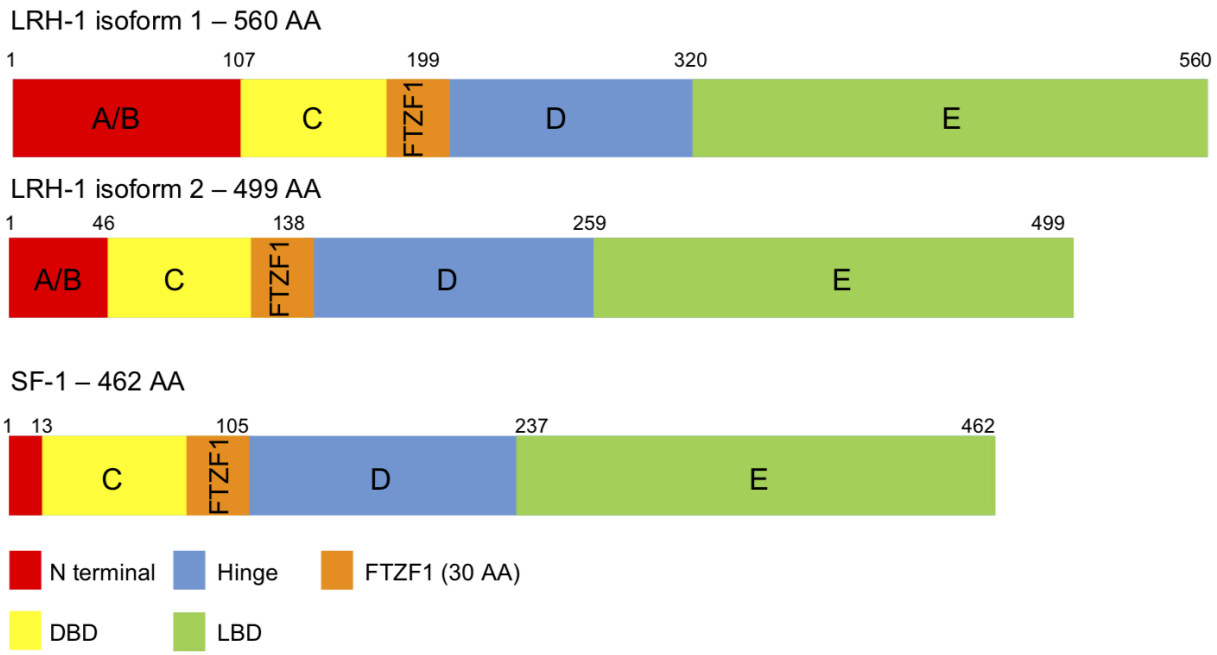

Figure 1.5

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.

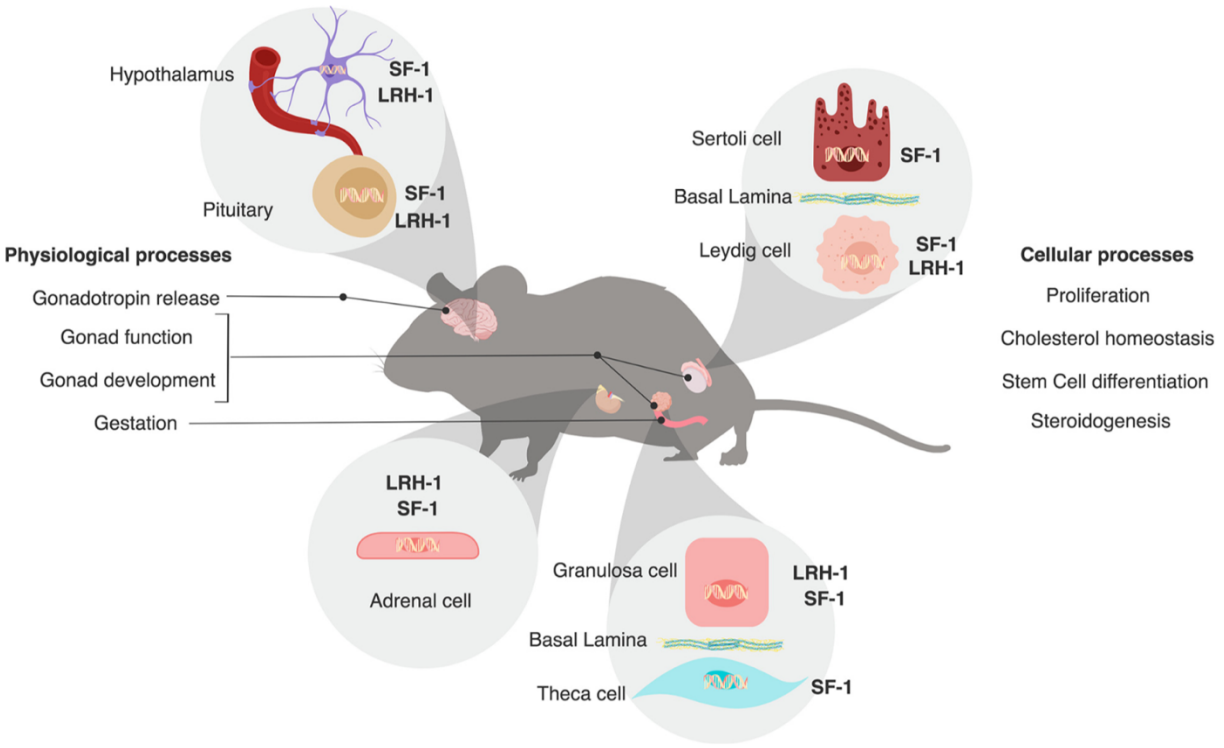

Figure 1.6

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.

1.15 Tables

Table 1.1 The principal mechanism for regulation of the transcriptional activity of the NR5A receptors is interaction with cofactors.

Co-regulator	Regulation	Interaction Site/Mode	Nuclear Receptor	Reference Nos.
SHP (NROB2)	Negative	Attaches COOH-terminal domain and interacts with AF-2 domain	SF-1, LRH-1	88, 159
DAX1 (NROB1)	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, 286, 320
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

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.

Table 1.2 Both LRH-1 and SF-1 regulate the process of steroid synthesis in reproductive target tissues, sometimes in the same cells.

Steroidogenic Gene	Principal Reproductive Tissue Localization	Reference Nos.
	SF-1	
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	
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

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.

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 Nos.
Germline deletion	Homozygous		Embryo lethality at gastrulation period (E6–7.5)	Postnatal lethality ≤8 days after birth		176, 208
			Defective visceral endoderm development	Absence of adrenal gland and gonad regression at E11.5–12		148
			Impaired primitive streak morphogenesis	Adrenocortical insufficiency		
	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	Depletion in Leydig cells of testes	Depletion in granulosa cells of all follicles	68
			Infertile	Infertile	Infertile	214
			No ovulation	Arrested spermatogenesis	No ovulation	
			No luteinization	Delayed fetal testes organization	Absence of CL	
			Decrease in granulosa cells proliferation	Hypoplastic, undescended testes	Decrease in granulosa cells proliferation	
			No cumulus expansion	Decrease in testes somatic cell proliferation	Reduced follicle number	
	Cyp19a1	Fertile	Depletion in granulosa cells from antral follicles forward	Fertile	Fertile	14
			Infertile			
			Incomplete cumulus expansion			
			No ovulation			
			No luteinization			
	Pgr	Fertile	Depletion in corpus luteum	Fertile	Depletion in corpus luteum	318
			Infertile		Subfertile to infertile	249
			Corpus luteum unable to maintain pregnancy. Impaired dedicualization		Impaired luteinization	

LRH-1, liver receptor homolog-1; SF-1, steroidogenic factor-1

1.16 Recent scientific additions

Since the publication of the above review in early 2019, a number of novel studies have brought additional insight on the regulation of SF-1 and its effect on reproductive function. The following is a brief review of recent data that are worthy of mention in the context of this thesis.

1.16.1 SF-1 regulation in gonadotropes

Few studies have tried to understand the mechanism involved in the transcriptional regulation of SF-1 (*Nr5a1)*. As mentioned above, the *Nr5a1* promotor region contains an E-box motif that enhances its transcription when bound to upstream transcription factor (USF) 1 and 2 and is essential for SF-1 expression in adrenocortical, gonadotrope and steroidogenic cell lines (106, 309, 310) GnRH sensitivity has been associated to this USF binding site, present on *Cga* and *Fshb* promoter region as well (311-313). Moreover, a recent *in vivo* study in ovariectomized rats showed that endogenous GnRH and kisspeptin secretion from the hypothalamus regulate the transcriptional activity of SF-1 in the pituitary via the Wnt pathway. Indeed, pulsatile intracerebroventricular injections of GnRH in rat brains upregulated the β-catenin gene (*Cnntb1*) and protein expression, and kisspeptin injections induced DAX1 gene (*Nr0b1*) and protein expression, both well-established co-regulators of SF-1 (313).

1.16.2 SF-1 in stem cell differentiation

Because of its essential role in adrenal and gonad cell differentiation, many groups have used SF-1 and LRH-1 in combination with other factors to direct stem cells towards the steroidogenic cell lineages (314). Recently, Hou et al. successfully reprogrammed human fibroblasts into induced Leydig-like cells by overexpressing SF-1, GATA-4 and NGFI-B (315).

Though this *in vitro* reprogramming technique created cells with a transient state of steroidogenesis, from which testosterone production eventually stopped, the capacity to rapidly produce Leydig-like cells from somatic cells without the necessity of passing through a pluripotent state removes the risk of tumorigenesis and is promising for uses in regenerative medicine. Similarly, Sertoli-like cell were also derived from human fibroblast cells, in this case with NR5A1 and GATA-4 as reprogramming factors (316). These cells were able to sustain spermatogonia culture *in vitro*, and suppress the proliferation of human T lymphocytes, an encouraging aspect for future use in transplantation therapies. Likewise, the exogenous introduction of SF-1 in porcine preadipocytes together with cAMP treatment resulted in sex steroid-producing cells (317). These cells were subsequently used as an *in vitro* model to demonstrate that 11-ketotestosterone is the dominant androgen produced in female porcine steroidogenic cells. These studies provide additional evidence of the regulatory role of SF-1 in inducing the differentiation of stem cells into steroidogenic cells.

1.16.3 SF-1 in gonadal development

Though SF-1 is indispensable for the development of the genital ridge of both sexes (318), its expression is upregulated in testes and downregulated in ovaries after sex determination (205, 207). This suppression of SF-1 was recently found to allow the upregulation of *Notch2* and induce Notch signaling in female mice, a pathway known to promote follicular formation and maturation in fetal ovaries (319). In males, the upregulation of SF-1 inhibits the Notch signaling pathway, which is known to abrogate fetal Leydig cell differentiation (320). SF-1 and its co-factor SRY induce the expression of *Sox9* in Sertoli precursor cells (205), and SOX9 in turn acts as a co-factor of SF-1 to induce *Amh* expression (321). A recent study has shown that the Sertoli cell-specific

recombination of SF-1 between E12.5 and E14.5, using a *Sox9*-Cre transgenic mouse model, caused reduced AMH levels in the gonads of XY animals and resulted in postnatal male-to-female gonadal sex reversal. These cKO gonads had a reduced Leydig cell number and FOXL2 expression was measured in certain cells, indicating that Sertoli progenitor cells were redirected towards the ovarian pathway, differentiating into granulosa cells (322). Another study, inducing the Sertoli cell-specific ablation of SF-1 at E14.5 with a *Amh*-Cre mouse model, demonstrated that loss of SF-1 increased Sertoli and germ cell apoptosis in cKO testes (323). Molecular analysis indicated that SF-1 might directly regulate murine double minute 2 (MDM2) expression, which in turn inhibits tumor suppressor TP53, and thus is required for fetal Sertoli cell survival. The importance of SF-1 in sex-specification is further supported by time series single-cell RNA sequencing (scRNA-seq) performed on ovarian SF-1-positive somatic cells isolated during sex determination (324). These SF-1 positive progenitor cells gave rise to both pre-granulosa cells and potential steroidogenic precursor cells. These recent advances provide additional insight on how SF-1 regulates fetal gonad development.

1.16.4 SF-1 in gonad function

1.16.4.1 Testis

A recent study demonstrated the role of SF-1 in regulating the expression of *GnRHR2*, the receptor with high affinity to GnRH2, in porcine Leydig cells (325). Unlike GnRHR1 that is mainly found in the pituitary, *GnRHR2* expression is found in peripheral tissues of the pig such as the testis, where it regulates testosterone secretion (326). Using ChIP and electrophoretic mobility shift assays (EMSA), Ding et al. confirmed that SF-1 does bind to the porcine *GnRHR2* promoter to induce its expression, and preliminary results from testicular Leydig cell *in vitro* culture indicate

that the binding of GnRH2 to GnRHR2 also induces the expression of *SF-1* and other steroidogenic enzymes to induce the biosynthesis of testosterone and ultimately, spermatogenesis and fertility.

Another study used ChIP, demonstrated that SF-1 binds to the promoter region of connexin 43 (CX43), the most abundant connexin protein in the testis (327). Connexins help form gap junctions between neighboring cells, and play an important role in the formation of the blood-testis barrier (BTB) in the seminiferous tubule of the testis (328). Couture et al. found that SF-1 cooperates with SOX8 to activate *Cx43* transcription in mouse TM4 Sertoli cells, and that LRH-1 can also bind to SOX8 and compensate for SF-1 absence. These novel findings highlight the essential role of SF-1 BTB formation and non-steroidogenic testicular function.

1.16.4.2 Ovary

A study in the *Drosophila* showed that *fushi tarazu-factor 1* (*ftz-f1;* the *Drosophila* homologue of SF-1) was required in the late stages of oogenesis to induce ovulation and follicle rupture (329). *Ftz-f1* was found to bind directly to the *single-minded* (*sim*) gene, a transcription factor involved in *Drosophila* embryonic neuronal development, to which this study now added a role in mediating follicle cell differentiation to induce ovulation. The loss of *Ftz-f1* in follicle cells at late stages blocked the final steps of folliculogenesis, rendering the follicles incompetent for ovulation and rupture. These results offer a glimpse into possible regulatory actions of SF-1 in important ovarian functions outside of its well-established role in steroidogenesis.

Chapter 2. Hypothesis and objectives

Steroidogenic factor 1 is a nuclear transcription factor expressed during cell differentiation stages of the male and female reproductive organ development and most studies to date demonstrate the impact of SF-1 absence in reproductive endocrine organ development and fetal or pre-pubertal gonad function. The purpose of this investigation was to identify the role of SF-1 in both male and female adult reproductive function, specifically in the regulation of steroidogenesis and the hormonal loop between the gonads and the pituitary-hypothalamic endocrine axis.

Based on evidence from the literature demonstrating that SF-1 is involved in hypothalamic formation, gonadotropin release from the pituitary gland and is required for normal gonad development and sex differentiation, our general hypothesis was that SF-1 is an essential regulator of key events in male and female mouse reproductive function.

To study this hypothesis, our primary objectives were to 1) identify the role of SF-1 in peri-ovulatory events between the ovary and the pituitary-hypothalamic endocrine axis, and 2) identify the role of SF-1 in male and female gonadal steroidogenesis. Results pertaining to objective 1 are presented in Chapter 3 and results pertaining to objective 2 are presented in Chapter 3 and 4.

Chapter 3. Article 1

Steroidogenic factor 1 plays an essential role in the hypothalamic-pituitary-ovarian axis of adult female mice

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This work was supported by CIHR operating grants PJT-166020 to BDM and PJT-169184 to DJB. OS is funded by a doctoral training grant from FRQS.

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Keywords: Infertility, SF-1, ovary, pituitary gland, ovarian transplantation, conditional knock-out.

Status: This manuscript is to be submitted to *Endocrinology*.

Author contributions: OES contributed to the experimental design, performed experiments, analyzed the results, and wrote the article; representing a relative contribution of 90%. VR and FM performed experiments. LO and XZ conducted the gonadotropin assays. MB and DJB contributed to the experimental design, data analysis and article editing. BDM contributed to the experimental design, data analysis, and article editing.

3.1 ABSTRACT

The orphan nuclear receptor steroidogenic factor-1 (SF-1 or NR5A1) is an indispensable regulator of adrenal and gonadal formation, playing roles in sex determination, hypothalamic development, and pituitary function. This study aimed to identify the roles of SF-1 in post-natal female reproductive function. Using a progesterone receptor-driven Cre recombinase, we developed a novel murine model characterized by the conditional depletion of SF-1 (PR-Cre;*Nr5a1*$^{f/f}$; cKO) in the hypothalamic-pituitary-gonadal axis. Mature female cKO were infertile due to the absence of ovulation. Although sufficient to induce regular estrous cycles and sexual behavior, reduced gonadotropin synthesis in the pituitary gland was observed in cKO mature females. Ovaries in cKO females showed abnormal lipid accumulation in the stroma, due to irregular expression of cholesterol homeostasis genes such as *Star, Scp2,* and *Acat1*. The depletion of SF-1 in granulosa cells prevented effective cumulus expansion, characterized by reduced expression of *Areg*, *Ereg,* and *Ptgs2*. Exogenous delivery of gonadotropins to cKO females to induce ovulation did not restore fertility, and was associated with impaired formation and function of corpora lutea accompanied by reduced expression of the steroidogenic genes *Cyp11a1* and *Cyp19*, and reduced progesterone production. Surgical transplantation of cKO ovaries to ovariectomized control animals (*Nr5a1*$^{f/f}$) resulted in two separate phenotypes, with some exhibiting apparently normal fertility, whereas others were sterile. The deletion of SF-1 in the pituitary and in granulosa cells near the moment of ovulation, demonstrated that this nuclear receptor plays essential roles in gonadotropin synthesis, cumulus expansion, and luteinization.

3.2 INTRODUCTION

Female mammalian reproductive function is orchestrated by the hypothalamic-pituitary-gonadal (HPG) axis. Pulsatile gonadotropin-releasing hormone (GnRH) secretion from the hypothalamus stimulates the synthesis and secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in the pituitary gland. These gonadotropins induce the growth of ovarian follicles, where granulosa and theca cells transform cholesterol into steroids that regulate all organs of the HPG axis. The expression of the enzymes involved in cholesterol homeostasis and steroidogenesis is regulated by a variety of transcription factors, among which the family of nuclear receptors play a key role (330).

While many nuclear receptors have known ligands, such as the estrogen (ER) and progesterone receptors (PR), 24 mammalian nuclear receptors defined as orphan do not yet have defined ligands and/or show ligand-independent activity (331). Steroidogenic factor-1 (SF-1; NR5A1) is one such orphan nuclear receptor, initially identified in the mouse with essential roles in steroidogenic tissues such as the adrenal gland and the gonads (13). In humans, SF-1 heterozygous mutations are linked to adrenal insufficiency and gonadal dysgenesis, and are believed to be the cause of up to 20% of 46,XY disorders of sex development (DSD) (153, 251). A wide range of clinical phenotypes are observed in women that carry SF-1 mutations, including 46,XX DSD, characterized by primary or secondary amenorrhea, elevated gonadotropin secretion, hypoestrogenism, precocious depletion of the ovarian reserve, infertility, or, in some cases, no apparent symptoms (332, 333). Many factors have been suggested to explain the variation in phenotypes, including epigenetic dysregulation, developmental compensation, and, recently, oligogenic inheritance (334, 335).

Several lines of evidence implicate SF-1 in gonadotropin regulation. SF-1 is expressed prior to the appearance of the FSH and LH ß-subunit genes (*Fshb* and *Lhb*) in the developing gonadotrope cells of the murine pituitary gland, but after the expression of the gonadotropin α-subunit (αGSU; product of the *Cga* gene) (336). Conditional ablation of SF-1 in *Cga* expressing cells results in hypogonadotropic hypogonadism and impaired gonadal steroidogenesis (337). Both germline and central nervous system (CNS)-specific deletion of SF-1 affects ventromedial hypothalamus (VMH) organization and cell populations (16, 202). SF-1 positive neurons express ER, and ER has been localized in VMH neurons that impinge on GnRH-neurons, leading some to hypothesize that SF-1 plays an essential role in GnRH secretion regulation (210, 211).

SF-1 has been implicated in the transcriptional regulation of various factors involved in steroidogenesis (13). Steroids such as estrogen and progesterone play essential roles in follicle growth and corpus luteum (CL) function, respectively, and exert positive and negative feedback on the hypothalamus and pituitary gland to regulate the gonadotropins. To date, few studies have investigated SF-1 absence specifically in the ovary. Pelusi et al. (225) conditionally ablated SF-1 expression in granulosa cells using the *Amhr2*-Cre driver and showed that females were infertile due to absence of ovulation and impaired estradiol secretion. Ovarian follicles were significantly reduced in number and CLs were absent, while expression of *Amh*, a gene correlated with the abundance of primordial follicles in the ovarian follicle reserve, was reduced.

PR is expressed in all organs of the female HPG axis. In the hypothalamus, PR is expressed in the kisspeptin neurons of the anteroventral periventricular nucleus (AVPV), where it regulates the estradiol/progesterone positive feedback-induced GnRH and subsequent LH surge (338, 339). In the pituitary, estrogen induces PR expression in gonadotropes, and absence of PR in these cells

impairs the amplitude of the LH surge (340). In the ovary, PR expression is induced in granulosa cells of antral follicles by the LH surge, where it is essential for follicle rupture, ovulation, and luteinization, and is also expressed in the CL where it regulates steroidogenesis (341). Therefore, our approach was to employ the PR-Cre driver to induce SF-1 depletion at multiple levels of the HPG axis to determine the role of SF-1 in sexually mature female mouse reproductive function. We set out to determine the role of SF-1 in peri-ovulatory events, and hypothesized that its absence in granulosa cells of mature follicles will have a negative impact on female reproduction in mice. The PR-cre activity in other relevant cell types in the HPG axis led to a complex set of phenotypes, from impaired LH secretion in the pituitary gland to inefficient ovulatory processes and steroidogenesis in the ovary.

3.3 MATERIAL AND METHODS

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. The conditional KO (cKO) mouse model was obtained by crossing SF-1 floxed (*Nr5a1*^{tm2Klp} MGI: 1346833) females [described in (337)] with males expressing Cre-recombinase driven by the progesterone receptor (Pgr$^{tm2(cre)Lyd}$ MGI: 3576366) (342). Following DNA extraction from tails, males heterozygous for the floxed SF-1 allele (*Nr5a1*$^{f/+}$) and PR-Cre were crossed with *Nr5a1*$^{f/f}$ females. Since PR$^{Cre/+}$;*Nr5a1*$^{f/f}$ males are fertile, they were subsequently mated with SF-1$^{f/f}$ females to produce cKO females (genotype PR$^{Cre/+}$;*Nr5a1*$^{f/f}$) and non-mutant female littermate controls (genotype PR$^{+/+}$;*Nr5a1*$^{f/f}$, hereafter CON). Mice were genotyped using primers listed in Table S3.1. All mutant and control mice were maintained on the C57BL/6 genetic background under a 14-hour

light, 10-hour dark cycle and provided food and water *ad libitum*. Euthanasia was performed by cervical dislocation following anesthetizing the animals with isoflurane.

Breeding trial, mating frequency and estrous cycle assessment

For breeding assays, 8-week-old CON and cKO females were housed with reproductively proven wild-type C57BL/6 males for 6 months. Cages were inspected daily, and parturition dates and litter sizes were recorded. To assess mating frequency, 12-week-old cKO and CON females were housed with vasectomized males for 40 days to establish the frequency of mating. The occurrence of copulatory plugs was verified by visual examination at 0900 each day. For vasectomies, male mice were anesthetized with isoflurane, then an incision of 1 mm through the skin along the midline of the lower abdomen was made, and 1 cm of vas deferens on each side was removed. Relative fecundity was assessed following Silver's overall fecundity equation: frequency of mating (% of matings that produce offspring) x average litter size x average number of litter in the six-month breeding trial (343).

Estrous cycle staging was assessed by daily vaginal swabbing with 1X phosphate-buffered saline (PBS) for 28 consecutive days; smears were placed on glass slides and cytology was evaluated under the microscope following the criteria defined by Bertolin et al. (344).

Adult female tissue collection

Hypothalami, pituitaries, ovaries, and blood (by cardiac puncture) were collected following isoflurane anesthesia and cervical dislocation of adult (> 8 weeks old) CON and cKO female mice on the morning of the first day of estrus. Blood was kept at 4ºC overnight then centrifuged at 10,000 rpm for 1 min and isolated serum was kept at -20º C for hormone assay. Tissues were either snap

frozen in liquid nitrogen and stored at -80ºC for gonadotropin assay or mRNA extraction, or fixed in 10% formalin (Sigma-Aldrich) for histological analysis and IHC analyses. Ovulated cumulus-oocyte complexes were collected and counted from oviducts of CON and cKO females at 0800 on the day that copulatory plug was observed (estimated 5 h post mating).

Superstimulated female tissue collection and mating

Immature female mice, 25 to 27 days old, were injected intraperitoneally (IP) with 5 IU of equine chorionic gonadotropin (eCG) (Folligon, Intervet, Whitby, ON, Canada) to stimulate follicular development, followed by the injection of 5 IU of human chorionic gonadotropin (hCG) (Chorulon, Intervet, Whitby, ON, Canada) 44 to 48 h post eCG, to induce ovulation. Ovaries and blood (collected by cardiac puncture) were collected at 11 h and 24 h post-hCG injection (hCG 11h and hCG 24h) and were either snap frozen in liquid nitrogen and stored at -80ºC for mRNA extraction or fixed in 10% formalin and embedded in paraffin for histological examination. Ovulated cumulus-oocyte complexes were collected from oviducts at hCG 16h and counted. Pure granulosa cells were isolated from CON and cKO ovaries at hCG 11 h by puncturing both ovaries with 25G needles in 1X PBS, and filtered (BD Falcon, Cell Strainer, 40 μm Nylon, Mexico). Cells were pelleted and snap frozen for mRNA extraction and RT-qPCR analysis.

For mating evaluation following hormonal stimulation, mature (> 6 weeks) female mice were injected IP with either 5 IU of eCG around 1 p.m.; 5 IU of hCG at 3 p.m. on the day of proestrus; or 5 IU of eCG followed by the injection of hCG 48 h later. Females were then mated with WT males overnight. Males were removed the next morning and the presence of copulatory plug was verified by visual examination. Females were visually monitored for presence of pregnancy, cages were inspected daily to record parturition dates and litter sizes.

Histology and follicle count

Tissue processing and embedding were performed using standard histological techniques as previously described (160). Ovaries were serially sectioned at 5 μM and paraffin sections stained with hematoxylin and eosin (HE) (Thermo Fisher Scientific #72511, Thermo Fisher Scientific #71311). Cryostat sections were stained with Oil-red-O.

For evaluation of cumulus-oophorus expansion, HE stained ovaries from superstimulated mice (1 slide from middle of ovary from 6 CON and 6 cKO, hCG 11 h) were assessed and follicles containing expanded cumulus-oophorus complexes (COC) were counted to obtain an average per genotype. Similarly, for assessment of luteal formation in ovarian sections from superstimulated mice, the CL were counted to obtain an average per genotype from 1 slide from the middle of each ovary from 5 CON and 5 cKO, hCG 24h; 3 CON and 3 cKO, hCG 36h).

RNA extraction and real-time PCR

RNA was extracted from tissues with PureLink RNA mini extraction kit according to the manufacturer's instructions (Invitrogen #12102385). Reverse transcription was performed using the SuperScript III reverse transcription enzyme (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). All transcripts were amplified following the 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. Relative mRNA levels were determined using Pfaffl's mathematical model (345), with *B2m*, *Hprt*, and *Ywha2* as the housekeeping genes. Primer sequences for the transcripts measured can be found in Table S3.1.

Immunofluorescence

Slides of formalin or paraformaldehyde-fixed paraffin-embedded ovaries from CON and cKO mice were rehydrated, boiled in 10 mM sodium citrate (pH 6) for 30 minutes, and cooled to room temperature, then blocked 1 hour with 5% bovine serum albumin (Jackson ImmunoResearch Laboratories) in 0.1% PBST. Slides were then incubated overnight at 4°C with antibody against SF-1 (1:100 in BSA5% / PBST 0.1%, Cell Signaling #12800, RRID AB_2798030), then incubated 1 hour with second antibody CY3 conjugated goat anti-rabbit IgG (1:300 in 5% BSA/0.1% PBST, Jackson ImmunoResearch #111-165-144 or Invitrogen #A11008). Finally, slides were counterstained with DAPI (Thermo Fisher Scientific #D1306), diluted 1:1000 in PBS, for 5 minutes before being washed three times in PBS and mounted with Permafluor (Thermo Fisher Scientific #TA006FM).

Steroid hormone measurement

Serum progesterone (P4) levels were measured by enzyme-linked immunosorbent assay (Cayman Chemical #582601) following manufacturer's instructions. Pituitary and serum FSH levels were measured using an in-house sandwich ELISA protocol as previously described (346). Pituitary LH levels were assessed with Abcam kit (Abcam #235648) following the manufacturer's instructions. Serum LH levels were determined as previously described (347).

Ovariectomy and ovarian transplant

For ovariectomy, CON and cKO females were anesthetized, the ovarian fat pad with the ovary exteriorized, the bursa opened, and ovaries removed. Following a 3-week recovery period, ovariectomized (Ov^x) females were euthanized and hypothalami, pituitary glands, and serum were

collected.

For ovarian transplant, ovaries were collected from CON or cKO females and maintained in PBS at room temperature. Recipient females were anesthetized and ovariectomized and donor ovaries were then grafted into the bursal cavity of recipients to produce CON-CONov, CON-cKOov or cKO-CONov animals (see schema in Figure 3.8A). After a 3-week recovery, the mice were mated with WT males for a period of 4 months to assess reproductive performance. Pups from gestation were genotyped to confirm that products of conception were derived from the transplanted ovary.

Statistical analyses

All values are expressed as mean ± SEM. To determine significance between means of normally distributed data sets, comparisons were made using Student's t-test analysis with Welch's correction. Non-normally distributed data sets were compared using Mann-Whitney test on Graphpad Prism V7.0 (Graphpad Software). When justified, log transformation was employed. $P < 0.05$ was considered significant.

3.4 RESULTS

SF-1 depletion is specific to ovary and pituitary

Adult (> 8 weeks-old) female cKO mice showed tissue-specific depletion of *Nr5a1* mRNA levels in the pituitary gland and not in other organs that express SF-1. Relative *Nr5a1* expression in whole ovaries from cycling cKO females was not altered (Figure 3.1A). SF-1 depletion was detected, however, in granulosa cells hCG 11 h (Figure 3.1B), a time point when the superstimulated ovary contains a large number of antral follicles with expanded cumulus-oophorus

complexes (COCs) on the verge of ovulation, and some 7 h after initiation of the ovulatory expression of PR specifically in the granulosa cells (342). Whole ovaries collected 24 h post hCG injection, a timepoint when superstimulated ovaries contain CL, also showed a significant reduction in *Nr5a1* expression compared to CON (Figure 3.1B). Immunofluorescent staining of SF-1 in the pituitary gland showed a smaller number of SF-1 positive cells in cKO than in CON animals (Figure 3.1C). Similar reduction in SF-1-positive cell number was observed in granulosa cells of antral follicles (Figure 3.1D). In the CL, many luteal cells showed absence of nuclear SF-1 staining, in contrast to what was observed in the CON (Figure 3.1E). These results demonstrate that while the PR-Cre$^{(+)}$;*Nr5a1*$^{f/f}$ mouse model induces pituitary- and ovary-specific depletion of SF-1, the recombination does not occur in all targeted cells and a few SF-1 positive cells remain present in cKO animals.

Reproductive performance of cKO female mice

To assess reproductive capability, cKO and CON females were coupled with males for a six-month breeding trial. Four of the seven cKO females in the trial were sterile; the other three cKO females had significantly lower numbers of litters and total number of pups with a lower relative fecundity number (1.58) when compared to CON females (24) (Table 3.1, Figure 3.2A-B). To determine their ovulatory capacity, mature CON and cKO females were mated with wild-type males and oviducts were collected from females with a copulatory plug, approximately 5 h post mating. Few to no oocytes were retrieved from the oviduct ampulla of cKO females (Figure 3.2C). Daily assessment of the vaginal smears showed an irregular pattern in the estrous cycles of cKO females, with longer periods in estrus, resulting in fewer cycles in a 28-day evaluation period (Figure 3.2D-E, Figure. S3.1A). Presence of copulatory plugs when mated with males indicated

that cKO females demonstrated mating behavior, and the frequency of copulation was higher for cKO than CON females (Figure 3.2F, Figure S3.1B).

Absence of ovulation and ineffective cholesterol transport in mature cKO ovaries.

The weights of ovaries collected from mature female cKO mice were significantly reduced relative to CON (Figure 3.3A). Hematoxylin-eosin staining showed the presence of primary, secondary, and antral follicles, but no CLs in cKO ovaries (Figure 3.3B-C). Ovaries collected from mature cKO females injected with hCG only did not rescue CL formation, suggesting that luteinization is affected by absence of SF-1 in the granulosa cells (Figure 3.3D). Abnormal hypertrophic structures were observed in the interstitial tissue of the cKO ovaries, and Oil-red-O staining showed strong levels of lipid accumulation in the stroma compared to CON (Figure 3.3E-F), hinting that cholesterol homeostasis is severely affected in the cKO ovaries.

While *Nr5a1* levels in whole ovaries of cKO mature mice were comparable to CON (Figure 3.1E), a significant reduction in the expression of genes that code for cholesterol transport proteins *Scarb1*, *Star*, *Ldlr,* and *Scp2* was observed (Figure 3.3G). The gene *Lipe*, which codes for the hormone-sensitive lipase that hydrolyses cholesteryl esters found in lipid droplets into free cholesterol for steroidogenesis, was also underexpressed, while the *Acat1* gene, coding for the mitochondrial acetyl-CoA acetyltransferase that transforms free cholesterol into lipid droplets, was significantly upregulated when compared to CON mature mouse ovaries (Figure 3.3G). Additionally, *Cyp11a1,* coding for the mitochondrial enzyme p450scc that catalyzes the initial transformation of cholesterol into pregnenolone, as well as *3bHsd1*, which codes for the 3β-hydroxysteroid dehydrogenase, were significantly reduced in the cKO female ovaries (Figure 3.3H). A non-significant reduction was observed in the expression of *Cyp19a1,* which codes for

aromatase, the enzyme that transforms androgens into estrogens, and is often used as an indicator of estradiol synthesis. The aldo-keto reductase family 1 member, C18 gene *Akr1c18* (also known as 20α-reductase), required for the inactivation of progesterone, was completely absent in cKO ovaries, possibly due to the absence of CL formation in the ovaries of mature cKO mice (Figure 3.3H).

Impaired pituitary function in cKO females.

Both SF-1 and PR are expressed in gonadotropes, and so the pituitary glands of mature CON and cKO females were collected to evaluate gonadotropin synthesis. The expression of the gonadotropin subunits (*Cga*, *Fshb*, and *Lhb*) was significantly reduced (Figure 3.4A), which was associated with decreased serum FSH levels and pituitary LH content cKO females, and a tendency towards decrease in pituitary FSH content (Figure 3.4B-C). Interestingly, expression of the gonadotropin-releasing hormone receptor (*Gnrhr*) was also significantly reduced in cKO pituitary (Figure 3.4A).

To ensure that the observed phenotype in the pituitary gland was not the result of impaired GnRH neuron function, hypothalami were collected from CON and cKO females for mRNA abundance analysis. The expression of *Nr5a1* in the hypothalami of cKO females did not differ from that observed in the CON animals (Figure 3.1A), and *Gnrh* and the kisspeptin receptor gene *Kiss1r* expression in cKO were comparable to CON (Figure 3.4E). Interestingly, a significant increase in *Kiss1* and decrease in progesterone receptor gene *Pr* were observed in the hypothalamus of cKO females (Figure 3.4E), likely due to decreased estrogen negative feedback from the ovary.

To confirm the observed phenotype in the pituitary gland was not due to a possible influence of SF-1 depletion in the ovary, CON and cKO mature females were ovariectomized (Ov^x) and their

serum and pituitary glands subsequently collected. Comparison of pituitary gland gene expression in mice before and after Ov^x showed that *Nr5a1* mRNA abundance was significantly lower in CON-Ov^x females than in cKO, and levels were nearly null in cKO-Ov^x mice (Figure 3.4F). On the other hand, *Gnrhr* levels were significantly lower in cKO females than in CON-Ov^x, and lowest in cKO-Ov^x females (Figure 3.4F). The expected increase in *Cga* expression levels post Ov^x was observed in both CON and cKO animals, though cKO-Ov^x levels were significantly lower than CON-Ov^x (Figure 3.4F). Interestingly, the expected increases in both *Lhb* expression and serum LH levels observed in CON-Ov^x mice was absent in cKO-Ov^x females (Figure 3.4, D and F). The *Fshb* expression levels were slightly increased in cKO females after Ov^x, but were significantly lower that CON-Ov^x, and serum FSH levels were not increased to the level observed in CON-Ov^x animals (Figure 3.4, D and F). These results indicate that reduction of SF-1 expression in female mice gonadotropes leads to impaired pituitary gland function.

Impaired cumulus expansion in stimulated cKO ovaries.

To assess the presence of an ovarian phenotype and remove the impact of reduced gonadotropin secretion in cKO females, exogenous gonadotropins were administered to both CON and cKO mature females. Ovaries collected from treated cKO females showed normal antral follicle formation when collected 48 h post eCG injection (data not shown). When oviducts were collected from superstimulated mice 16 h after hCG injection, the number of oocytes retrieved from the ampulla was substantially lower than from CON (Figure 3.5A).

A delay was observed in the ovarian Cre expression of the PR-Cre transgenic mouse used to produce this model, where recombination activity was described as being active in granulosa cells at hCG 12 h in superstimulated females, and not at the moment of PR expression initiation,

between hCG 4-8 h (342). Consistent with this Cre expression pattern, SF-1 depletion was significant in granulosa cells of the peri-ovulatory follicle (hCG 11 h) (Figure 3.1B). Analysis of histological sections of ovaries collected hCG 11 h, just prior to expected ovulation (12-14 h after hCG) showed a reduced number of follicles containing oocytes with expanded cumuli in cKO compared to CON ovaries (Figure 3.5B-D). Gene expression analysis of isolated granulosa cells collected 11 h post-hCG injection showed a significant decrease in the expression of essential cumulus expansion stimulators amphiregulin *(Areg)*, epiregulin *(Ereg),* and prostaglandin synthase 2 *(Ptgs2)*, but not betacellulin *(Btc)* (Figure 3.5E). There was a reduction in *Pr* expression, a further key regulator of these cumulus expansion genes (348). Of interest, a significant reduction of the vascular endothelial growth factor A (*Vegfa*) was also observed in cKO animals at this time point (Figure 3.5E). Also, while the cumulus hyaluronan matrix stabilizer *Tnfaip6* was not affected, the hyaluronan synthase 2 (*Has2*) was significantly increased in the cKO granulosa cells, indicating abnormal extra cellular matrix stability (Figure S3.2A).

Impaired luteinization in stimulated cKO ovaries

Ovaries of immature superstimulated females were collected 24 h and 36 h post-hCG injection to observe the frequency of luteinization of cKO granulosa cells and consequent CL formation. Ovarian histology showed presence of many large CLs in CON ovaries at both post-hCG 24 h and 36 h (Figure 3.6, A and D). In the cKO ovary, the CLs were less frequent, of smaller volume, and structurally aberrant compared to CON ovaries, with an oocyte trapped in some (Figure 3.6B, C, E and F). Quantification of CL numbers in CON and cKO ovaries showed that SF-1 depletion in the ovary resulted in a significant reduction at hCG 24 h, but not at hCG 36 h

(Figure 3.6G). The CL observed in the ovaries at hCG 36 h, however, were smaller and some presented incomplete luteinization or trapped oocytes (Figure 3.6E-F).

To assess the steroidogenic capacity of these abnormal CL, progesterone levels were assessed in the superstimulated CON and cKO females at hCG 16 h, 24 h, 36 h, 48 h, and 72 h (Figure 3.6H). While superstimulated CON animals showed an increase of progestrerone levels at hCG 36h, progesterone levels in cKO females remained constant from hCG 16 h to 48h, and were significantly lower than in CON at hCG 24 h, 36 h, and 48 h (Figure 3.6H).

To further analyze the role of SF-1 in ovarian steroidogenesis during peri-ovulatory events and in luteal cells, mRNA levels for genes regulating cholesterol homeostasis and steroidogenesis were quantified in CON and cKO granulosa cells collected at hCG 11 h and whole ovaries collected at hCG 24 h and 36 h, time points where *Nr5a1* levels were significantly reduced (Figure 3.7A). While no difference in expression was observed between genotypes in gonadotropin receptors in granulosa cells at hCG 11 h, *Fshr* was significantly increased in whole ovaries at both hCG 24 h and 36 h, and *Lhr* was upregulated at hCG 36 h of cKO ovaries (Figure 3.7B). Cholesterol transport genes that code for scavenger receptor class B type I (*Scarb1*) and sterol carrier protein 2 (*Scp2*) were upregulated in cKO granulosa cells (hCG 11h) when compared to CON, while the low-density lipoprotein receptor gene (*Ldlr*) was reduced (Figure 3.7C). Similarly, *Scp2* was increased, and the gene coding for steroidogenic acute regulatory protein (*Star*) were decreased in cKO ovarian cells compared to CON at hCG 24h. In contrast, *Scp2* levels were significantly downregulated and acetyl-coA acetyltransferase 1 (*Acat1*) upregulated at hCG 36h when compared to CON. The rate limiting enzyme in intracellular *de novo* cholesterol synthesis 3-hydroxy-3-methylglutaryl-coenzyme A (*HmgCoA*), was unaffected by SF-1 depletion at any of these three time points.

In accordance with the measured increase in cholesterol transport protein gene expression, steroidogenic genes *Cyp11a1* and *3bHsd* were significantly increased in granulosa cells at hCG 11 h, and *Cyp19a1* showed a tendency towards upregulation as well (Figure 3.7D), suggesting increased steroidogenesis in cKO granulosa cells at this time point compared to CON, though serum hormone levels were not measured to confirm this. At hCG 24 h, steroidogenic genes *Cyp11a1* and *Cyp19a1* were significantly reduced and *17b1Hsd* increased, while *3bHsd* and 17α-hydroxylase (*Cyp17a1*) cKO expression levels remained comparable to CON (Figure 3.7D). At hCG 36 h, both *17bHsd1* and *Cyp17a1* were significantly increased, while *3bHsd* and *Cyp19a1* showed a tendency towards downregulation in the absence of SF-1. Interestingly, *Akr1c18* expression was strongly reduced at hCG 36 h, a reduction also observed above in whole ovaries collected from mature, non-stimulated mice.

Exogenous gonadotropins do not rescue fertility in cKO females

In order to evaluate the impact of the abnormal ovarian function caused by SF-1 depletion in the peri-ovulatory ovary, *in vivo* mating experiments using exogenous gonadotropin administration were carried out. To determine if fertility could be restored in mature cKO mice, females were injected with either eCG, to induce follicular growth, hCG, to mimic LH surge and induce ovulation, or both eCG and hCG (Table 3.2). Doses of both eCG and hCG were also administered to CON females as positive control of our injection protocol. Females were housed with males overnight, then separated in the morning. The presence of a copulatory plug was considered evidence of mating for the reproductive assessment. While two out of the three CON females with copulatory plugs had offspring following these injections, only one of the 17 cKO

females with copulatory plugs after receiving eCG-only injection birthed pups. The hCG only and eCG + hCG injections were likewise insufficient to restore fertility in cKO females.

Loss of fertility in some ovariectomized CON females with transplanted cKO ovaries.

To further discriminate the relative contributions of pituitary versus ovarian defects to the phenotypes observed in cKO females, CON and cKO ovaries were transplanted under the ovarian bursa of Ov^x females of the opposite genotype (see the schematic representation in Figure 8A). A separate group of Ov^x-CON received CON ovaries (CON-CONov) as a technical control group to assess the effect of surgical manipulation. The reproductive capacity of females bearing transplanted ovaries was assessed in a fertility trial over four months with WT males. While all Ov^x-cKO females with CON ovaries (cKO-CONov) were infertile and all CON-CONov were fertile, four of the nine (43%) Ov^x-CON females with cKO ovaries (CON-cKOov) were infertile (Table 3.3). The five fertile CON-cKOov females produced comparable numbers of litters and pups per litter to CON-CONov females (Figure 3.8B-D).

Gene expression in pituitary glands collected from the transplanted females showed that *Nr5a1* and *Lhb* was significantly decreased in both fertile and infertile CON-cKOov females when compared to CON-CONov (Figure 3.8E). The transcript abundance of *Gnrhr*, *Cga* and *Fshb* was unaffected by the transplantation of a cKO ovary in a CON host (Figure 3.8E). Surprisingly, the fertility status of the CON-cKOov females did not affect the pituitary gland gene expression profile (data not shown). Similar to what was observed in the pituitary gland of cKO females (Figure 3.3A), *Gnrhr*, *Fshb* and *Lhb*, but also *Cga*, mRNA levels were significantly under-expressed in the cKO-CONov transplanted animals. Interestingly, the recombination of *Nr5a1* in cKO-CONov

mice was more effective (over 80%) than what was observed in mature (non-transplanted) cKO females (average 50% recombination) (Figure 3.1A).

Ovaries collected from CON-CONov and fertileCON-cKOov females showed presence of CL (Figure 3.9A-B), while ovaries from infertile CON-cKOov and cKO-CONov were hypomorphic and did not show presence of late stage folliculogenesis (Figure 3.9C-D). Interestingly, hypertrophic cells structures similar to those observed in ovaries in non-stimulated cKO females (Figure 3.3C) were present in the ovary medulla of both fertile and infertile CON-cKOov and cKO-CONov females (Figure 3.9B-F). While most ovaries of the transplanted females were collected on the first day of estrus after completion of the fertility trial, females were over seven months old at that point, and some were no longer cycling (data not shown). Nevertheless, the collected ovaries showed some interesting results. Ovaries were collected from all groups, including fertile and infertile CON-cKOov females. Similar to what was observed in mature (non-stimulated) cKO females (Figure 3.4G-H), gene expression analysis in the ovaries collected from the transplanted cKO-CONov females showed a significant reduction in *Cyp11a1*, *3bHsd* and *Scp2* when compared to CON-CONov (Figure S3.2B). Due to large variation in the CON-CONov group, the reduced expression of *Star* in ovaries from CON-cKOov and cKO-CONov females was not significant (Figure S3.2B). As observed in the ovaries of mature (non-transplanted) cKO females (Figure 3.1A), *Nr5a1* expression in CON-cKOov and cKO-CONov females were comparable to control, though there was a wide variation of the expression levels in both groups.

3.5 DISCUSSION

Abundant evidence implicates SF-1 in mediating VMH and pituitary specialization and gonad development *in utero* (41, 198, 273, 337, 349). Much less is understood about the role of

SF-1 in ovulation and luteinization. We generated a novel transgenic mouse line where the expression of SF-1 is depleted exclusively in cells that express PR. SF-1 has been identified as the earliest transcription factor involved in gonadotrope cell specification, detected as early as embryonic day 13.5 and initiating *Gnrhr*, *Fshb* and *Lh*b transcription (37, 350). As such, it was unsurprising to observe that the PR-Cre$^{(+)}$;*Nr5a1*$^{f/f}$ cKO females were infertile, due in large part to the pituitary phenotype. Exogenous gonadotropin treatments and ovarian transplantation experiments allowed us to circumvent the pituitary dysfunction to demonstrate, for the first time, that SF-1 plays an important role in granulosa and luteal cells, where it regulates the transcription of cumulus expansion genes to induce ovulation. We further showed that it is required for normal luteinization in the post-ovulation ovary. In addition, we showed that the absence of SF-1 in ovaries of mature mice can lead to female infertility, independent of the pituitary phenotype.

It was previously shown that pituitary specific ablation of SF-1 results in secondary hypogonadism and impaired steroidogenesis in both the testis and the ovary (337). Particularly, αGSU-cre;SF1 cKO females have severely hypoplastic ovaries and are sexually immature (351). While the ovarian phenotype observed in PR-Cre$^{(+)}$;*Nr5a1*$^{f/f}$ cKO females is caused in part, and compounded by pituitary SF-1 depletion, it is important to note that these females were sexually mature and did copulate with males despite the significant decrease in gonadotropin production. This can be explained by the minimal expression of the Cre recombinase in sexually immature PR-Cre$^{(+)}$ females (342). Another explanation might be the partial recombination observed in the pituitary gland of cKO females, where *Nr5a1* levels where reduced approximatively 50% compared to control mice and still permitted reproductive behavior. Though the level of SF-1 depletion in the αGSU-cre;SF1 cKO pituitary gland was not quantified, it is possible that it was more pronounced than in our PR-Cre$^{(+)}$;*Nr5a1*$^{f/f}$ cKO females. Still, in contrast to the αGSU-

cre;SF1 cKO model, exogenous gonadotropin stimulation to mimic pituitary function did not restore ovarian function to the level observed in CON females. In fact, ovulatory capacity of the cKO female was still hindered after gonadotropin stimulation.

Most studies attribute a role in ovulation to SF-1 because of its regulation of hormone biosynthetic enzymes, including STAR, CYP11A1, CYP19, and 3BHSD1, in steroidogenic cells of the ovary [Reviewed in (331)]. Here, we demonstrate that SF-1 recombination in granulosa cells of the pre-ovulatory follicle results in impaired cumulus expansion, which could have contributed to the reduced number of ovulated oocytes in cKO females following exogenous gonadotropin administration. Expression of epidermal growth factor (EGF)-like factors *Areg* and *Ereg*, but not *Btc*, was significantly reduced in the granulosa cells of cKO females. The expression of these genes is regulated by LH stimulation of cAMP in granulosa cells (352). It has been reported that the expression of *Areg* and *Ereg* is activated via the PKA-CREB pathway, where phosphorylated CREB binds the CRE site on the *Areg* promoter and the SP1 site on the *Ereg* promoter (353, 354). SF-1 interacts with CREB and SP1 to induce the expression of steroidogenic genes such as *Star*, *Cyp11a1,* and *Cyp19* (355-357). It is possible that SF-1 regulates the expression of EGF-like factors through CREB and SP1 interaction as well, though this hypothesis remains to be tested.

Another pathway through which SF-1 may regulate cumulus expansion is via prostaglandin biosynthesis. *Ptgs2* expression is downregulated in peri-ovulatory granulosa cells of cKO females. COX2 (product of the *Ptgs2* gene) regulates the synthesis of prostaglandin E2 (PGE2), which has been identified as the key prostaglandin in murine cumulus expansion and ovulation (358). The reduction of both AREG and EREG in the cKO females could explain the observed reduction of *Ptgs2*, a downstream target of the ERK1/2 pathway triggered by activated EGFR receptors in

granulosa cells (348). Finally, a growing body of research has demonstrated that PR is essential for follicle rupture in mammals (reviewed in (359), and the observed decrease in *Pr* expression in the granulosa cells of cKO females could contribute to the impaired release of oocytes in this model. Additionally, *Pr* drives the recombination level of *Nr5a1* in this model, and its reduced expression could explain the low recombination observed in granulosa cells. The presence of entrapped oocytes in the CL of ovaries at hCG 24 h and 36 h further supports the essential role of SF-1 in follicle rupture and ovulation. Recently, a study in *Drosophila* showed that *fushi tarazu-factor 1* (*ftz-f1;* the *Drosophila* homologue of SF-1) was required in the late stages of oogenesis to induce ovulation and follicle rupture (329). The loss of *Ftz-f1* in follicular cells blocked the final steps of folliculogenesis, rendering the follicles incompetent for ovulation and rupture. We contend that, in accordance with the novel findings of Knapp et al. (329), the results obtained with our cKO mice demonstrate a role for SF-1 in regulating mammalian follicle maturation and ovulation.

To our knowledge, no studies have reported ovarian lipid accumulation in the interstitial tissue due to impaired pituitary gonadotropin secretion. The hypertrophy-hyperplasia in the interstitial space of cKO ovaries might have been due to the combination of reduced gonadotropin secretion and inefficient cholesterol uptake in steroidogenic cells of the ovary. The presence of such structures in both cKO ovaries transplanted in CON hosts and vice versa demonstrates that both the absence of SF-1 in pituitary gland and in the ovary can independently cause ovarian hypertrophy-hyperplasia. The expression of genes involved in cholesterol homeostasis and steroidogenesis were altered in cKO ovaries before exogenous gonadotropin injections as well as after, though to a lesser extent. Nevertheless, a significant decrease in the number of CL formed and reduced progesterone production was observed in superstimulated cKO females. A possible cause is the abnormal expression of essential luteinizing factors such as the vascularization factor

Vegfa in luteinizing granulosa cells prior to ovulation, and important steroidogenic factors like *Star* and *Cyp11a1* at hCG 24 h. Reduced expression of *Cyp19a1* was also observed at hCG 24h, indicating impaired estrogen biosynthesis, which an essential role in stimulating cholesterol synthesis and uptake in the CL (reviewed in (360). Interestingly, an increase of *17bHsd1* at both hCG 24 h and 36 h, as well as an increase of *Cyp17a1*, which codes for 17α-hydroxylase, at 36 h only, was observed in cKO luteal cells. 17βHSD -1 is involved in different steps of androgen and estrogen synthesis, including the transformation of androstenedione into testosterone and the transformation of estrone into estradiol, and 17α-hydroxylase hydroxylates both pregnenolone and progesterone (361). The activity of 17βHSD-1 is specific to the granulosa cells and disappears in the CL (362), further suggesting impaired luteinization in the cKO ovaries. The increased expression of 17α-hydroxylase could be an indicator of upstream substrate accumulation due to reduced aromatase expression, though this remains to be verified. Nevertheless, it is clear from these results that SF-1 absence during luteinization leads to abnormal luteal steroidogenic activity and ultimately, abnormal steroid production in the CL. This, together with the reduced ovulation and compromised cumulus expansion, evidently demonstrate that the responses of the ovary to gonadotropins are impaired by the loss of SF-1 in granulosa and luteal cells.

The exogenous gonadotropin injections and ovarian transplantation experiments in this study allowed us to further evaluate the ovarian phenotype of cKO females by removing the contribution of an upstream effect of SF-1 depletion in the pituitary gland. The absence of pregnancy in cKO females that were mated after exogenous gonadotropin stimulation suggested there was an ovarian phenotype preventing normal fertility. Only one cKO female successfully birthed pups following eCG only injection, which could be explained by a less effective recombination of SF-1 in this particular mouse. This contrasted with the two separate phenotypes

ensued from the ovarian transplantation, where some of the CON-cKOov females became infertile (4 out of 9), whereas the others maintained fertility (5 out of 9). One interpretation for this divergence is that poor recombination of PRCre-SF-1 occurs in the fertile group, whereas a better recombination resulted in the sterile group. This was not evidenced by ovarian gene expression levels measured from these animals, though the tissues analyzed were collected from aged (non-stimulated) transplanted females after they underwent a four-month long fertility trial. Inefficient PRCre-SF-1 recombination in the ovary could also explain the discrepancy observed between the exogenous gonadotropin treatment experiments and the fertile CON-cKOov females. Treating females with eCG and hCG may have driven the system to induce a stronger recombination in the granulosa cells. As mentioned above, reduced *Nr5a1* expression is only measured in the ovary when females were treated with gonadotropins, not in untreated cKO mice. A greater level of recombination might therefore occur more readily with eCG and hCG injections than naturally, revealing the ovarian phenotypes. It would be important to find a different Cre driver, one that is directed at granulosa cells of mature follicles, or a novel directed gene ablation technique, to truly assess the effects of SF1 loss in these cells without the confounded pituitary defects.

3.6 CONCLUSION

In conclusion, our findings demonstrate that SF-1 is required at different levels of the HPG axis for normal female reproduction function. Infertility in cKO females is principally due to the reduced gonadotropin secretion caused by the reduced expression of SF-1 in the pituitary gland. There is also an ovarian phenotype that manifests as impaired ovulatory capacity due to the failure of cumulus expansion and reduced progesterone production due to insufficient luteinization. The absence of SF-1 specifically in granulosa and luteal cells of the ovary can lead to infertility in some

females, though the complexities of why this is not always the case remain to be elucidated. Further studies using this novel mouse model will provide valuable insights into the mechanisms of action of SF-1 in female reproductive function with the possibility of developing new, more targeted infertility treatments for women.

3.7 ACKNOWLEDGEMENTS

The authors thank Marie-Charlotte Meinsohn and Camilla C. Hughes for valuable scientific discussions and Rodrigo Gonzalez-Lopez for technical assistance. This study was funded by an operating grant from the Canadian Institutes of Health Research (PJT 166020) to BDM. OES was supported by doctoral training scholarships from the Fonds Québécois de la Recherche en Sciences (FQRS) and the Fonds Québécois de la Recherche en Nature et Technologies (FQRNT).

3.8 FIGURES

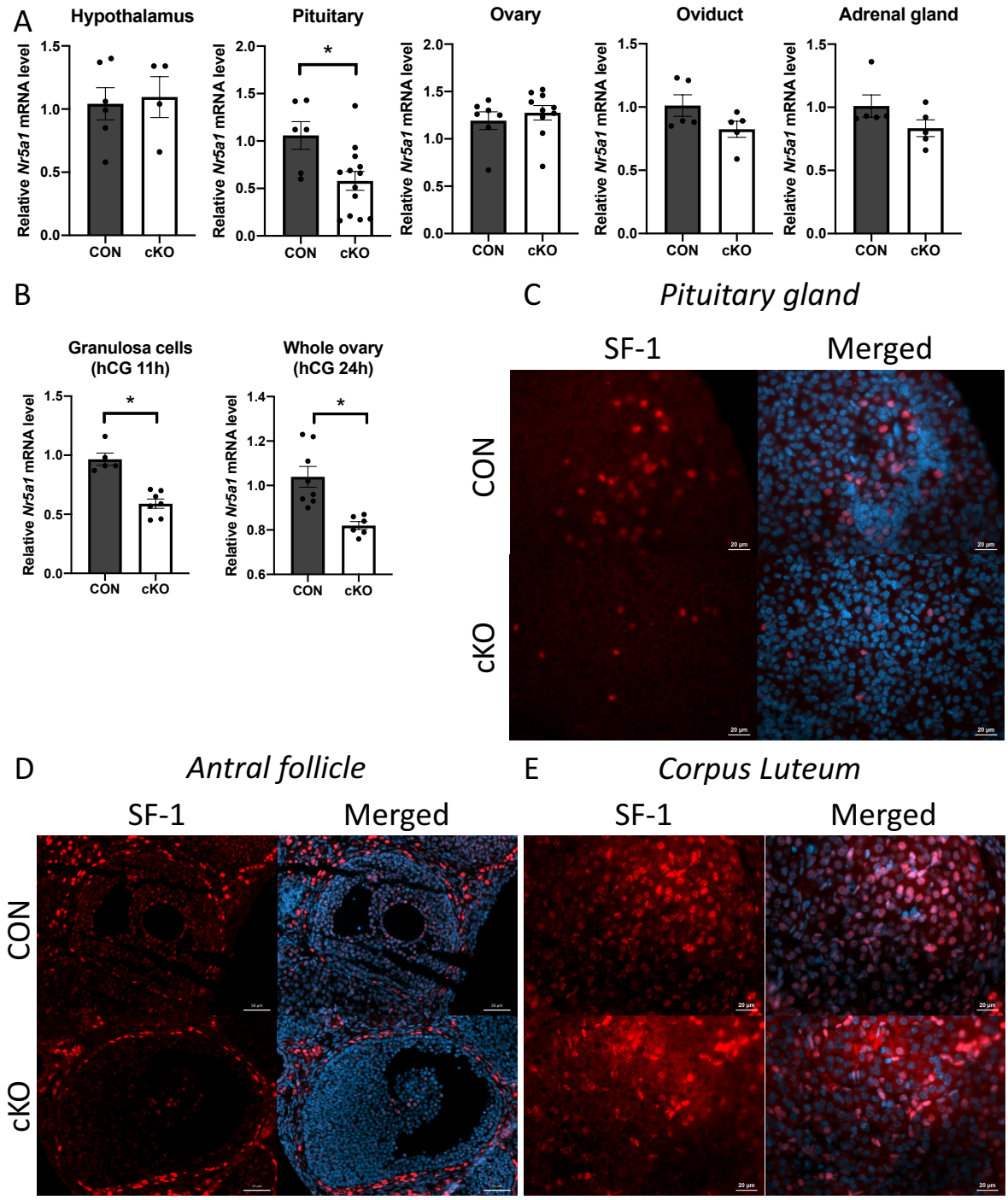

Figure 3.1 Reduced gene and protein expression of SF-1 in pituitary gland and ovary of the PR-Cre$^{(+)}$;*Nr5a1*$^{f/f}$ conditional knockout mouse.

(A-B) Tissue specific *Nr5a1* relative mRNA abundance of (A) mature CON and cKO females, as well as in (B) granulosa cells and whole ovaries of superstimulated immature CON and cKO females. Minimum of n=5 per genotype per tissue. Asterisks indicate statistically significant differences ($P < 0.05$). (C-D) Representative images showing the expression of SF-1 in (C) the pituitary gland, (D) antral follicles and (E) corpora lutea in the superstimulated ovary of CON and cKO female mice 24 h after hCG treatment. Scale bar 50 μM (antral follicle) and 20 μM (corpora lutea).

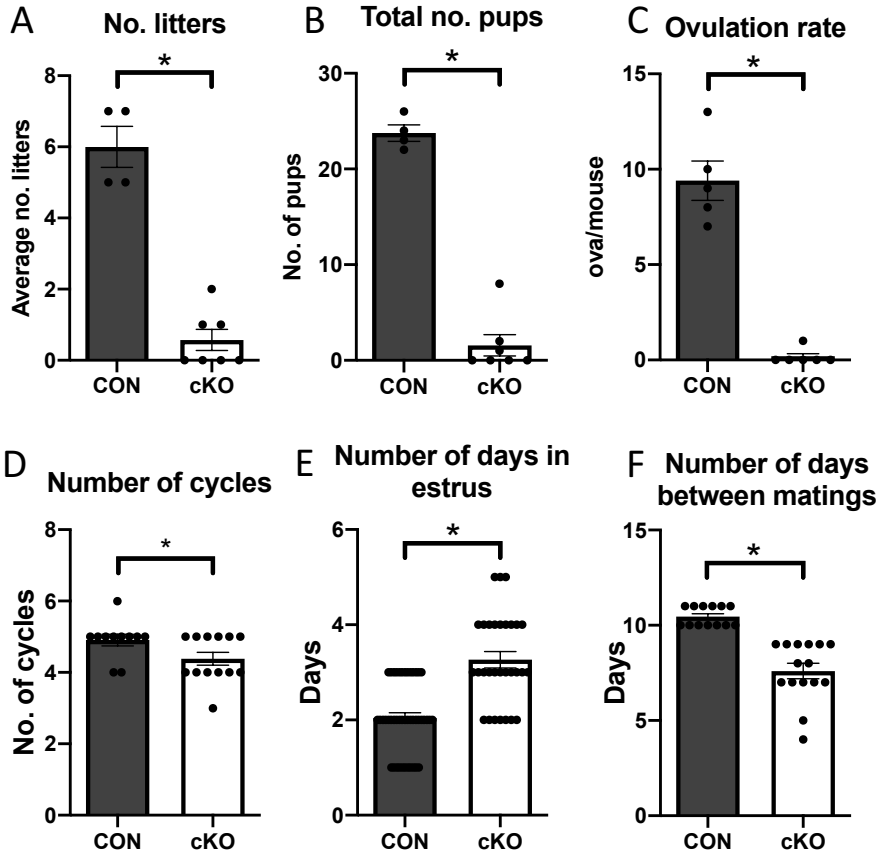

Figure 3.2 PR-Cre$^{(+)}$;*Nr5a1*$^{f/f}$ cKO females show reduced reproductive capacity.

(A) Number of litters and (B) total number of pups born from CON and cKO females. (C) Mean number of oocytes recovered from CON (n=5) and cKO (n=6) oviducts in non-stimulated mice 5 h post-mating with WT males. (D) Number of days in estrus phase and (E) number of cycles completed in CON (n=11) and cKO (n=13) mature females obtained from daily vaginal smear profiles collected over 28 days. (F) Number of days between matings in CON (n=13) and cKO (n=15) females mated with vasectomised C57BL/6 males for a 40-day breeding trial. Asterisk indicates statistically significant difference ($P < 0.05$).

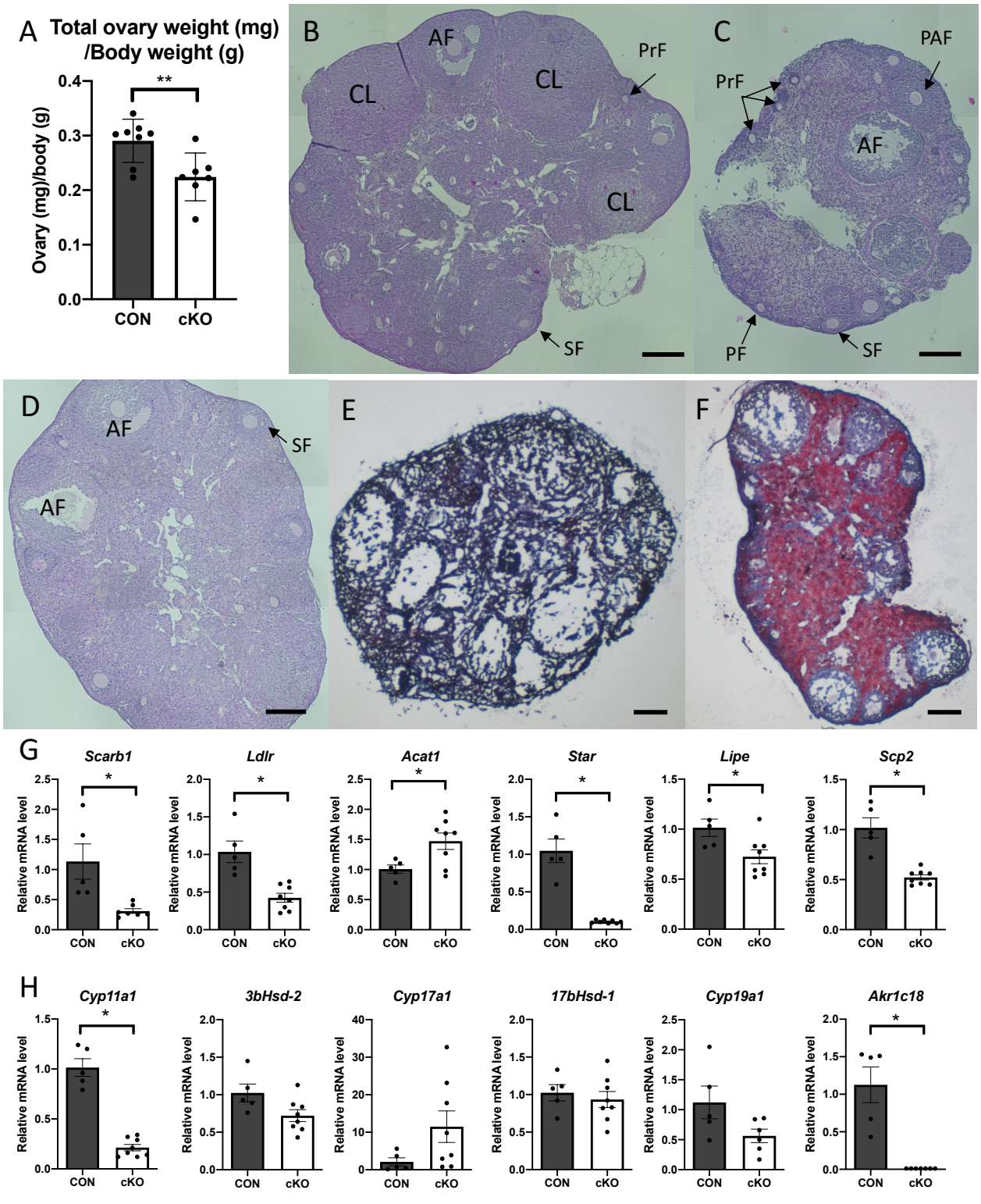

Figure 3.3 Absence of corpora lutea and abnormal cholesterol homeostasis in cKO ovaries.

(A) Total ovary weight normalized to bodyweight of CON and cKO females. (B-D) Bright field microscopy images of hematoxylin-eosin-stained sections of ovaries from 6-month-old (B) CON and (C) cKO mice collected in the estrus phase, and (D) cKO ovary collected 24 h after hCG only injection. (E-F) Bright field microscopy images of Oil-Red-O-stained sections of ovaries from 6-month-old (E) CON and (F) cKO mice collected in the diestrus phase. Scale bar, 200 μm. (G) Relative gene expression of cholesterol homeostasis factors in CON and cKO whole ovaries. (H) Relative mRNA abundance of steroidogenic genes in CON and cKO whole ovaries. Asterisk indicates statistically significant difference ($P < 0.05$). Minimum of n=7 per genotype. Unless stated otherwise, all samples were collected from mature females on the morning of the first day of estrus. PAF, preantral follicle; AF, antral follicle; CC, cumulus cells; O, oocyte.

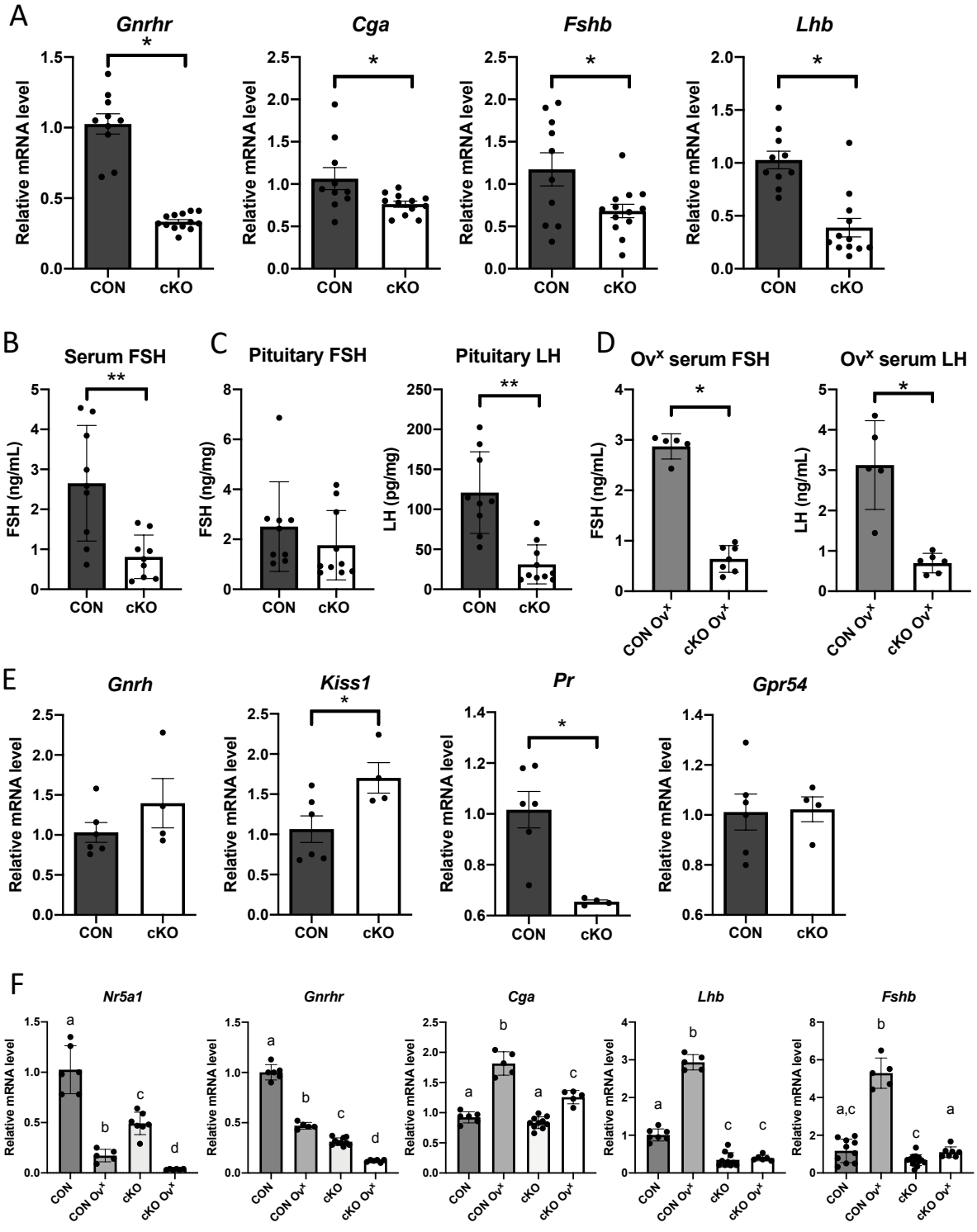

Figure 3.4 Abnormal gonadotropin gene expression and hormone secretion in cKO mature female pituitary gland.

(A) Relative mRNA abundance of gonadotropin subunits and regulators in the pituitary gland of CON and cKO mice (minimum of n=10 per genotype). (B) FSH concentration in serum collected from CON and cKO mice (n=9 per genotype). (C) FSH and LH content in pituitary glands of CON (n=10) and cKO (n=10) mature females collected the morning of the first day of diestrus. (D) FSH and LH concentration level in serum of CON (n=5) and cKO (n=7) ovariectomised females. (E) Relative abundance of *Gnrh*, *Kiss1*, *Pr* and *Gpr54* in hypothalami of CON (n=6) and cKO (n=4) females. (F) Pituitary gland gene expression before and after ovariectomy in CON and cKO mice (minimum of n=5 per group). Unless stated otherwise, all samples were collected from mature females on the morning of the first day of estrus. Asterisks and letters indicate statistically significant difference ($P > 0.05$).

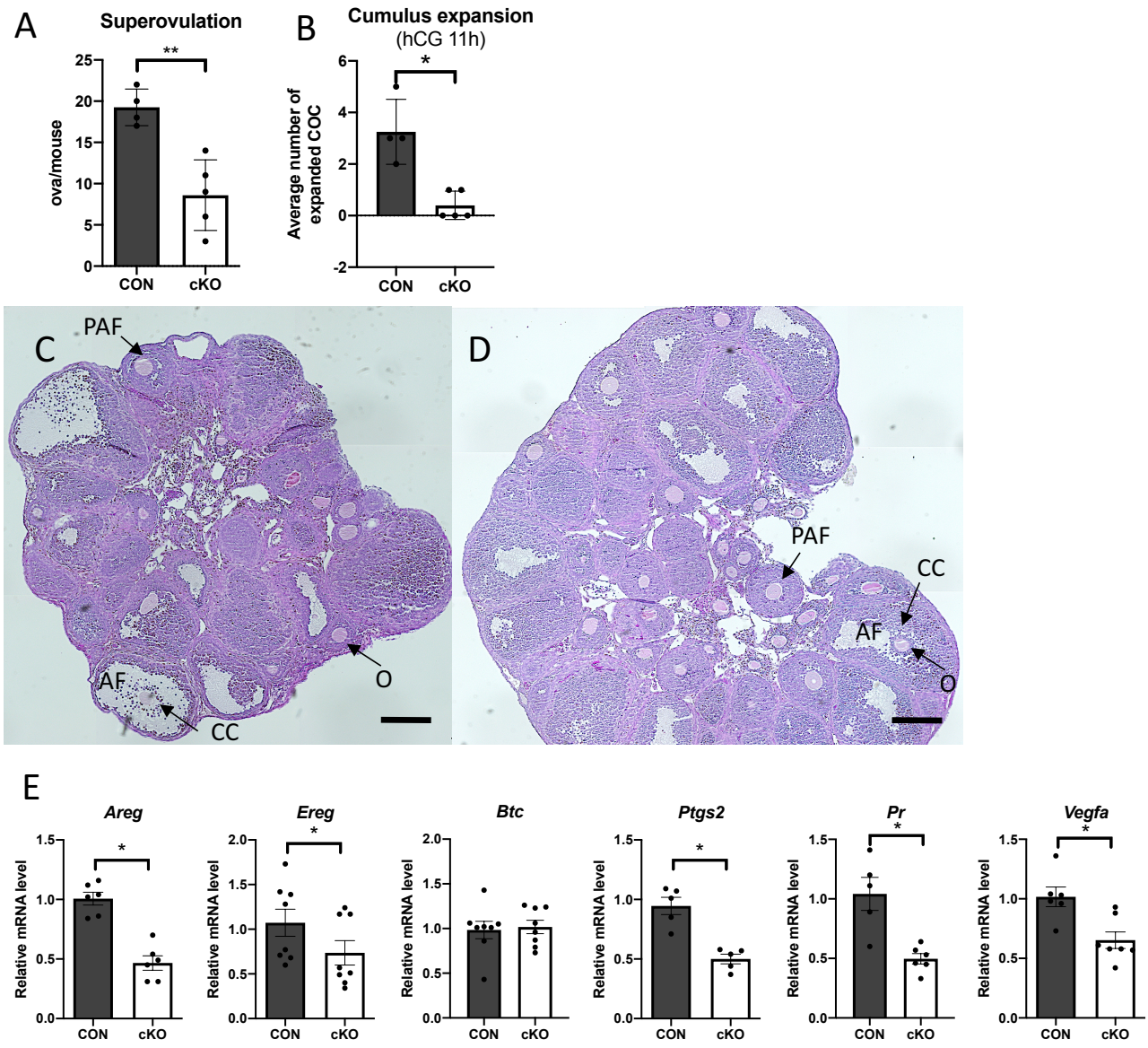

Figure 3.5 Impaired ovulation in superstimulated cKO females.

(A) Average number of oocytes recovered from CON (n=4) and cKO (n=5) oviducts in superstimulated mice 16 h after the ovulatory stimulus, hCG. (B) Evaluation of cumulus expansion by averaging the number of follicles containing expanded COCs in ovarian sections of CON (n=3) and cKO (n=5) mice collected at hCG 11 h. (C-D) Brightfield microscopy images of hematoxylin-eosin-stained sections of superstimulated ovaries from (C) CON and (D) cKO mice collected 11 h

post hCG. Scale bar, 200 μm. (E) Relative mRNA abundance of ovulation factors in granulosa cells of superstimulated CON and cKO (n=8 per genotype), collected 11 h post hCG. Asterisk indicates statistically significant difference ($P < 0.05$). PAF, preantral follicle; AF, antral follicle; CC, cumulus cells; O, oocyte.

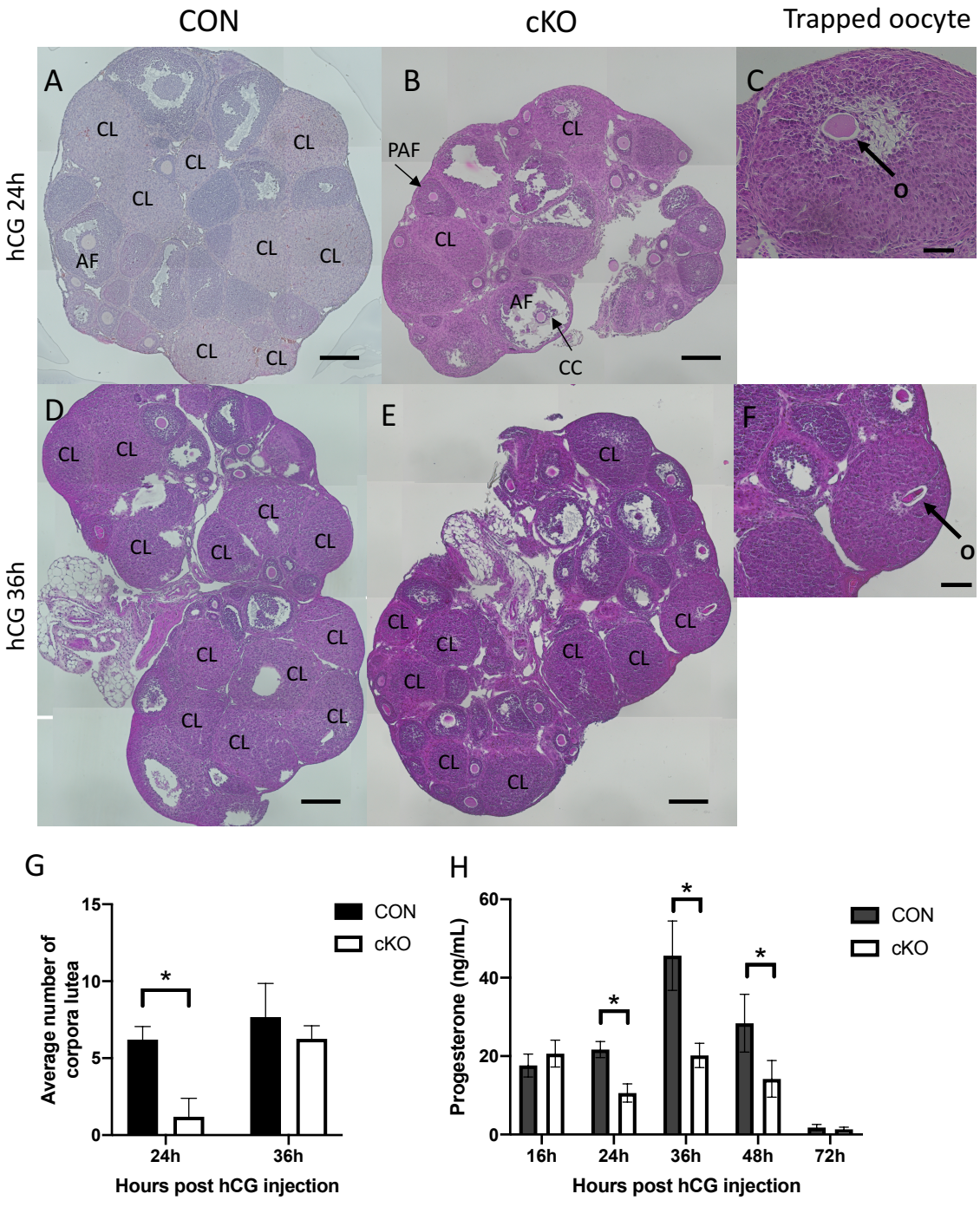

Figure 3.6 Impaired corpus luteum formation in cKO females.

(A-F) Brightfield microscopy images of hematoxylin-eosin-stained sections of superstimulated ovaries from (A) CON and (B) cKO mice collected 24 h post hCG injection, (D) CON and (E) cKO mice collected 36 h post hCG injection. Scale bars, 200 μM. Magnification of a CL showing an entrapped oocyte (O) in cKO ovary (C) 24h post hCG and (F) 36h post hCG indicated by the arrow. Scale bars, 50 μm. (G) Average number of CL present in superstimulated CON and cKO ovary sections at 24 h (n=5 per genotype) and 36 h (n=3 per genotype) post hCG injection. (H) Progesterone concentration in serum collected from superstimulated mice 16 h, 24 h, 36 h, 48 h, and 72 h post-hCG injection (minimum of n=5 per genotype, per timepoint, except for 72h: n=3). Asterisk indicates statistically significant difference ($P < 0.05$). CL, corpus luteum; PAF, preantral follicle; AF, antral follicle; CC, cumulus cells; O, oocyte.

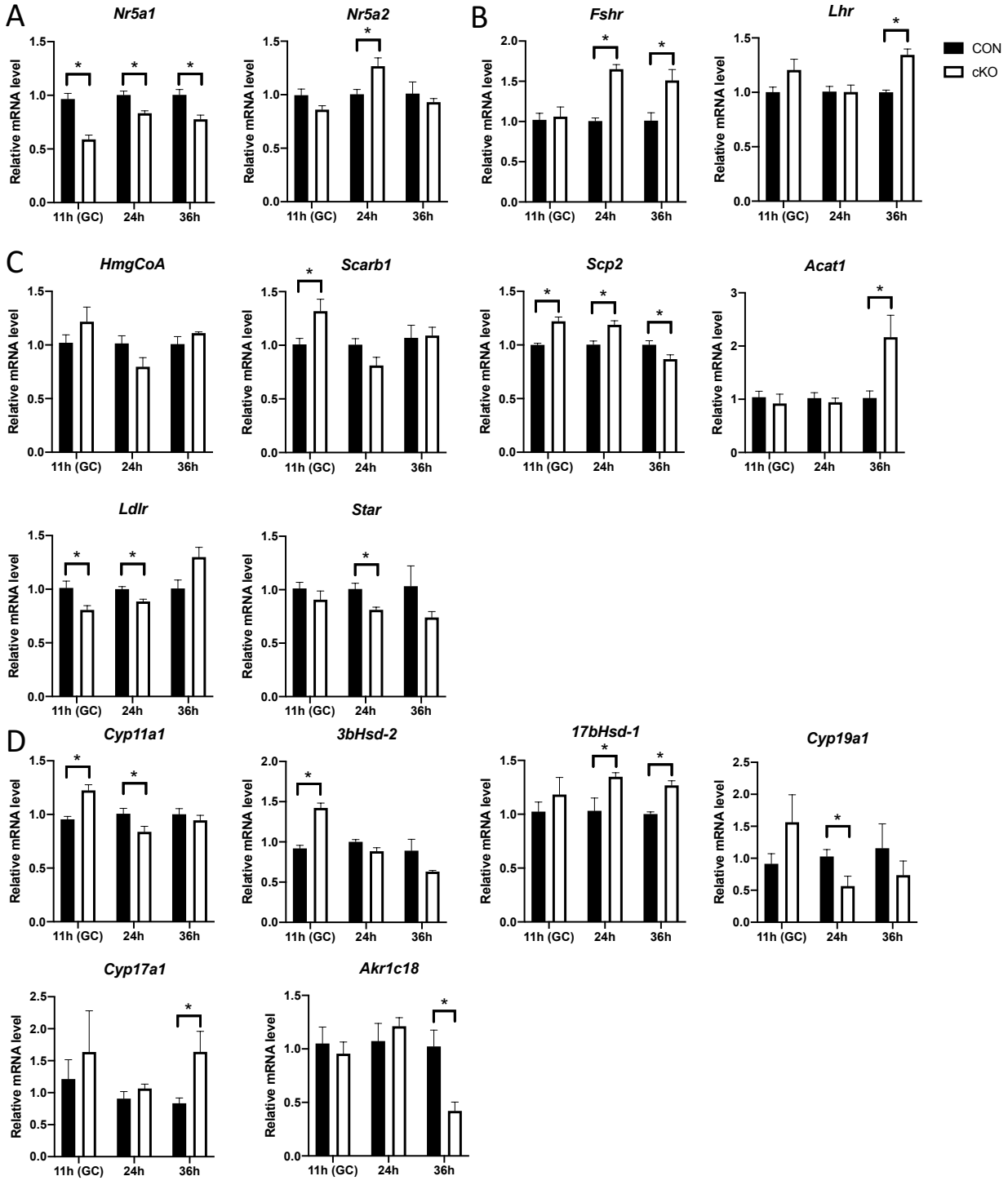

Figure 3.7 Impaired steroidogenesis and cholesterol homeostasis during granulosa cell luteinization and in luteal cells of the corpus luteum of cKO mice.

(A-D) Relative gene expression of (A) orphan nuclear receptors SF-1 and LRH-1, (B) gonadotropin receptors FSHR and LHR, factors involved in (C) steroidogenesis and (D) cholesterol homeostasis in superstimulated CON and cKO granulosa cells collected 11 h (11h GC) and whole ovary cells collected 24 h and 36 h post-hCG injection. Asterisk indicates statistically significant difference ($P < 0.05$).

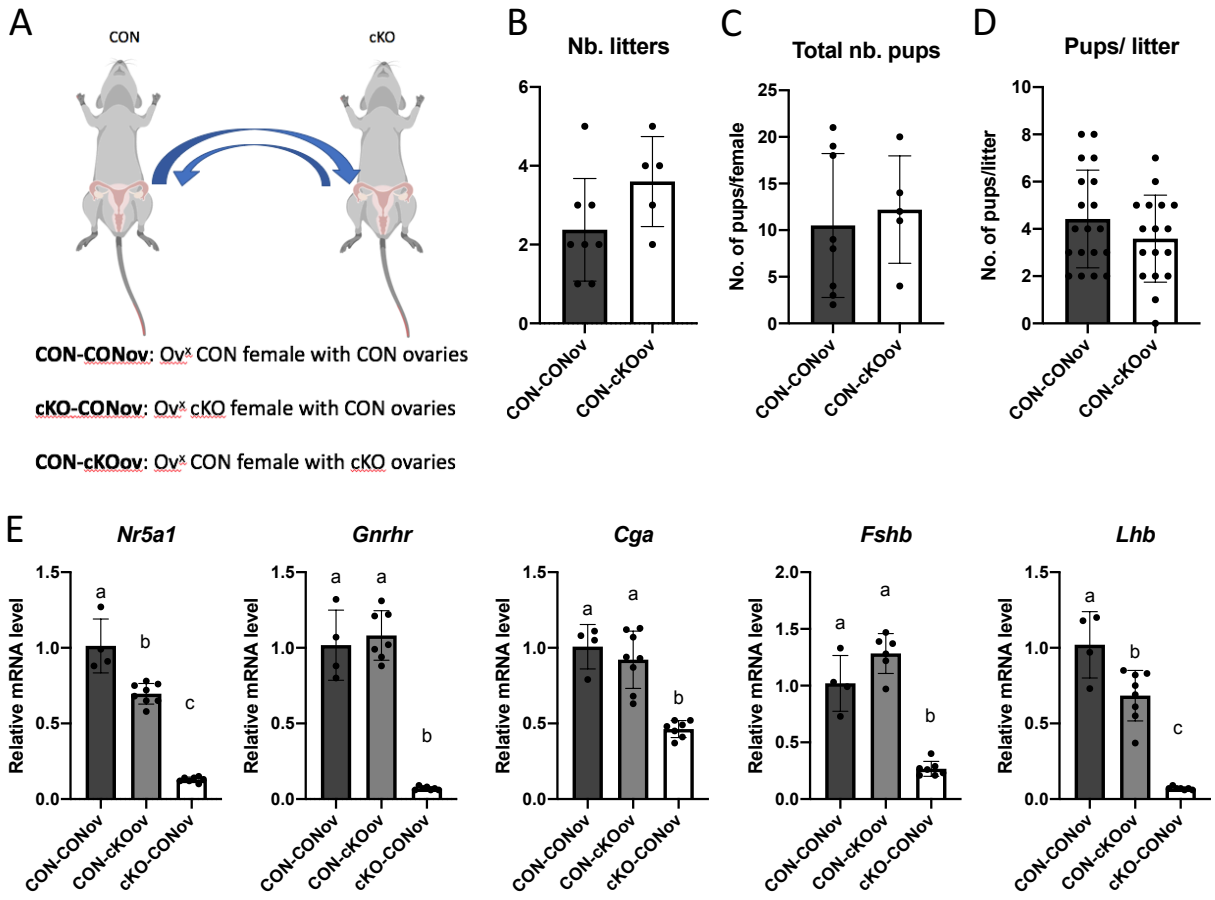

Figure 3.8 Ovarian transplantation of cKO ovaries in ovariectomized CON females affects pituitary gene expression and shows presence of hypertrophic structures in the ovary.

(A) Ovarian transplantation schematic representation. Ovariectomized CON females with cKO ovaries transplanted under the ovarian bursa (CON-cKOov); ovariectomized cKO females with CON ovaries transplanted under the ovarian bursa (cKO-CONov); ovariectomized CON females with CON ovaries transplanted under the ovarian bursa (CON-CONov). (B-D) Average (B) number of litters, (C) total number of pups and (D) pups per litter born from CON-CONov and CON-cKOov (fertile females only) mice. (E) Relative expression of gonadotrope genes in the

pituitary gland of CON-CONov (n=5), cKO-CONov (n=10), and CON-cKOov (n=9) females collected the first day of estrus. Asterisk indicates statistically significant difference ($P < 0.05$).

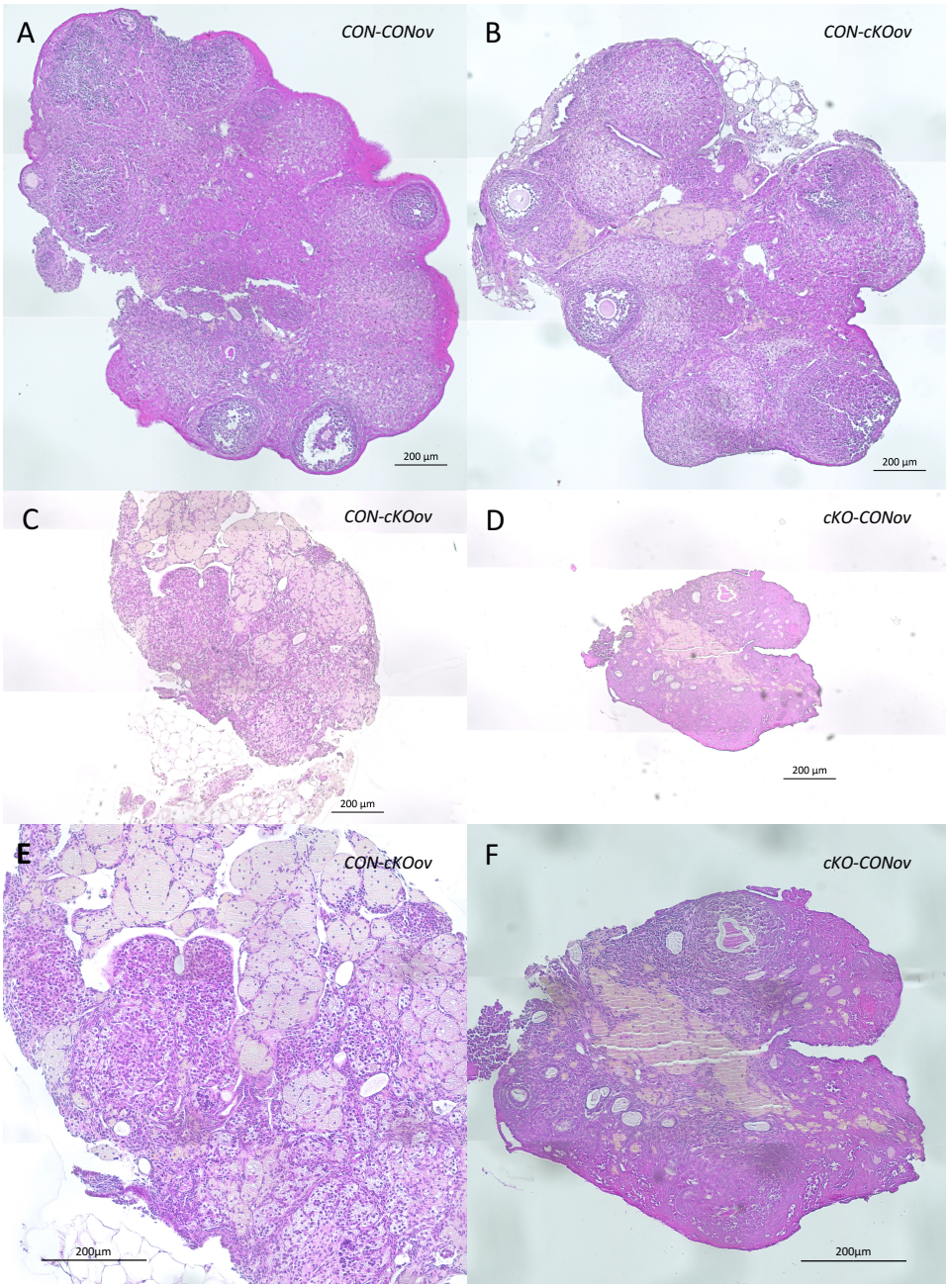

Figure 3.9 Abnormal ovarian morphology of cKO ovaries after transplantation in CON mice.

(A-D) Bright field microscopy images of hematoxylin-eosin-stained sections of ovaries from (A) CON-CONov, (B) fertile CON-cKOov, (C) infertile CON-cKOov and (D) cKO-CONov

transplanted mice. (E-F) Higher magnification of ovaries from (E) infertile CON-cKOov and (F) cKO-CONov females. Scale bar, 200 µM.

3.9 SUPPLEMENTAL FIGURES

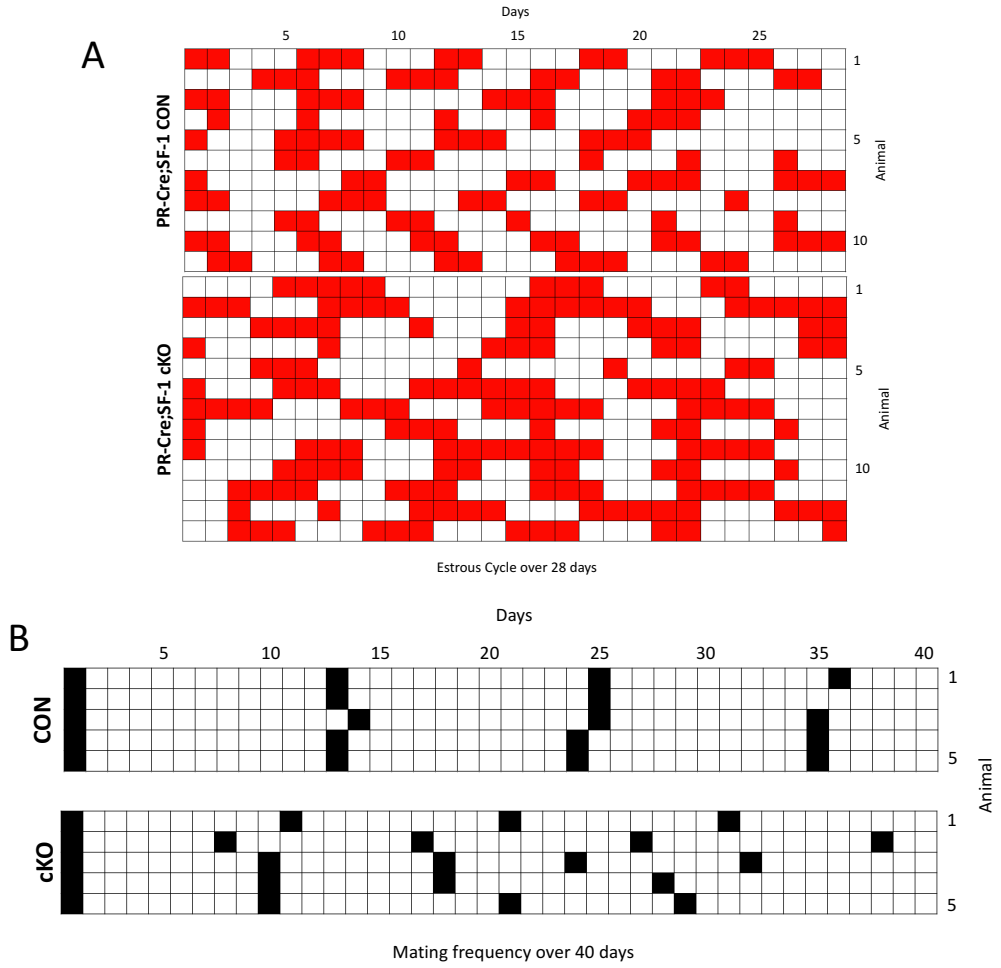

Figure S3.1

(A) Estrous cycle representation, where red squares indicate female in estrus and white squares are either metestrus, diestrus of proestrus. (B) Mating frequency representation of CON (n=5) and cKO (n=5) mature females, where black squares indicate the observation of a copulatory plug.

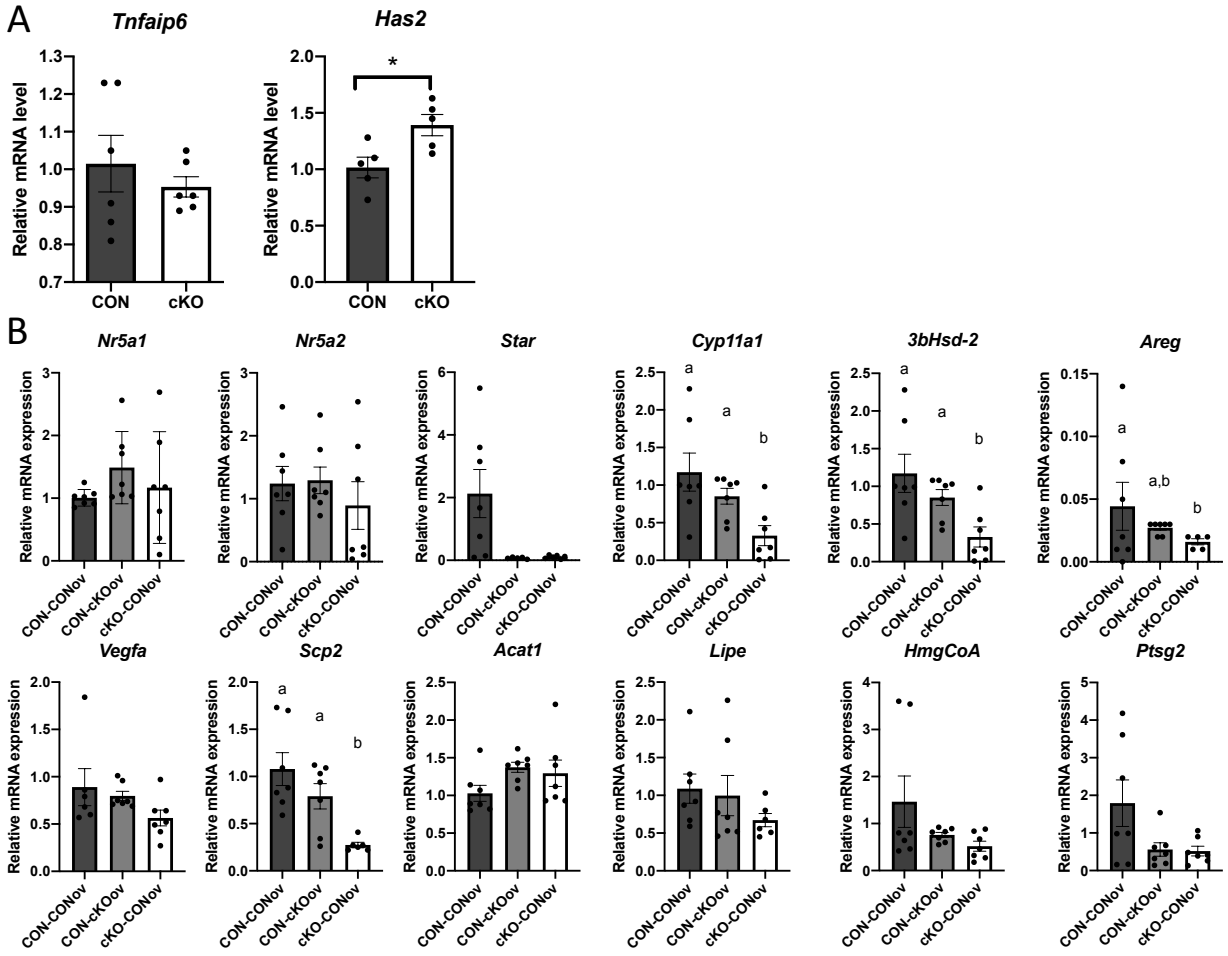

Figure S3.2

(A) Relative expression of cumulus expansion genes in granulosa cells of superstimulated CON and cKO (n=8 per genotype), collected 11 h post hCG. Asterisk indicates statistically significant difference ($P < 0.05$). (B) Relative expression of steroidogenic genes in CON-CONov (n=5), CON-cKOov (n=10), and cKO-CONov (n=9) whole ovaries. Letters indicates significant statistical difference ($P < 0.05$).

3.10 TABLES

Table 3.1 Reproductive performance of cKO female mice.

Six-month breeding trial of CON (n=4) and cKO (n=7) mature females mated with reproductively proven C57BL/6 males. Only fertile females were used in the calculation of the average number of litters/female and average litter size. Silver's relative fecundity number = (productive matings) x (average nb of litters/ female) x (average litter size). Asterisks indicate statistical significance.

Reproductive performance	**Genotype**	
	CON	**cKO**
Number of females	4	7
Productive matings	100%	43%
Average nb litters/ female	6 ± 0.58	1.34 ± 0.34*
Average litter size	4 ± 0.43	2.75 ± 1.11*
Silver's relative fecundity	24	1.58

Table 3.2 Reproductive performance of superstimulated CON and cKO females.

CON and cKO females mated overnight with WT males after exogenous gonadotropin treatment to stimulate follicle growth (eCG only), ovulation (hCG only), or both (eCG + hCG). CON females were injected with both eCG and hCG before mating.

	Genotype			
	CON	cKO		
Treatment	eCG + hCG	eCG only	hCG only	eCG + hCG
Females injected	**5**	**29**	**20**	**14**
Copulatory plug	3	17	9	6
Birth	2	1	0	0
Average nb of pups	8 ± 1.41	8	-	-

Table 3.3 Reproductive performance CON and cKO females after ovarian transplantation.

Reproductive performance of CON-CONov (n=8), cKO-CONov (n=9) and CON-cKOov (n=9) females mated over 4 months with reproductively proven C57BL/6 males. Only fertile females were used in the calculation of the average number of litters/female and average litter size. Silver's relative fecundity number = (productive matings) x (average nb of litters/ female) x (average litter size).

Reproductive performance	**Genotype**		
	CON-CONov	**CON-cKOov**	**cKO-CONov**
Number of females	8	9	9
Productive matings	100%	56%	0%
Nb litters/female	2 ± 1.30	2 ± 2.05	-
Litter size	4 ± 2.12	3 ± 2.22	-
Silver's relative fecundity	8.00	3.36	0.00

3.11 SUPPLEMENTAL TABLES

Table S3.1 List of primer sequences used in this study. All primers are 5' to 3'.

	Gene	Forward	Reverse
Genotyping	*Sf-1*	AGACAAGTGCACCCCATTTC	ACCATCACCAACCGCTAAAC
	PrCre	GTCCCGAGCTCCCAGACGGA	ACGCCTGGCGATCCCTGAACA
Housekeeping	*B2m*	GACCGGCCTGTATGCTATCC	TTTCAATGTGAGGCGGGTGG
	Hprt	ATGATCAGTCAACGGGGGAC	GAGAGGTCCTTTTCACCAGCA
	Ywhaz	CAGTCTGCCTGGGATTCTATTC	TGGTTGGTGACAAGACAGTAAA
Receptors	*Nr5a1*	GTCTCAAGTTCCTCATCCTCTTC	GGGTAGTGACACAAGGTGTAAT
	Nr5a2	TCATGCTGCCCAAAGTGGAGA	TGGTTTTGGACAGTTCGCTT
	Fshr	AGAAAGCAGGTGGATGGATAAA	GAAGACCCTGTTAGAGCAATGA
	Lhr	GCATCTGTAACACAGGCATCC	AAAGCGTTCCCTGGTATGGT
	Pr	AGGTCTACCCGCCATACCTT	CAGGTAAGCACGCCATAGTG
Hypothalamus	*Gnrh*	GATCCACAACACCCGAGTATAA	AATCTACGCTGCTGGGTATAAA
	Gpr54	CCCTCTGAGTCTCACCTAGAAA	CCTTGCTCCCAAAGAGGTTAG
	Kiss1	CCTCCCAGAATGATCTCAATGG	GTGGATCCAGGCTTCACTTT
Pituitary gland	*Gnrhr*	CACGGGTTTAGGAAAGCAAA	TTCGCTACCTCCTTTGTCGT
	Cga	TGTGTGGCCAAAGCATTTAC	GCGCTCAGAAGCTACGACTT
	Fshb	GTGCGGGCTACTGCTACACT	CAGGCAATCTTACGGTCTCG
	Lhb	AGCAGCCGGCAGTACTCGGA	ACTGTGCCGGCCTGTCAACG
Ovulation	*Areg*	CATCGGCATCGTTATCACAG	TGTCGAAGCCTCCTTCTTTC
	Ereg	CCATCATGCATCCCAGGAGAA	TAGCCGTCCATCGTCAGAAACTCACT
	Btc	AACTGCACAGGTACCACCCCTAGA	ACAGATGCAGGAGGGAGTTTGC
	Ptgs2	A TCCTGAGTGGGGTGA TGAG	AAGTGGTAACCGCTCAGGTG
	Tnfaip6	TGAAGGTGGTCGTCTGCTAACC	TCCACAGTTGGGCCCAGGTTTCA
	Has2	CAAAAATGGGGTGGAAAGAG	TCCAGTATCTCACGCTGCTG
	Vegfa	GCGAGGCAGCTTGAGTTAAA	TCTTTCCGGTGAGAGGTCTG
Cholestrol homeostasis	*Scarb1*	TTGGCCTGTTTGTTGGGATG	GGATTCGGGTGTCATGAAGG
	Star	TTGGGCATACTCAACAACCA	GCGGTCCACAAGTTCTTCAT
	Ldlr	AATGGTGGTTGCCAGTACCT	CCCTGGGTGGTCAGTACAGT
	Acat1	CTAAAGCCCTGGGTTCTTTCT	CTCGGTTCGTTTCTCTCTATGG
	Lipe	CTTTGTGGCACAGACCTCTAA	GGGAGTAGTCGATGGAGAAGATA
	Scp2	CGGACCACTCCAACTGATAAA	TCCCAGCATACCCAAACATC
	HmgcoA	CAGAGAAGACAGTGCTCGTTAG	GCTCCATCATTGGCTCTGTAA
Steroidogenesis	*Cyp11a1*	CTGCCTCCAGACTTCTTTCG	TTCTTGAAGGGCAGCTTGTT
	3bHsd-2	GGTTTTTGGGGCAGAGGATCA	GGTACTGGGTGTCAAGAATGTCT
	Cyp17a1	ACCAGCCAGATCGGTTTATG	CAGCTCCGAAGGGCAAATA
	17bHsd-1	GGTGGTCTGCATCGGATAAA	GCAACACAGGGCTACATAGT
	Cyp19a1	ATGTTCTTGGAAATGCTGAACCC	AGGACCTGGTATTGAAGACGAGC
	Akr1c18	GAGCAGTGGCTGAGAATGAA	AGATGCTCTTCAGTTGCATAGG

Chapter 4. Article 2

The role of steroidogenic factor 1 (SF-1) in steroidogenic cell function of the testes and ovaries of mature mice.

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This work was supported by CIHR Project Grant 419226 to BDM. OES is funded by a Doctoral Training Grant from FRQS.

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Keywords: SF-1, Gonads, Leydig cells, Sertoli cells, Infertility, Conditional knock-out, Steroidogenesis

Status: This manuscript is in the final phase of preparation to be submitted to *Reproduction*.

Author contributions: OES contributed to the experimental design, performed experiments, analyzed the results, and wrote the article; representing a relative contribution of 90%. VR and FM performed experiments. MB contributed to the experimental design. BDM contributed to the experimental design, data analysis, and article editing.

4.1 ABSTRACT

Steroidogenic factor 1 (SF-1) is an orphan nuclear receptor that plays an essential role in the development of fetal gonads and regulates the expression of genes involved in steroid biosynthesis. Since SF-1 is expressed in multiple cell types of the mouse gonads, we developed three novel conditional KO (cKO) mouse models employing Cre-recombinase and floxed alleles of SF-1 (SF-$1^{f/f}$) to identify its role in steroid producing cells of testes and ovaries of mature mice: Cytochrome P450 17α-hydroxylase (Cyp17-Cre; SF-$1^{f/f}$, Leydig and theca cell specific), aromatase (Cyp19-Cre;SF-$1^{f/f}$, Sertoli and granulosa cell specific), as well as a combination of these two (Cyp17+Cyp19-Cre;SF-$1^{f/f}$). When compared to control animals, Cyp19-Cre;SF-$1^{f/f}$ cKO males showed normal fertility and testicular function. The Cyp17-Cre;SF-$1^{f/f}$ cKO males had smaller testis, with drastically reduced Leydig cell volumes and impaired steroidogenesis, though their reproductive performance remained comparable to controls. Some 50 % of Cyp17+Cyp19-Cre/SF-1 double-cKO (dKO) males were infertile, while the remaining 50 % showed significantly reduced fertility. These dKO males also had smaller testis with degenerative seminiferous tubules, reduced Leydig cell population and lower levels of intra-testicular testosterone. Abnormal Sertoli cell localization was noted in the dKO testis, with increased *Sox9* expression and decreased aromatase and inhibin A transcript abundance. Female mice from all genotypes showed normal reproductive capacity, though steroidogenic gene expression levels were significantly decreased in both Cyp17-Cre;SF-$1^{f/f}$ cKO and dKO females, while liver receptor homologue 1 (*Lrh1*) and anti-Müllerian hormone (*Amh*) were increased in granulosa cells of Cyp19-Cre;SF-$1^{f/f}$ cKO mice. These results show the essential role of SF-1 in mature mouse gonad steroidogenic gene expression is required for Leydig and Sertoli cell function, and that the depletion of SF-1 in all steroidogenic cells of the testis compromises steroidogenesis, spermatogenesis, and male fertility.

4.2 INTRODUCTION

The orphan nuclear receptor steroidogenic factor 1 (SF-1 encoded by the *NR5A1* gene) stimulates the expression of several genes required for the development and maintenance of the male differentiation cascade. In addition to regulating the expression steroidogenic enzymes in mature testes, SF-1 regulates the testis-determining genes *SRY* and SRY-box transcription factor 9 (*SOX9*) in fetal gonads, where it then stimulates the expression of anti-Müllerian hormone (AMH) at embryo day (E)12.5, inducing the regression of the Müllerian ducts in the developing fetus, and inhibiting female genitalia formation (198, 219). A sex reversal phenotype is observed in the absence of SF-1 in the male germline, further demonstrating the essential role of SF-1 in testis formation (36, 41, 220). SF-1 also increases the expression of insulin-like polypeptide 3 (INSL3), which regulates testicular descent and is a survival factor for male germ cells in adults (363, 364).

In humans, heterozygous mutations in SF-1 account for 4% of infertile men with severe spermatogenic failure that do not have chromosomal anomalies, and are the cause of up to 20% of 46,XY disorder of sex developmental cases (253, 365). Single nucleotide variations and missense mutations in the SF-1 gene have been associated with low testosterone levels, azoospermia, oligozoospermia, hypospadias and elevated gonadotropin secretion (Köhler et al. 2009). Transgenic mouse models have been designed to study the role of SF-1 in testicular cells. The inactivation of SF-1 in cells expressing the AMH type 2 receptor (AMHr2) led to infertile male mice that showed undescended, hypoplastic testes with abnormal seminiferous tubule structure, and spermatogonia that failed to develop into sperm (198). This model induced SF-1 deletion at mouse E11.5 in both Leydig and Sertoli cells, before normal testis development has been initiated (198, 366). A recent study, using the Sertoli-specific Amh-cre model, demonstrated the essential

role of SF-1 in Sertoli cell survival in fetal testes, where loss of SF-1 post sex determination prevented Sertoli cell maturation in adult testis, inducing germ cell apoptosis and resulting in testis cord dysgenesis (323). Another transgenic mouse model using a Cyp11a1-Cre recombinase induced effective SF-1 depletion in differentiated Leydig cells of the testis, theca cells of the ovary as well as in steroidogenic cells of the fetal adrenal gland (367). This study showed that, while SF-1 depletion in these cell types caused defects in steroidogenic gene expression and resulted in abnormal Leydig cell morphology in the testis, it had no impact on mouse fertility.

In the present study, we developed three novel conditional knockout mouse models to deplete SF1 expression using Cre-recombinase under regulation of the *Cyp17* promoter, the Aromatase (Cyp19A1) promoter, and a combination of both, for the study of this transcription factor specifically in the Leydig cells and Sertoli cells of the testis and in the theca cells and granulosa cells of the ovary. We demonstrate that SF-1 is required for steroidogenesis in Leydig and theca cells of the mouse gonads, and that its depletion in all steroidogenic cells of the testis is detrimental to male fertility.

4.3 MATERIAL AND METHODS

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 genetic 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. Mice were generated by crossing *Nr5a1*$^{f/f}$

(*Nr5a1*^{tm2Klp} MGI: 1346833) females (described in (337)) with transgenic males expressing Cre-recombinase under control of either the *Cyp17a1* promoter (*Cyp17*-Cre, founder line Cyp17iCre) (368) or the *Cyp19a1* promoter (*Cyp19*-Cre, transgenic line tgCyp19a1$^{Cre/+}$) (369). Following DNA extraction from tails, males heterozygous for SF-1 and *Cyp17*-Cre or *Cyp19*-Cre were crossed with *Nr5a1*$^{f/f}$ females. Since *Cyp17*$^{Cre/+}$;*Nr5a1*$^{f/f}$ and *Cyp19*$^{Cre/+}$;*Nr5a1*$^{f/f}$ males are fertile, they were subsequently mated with *Nr5a1*$^{f/f}$ females to produce homozygous conditional knockout (cKO) offspring (genotype *Cyp17*$^{Cre/+}$;*Nr5a1*$^{f/f}$ or *Cyp19*$^{Cre/+}$;*Nr5a1*$^{f/f}$, referred to as Cyp17 cKO and Cyp19 cKO respectively) and non-mutant littermates were used as controls (genotype *Cyp17*$^{+/+}$;*Nr5a1*$^{f/f}$ or *Cyp19*$^{+/+}$;*Nr5a1*$^{f/f}$, referred to as CON). Additionally, Cyp17 cKO females were crossed with Cyp19 cKO males to produce *Cyp17*$^{Cre/+}$+*Cyp19*$^{Cre/+}$;*Nr5a1*$^{f/f}$ double cKO animals, (referred to as dKO). Mice were genotyped using primers listed in Table S4.1.

For breeding assays, 8-week-old Cyp17 cKO, Cyp19 cKO, dKO and CON males and females were housed with reproductively proven C57BL/6 mates for 6 months. Cages were inspected daily, and parturition dates and litter sizes were recorded. Relative fecundity was assessed following Silver's overall fecundity equation: frequency of mating (% of matings that produce offspring) x average litter size x average number of litter in the six-month breeding trial (343).

Tissue collection

Gonads and adrenal glands were collected from mature (2-5 month old) Cyp17 cKO, Cyp19 cKO, dKO and CON males and female mice and flash-frozen followed by homogenization for hormone dosing or RT-qpCR analyses, or fixed in 10% formalin or Bouin's fluid for morphological analysis and immunofluorescence staining.

To avoid the complexity of ovarian functions associated with estrous cycles and endogenous surges of gonadotropins, the response of 25-day-old immature females to exogenous gonadotropins was analyzed. Specifically, mice were injected intraperitoneally with 5 IU eCG (equine chorionic gonadotropin; Calbiochem) followed 48 hours later with 5 IU hCG (human chorionic gonadotropin; American Pharmaceutical Partners, Schaumburg, IL). Pure granulosa cells were isolated from CON and Cyp19 cKO ovaries at 4 h post-hCG by puncturing both ovaries with 25G needles in 1X PBS, and filtered (BD Falcon, Cell Strainer, 40 μm Nylon, Mexico). Cells were pelleted and snap frozen for mRNA extraction and RT-qPCR analysis.

Histology and degenerated seminiferous tubule count

Tissue processing and embedding were performed using standard techniques. Tissues were sectioned at 5μM and stained with hematoxylin and eosin (Thermo Fisher Scientific #72511, Thermo Fisher Scientific #71311). Three mice per genotype were used for histological analysis.

Using GIMP 2.10 software, a grid was superposed on HE stained testis slides from the four genotypes (n=3 per genotype) at the same magnification and the seminiferous tubules where the grid lines crossed were assessed as either normal (N) or abnormal (Ab) and counted. Grid interval was 600 pixels, and between 33 and 57 points where grid crossing points coincided with a seminiferous tubule were analyzed on one testicular cross-section per animal.

RNA extraction and real time PCR

RNA was extracted from tissues 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). All transcripts were amplified following the 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. Relative mRNA levels were determined using Pfaffl's mathematical model (345), with *B2m*, *Hprt*, *Ywha2* as the housekeeping genes. As the number of Leydig cells was reduced in Cyp17 cKO and dKO testes whereas Sertoli cell number remained unchanged, leading to an increase in the proportion of Sertoli cells in the testis, the mRNA expression of Sertoli-specific markers was normalized relative to the endogenous Sertoli cell marker *Gata6* mRNA, as previously described (370, 371). Primer sequences for genes measured in this manuscript can be found in Table S4.1.

Immunohistochemistry and Immunofluorescence

Slides of formalin or paraformaldehyde-fixed paraffin-embedded testes, adrenal glands and ovaries from CON, Cyp19 cKO, Cyp17 cKO and dKO mice were rehydrated, boiled in 10 mM sodium citrate (pH 6) for 30 minutes, and cooled to room temperature, then blocked 1 h with 5% bovine serum albumin (Jackson ImmunoResearch Laboratories) in PBST 0.1%. Sections were probed overnight at 4°C with primary antibodies against SF-1 (1:100), WT1 (1:100) and Cleaved Caspase-3 (1:300) (Cell Signaling Technology, #12800, 83535, 9661), as well as Connexin 43 (1:100), CYP17 (1:100), 3βHSD (1:400) and StAR (1:200) (Santa Cruz, #9059, 46085, 30820 and 25806) diluted in BSA5% / PBST 0.1%. CY3 conjugated goat anti-rabbit IgG (1:300 in BSA 5%/PBST 0.1%, Jackson ImmunoResearch #111-165-144) was used as second antibody for SF-1, WT1, Cleaved Caspase-3, Connexin 43 and STAR and CY3 conjugated donkey anti-goat IgG (1:300 in BSA 5%/PBST 0.1%, Jackson ImmunoResearch #111-165-003) was used for CYP17 and 3βHSD immunolocalization. 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) and viewed in a Zeiss ZenPro microscope.

Steroid hormone measurement

Serum and testis samples were collected from mature males. Testis samples were weighed and homogenized in DPBS. Serum and intra-testis testosterone levels were measured by enzyme-linked immunosorbent assay (Calbiotech), performed by the Ligand Assay and Analysis Core at the University of Virginia.

Statistical analyses

Data are presented as mean ± SEM, and were analyzed by Brown-Forsythe and Welch ANOVA test or Student's t-test, Kruskal-Wallis or Mann-Whitney tests were used when not normally distributed. $P \leq 0.05$ was considered statistically significant. Data were analyzed using GraphPad Prism version 7.0 (GraphPad Software).

4.4 RESULTS

Conditional depletion of SF-1 in testicular cells of Cyp17 cKO, Cyp19 cKO and dKO mice

We used previously generated transgenic mouse strains expressing Cre recombinase under the control of the *Cyp17a1* and *Cyp19a1* transcriptional regulatory elements to induce SF-1 depletion in steroidogenic cells and define the functional role of this transcription factor in gonadal cells (368, 369). Immunolocalization of SF-1 in the testis of CON mice showed strong nuclear expression in Leydig cells of the interstitial space, as well as in the Sertoli cells along the basal membrane of the seminiferous tubules (Figure 4.1A). The number of SF-1 positive cells in the

seminiferous tubules of Cyp19 cKO testis was significantly reduced when compared to CON, while SF-1 expression in Leydig cell was maintained (Figure 4.1C). *Nr5a1* expression level in the Cyp19 cKO testis was comparable to CON, and the proportion of SF-1 positive cells in the interstitium was smaller than CON (Figure 4.1, B and D). On the other hand, immunolocalization of SF-1 in the Cyp17 cKO testis showed a decrease proportion of SF-1-positive cells in the interstitial space, as well as a slight decrease in the number of SF-1-positive cells at the basal membrane of seminiferous tubules. A significant reduction in *Nr5a1* mRNA abundance was measured in the Cyp17 cKO and dKO testis (Figure 4.1B). As expected, the depletion of SF-1 in both steroidogenic cell types of the testis in the dKO male induced a significant reduction in the number of SF-1-positive cells in both the seminiferous tubules and interstitial space (Figure 4.1, A, C and D).

The Cyp17iCre founder mouse line expression analysis showed that Cre activity was observed in the male but not the female embryonic adrenal glands (368). SF-1 expression was therefore measured in adrenal glands collected from CON, Cyp17 cKO and dKO males, as well as steroidogenic enzymes that are known targets of this transcription factor. No significant decrease was measured in *Nr5a1* mRNA abundance of the mutant males (Figure S4.1A). While most steroidogenic genes were comparable to CON in the mutant males' adrenal gland, *3βHsd* expression was significantly reduced in Cyp17 cKO and *Cyp11a1* was significantly reduced in dKO males (Figure S4.1A). SF-1 and CYP17 immunofluorescent staining confirmed the gene expression observations (Figure S4.1, B-C).

Loss of SF-1 in steroidogenic cells of the testes impairs male mouse reproductive function

To assess their reproductive capacity, Cyp19 cKO, Cyp17 cKO, dKO and CON males were housed with adult females for a six-month breeding trial. Cyp19 cKO and Cyp17 cKO fertile males

produced comparable number of litters and pups to CON males, though one of the six Cyp19 cKO males in the trial was infertile even after mating with two different CON females (Table 4.1). In contrast, half of the dKO males in the fertility trial were infertile, and the other half had significantly lower frequency of litters and total number of pups with a lower relative fecundity number when compared to CON or cKO (Table 4.1) (343).

Next, the body and testis weight were compared to examine the phenotype associated with SF-1 depletion in the male gonad. While bodyweight remained comparable between all four genotypes, the testis-body weight ratio of Cyp17 cKO and dKO males was significantly lower than CON and Cyp19 cKO, indicating smaller testis sizes when SF-1 is reduced in Leydig cells (Figure 4.2, A-B). A significant reduction in the number of sperm counted from the epididymides was observed in Cyp17 cKO and dKO males when compared to CON and Cyp19 cKO (Figure 4.2C). To understand this reduction in sperm count, intra-testicular testosterone levels were measured. No difference was observed in Cyp17 cKO male testosterone levels when compared to the Cyp19 cKO and CON males, while it was significantly reduced in the dKO testis (Figure 4.2D).

Loss of SF-1 in the testes leads to abnormal seminiferous tubule morphology

We analyzed the histology of mature testes to determine the impact of SF-1 depletion on testicular structure in the Cyp19 cKO, Cyp17 cKO and dKO males. Hematoxylin-eosin staining revealed clear signs of abnormal seminiferous tubule structures in the testis of the all three KO genotypes. Presence of seminiferous tubules with closed lumina and presence of nucleated cells at the center of the seminiferous tubule was observed in Cyp19 cKO and Cyp17 cKO (Figure 4.3A). Atrophy of the seminiferous tubules was particularly apparent in dKO testes, with some seminiferous tubules showing decreased luminal width accompanied with abnormal accumulation

of cells in the tubular lumen, while others showed wide and empty lumina with evident germ cell loss (Figure 4.3A, dKO). In contrast, testes from CON mice had largely normal germ cell arrangement and morphology. Immunostaining for DDX4 identified germ cells localized in the lumen of severely disrupted tubules of Cyp17 cKO and dKO testes, a cell population usually localized in the basal membrane and adluminal layer (Figure S4.2).

The percentage of abnormal seminiferous tubules was evaluated in the four genotypes and showed that testes from the Cyp19 cKO and Cyp17 cKO males tended to have a higher proportion of abnormal seminiferous tubules, while the majority of seminiferous tubules in the dKO testes were abnormal (Figure 4.3B). Additionally, close inspection of the interstitium of Cyp17 cKO and dKO testes showed a noticeable change in the Leydig cell morphology (Figure 4.3C) and the population of nucleated cells seemed reduced compared to CON testes (Figure 4.3A, middle panels), indicating a possible effect of SF-1 depletion on Leydig cell differentiation and survival.

Loss of SF-1 in testes impairs Leydig steroidogenic gene expression

In order to understand the abnormal spermatogenesis observed in seminiferous structures and the measured reduction in sperm count and testosterone level in our mutant males, we analyzed the expression of enzymes involved in steroidogenesis. In the male gonad, the initial steps in the transformation of cholesterol into steroids occur in the Leydig cells, where cholesterol is transported into the mitochondria by steroidogenic acute regulatory protein (STAR) to be transformed into pregnenolone by P450 side-chain cleavage enzyme (coded by the *Cyp11a1* gene) (372). In both Cyp17 cKO and dKO males, testicular expression of both *Star* and *Cyp11a1*, as well as genes coding for Leydig cell steroidogenic enzymes 3-hydroxysteroid dehydrogenase (*3*β*Hsd*) and 17α-hydroxylase (*Cyp17a1*), was significantly reduced (Figure 4.4A). LH receptor (*Lhr*)

mRNA abundance was significantly decreased in Cyp17 cKO and dKO testes, supporting the observation in Figure 4.1D and 4.3A of a reduction in the number of Leydig cells. CYP17, STAR and 3βHSD immunostaining support the steroidogenic gene quantification and demonstrate that most cells present in the interstitial space of Cyp17 and dKO testes, between seminiferous tubules, are not functionally steroidogenic (Figure 4.4, B-C, Figure S4.3A). Interestingly, while some STAR immunostaining was detected in cells of the interstitial compartment of dKO testes, these cells demonstrated abnormal morphology compared to STAR-positive cells in the CON testis (Figure 4.4C). Leydig cell morphology and steroidogenic gene and protein expression levels in Cyp19 cKO testes were comparable to CON (Figure 4.4 A-C, Cyp19 cKO). Interestingly, 3-hydroxy-3-methylglutaryl-coenzyme A (*HmgCoA*), the rate limiting enzyme in intracellular *de novo* cholesterol synthesis, was significantly reduced in dKO testes (Figure S4.3B).

Reduced expression of SF-1 in mouse testes induces programmed cell death of germ cells but not Leydig cells

The reduced expression of CYP17, a Leydig cell marker as well as the reduced expression of the Leydig specific gonadotropin receptor *Lhr* in Cyp17 cKO and dKO testes suggests that Leydig cell survival may be negatively affected by the depletion of SF-1. To test this hypothesis, we performed TUNEL staining on mature testis from CON, Cyp19 cKO, Cyp17 cKO and dKO males to measure apoptosis. Apoptotic cells were spotted in abnormal seminiferous tubules, more specifically in spermatogonia, primary spermatocytes and spermatids (Figure 4.5A, seen in Cyp19 cKO, Cyp17 cKO and dKO). No TUNEL-positive cells were found in the interstitium of the testis of either Cyp17 cKO or dKO males, indicating that Leydig cells are not undergoing apoptosis in the adult testes. Though DNA fragmentation was observed in the four genotypes, the number of

seminiferous tubules containing 3 or more TUNEL-positive cells was significantly higher in dKO testes, and showed a tendency to be increased in Cyp17 cKO testes (Figure 4.5B). Cleaved caspase-3 immunofluorescence was also performed and showed presence of apoptotic cells within the seminiferous tubules of the dKO testes (Figure S4.4A). Again, no apoptosis was observed in Leydig cells of the interstitial space of Cyp17 cKO and dKO males. Further supporting the observed increase in programmed cell death in the testes of Cyp17 cKO and dKO males, pro-apoptotic genes *Bad* and *Bax*, as well as the autophagy marker *Mtor* were significantly upregulated in these mice compared to CON (Figure 4.5C). Additionally, cell cycle inhibitors *Cdkn1a* and *Lin28a* were also significantly upregulated, indicating an increased frequency of non-dividing cells. Together, these results indicate that SF-1 depletion in steroidogenic cells of the mouse testis leads to programmed cell death in germ cells of the seminiferous tubules, with increased apoptosis, autophagy, and cell cycle arrest. Interestingly, no increased DNA damage was observed in the testes of Cyp19 cKO males, other than a significant reduction in the anti-apoptotic factor *Mcl1* (Figure 4.4B).

Abnormal Sertoli cell function and localization in dKO testes

Next, we measured Sertoli cell function to see if it could explain the decreased spermatogenesis and abnormal seminiferous tubule structure observed in the mutant males. Sertoli cells play an essential role in spermatogenesis, providing nutrients and growth factors to induce the production of viable and mature gametes. These nutrients are delivered through the blood-testis barrier from Sertoli cells to spermatogonia via anchoring junctions, tight junctions and gap junctions (373). Connexin 43 (CX43, *Gja1*), a constitutive protein in gap junctions, is the most expressed connexin in the testis and has been shown to regulate numerous physiological functions

like differentiation, proliferation and apoptosis (374). A recent *in vitro* study showed that SF-1 cooperates with other transcription factors such as SOX8 and SOX9 to induce *Gja1* expression in mouse Sertoli cells (327). Here we show normal *Gja1* expression in both Cyp17 cKO and Cyp19 cKO testes and surprisingly, a marked upregulation in dKO testes (Figure S4.5A). This upregulation was not translated to the protein level, as seen by CX43 immunostaining (Figure S4.5B). While testicular immunostaining in CON, Cyp19 cKO and Cyp17 cKO males shows CX43 in Leydig cells, Sertoli cells and between Sertoli cells and germ cells, CX43 staining in testes from dKO mice was present mainly in the cells of the seminiferous tubules, possibly due to the reduced number of Leydig cells and increased apoptosis in germ cells in this model. While CX43 signal in adult male mouse testis is expected to appear as a focal band distributed close to the basal membrane, the dKO testes show spotty signal in many cells of the adluminal compartment.

Similar to *Gja1*, Sertoli markers *Sox9* and Inhibin A (coded by the *Inha* gene) were also significantly higher in the dKO testes, as well as mature Sertoli cell marker *Cdkn1b*, while *Cyp19a1* and immature Sertoli cell marker *Amh* were significantly increased in both Cyp17 cKO and dKO (Figure S4.5D). We suspected the relative contribution of Sertoli cells in the Cyp17 cKO and dKO testis becomes higher due to the reduction in Leydig cell number. Indeed, previous studies have shown that normalizing the mRNA levels of Sertoli cell markers by using *Gata-6*, a member of the GATA-binding protein family that is specifically expressed in Sertoli cells, as an endogenous reference allowed a more representative evaluation of Sertoli cell function (370, 371). Interestingly, normalization with *Gata-6* showed *Sox9* was downregulated in Cyp19 cKO Sertoli cells and remained upregulated in dKO (Figure 4.6A). The mRNA abundance of *Gja1* in dKO was comparable to CON when normalized to *Gata-6*, supporting the CX43 immunostaining observed above (Figure S4.5C). The expression levels of *Amh* and *Cdkn1b* in all mutant males were also

comparable to CON, indicating no effect of SF-1 depletion on Sertoli cell maturation (Figure 4.6B). In contrast, *Cyp19a1* and *Inha* were significantly downregulated in dKO when normalized with *Gata-6,* indicating that Sertoli cell function in this mouse model is impaired (Figure 4.6C). Immunofluorescent staining of another Sertoli specific transcription factor, Wilms tumor 1 (WT1), showed abnormal presence of Sertoli cells in the lumen of some seminiferous tubules in dKO testes, in addition to the expected basal membrane location (Figure 4.6D). The WT-1 positive cell count in seminiferous tubules showed that the number of Sertoli cells was comparable to CON, while the number of Sertoli cells in Cyp19 cKO and Cyp17 cKO testis was reduced (Figure 4.6E).

Depletion of SF-1 in steroidogenic cells of the ovary does not affect female mouse reproductive function but impairs steroidogenic gene expression

Cyp19 cKO, Cyp17 cKO, and dKO female mice were coupled with males for a six-month breeding trial in order to assess the effect of ovary-specific SF-1 depletion on female reproductive performance (Table 4.2). Females from all three genotypes were fertile and produced comparable number of litter and pups when compared to CON females. Body and ovarian weights were also comparable to CON mice (data not shown), and hematoxylin-eosin staining of mature female ovaries showed presence of primary, secondary and antral follicles, as well as corpora lutea (Figure 4.7A).

Next, we measured ovarian steroidogenic gene expression in the three SF-1 cKO models. SF-1 expression in the female gonad is strongest in theca cells and cells of the interstitium and weaker in granulosa cells and luteal cells (375) (Figure 4.7E, CON). The Cyp19-Cre mouse has been shown to induce recombination specifically in mural granulosa cells of the antral follicle and in luteal cells of the corpus luteum (160, 369). Though we were unable to detect a reduction in

Nr5a1 expression of granulosa cells collected from mature Cyp19 cKO females, *Nr5a2* and *Amh* were significantly upregulated, while *Fshr* and *Cyp19a1* remained comparable to CON (Figure 4.7B). This led us to measure *Nr5a1* expression in isolated granulosa cells collected from superstimulated immature female mice 4h post hCG injection to ensure a more uniform cell population. While both *Nr5a1* levels (Figure 4.7C) and SF-1 immunostaining were reduced in isolated granulosa cells (Figure 4.7E, Cyp19 cKO), no reduction in *Cyp19a1* was measured (data not shown). Theca cell specific steroidogenic enzymes mRNA abundance in Cyp19 cKO ovaries were comparable to controls (Figure 4.7D).

Theca cells isolated from mature Cyp17 cKO ovaries showed significant downregulation of *Nr5a1* as well as ovarian steroidogenic genes, *Cyp11a1*, *Cyp17a1* and *3*β*HSD* (Figure 4.7, D). Ovarian immunofluorescent staining shows reduced SF-1 expression in theca, but not granulosa or interstitial cells of the follicle (Figure 4.7F, Cyp17 cKO). Cholesterol transport proteins *Star* and scavenger receptor class B type I (*Scarb1*) gene expression were also significantly reduced, and StAR immunostaining in Cyp17 cKO showed that this reduction was also observed in corpora lutea, further supporting the observation that SF-1 is required for steroid gene expression in the ovary (Figure 4.7, D-E, Figure S4.6A). Similar to Cyp17 cKO theca cells, ovaries collected from mature dKO female also showed reductions in *Nr5a1*, *Scarb1*, *Star* and *Cyp11a1* expression levels (Figure S4.6B). *Cyp17a1* and *3*β*Hsd* mRNA levels, however, were comparable to controls. No variation was detected in *Nr5a2* expression in the dKO ovary, though *17*β*Hsd*, aromatase and even FSH receptor (*Fshr*), genes that are expressed in granulosa cells, showed a tendency towards upregulation. Interestingly, the aldo-keto reductase family 1 member C18 (*Akr1c18*), also referred to as 20-α reductase, was strongly reduced in the dKO ovary, suggesting a possible role of SF-1 in luteal function.

4.5 DISCUSSION

While the essential role of SF-1 in sex determination and steroidogenic cell differentiation has been extensively studied since its discovery three decades ago, much less is understood of its role in regulating mature mouse reproductive function. The Cyp19 cKO mouse, in which SF-1 expression is depleted in Sertoli and granulosa cells of the male and female respectively, and the Cyp17 cKO mouse, where the SF-1 depletion is specific to Leydig and theca cells, provide interesting models for comparing the importance of this transcription factor in steroidogenic cell function. In the present study, we have also examined the impact of SF-1 depletion in all steroidogenic cells of adult mice gonads, using the dKO model. We show for the first time that depletion of SF-1 in both mature Sertoli and Leydig cells can result in male infertility, suggesting complementary actions of SF-1 between the two types of steroidogenic cells of the adult male gonad. Though the depletion of SF-1 in theca cells impaired steroidogenic gene expression, females of these three cKO mouse lines were fertile.

Similar to another Leydig cell-specific SF-1 knockout model using Cyp11a1-Cre mice (367), adult Cyp17 cKO males were fertile, showed reduced steroidogenic gene expression in the testis that did not correlate to a significant decrease in the intra-testicular production of testosterone when compared to control males. The persistent reproductive performance of these mice, regardless of the marked reduction in testicular steroidogenic gene expression, might be due to residual steroidogenesis occurring in Leydig cells where the Cre recombinase transgene is not expressed, or where both *Sf-1* alleles were not successfully depleted, as was observed in the Cyp11-Cre model. Yet, in contrast to the Cyp11a1-Cre SF-1 mutant males, decreased testis size and sperm count was observed in the Cyp17 cKO males and closer morphological analysis of the testis showed presence

of disrupted seminiferous tubules with impaired spermiogenesis, demonstrating that SF-1 depletion in Leydig cells can indirectly affect spermatogenesis. Indeed, genes coding for apoptotic markers were upregulated in Cyp17 cKO testes, and DNA fragmentation was observed in spermatogonia and spermatids of disrupted seminiferous tubules. Still, the reduced expression of SF-1 in Leydig cells specifically remains insufficient to impair the reproductive capacity of the Cyp17 cKO male. Interestingly, the male fertility performance of dKO males is markedly lower than Cyp17 cKO, to the point that 50% of males evaluated were infertile. This indicates that the depletion of SF-1 in CYP19 expressing cells in addition to CYP17 expressing cells induces additional testicular malfunction that significantly impairs male reproductive capacity.

Previous studies have demonstrated the crucial role SF-1 plays in fetal Sertoli cell survival, and the reduced adult testis size observed in the Amh-Cre SF-1 knockout model has been linked to increased apoptosis in both Sertoli and germ cell population (323). This reduction in testis size and increased apoptosis in seminiferous tubules was not observed in our Cyp19 cKO males. Another study, using the Amhr2-Cre SF-1 KO mouse model, showed that absence of SF-1 in Sertoli cells of prepubertal males impaired Sertoli cell survival, maturation and function (366). In the Cyp19 cKO male, the number of SF-1 positive cells in the seminiferous tubules was also significantly reduced and a moderate reduction in sperm production was observed, but the reproductive phenotype was comparable to CON. We do not know exactly when the Cyp19-cre recombination is initiated in our mouse model, and the reduced number of WT1-positive cells in the basal membrane and reduced expression of Sox9 suggest that Sertoli cell survival might be affected, though not enough to significantly impair spermatogenesis.

Another group using the Cyp19a1-Cre transgenic mice to inhibit Neuregulin 1 expression showed that the Cre recombinase was expressed in Leydig cells of the infant testis at one week of age, but not in the adult Leydig cells (376). The adult Leydig cell population is derived from progenitor Leydig cells in infant testis that initiate proliferation in response to increased LH levels (377). In addition, a recent study has shown that a proportion of fetal Leydig cells dedifferentiate to later form adult Leydig cells at puberty and that these dedifferentiated cells also contribute to the peritubular myoid cell and vascular pericyte populations in the neonatal testis (378). It is possible, then, that the absence of SF-1 at that early post-natal stage might interfere with the proliferation of adult Leydig cell and thus their population in later stages. Since Cyp19 cKO males are fertile and show strong expression of SF-1 in Leydig cells of the interstitium as well as normal steroidogenesis, we considered this possible recombination of SF-1 in Leydig cells of the infant testis to be inconsequential. The infertility observed in the dKO male indicates that this initial hypothesis might be erroneous. A reduced adult Leydig cell population caused by SF-1 depletion in proliferating progenitor Leydig cells, as minimal as it may have seemed to be, could be sufficient to affect the steroidogenic capacity of the male testis and might explain the difference in reproductive capacity observed between Cyp17 cKO and dKO males.

In many aspects (testis weight, sperm count, steroidogenic and apoptotic gene expression levels), the dKO male phenotype resembled the Cyp17 cKO male. Unlike the Cyp19 cKO, Sertoli cell function was significantly impaired in the dKO testes, as demonstrated by the reduced expression of *Cyp19a1* and *Inha*. The role of SF-1 in regulating *Cyp19a1* expression in gonadal cells is well established (379), and *Inha* expression is stimulated by SF-1 in synergy with the cAMP pathway (380). Surprisingly, a normal number of Sertoli cells were counted in dKO testes, though

this might be due to the severely disorganized seminiferous tubules, where Sertoli cells were observed in the lumen as well as in its usual location, the basal membrane.

Similar to the observed testicular phenotype in the dKO model, the inactivation of SF-1 in cells expressing Amhr2 produced infertile male mice with hypoplastic testes where the lumina of the seminiferous tubules were closed and spermatogonia failed to develop into sperm, and where Leydig cell steroidogenic gene expression and testosterone production was impaired (198). Amhr2-Cre is expressed in both the Sertoli and Leydig cells of fetal testes (366), and recent studies in females have shown evidence that Amhr2-Cre recombination occurs as early as the primordial follicle stage of the follicle development trajectory, indicating that this mouse model might impair the normal development of both the testis and the ovary (381). Based on the literature, we consider the Cyp17-Cre and Cyp19-Cre recombination of SF-1 to occur after gonadal cells have differentiated into steroidogenic cells, providing novel models to understand the role of SF-1 in mature cells (368, 369).

Finally, females from these three novel mouse lineages showed normal fertility and ovarian morphology. Impaired steroidogenic gene expression was observed in theca cells of the Cyp17 cKO females, and granulosa cells of the Cyp19 cKO female showed increased expression of SF-1 homologue *Nr5a2* (LRH-1) and *Amh.* LRH-1 is structurally similar to SF-1, and regulates many of the same genes, particularly those associated with steroidogenesis (375). These two closely related nuclear receptors are co-expressed in the ovary at all stages of the estrous cycle, and despite their structural conservation, differences in their ligand binding domains recruit distinct cofactors known to regulate different biological functions (13, 382). Previous transgenic mouse studies inducing ovary specific LRH-1 depletion at different follicular stages have recorded no variation in SF-1

mRNA levels caused by LRH-1 absence (46, 160, 161). Similarly, no variation in LRH-1 expression was observed in granulosa cells of early stages of folliculogenesis where SF-1 was depleted (225). Here, we report a significant increase of LRH-1 in granulosa cells of Cyp19 cKO mice, indicating that LRH-1 may be able to compensate for SF-1 depletion and maintain normal levels of steroidogenic activity. Interestingly, the aldo-keto reductase family 1 member C18 (*Akr1c18*), also referred to as 20-α reductase, an enzyme required for progesterone breakdown into an inactive steroid, was strongly reduced in the dKO ovary, suggesting a possible role of SF-1 in luteal function (383). Yet, preliminary data with the female dKO shows that the reduced expression of SF-1 from both theca and mural granulosa cells of the antral follicle does not negatively affect female mouse fertility, unlike the phenotype observed in the male.

4.6 CONCLUSION

The principal aim of the present study was to evaluate the importance of the orphan nuclear receptor SF-1 in adult gonad function and fertility. Our models show that the Cyp17 cKO and Cyp19 cKO models do not induce complete recombination of SF-1 in steroidogenic cells of the testis, and that the combination of both models is required to disrupt steroid production in Leydig cells. The testis cord dysgenesis caused by SF-1 depletion in both Leydig and Sertoli cells affects spermatogenesis and causes infertility in the male mouse, representing *in vivo* evidence that SF-1 plays an essential role in mature testicular function. Further studies using microarray analysis in the gene expression pattern observed in each cKO model will offer new insight on the mechanisms underlying male infertility.

4.7 ACKNOWLEDGEMENTS

The authors thank Alexandre Boyer for valuable scientific discussions and for carefully reading this manuscript, as well as Laura Benoit-Barné, Rodrigo Gonzalez-Lopez, Marie-Charlotte Meinsohn and Emmanuelle Martinot for technical assistance. This study was funded by an operating grant from the Canadian Institutes of Health Research (PJT 166020) to BDM. Olivia Eilers Smith was supported by the Fonds Québécois de la Recherche en Sciences (FQRS) –doctoral training scholarship and the Fonds Québécois de la Recherche en Nature et Technologies (FQRNT) –doctoral training scholarship.

4.8 FIGURES

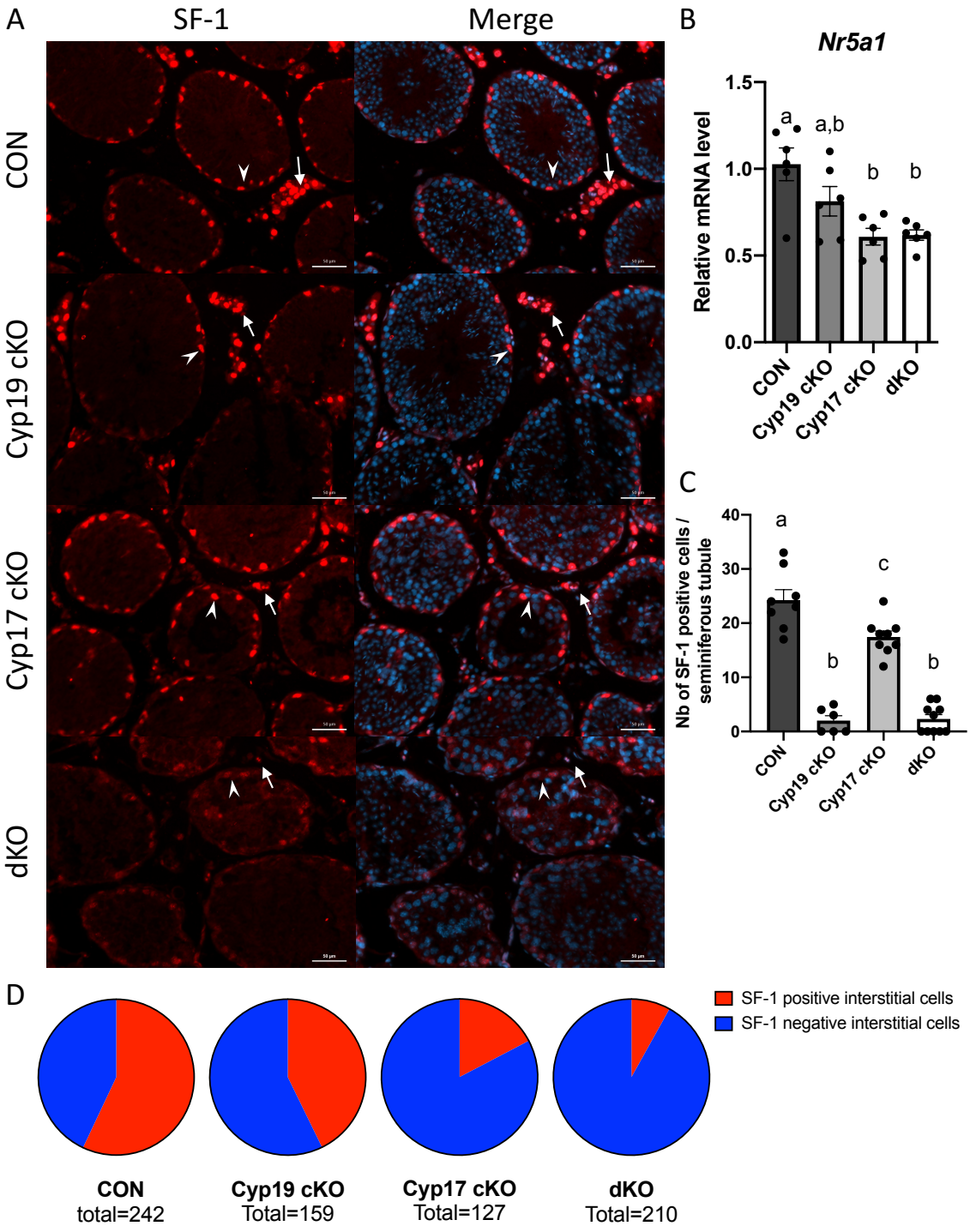
A
SF-1
Merge
CON
Cyp19 cKO
Cyp17 cKO
dKO
B
Nr5a1
Relative mRNA level
1.5
1.0
0.5
0.0
a
a,b
b
b
CON
Cyp19 cKO
Cyp17 cKO
dKO
C
Nb of SF-1 positive cells / seminiferous tubule
40
30
20
10
0
a
b
c
b
CON
Cyp19 cKO
Cyp17 cKO
dKO
D
SF-1 positive interstitial cells
SF-1 negative interstitial cells
CON
total=242
Cyp19 cKO
Total=159
Cyp17 cKO
Total=127
dKO
Total=210

Figure 4.1 Characterization of the SF-1$^{f/f}$ (CON), Cyp17-cre;SF-1$^{f/f}$ cKO (Cyp17 cKO), Cyp19-cre;SF-1$^{f/f}$ cKO (Cyp19 cKO) and Cyp17+Cyp19-cre;SF-1$^{f/f}$ cKO (dKO) conditional knockout male mouse.

(A) Protein expression of the nuclear receptor SF-1 in CON, Cyp19 cKO, Cyp17 cKO and dKO mature testes. SF-1 positive cells (red) are present in the interstitium where Leydig cells are found (arrows), as well as in the testicular cords where Sertoli cells are found (arrowheads). Merged images show both SF-1 (red) and DAPI (blue) staining. Scale bars 50μm. (B) Relative abundance of *Nr5a1* mRNA in the testis of CON, Cyp19 cKO, Cyp17 cKO and dKO mature males (n= 6 per genotype). Data are represented as mean ± SEM. Letters indicate significant statistical difference ($P < 0.05$). (C) Quantitative analysis of SF-1 staining in tubules was calculated as the ratio of SF-1 positive cells per seminiferous tubule. Data are represented as mean ± SEM. Letters indicate significant statistical difference ($P < 0.05$). (D) Quantitative analysis of SF-1 staining in testis interstitium was calculated as the ratio of SF-1 positive cells (red and blue nuclear staining) versus the number of SF-1 negative cells (only blue nuclear staining). Data are represented as a pie chart of total number of cells counted.

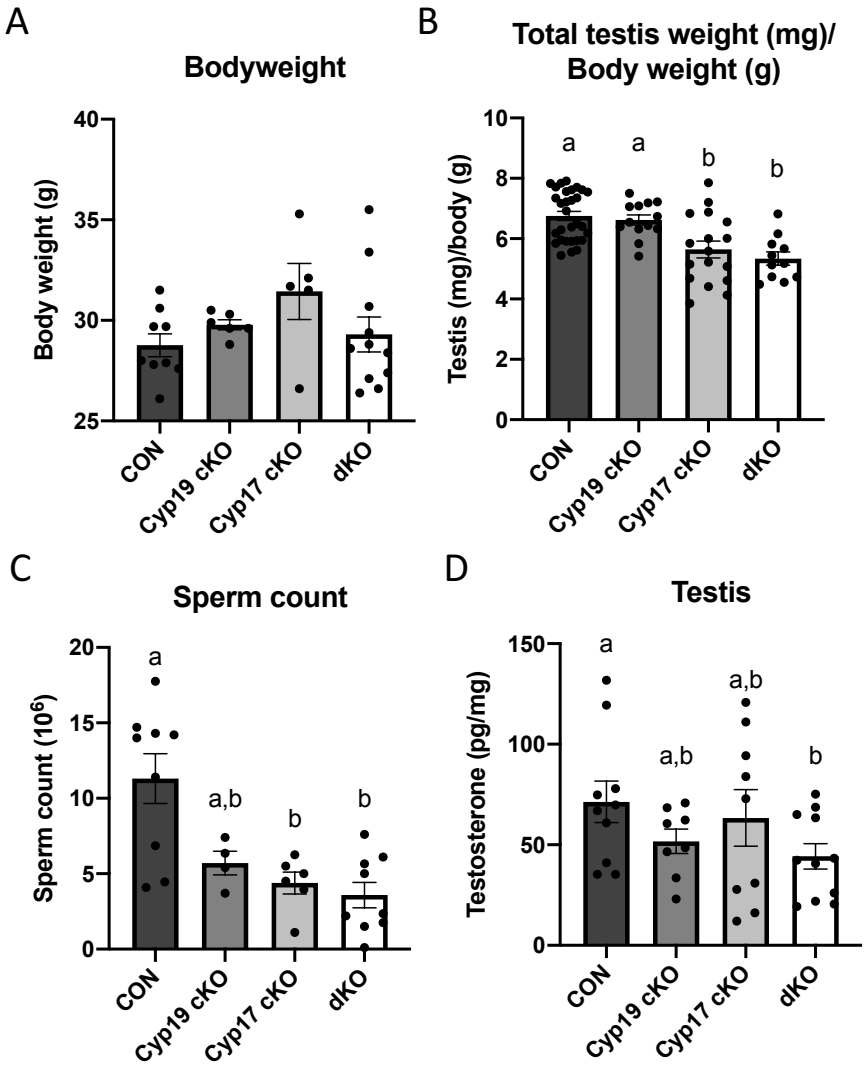

Figure 4.2 Effect of steroidogenic cell-specific SF-1 depletion on male reproductive function

(A) Body weight and (B) total testis weight over body weight ratio of mature males from the 4 genotypes. (C) Total sperm number counted from 1 epididymis collected from mature (between 4 and 6 months) males of the 4 genotypes. (D) Intra-testicular testosterone levels of mature males from the 4 genotypes. Data are represented as mean ± SEM. Letters indicate significant statistical difference ($P > 0.05$).

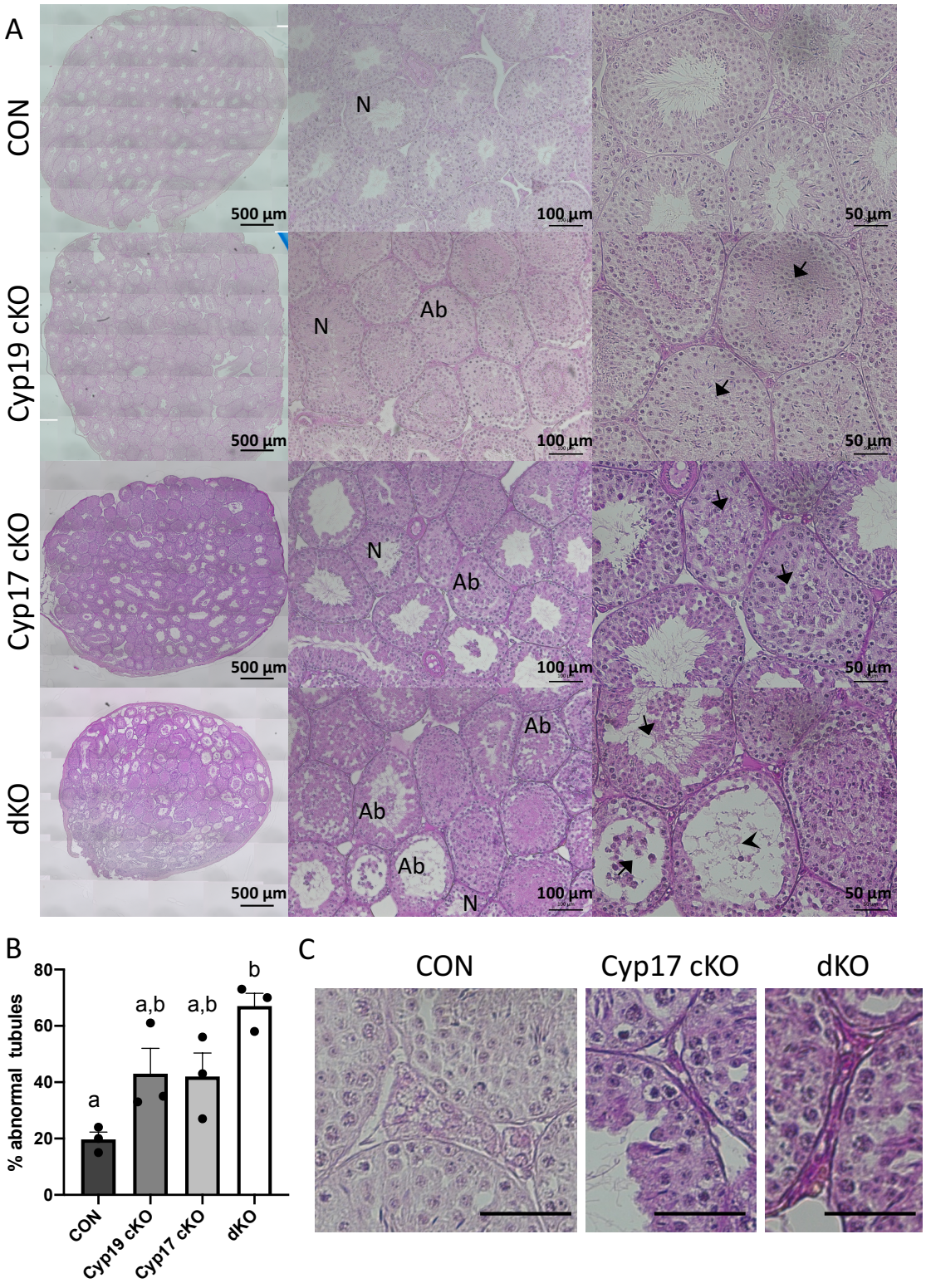
A
CON
Cyp19 cKO
Cyp17 cKO
dKO
500 µm
100 µm
50 µm
N
Ab
B
% abnormal tubules
80
60
40
20
0
a
a,b
a,b
b
CON
Cyp19 cKO
Cyp17 cKO
dKO
C
CON
Cyp17 cKO
dKO

Figure 4.3 Abnormal testis morphology in mice with steroidogenic cell-specific SF-1 depletion.

(A) Hematoxylin-eosin-stained testis sections of CON, Cyp19 cKO, Cyp17 cKO and dKO mature mice. Examples of normal (N) seminiferous tubules that show different stages of spermatogenesis, and abnormal (Ab) seminiferous tubules with presence of nucleated cells in the lumina (arrows) or absence of elongated spermatids (arrowheads). (B) Quantification of abnormal testis morphology by averaging the number of tubules with normal (N) and abnormal (Ab) structure in testis sections (n=3 per genotype) of CON, Cyp17 cKO and dKO adult male mice. Data are represented as mean ± SEM. Letters indicate significant statistical difference ($P > 0.05$). (C) Examples of morphology of cell in the interstitial space of CON, Cyp17 cKO and dKO testes. Scale bar, 50 μM.

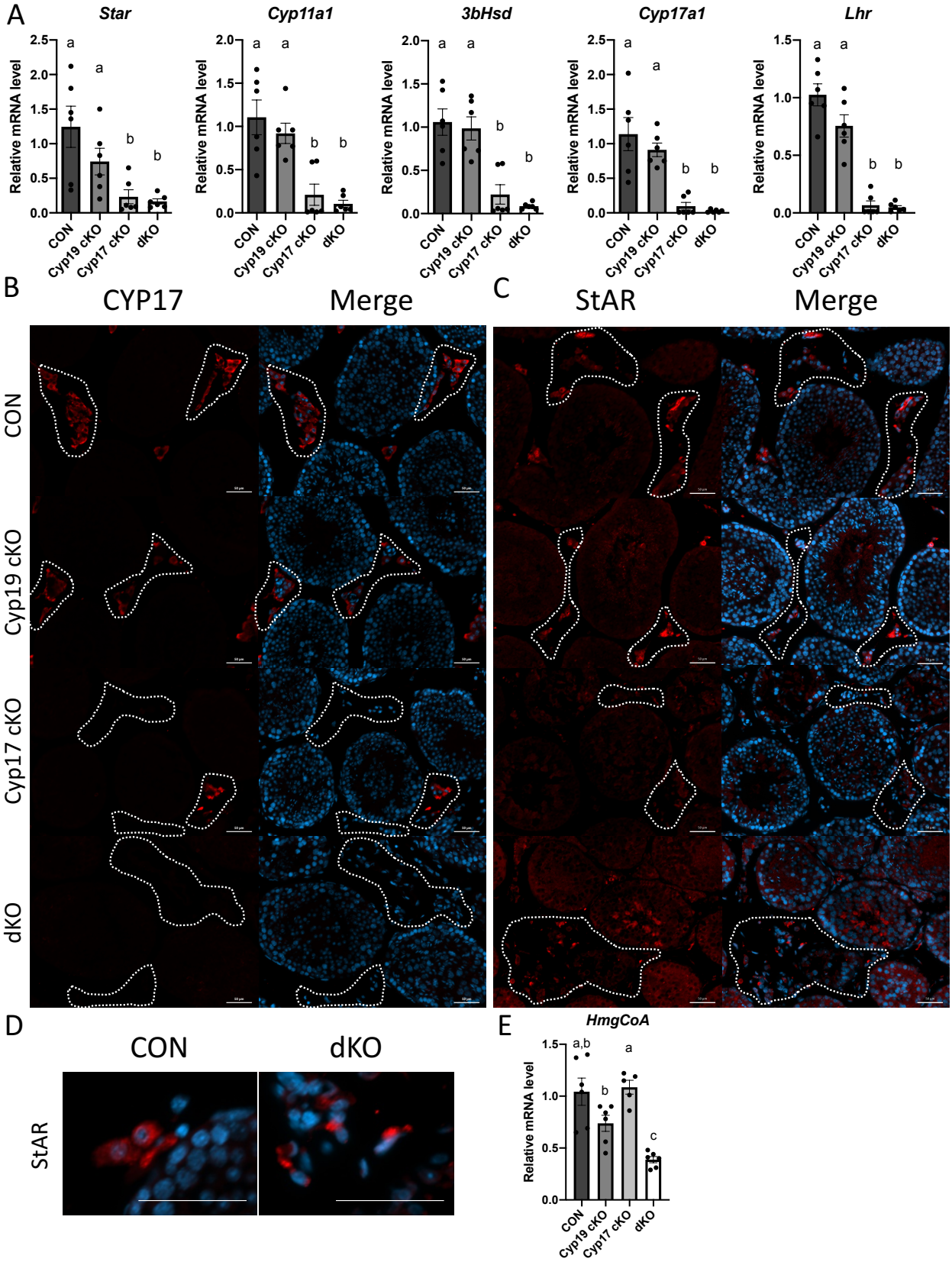
A
Star
Cyp11a1
3bHsd
Cyp17a1
Lhr
Relative mRNA level
CON
Cyp19 cKO
Cyp17 cKO
dKO
B
CYP17
Merge
C
StAR
Merge
D
CON
dKO
StAR
E
HmgCoA

Figure 4.4 Reduced steroidogenesis in Leydig cells of Cyp17 cKO and dKO mature males.

(A) Relative abundance of Leydig specific steroidogenic genes (*Star*, *Cyp11a1*, *3bHsd* and *Cyp17*) and Leydig cell-specific *Lhr* in the testes of CON, Cyp19 cKO, Cyp17 cKO and dKO mature males (n= 6 per genotype). Letters indicate significant statistical difference ($P < 0.05$). Protein expression of (B) CYP17 and (C) STAR in CON, Cyp19 cKO, Cyp17 cKO and dKO mature testes. CYP17 or STAR positive cells (red) are present in the interstitial space (circled by dotted white line). Merged images show both CYP17 or STAR (red) and DAPI (blue) staining. Scale bars 50μm.

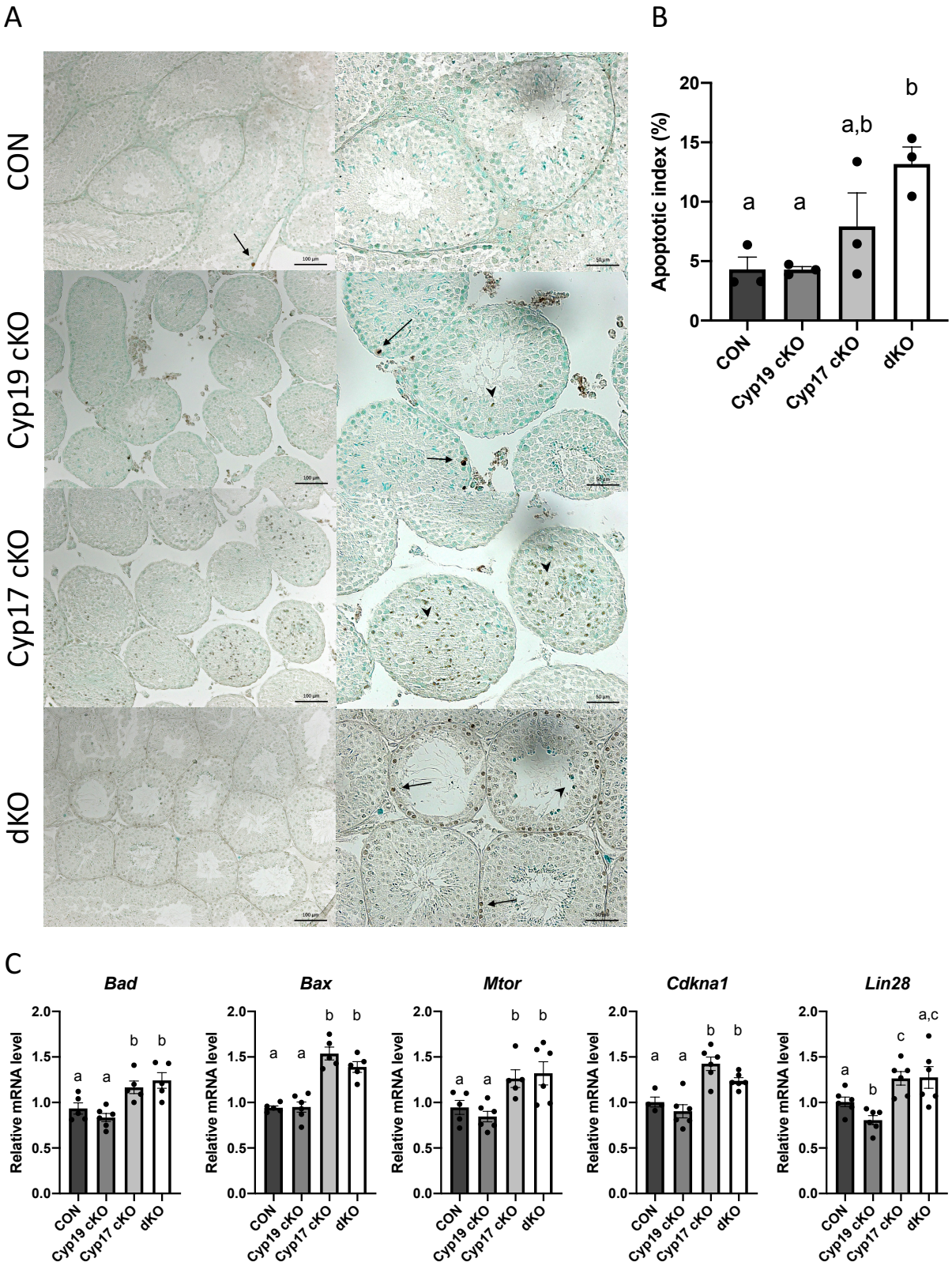
A
CON
Cyp19 cKO
Cyp17 cKO
dKO
B
Apoptotic index (%)
20
15
10
5
0
a
a
a,b
b
CON
Cyp19 cKO
Cyp17 cKO
dKO
C
Bad
Bax
Mtor
Cdkna1
Lin28
Relative mRNA level
2.0
1.5
1.0
0.5
0.0
a
a
b
b
a,c
c
CON
Cyp19 cKO
Cyp17 cKO
dKO

Figure 4.5 Increased apoptosis in dKO testes.

(A) Representative images of TUNEL staining in mature testes sections of CON, Cyp19 cKO, Cyp17 cKO and dKO mature mice. Presence of TUNEL staining positive cells in basal compartment (black arrows) and adluminal compartment (black arrowheads). Scale bars 100 μm (left pictures) and 50 μm (right pictures). (B) Quantitative analysis of apoptosis in testis. The apoptosis percentage was calculated as the ratio of apoptosis-positive seminiferous tubules (3 or more apoptotic cells) to the total number of seminiferous tubules. Data (n=3) are represented as mean ± SEM. (C) Relative abundance of pro-apoptotic (*Bad, Bax*), autophagy (*Mtor*), and cell cycle arrest (*Cdkna1* and *Lin28)* markers mRNA in the testes of CON, Cyp19 cKO, Cyp17 cKO and dKO mature males (n= 6 per genotype). Letters indicate significant statistical difference ($P < 0.05$).

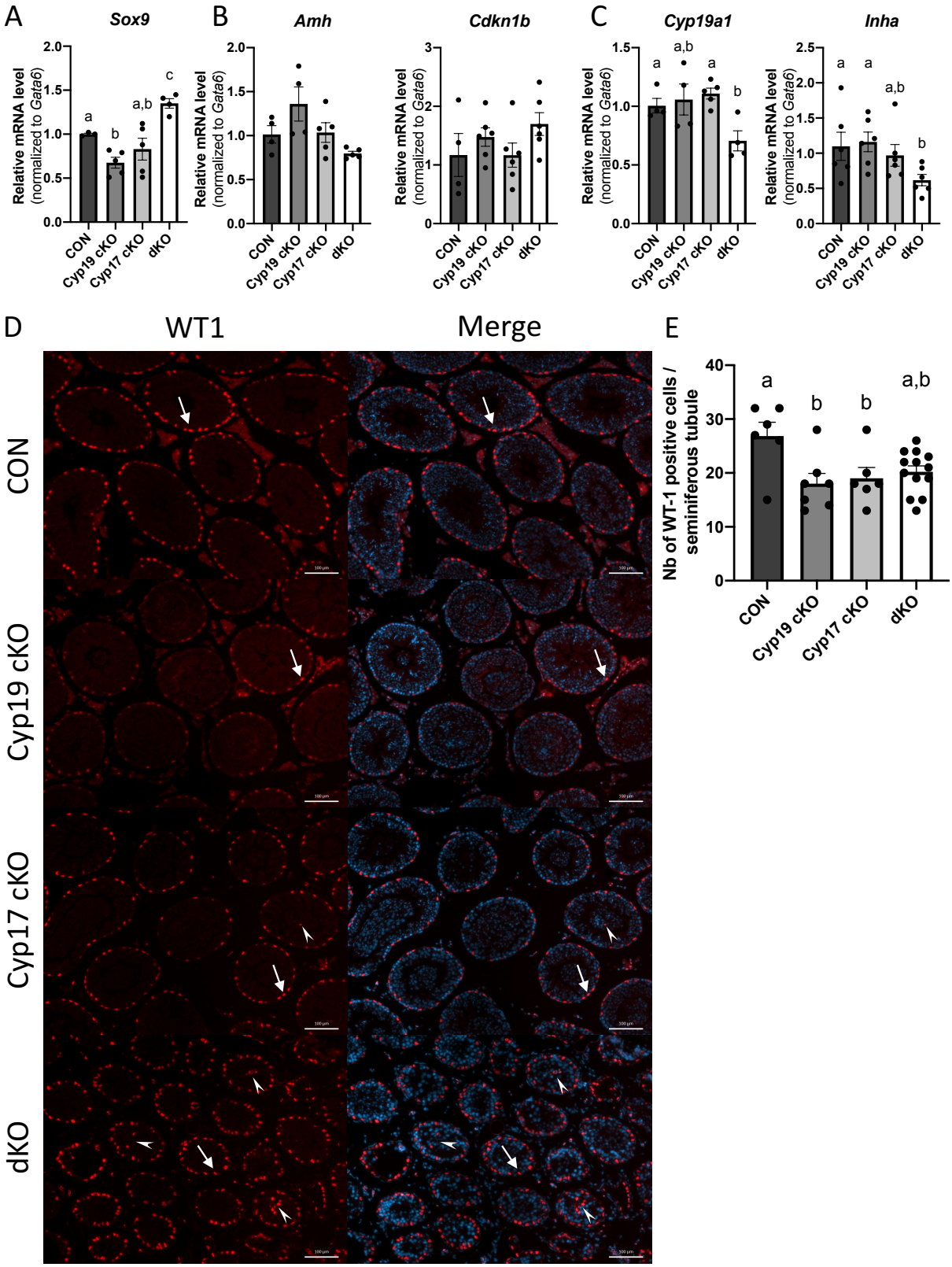
A
Sox9
B
Amh
Cdkn1b
C
Cyp19a1
Inha
Relative mRNA level (normalized to Gata6)
CON
Cyp19 cKO
Cyp17 cKO
dKO
D
WT1
Merge
E
Nb of WT-1 positive cells / seminiferous tubule

Figure 4.6 Abnormal Sertoli cell function and localization in dKO testes .

(A-C) mRNA abundance of Sertoli-specific cell markers (A) *Sox9*, (B) *Amh* and *Cdkn1b*, and (C) *Cyp19a1* and *Inha* normalized to Sertoli-specific GATA6 (in the testes of CON, Cyp19 cKO, Cyp17 cKO and dKO mature males (n= 6 per genotype). (D) Protein expression of WT1 in CON, Cyp19 cKO, Cyp17 cKO and dKO mature testes. WT1 positive cells are present in the basal compartment of the testicular cords (arrows) of all genotypes as well as in the adlumina (arrowhead) of dKO testis. Merged images show WT1 (red) and DAPI (blue) staining. Scale bars 100μm. (E) Quantitative analysis of WT-1 staining in tubules was calculated as the ratio of SF-1 positive cells per seminiferous tubule. Data are represented as mean ± SEM. Letters indicate significant statistical difference ($P < 0.05$).

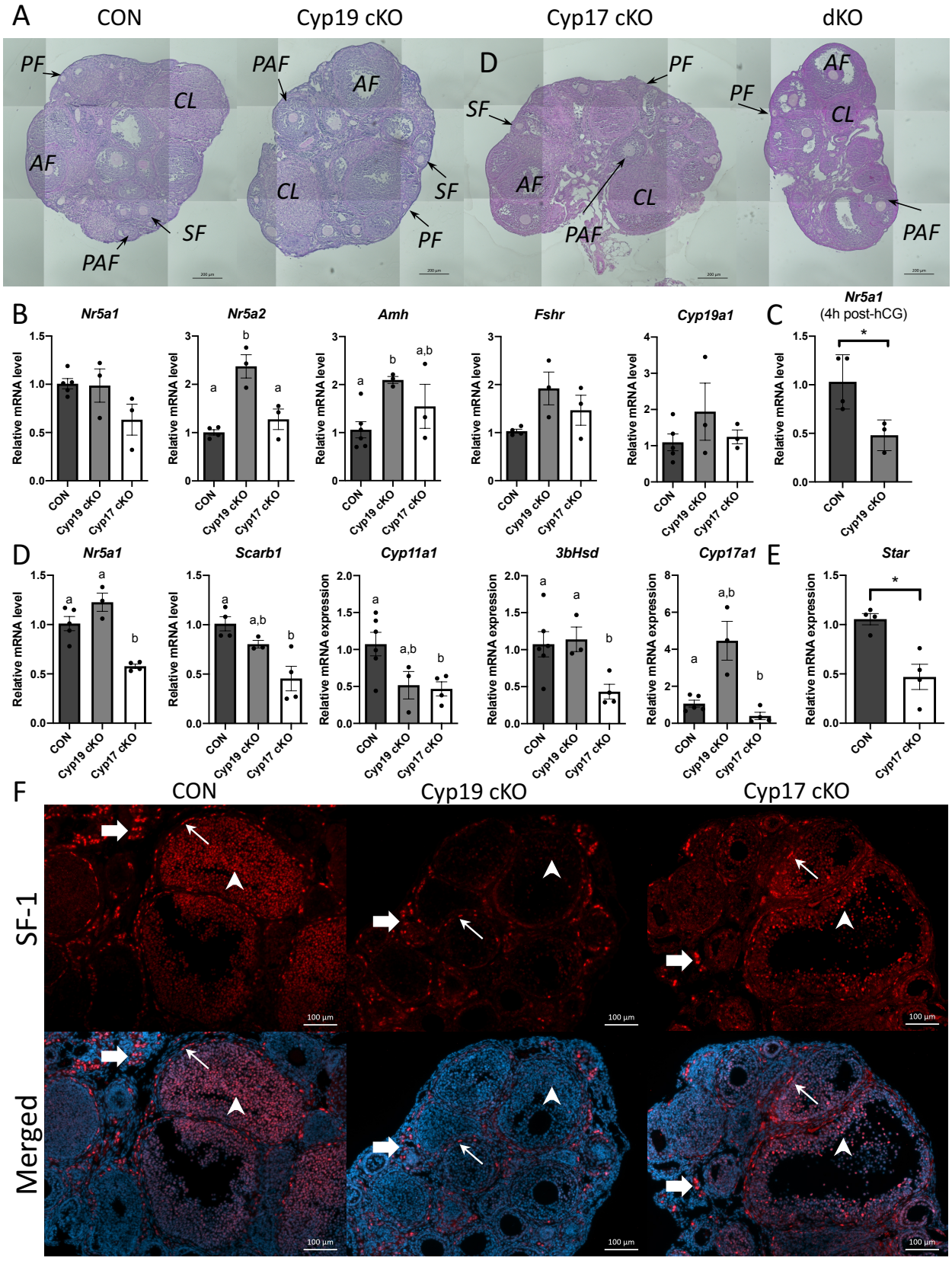
A
CON
Cyp19 cKO
Cyp17 cKO
dKO
PF
CL
AF
SF
PAF
D
B
Nr5a1
Nr5a2
Amh
Fshr
Cyp19a1
C
Nr5a1
(4h post-hCG)
Relative mRNA level
CON
Cyp19 cKO
Cyp17 cKO
D
Nr5a1
Scarb1
Cyp11a1
3bHsd
Cyp17a1
E
Star
Relative mRNA expression
F
CON
Cyp19 cKO
Cyp17 cKO
SF-1
Merged
100 µm

Figure 4.7 Effect of steroidogenic cell-specific SF-1 depletion on female reproductive function and ovarian morphology.

(A) Bright field microscopy images of hematoxylin-eosin-stained sections of ovaries from mature mice of the 4 genotypes collected on the morning of estrus. Scale bar, 200 μm. PF, primary follicle; SF, secondary follicle; PAF, preantral follicle; AF, antral follicle; CL, corpora lutea. (B) Relative mRNA abundance of granulosa specific factors in granulosa cells isolated from the ovaries of mature CON (n=6), Cyp19 cKO (n=3) and Cyp17 cKO (n=3) female mice. (C) Relative mRNA abundance of *Nr5a1* in granulosa cells isolated from superstimulated CON (n=4) and Cyp19 cKO female mice 4h post hCG injection. (D) Relative mRNA abundance of theca specific steroidogenic factors in theca cells isolated from the ovaries of mature CON (n=6), Cyp19 cKO (n=3) and Cyp17 cKO (n=4) female mice. Letters indicates significant statistical difference ($P < 0.05$). (D) Protein expression of nuclear specific SF-1 in CON, Cyp19 cKO and Cyp17 cKO superstimulated ovaries. Merged images show SF-1 (red) and DAPI (blue) staining in theca cells (small white arrows), granulosa cells (white arrowheads) and cells of the interstitial tissue (large white arrow). Scale bars 100μm.

4.9 SUPPLEMENTAL FIGURES

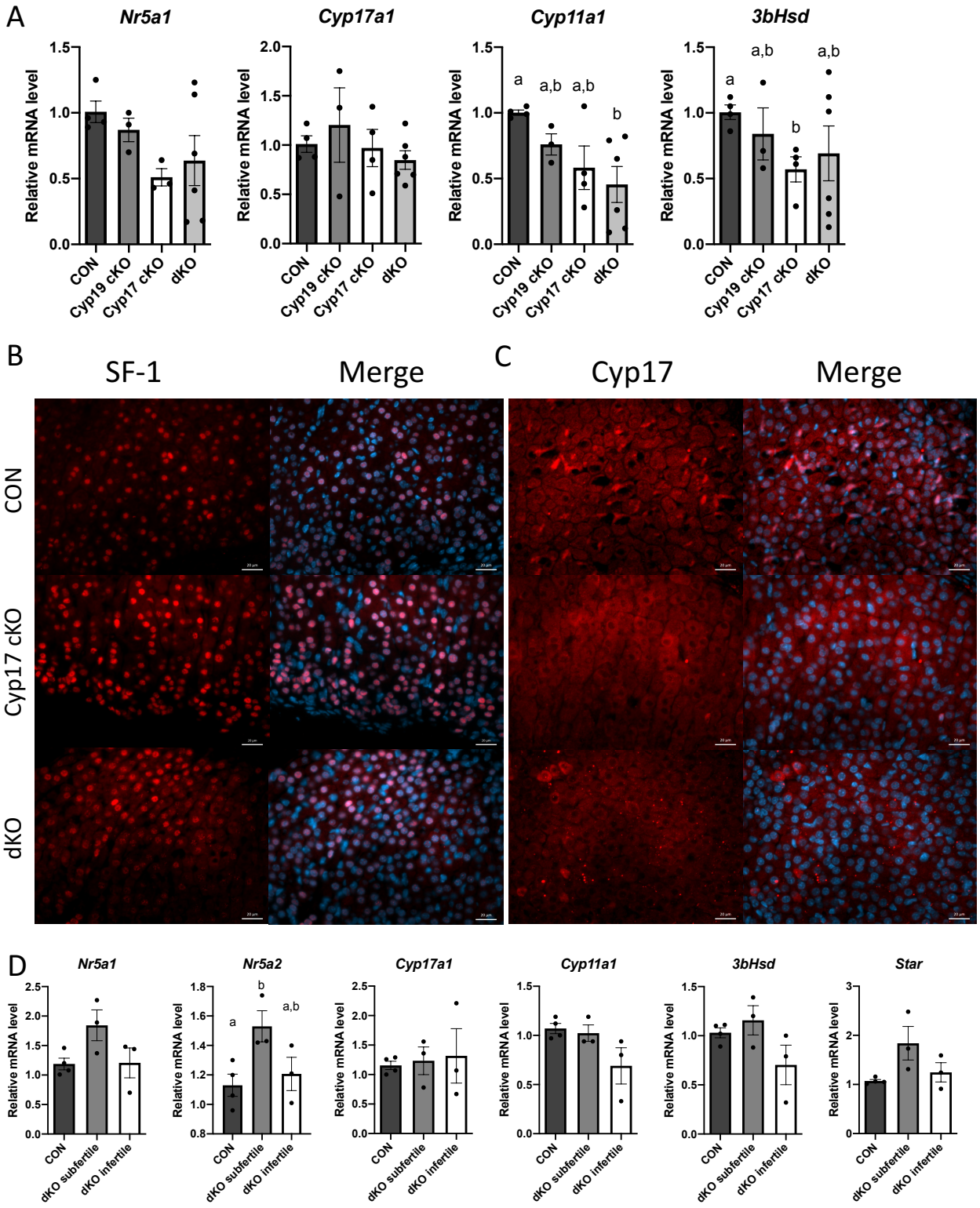

Figure S4.1

(A) Abundance of steroidogenic enzyme mRNA in the adrenal glands of CON, Cyp19 cKO, Cyp17 cKO and dKO mature males (n= 6 per genotype). (B-C) Protein expression of (B) nuclear specific SF-1 and (C) cytoplasmic CYP17 in CON, Cyp17 cKO and dKO mature adrenal gland. Merged images show both SF-1 or CYP17 (red) and DAPI (blue) staining. Scale bars 20μm. (D) Abundance of *Nr5a1*, *Nr5a2* and steroidogenic enzymes mRNA in the adrenal glands of CON (n=4) mature males and subfertile (n=3) and infertile (n=3) dKO males after completing fertility trial. Letters indicate significant statistical difference ($P < 0.05$).

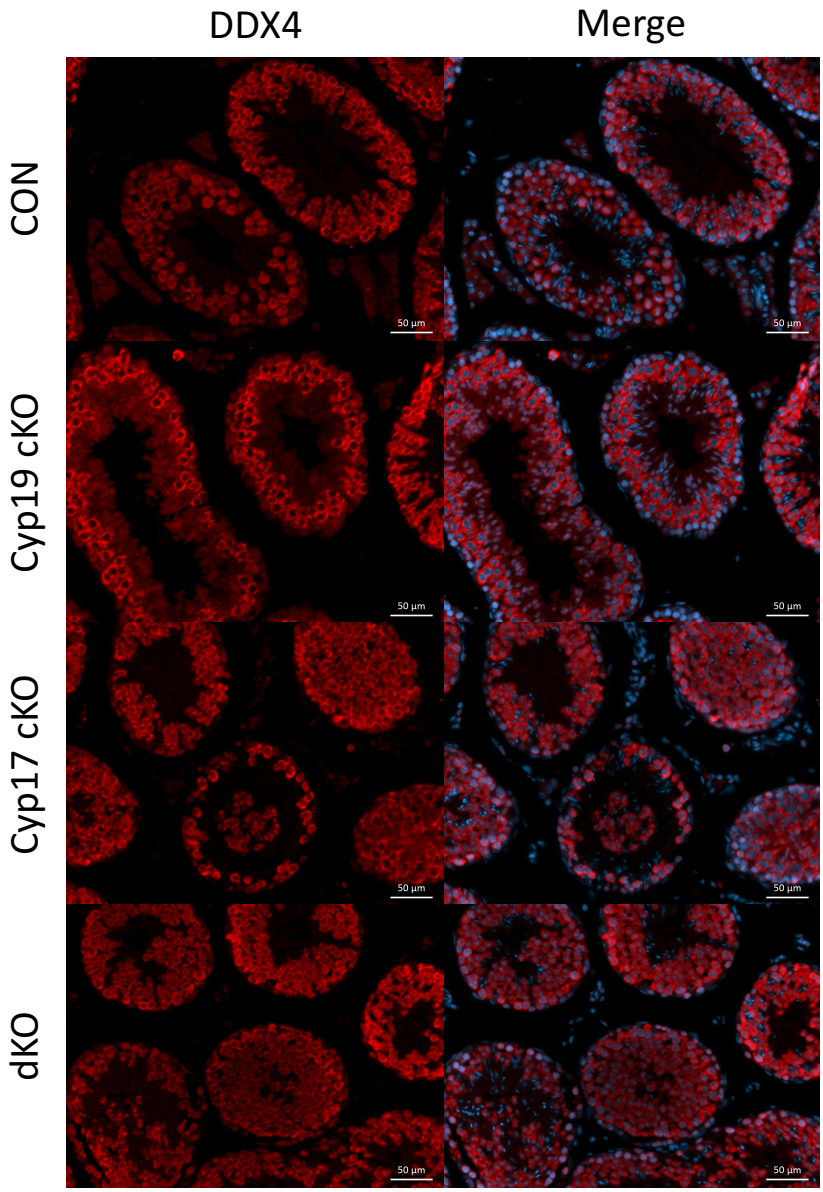

Figure S4.2

Protein expression of germ line-specific DDX4 in CON, Cyp19 cKO, Cyp17 cKO and dKO mature testes. DDX4 positive cells (red) are present within seminiferous tubules. Merged images show both cytoplasmic DDX4 (red) and nuclear DAPI (blue) staining. Scale bars 50µm.

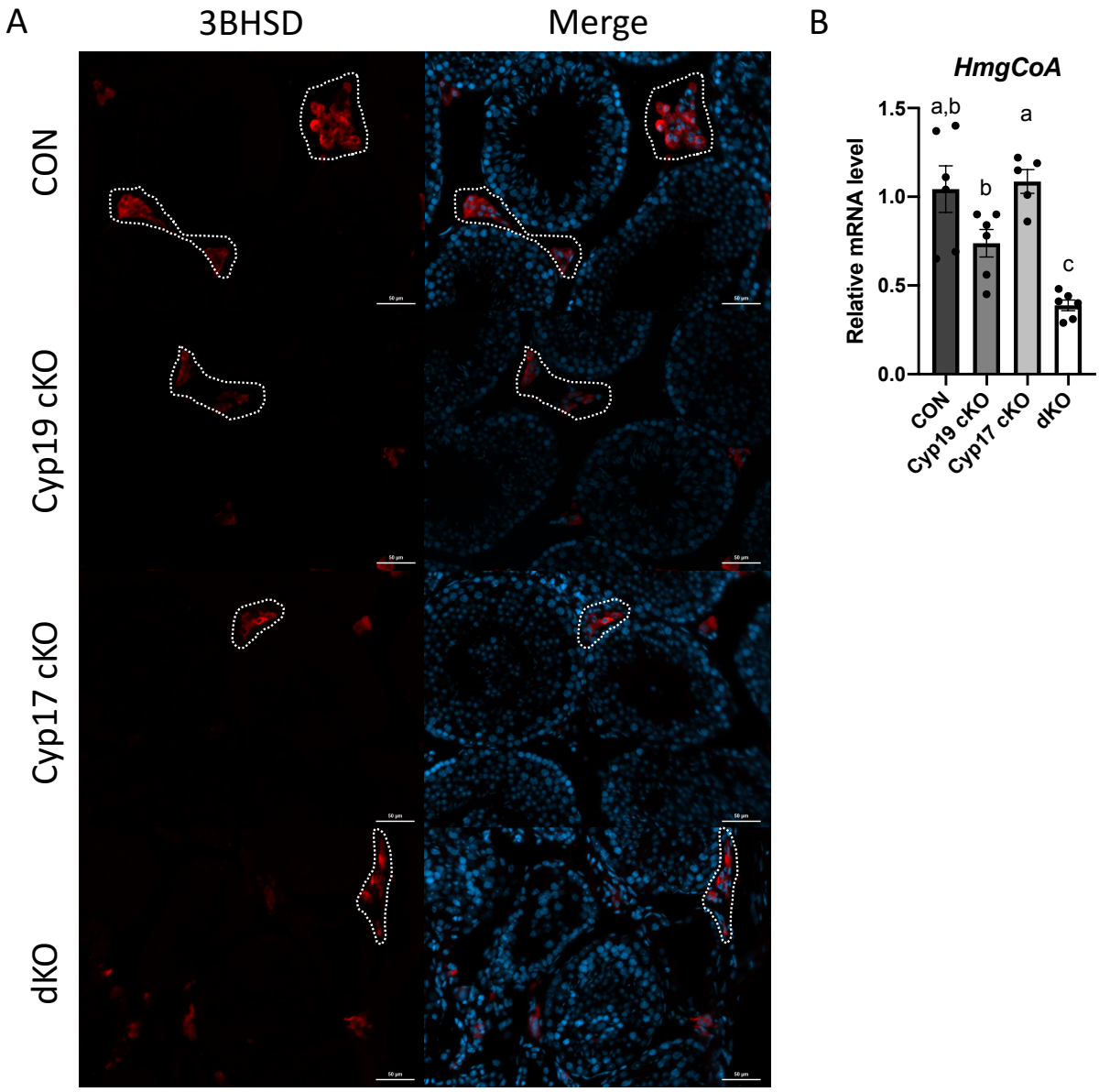

Figure S4.3

(A) Protein expression of cytoplasmic 3BHSD in CON, Cyp19 cKO, Cyp17 cKO and dKO mature testes. 3BHSD positive cells (red) are present in the interstitial space. Merged images show both 3BHSD (red) and DAPI (blue) staining. Scale bars 50µm. (B) mRNA abundance of cholesterol homeastasis markers (*HmgCoA*, *Scp2*, *Acat1* and *Lipe*) in the testes of CON, Cyp19 cKO, Cyp17

cKO and dKO mature males (n= 6 per genotype). Letters indicate significant statistical difference ($P < 0.05$).

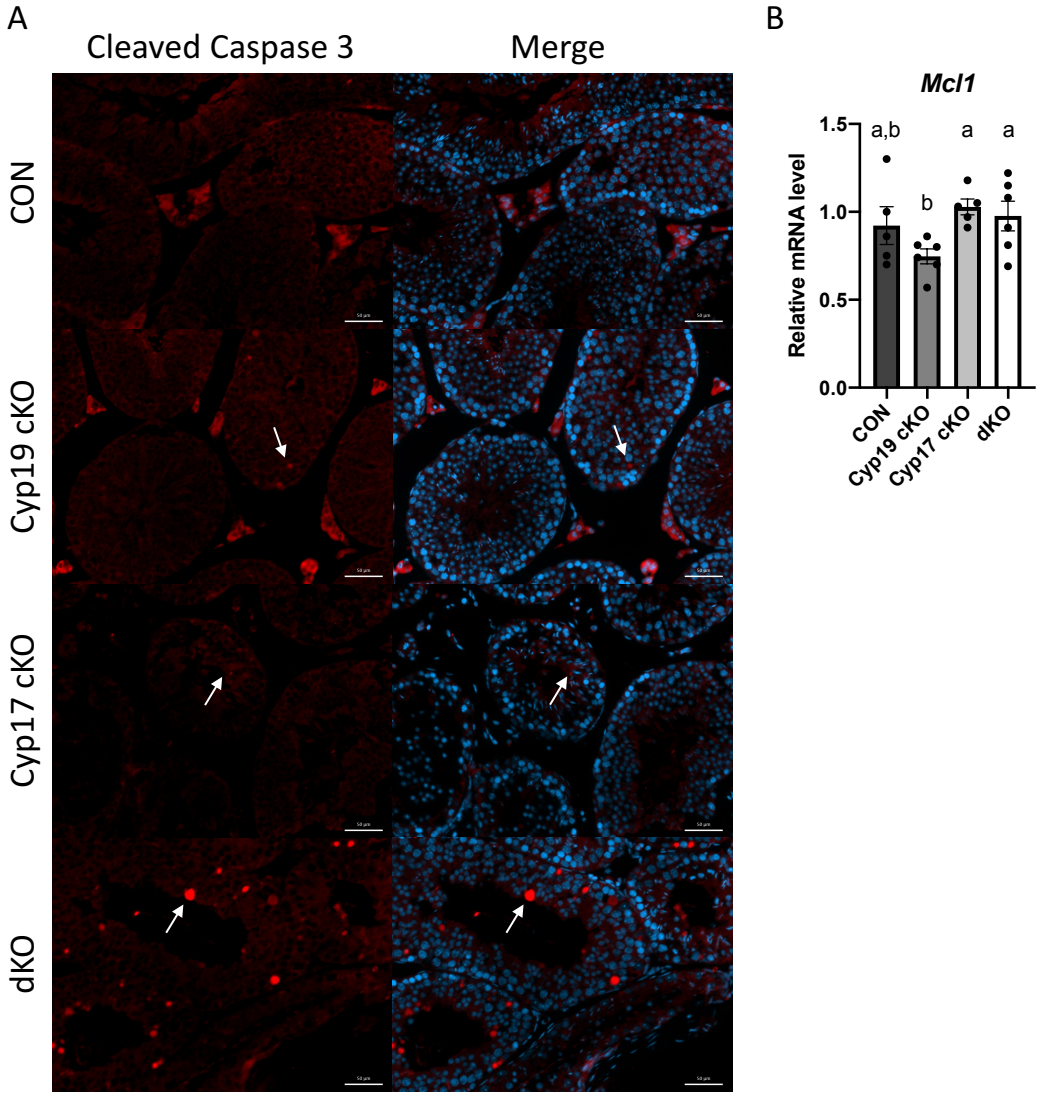

Figure S4.4

(A) Protein expression of nuclear specific cleaved caspase-3 in CON, Cyp19 cKO, Cyp17 cKO and dKO mature testes. Cleaved caspase-3 positive cells (red) are present in the testicular cords (white arrow). Merged images show both cleaved caspase-3 (red) and DAPI (blue) staining. Scale bars 50µm. (B) Relative abundance of anti-apoptotic marker *Mcl1* in the testes of CON, Cyp19 cKO, Cyp17 cKO and dKO mature males (n= 6 per genotype). Letters indicate significant statistical difference ($P < 0.05$).

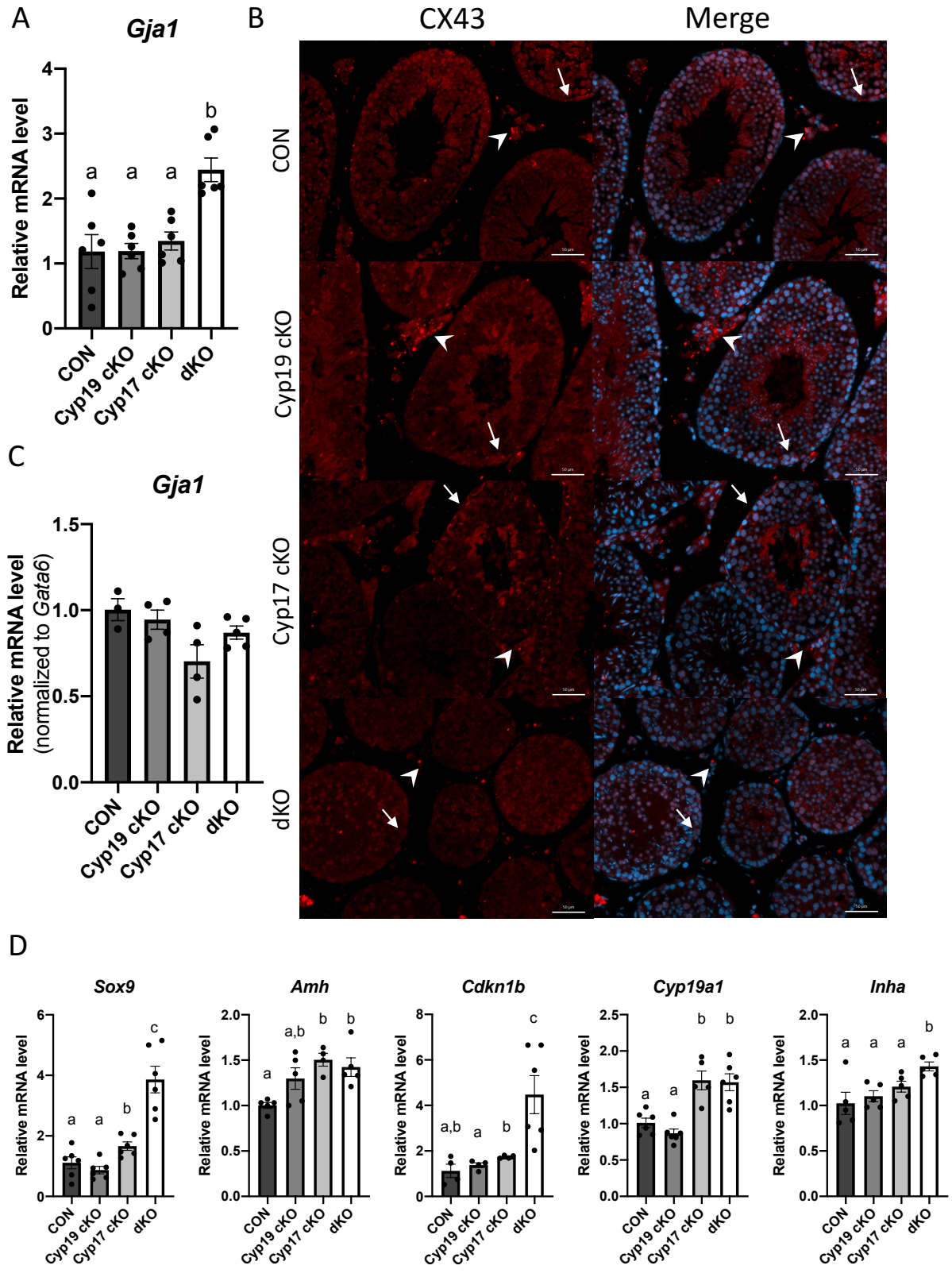
A
Gja1
Relative mRNA level
CON
Cyp19 cKO
Cyp17 cKO
dKO
B
CX43
Merge
C
Gja1
Relative mRNA level
(normalized to Gata6)
D
Sox9
Amh
Cdkn1b
Cyp19a1
Inha

Figure S4.5

(A) mRNA abundance of gap junction marker CX43 (*Gja1)* mRNA in the testes of CON, Cyp19 cKO, Cyp17 cKO and dKO mature males (n= 6 per genotype). Letters indicate significant statistical difference ($P < 0.05$). (B) Protein expression of CX43 in CON, Cyp19 cKO, Cyp17 cKO and dKO mature testes. CX43-positive cells are present in interstitial space (arrowheads) and in the testicular cords (arrows). Merged images show CX43 (red) and DAPI (blue) staining. Scale bars 50μm. (C) mRNA abundance of *Gja1* normalized to *Gata6* in the testes of CON, Cyp19 cKO, Cyp17 cKO and dKO mature males (n= 6 per genotype). (D) mRNA abundance of Sertoli-specific cell markers normalized to housekeeping genes in the testes of CON, Cyp19 cKO, Cyp17 cKO and dKO mature males (n= 6 per genotype). Letters indicate significant statistical difference ($P < 0.05$).

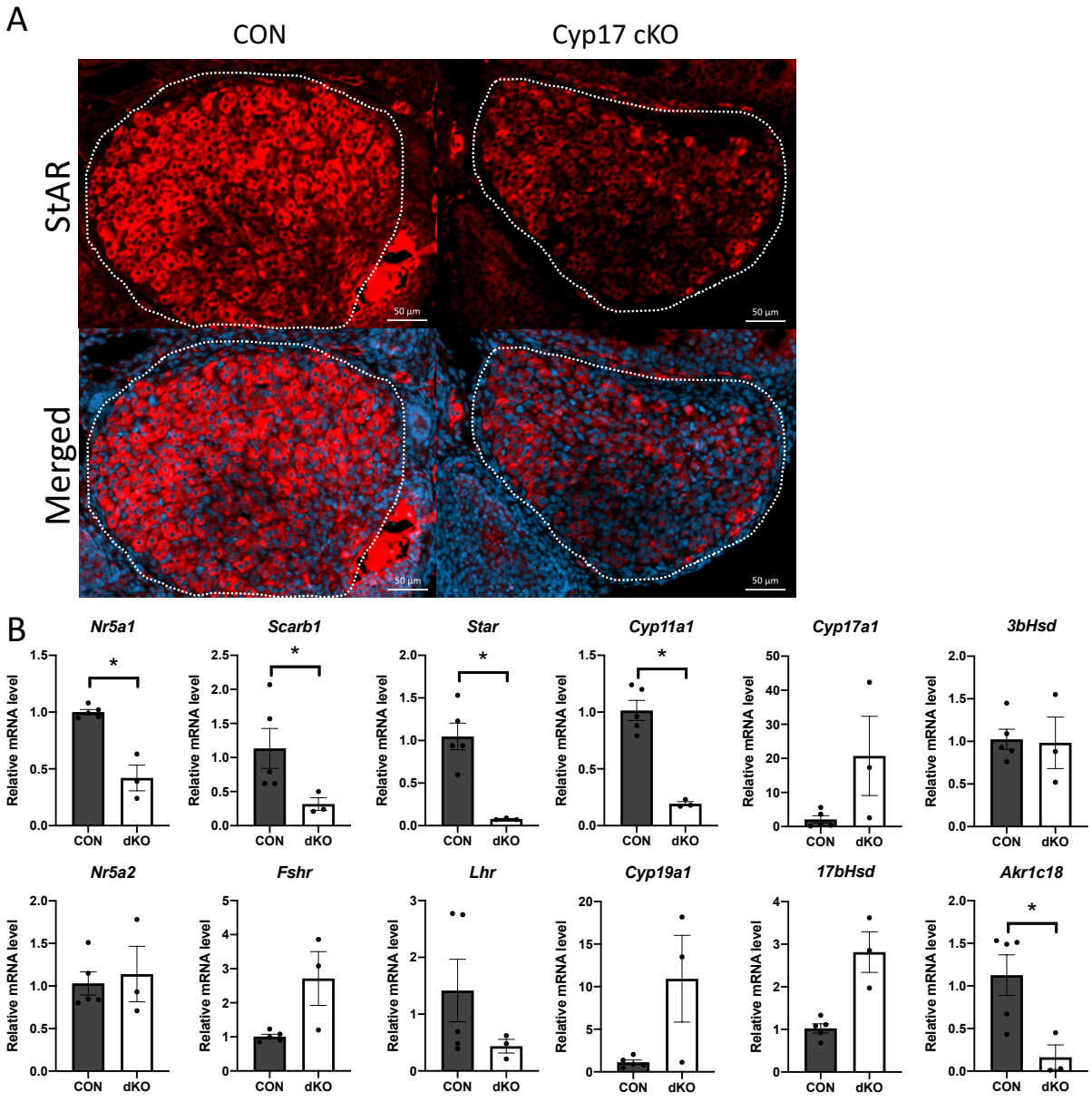

Figure S4.6

(A) Protein expression of cytoplasmic StAR in corpora lutea of CON and Cyp17 cKO superstimulated ovaries. Merged images show StAR (red) and DAPI (blue) staining in luteal cells. Scale bars 50µm. (B) Relative mRNA abundance of factors involved in steroidogenesis in CON (n=5) and dKO (n=3) whole ovaries. Asterisk indicates significant statistical difference ($P < 0.05$).

4.10 TABLES

Table 4.1 Reproductive performance of CON, Cyp19 cKO, Cyp17 cKO and dKO mature male mice.

Six-month breeding trial of males mated with reproductively proven C57BL/6 females. Only fertile males were considered to calculate the average number of litters / male and average litter size. Silver's relative fecundity number = (productive matings) x (average nb of litters / female) x (average litter size).

Reproductive performance	Genotype			
	CON	**Cyp19 cKO**	**Cyp17 cKO**	**dKO**
Number of males	5	6	5	6
Productive matings	100%	83%	100%	50%
Nb litters/male	5.5 ± 0.5	5.8 ± 1.1	4.6 ± 1.5	2.7 ± 1.15 *
Litter size	5.3 ± 1.6	5.2 ± 1.1	5.6 ± 1.7	6 ± 0.9
Total number of pups/male	28.2 ± 7.4	30.2 ± 10.5	26 ± 9.9	16 ± 9.8 *
Silver's relative fecundity	28.6	25.0	25.8	8.1

Table 4.2 Reproductive performance of CON, Cyp19 cKO, Cyp17 cKO and dKO mature female mice.

Six-month breeding trial of females mated with reproductively proven C57BL/6 males. Only fertile females were considered to calculate the average number of litters / female and average litter size. Silver's relative fecundity number = (productive matings) x (average nb of litters / female) x (average litter size).

Reproductive performance	Genotype			
	CON	**Cyp19 cKO**	**Cyp17 cKO**	**dKO**
Number of females	5	4	5	3
Productive matings	100%	100%	100%	100%
Nb litters/female	5.5 ± 0.5	5.3 ± 1.9	4.5 ± 1.6	6.0 ± 1.0
Litter size	5.3 ± 1.6	4.7 ± 0.7	6.1 ± 1.6	6.2 ± 1.1
Total number of pups/female	28.2 ± 7.4	26.3 ± 11.8	29.3 ±14.9	37.3 ± 9.3
Silver's relative fecundity	28.6	24.9	27.5	37.2

4.11 SUPPLEMENTAL TABLES

Table S4.1 List of primer sequences used in this study. All primers are 5' to 3'.

	Gene	Forward	Reverse
Genotyping	*Sf-1*	AGACAAGTGCACCCCATTTC	ACCATCACCAACCGCTAAAC
	Cyp19Cre	ACAGCACCCTCTGAAGCAAC	CACAGTCAGCAGGTTGGAGA
	Cyp17Cre	AAACTTCTGCAGGCCAAGAG	TCCCTCACATCCTCAGGTTC
qPCR	*B2m*	GACCGGCCTGTATGCTATCC	TTTCAATGTGAGGCGGGTGG
	Hprt	ATGATCAGTCAACGGGGGAC	GAGAGGTCCTTTTCACCAGCA
	Ywhaz	CAGTCTGCCTGGGATTCTATTC	TGGTTGGTGACAAGACAGTAAA
	Rpl19	CTGAAGGTCAAAGGGAATGTG	GGACAGAGTCTTGATGATCTC
	Nr5a1	GTCTCAAGTTCCTCATCCTCTTC	GGGTAGTGACACAAGGTGTAAT
	Nr5a2	TCATGCTGCCCAAAGTGGAGA	TGGTTTTGGACAGTTCGCTT
	Fshr	AGAAAGCAGGTGGATGGATAAA	GAAGACCCTGTTAGAGCAATGA
	Lhr	GCATCTGTAACACAGGCATCC	AAAGCGTTCCCTGGTATGGT
	Cyp11a1	CTGCCTCCAGACTTCTTTCG	TTCTTGAAGGGCAGCTTGTT
	3βHsd-2	GGTTTTTGGGGCAGAGGATCA	GGTACTGGGTGTCAAGAATGTCT
	Cyp17a1	ACCAGCCAGATCGGTTTATG	CAGCTCCGAAGGGCAAATA
	17βHsd-1	GGTGGTCTGCATCGGATAAA	GCAACACAGGGCTACATAGT
	Cyp19a1	ATGTTCTTGGAAATGCTGAACCC	AGGACCTGGTATTGAAGACGAGC
	Akr1c18	GAGCAGTGGCTGAGAATGAA	AGATGCTCTTCAGTTGCATAGG
	Bad	CAACATTCATCAGCAGGGAC	TCCATCCCTTCATCCTCCTC
	Bax	GCTGATGGCAACTTCAACTG	ATCAGCTCGGGCACTTTAG
	Mtor	CGGGACTACAGAGAGAAGAAGA	CATCAACGTCAGGTGGTCATAG
	Cdkna1	CAAGCTAGGATGACAGTGAAG	GAGTCGGGATATTACGGTTGAG
	Lin28	GGACTCAACTCCTTAGCCTTTC	GCTATCAACCATCACGCTACA
	Mcl1	TCGGACTTCAGAGCACTTTATG	GGAAGTCAGGCTCCTAGTAAAC
	Gata-6	CGGTGCTCCACAGCTTACA	CTCCGACAGGTCCTCCAAC
	Sox9	AGGAAGTCGGTGAAGAACGG	GGACCCTGAGATTGCCCAGA
	Amh	ACAGAACCTCTGCCCTACT	TGCTTGGTTGAAGGGTTAAGA
	Cdkn1b	GACATCCTGTATAAGCACTGAGAA	GCGCAATGCTACATCCAATG
	Inha	ATGCACAGGACCTCTGAACC	GGATGGCCGGAATACATAAG
	Gja1	TCCAAGGAGTTCCACCACTT	TGGAGTAGGCTTGGACCTTG
	Scarb1	TTGGCCTGTTTGTTGGGATG	GGATTCGGGTGTCATGAAGG
	Star	TTGGGCATACTCAACAACCA	GCGGTCCACAAGTTCTTCAT
	HmgcoA	CAGAGAAGACAGTGCTCGTTAG	GCTCCATCATTGGCTCTGTAA

Chapter 5. General discussion

The overall aim of my PhD research has been to investigate the role of SF-1 in mature mouse reproductive function, with focus on how its absence in steroidogenic cells of the gonads affects fertility. The major findings presented in this thesis demonstrate that SF-1 is involved in the regulation of ovulation and luteinization in mature females and is required for normal testicular function in males. Ablation of SF-1 at the onset of puberty (Chapter 3) caused ovarian lipid accumulation, anovulation and female infertility, due principally to impaired pituitary function. These results provide further evidence that SF-1 plays an essential role in regulating gonadotropin synthesis and secretion. Abnormal ovarian cholesterol homeostasis and reduced ovulation was observed in conditional knockout (cKO) females treated with exogenous gonadotropins, where the expression of genes involved in ovulation and luteinization was significantly decreased. Furthermore, data obtained from ovarian transplants presented in Chapter 3 demonstrate that SF-1 depletion, specifically in the ovary of adult mice, results in infertility, although not in all cases. The results from Chapter 4 suggest that depletion of SF-1 in either Leydig or Sertoli cells does not affect male fertility, but that reduced expression of the orphan nuclear factor in both testicular cell populations impairs steroidogenesis and spermatogenesis, and in some cases, leads to infertility. Surprisingly, the depletion of SF-1 in theca cells and mural granulosa cells of antral follicles, separately or combined, induced reduced steroidogenic gene expression but did not affect female fertility. In this final section, I will further discuss the results and questions that arose from the data presented in Chapters 3 and 4, describe the challenges that were met, and propose potential implications and perspectives of my results for future studies.

5.1 Role of SF-1 in the brain

5.1.1 The essential role of SF-1 in pituitary gland function

Female mammalian reproductive function is controlled by a hormonal loop between the ovary and the pituitary-hypothalamic endocrine axis. GnRH and FSH secretion in the brain is required to stimulate folliculogenesis and steroidogenesis in the ovary, and the secreted sex steroids will trigger the positive feedback induction of GnRH and LH surges in the hypothalamus and pituitary gland required for ovulation. As SF-1 is expressed at early stages of the mouse fetal development, studies have demonstrated the role of this nuclear transcription factor in reproductive endocrine organ development and function (16, 37, 318). Targeted disruption of SF-1 in the pituitary via αGSU-cre resulted in sexually immature cKO females with severely hypoplastic ovaries (351). Granulosa-cell specific depletion of SF-1 using the Amhr2-cre model also resulted in acyclic and anovulatory females with hypoplastic gonads (225). These models induce SF-1 depletion at early stages of gonadotropin synthesis and follicular development, and, to our knowledge, no studies have identified the ovarian role of SF-1 in later follicular functions such as ovulation and luteinization. Progesterone receptor (PR) plays a crucial role in female reproductive organ function and is expressed in all organs of the female HPG axis (342, 384). In Chapter 3, we selected the PR-Cre specifically to study the impact of SF-1 depletion on ovarian processes without affecting early stages of follicle growth. We found that the strong pituitary phenotype in this model prevented ovulation from occurring, a condition that could be reversed by exogenous gonadotropin treatment. We assume that the depletion of SF-1 in gonadotropes occurs around the onset of puberty since our PR-Cre;SF-1$^{f/f}$ female display sexual behavior. Indeed, progesterone receptor knockout (PRKO) mice develop normally to adulthood, at which point severe defects in female reproductive

tissues are observed (384). Nevertheless, it would be interesting to validate this in our model by measuring both gene and protein SF-1 expression in tissues collected from immature mice. It would also be worthwhile to determine the age at which vaginal opening onset occurs in cKO females to establish whether impaired gonadotropin secretion at the onset of puberty delays sexual maturity in females.

In Chapter 3, we show that SF-1 is essential for gonadotropin synthesis and secretion in the gonadotropes of mature female mice. A previous study showed that SF-1 induces the transcription of *Gnrhr* by binding to its promoter region with the gonadotrope specific element (GSE) (385). In addition, GnRH administration in the αGSU-cre SF-1 cKO mouse induced LHβ protein expression, indicating that SF-1 is not essential for LH synthesis (337). Nevertheless, a SF-1 response element is present in the murine promoter region of the *Cg*α, *Fsh*β and *Lh*β genes (214). A recent study in the orange-spotted grouper showed that Nr5a1b (a homologue of SF-1) binds to the *lhb*, but not *fshb*, promoter region to induce its expression in the pituitary (386). In contrast, a study on the goldfish showed that the *fshb* promoter region contained a sequence identical to the SF-1 binding site in the human *STAR* gene, next to the highly-conserved AP-1 binding site (387). In this thesis, we report a significant reduction in *Gnrhr* in the pituitary gland of our PR-Cre;SF-$1^{f/f}$ females. It would be interesting to verify, using chromatin immunoprecipitation (ChIP) or chromatin immunocleavage (ChIC) sequencing, whether SF-1 regulates *Fshβ* and *Lhβ* expression directly in the mouse gonadotropes or if the observed reduction in gonadotropin synthesis in the cKO pituitary gland is due to the reduced expression of *Gnrhr*.

In rodent models, ovariectomy (Ov^x) is employed to remove the principal source of endogenous estrogens. This technique allowed us to observe the impact of this estrogen deficiency

on the pituitary gland of the PR-Cre;SF-1$^{f/f}$ female. These results confirmed that the reduction in gonadotropin synthesis and secretion is specific to SF-1 depletion in the pituitary gland. In terms of RNA abundance, *Fshb* expression was increased in the pituitary gland of cKO-Ov^{x} mice compared to cKO, but this upregulation was much smaller than in CON-Ov^{x}. Similar *Fshb* upregulation occurs in wild-type Ov^{x} animals due to the loss of ovarian inhibin feedback (388). While this increase is attenuated in cKO-Ov^{x} females, supporting previous findings on the role of SF-1 in regulating the FSH subunit, other transcription factors may compensate for SF-1 depletion. On the other hand, *Lhb* remained underexpressed and LH secretion was also reduced in cKO-Ov^{x} females, demonstrating that SF-1 plays a crucial role in LH synthesis. Curiously, *Nr5a1* was significantly decreased in the pituitary of CON-Ov^{x} females, contrary to what has been reported in female rats and ewes (389, 390). Considering *Gnrhr* is also reduced in CON-Ov^{x} mice, whether GnRHR is involved directly or indirectly in the regulation of SF-1 expression is yet to be discovered. Nonetheless, it is known that GnRH increases cAMP levels in gonadotropes, a signaling pathway known to induce SF-1 acetylation and promote dissociation from repressors, thereby increasing its DNA binding capacity and transcriptional activity (120, 391). Further molecular analysis will bring insight in this possible regulation of SF-1 activity in the pituitary gland.

5.1.2 Possible presence of a hypothalamic phenotype in the PR-Cre;SF-1$^{f/f}$cKO

Both SF-1 and PR are expressed in the hypothalamus of the mouse. SF-1 is expressed exclusively in the ventromedial hypothalamus (VMH), and SF-1 positive neurons have been shown to regulate food intake and ovulation via regulation by estrogen receptors (ER) (210). It is not yet known if these SF-1 positive neurons also express PR. It has been shown that PR action in the

hypothalamus is upstream of the GnRH neurons, in the kisspeptin neurons of the anteroventral periventricular nucleus, where it plays a vital role in regulating the estradiol positive feedback-induced LH surge (338, 339). To date, there is no evidence of SF-1 expression in the kisspeptin neurons or GnRH neurons (392). In Chapter 3, we showed that the levels of *Nr5a1* were comparable to CON in the hypothalamus of the PR-Cre;SF-1$^{f/f}$ cKO females, indicating that either SF-1 positive neurons do not express PR, or the depletion is undetectable in the hypothalamus. Further analysis of the cKO hypothalamus gene expression pattern showed an increase of the kisspeptin gene *Kiss1* and reduction of *Pr* expression. Kisspeptin stimulates the HPG axis by binding directly to KISS1 receptors on GnRH neurons to induce GnRH secretion and the subsequent secretion of gonadotropins (393). In our study, all mature female reproductive tissues were collected on the morning of estrus, when the principal ovarian secretory hormone shifts from estrogens to progesterone (344). This shift induces a reduction in gonadotropin secretion via kisspeptin and GnRH inhibition in the hypothalamus (392). Since the PR-Cre;SF-1$^{f/f}$ cKO females neither ovulate nor form corpora lutea, the lack of this negative feedback from the ovary could explain this increase in hypothalamic kisspeptin expression. This hypothesis is further strengthened by the observation that Kiss receptor gene expression levels are unaffected in cKO relative to CON mice in Chapter 3. Estradiol also mediates *Pr* induction in rat kisspeptin neurons (394), and mouse neuron *in vitro* studies showed this regulation occurs specifically via nuclear ER signaling, rather than the estrogen membrane-initiated signaling that induces kisspeptin expression (395). This difference in estradiol pathway to mediate kisspeptin and PR gene expression might explain the opposing expression pattern observed in the hypothalamus of the PR-Cre;SF-1$^{f/f}$ cKO female, though this remains to be verified. Further experimentation with exogenous GnRH injection in the

cKO females would allow us to establish whether there is SF-1 depletion in the hypothalamus which is affecting gonadotropin synthesis and secretion.

The majority of the PR-Cre;SF-1$^{f/f}$ females were infertile, and we suspect that the few that successfully produced pups were able to do so due to inefficient SF-1 depletion, as observed in other SF-1 cKO models (225, 337). In many mouse models where pituitary specific ablation of a gene leads to significant reduction of gonadotropin secretion, female mice are acyclic and remain sexually immature throughout adulthood (199, 337, 396, 397). The loss of SF-1 in the Amhr2-Cre;SF-1 cKO females induced estrous cycles of prolonged length due to impaired estrogen positive and negative feedback (225). On the other hand, the PR-Cre;SF-1$^{f/f}$ females in the present study were sexually mature, in that they were cyclic, albeit with estrous phases slightly prolonged. Furthermore, they displayed sexual behavior which permitted mating with males. Interestingly, a study of the absence of SF-1 in the VMH via CNS-cre also showed copulatory plug number comparable to controls when mated with males, but expanded sexual behavior analysis showed the CNS-cre;SF-1 cKO females had reduced lordosis quotient and receptivity (Kim et al. 2010). While impaired steroidogenesis in the ovary of our mature PR-Cre;SF-1$^{f/f}$ female can explain the observed increased frequency of copulatory plugs, future experiment to further analyze sexual behavior in this model will help assess the presence of a possible VMH phenotype.

5.2 Role of SF-1 in ovarian function

To our knowledge, only one other group has demonstrated the impact of SF-1 ablation specifically in the ovary (225). Using a Amhr2-Cre;SF-1 cKO mouse, a model specific to granulosa cells from the initial stages of folliculogenesis, Pelusi et al. observed a significant decrease in follicle numbers at all stages in immature mice, indicating the importance of SF-1 in postnatal

gonad development. Unlike the PR-Cre;SF-1$^{f/f}$ cKO model described in Chapter 3, exogenous gonadotropin injections in the Amhr2-Cre;SF-1 cKO females did not rescue corpus luteum formation. Additionally, Amhr2-Cre;SF-1 cKO females remained anovulatory with gonadotropin injection, while our PR-Cre;SF-1$^{f/f}$ cKO ovulated, albeit at significantly lower numbers than CON. Thus, the PR-Cre;SF-1$^{f/f}$ cKO mouse provides a novel model to understand the role of SF-1 in ovarian functions such as ovulation and luteinization, though exogenous gonadotropin treatments were necessary to establish these roles. On the other hand, preliminary results from females of the Cyp17-Cre;SF-1$^{f/f}$ (Cyp17 cKO), Cyp19-Cre;SF-1$^{f/f}$ (Cyp19 cKO) and Cyp17-Cre+Cyp19-Cre;SF-1$^{f/f}$ (dKO) mouse lineages, described in Chapter 4, show that SF-1 is dispensable for functions of theca cells and granulosa cells of the antral follicle, though closer analysis of these specific ovarian cells types showed impaired steroidogenic gene expression. In contrast, SF-1 depletion specifically in granulosa cells of the pre-ovulatory follicle resulted in an increased expression of steroidogenic genes and impaired expression of genes required for ovulation to occur. These differences in phenotype demonstrate that SF-1 regulation of ovarian function varies not only on ovary cell type, but also on follicular stage.

5.2.1 Role of SF-1 in ovulation

Gene expression analyses of granulosa cells isolated from PR-Cre;SF-1$^{f/f}$ cKO mice after gonadotropin injection indicated that factors involved in cumulus expansion and follicle rupture were downregulated in the reduced presence of SF-1. The cKO females treated with exogenous gonadotropins ovulated a lower number of oocytes compared to controls, and histological analysis of their ovaries showed a significant reduction in the number of follicles with expanded oocytes and presence of trapped oocytes in corpora lutea. Together, these results provide strong evidence

that SF-1 plays a crucial role in the regulation of peri-ovulatory events. These results are reminiscent of the PR germline KO mice where follicular development to the preovulatory state and cumulus expansion is normal, but ovulation does not occur and matured oocytes are found entrapped in the corpus luteum rather than in the oviduct (348). To amplify the conclusions obtained from this model, one could compare CON and PR-Cre;SF-$1^{f/f}$ cKO cumulus-oocyte complexes isolated from punctured ovaries of superstimulated females and evaluate their capacity to expand *in vitro*. Alternatively, follicles dissected from cKO ovaries could be cultured *in vitro* to subsequently induce their ovulation and assess follicle rupture capacity, thereby ensuring that the impaired pituitary function of cKO females is not affecting the ovulation results. Additionally, next-generation transcriptome sequencing could be used to compare CON and PR-Cre;SF-$1^{f/f}$ cKO granulosa cells and clarify if and how SF-1 regulates the expression of genes involved in cumulus expansion and follicle rupture.

In Chapter 3, we used ovarian transplantation experiments to further evaluate the ovarian phenotype of the PR-Cre;SF-$1^{f/f}$ cKO female by removing the risk of a secondary effect of SF-1 ablation in the pituitary gland. Interestingly, two separate phenotypes resulted from these transplants, with roughly half of the CON-cKOov females found to be infertile, and the other half maintaining normal fertility levels. While the possibility of mishaps in the surgical manipulation can never be completely excluded, the absence of abnormal fertility levels in our CON-CONov group strengthens the conclusion that the infertility of the CON-cKOov females is a true phenotype resulting from SF-1 depletion in the peri-ovulatory ovary. Indeed, one explanation for the occurrence of two phenotypes in the CON-cKOov females could be variability in the level of recombination occurring in the cKO ovaries. We observed a wide variation in the ovarian SF-1 expression levels of the CON-cKOov ovaries, even when samples from infertile females were

separated from those of fertile females, rendering it difficult to assess the recombination. This may have been due to the fact that SF-1 is expressed in multiple cell types in the ovary, including the theca, stroma, granulosa and corpora lutea, which were present at variable frequency in the ovarian samples. Females were not stimulated prior to tissue collection and both ovaries were collected and kept whole for either histological evaluation or gene expression analysis to evaluate antral follicles and corpora lutea. We plan additional ovarian transplantations in CON and cKO females in order to isolate granulosa cells by ovarian puncture and luteal cells by luteal micro-dissection from ovaries selected to perform gene expression analysis. By these means it would become possible to reduce the presence of SF-1 expressing ovarian cells that are not targeted by the Cre-recombinase, thus providing us with a more representative analysis of the ovarian specific recombination occurring in the mice.

Finally, the Cyp19-Cre;SF-$1^{f/f}$ mouse model, where the Cre-recombinase linked to the aromatase gene, *Cyp19a1,* induces SF-1 depletion specifically in mural granulosa cells of antral follicles, offered another *in vivo* approach to observe the role of SF-1 in peri-ovulatory events without the presence of a pituitary phenotype (398). The Cyp19-Cre mouse was previously used in our laboratory to create a granulosa specific liver receptor homolog 1 (LRH-1) knockout model (Cyp19-Cre;LRH-$1^{f/f}$ cKO), which demonstrated that LRH-1 expression in antral follicles is required to provide the necessary ovulatory signals (160, 176). In Chapter 4 of this thesis, we showed that the Cyp19-Cre;SF-$1^{f/f}$ females produced a comparable number of litters and pups when compared to controls, suggesting normal cumulus expansion and ovulation rates. It would seem from these results that SF-1, unlike the closely-related orphan nuclear receptor LRH-1, does not play an essential role in the regulation of ovulatory factors in granulosa cells of antral follicles. Conversely, results from the PR-cre;LRH-$1^{f/f}$ cKO mouse, another model studied in our research

group which used the PR-Cre model to induce LRH-1 depletion, showed normal ovulatory rates, demonstrating that LRH-1 does not regulate ovulatory events after the LH surge, known to be the time when PR expression is initiated . We can thus conclude that SF-1 and LRH-1 are required in granulosa cells at different moments of development in the mouse follicle, LRH-1 being indispensable in antral follicles as granulosa cells proliferate and the follicle grows, and SF-1 playing a key role after the LH surge. Next, it would be critical to next evaluate if and how gonadotropins regulate the expression of these two orphan nuclear receptors of the NR5A family. It is important to note that the relative mRNA abundance in granulosa cells of superstimulated females showed that LRH-1 recombination was more effective in Cyp19-Cre;LRH-$1^{f/f}$ than SF-1 in Cyp19-Cre;SF-$1^{f/f}$ cKO mice. This finding might be another explanation as to why the Cyp19-Cre;SF-$1^{f/f}$ females show normal reproductive performance. Measuring Cre activity of isolated granulosa cells from both Cyp19-Cre;SF-$1^{f/f}$ and PR-Cre;SF-$1^{f/f}$ cKO animals cultured *in vitro* will allow us to better evaluate recombination levels in our mouse models. Future endeavors should aim to evaluate mating frequency and ovulation capacity of the Cyp19-Cre;SF-$1^{f/f}$ mice in order to confirm the absence of an ovarian phenotype in this model. Additionally, the use of an adenovirus Cre to induce the *in vitro* depletion of SF-1 and LRH-1 in granulosa cell cultures under different stimulatory conditions, followed by the evaluation of gene expression patterns at different time points would provide novel insight on the timely regulatory impact of these two orphan nuclear receptors.

5.2.2 Role of SF-1 in luteinization

Though SF-1 expression has been detected in the corpora lutea of humans, macaques, cows, ewes and rats, its presence in the mouse is less well defined (38, 39, 227). In cow luteal cells, SF-

1 has been show to induce the basal and c-AMP stimulated expression of CYP11A in combination with SP-1 (399). In ewes, SF-1 mRNA expression levels in luteal cells were linked to serum progesterone concentration, demonstrating the importance of this transcription factor in corpus luteum steroidogenesis (400). Most studies agree that SF-1 expression is highest in the interstitial tissue and theca cells of the mouse ovary, and, similar to what is observed in the human, that SF-1 expression decreases in ovarian follicles after ovulation (375). Here, we show SF-1 is present in the nucleus of luteal cells of our CON female mice, though at a much lower intensity than what is observed in interstitial tissue and theca cells, indicating that SF-1 might still be involved as a driver of steroidogenesis post ovulation. On the other hand, the SF-1 homologue LRH-1 is strongly and only expressed in granulosa and luteal cells of mouse ovaries (375). Depletion of LRH-1 specifically in luteal cells caused reduced expression of steroidogenic genes in corpora lutea and impaired progesterone secretion in both superstimulated and mated cKO females (46). A similar significant decrease in progesterone production is also observed in the superstimulated PR-Cre;SF-$1^{f/f}$ cKO female, where a reduced number of corpora lutea was measured as well as the abnormal expression of essential steroidogenic genes. In addition, the mating of PR-Cre;SF-$1^{f/f}$ cKO females after exogenous gonadotropin injections did not result in the birth of pups, suggesting that SF-1 depletion post ovulation leads to luteal insufficiency. These data provide evidence that SF-1 is required for normal luteinization and luteal cell function. A subsequent experiment would be to collect sera and ovaries from PR-Cre;SF-$1^{f/f}$ cKO females, mated after exogenous gonadotropin treatment, at dpc 4.5 to evaluate progesterone secretion and ovarian histology, and collect the blastocysts from uterine horns to ensure gametes are fertilizable. Another worthwhile experiment would be to treat the superstimulated PR-Cre;SF-$1^{f/f}$ cKO females with exogenous progesterone injections after mating to observe if fertility is rescued and confirm that the infertility derives from

luteal insufficiency caused by the depletion of SF-1 in the corpus luteum. Finally, SF-1 has been shown to interact directly with CCAAT/enhancer-binding protein β (C/EBPβ) to induce progesterone production in human granulosa tumor cell line KGN (170). Chromatin immunoprecipitation analysis in this study showed that both SF-1 and C/EBPβ strongly bind to the promoter region of *STAR*, *CYP11A1* and *3BHSD2*. In addition, granulosa-specific studies have shown that C/EBPβ is a critical factor involved in ovulation, luteinization and ovarian steroidogenesis (401, 402). In terms of future experimentation, it would be interesting to verify this interaction between SF-1 and C/EBPβ in mouse granulosa and luteal cells.

Studies in domestic ruminants have shown that theca cells are the precursors to small luteal cells, and granulosa cells to large luteal cells (360). Interestingly, SF-1 has been shown to induce oxytocin gene expression in bovine large luteal cells of the corpus luteum, shifting steroidogenesis towards progesterone production after the LH surge (38). In the primate corpus luteum, these two luteal cell types do not mix and have distinct functions, the granulosa-lutein cells being the main source of estrogens while the theca-lutein cells produce androgens (403). In contrast, both small and large luteal cells in rats express CYP17A1 and CYP19A1 and produce androgens and estrogens (404). How luteinizing cells populate the corpus luteum in the mouse has yet to be confirmed. The SF-1 cKO mouse models described in Chapter 4 of this thesis were designed to identify the role SF-1 plays in different cell types of the developing mouse ovary, including in luteal cells. We aimed to see how the absence of SF-1 in theca cells (Cyp17 cKO) and in granulosa cells (Cyp19 cKO) would have an impact on early regulation of luteinization, and, since both theca and granulosa cells populate the corpus luteum, if communication and compensation can occur between these two cell types (dKO). We observed that Cyp17 cKO, Cyp19 cKO and dKO females were capable of forming corpora lutea and produced litter numbers and sizes comparable to controls,

indicating that SF-1 presence in theca and granulosa cells of growing follicles is not essential for luteinization and pregnancy maintenance. Because a differential phenotype was observed in the male animals of these mouse lines, the evaluation of the ovarian phenotype was not further pursued. Nevertheless, we aim to verify SF-1 expression levels in luteal cells of the corpus luteum in the female Cyp17 cKO, Cyp19 cKO and dKO models, as well as measure progesterone production levels.

5.2.3 Comparison with liver receptor homologue 1 (LRH-1) cKO mouse models

Our understanding of the role SF-1 plays in important ovarian functions is based on the knowledge that SF-1 regulates the transcription of various factors involved in steroidogenesis. Many transcription factors and co-factors are capable of activating the expression of these genes, giving place to possible redundancy or compensation. As mentioned in Chapter 1 of this thesis, LRH-1 (encoded by the *Nr5a2* gene) is structurally similar to SF-1, and regulates many of the same genes, particularly those associated with steroidogenesis (331). Previous transgenic mouse studies inducing ovary specific LRH-1 ablation at three different stages of folliculogenesis (in primary follicles onwards, in antral follicles before ovulation and in the corpus luteum) have recorded no variation in *Nr5a1* expression levels caused by the depletion of LRH-1 (46, 160, 161). Similarly, no variation in *Nr5a2* expression was observed in granulosa cells of early stages of folliculogenesis where SF-1 was depleted (225). Interestingly, *Nr5a2* is significantly increased in luteal cells of PR-Cre;SF-1$^{f/f}$ cKO females at hCG 24 h, indicating a possible compensation for the depletion of SF-1 in the corpus luteum. Our results indicate that the intracellular cholesterol transport gene, *Scp2*, is also overexpressed in the PR-Cre;SF-1$^{f/f}$ cKO ovary at hCG 24 h. A recent study showed that SCP2, which transports phospholipids from the endoplasmic reticulum into the plasma membrane,

can regulate the expression of LRH-1 (405). Yet, *Star*, *Cyp11a1* and *Cyp19a1,* steroidogenic genes known to be regulated in luteal cells by LRH-1 (46), were downregulated in the PR-Cre;SF-1$^{f/f}$ cKO ovary at hCG 24 h*,* supporting the theory that LRH-1 does not compensate for the reduced expression of SF-1 in mouse luteal cells.

As a side note, Zhang et al. demonstrated the essential role of LRH-1 in pregnancy maintenance and uterine decidualization in both the mouse and humans. Uterine studies were not performed with our PR-Cre;SF-1$^{f/f}$ cKO females because recent studies have shown that SF-1 silencing in the endometrium is required to ensure pregnancy maintenance (244). Nevertheless, we do not dismiss the possibility of a uterine phenotype in this model, and future studies with both gonadotropin and progesterone administration to help maintain pregnancy could allow us to further understand the multiple functions of SF-1 in mammalian reproduction.

5.3 Role of SF-1 in testicular function

SF-1 regulates the expression of Amh in developing fetuses, which inhibits female reproductive tract formation by inducing the regression of the Müllerian ducts (219). Studies of transgenic mice have shown that absence of SF-1 in Leydig or Sertoli cells impairs testis development and function, in some cases leading to infertility (41, 198, 221). In Chapter 4 of this thesis, we evaluated the impact of SF-1 depletion in post-natal testes via our novel mouse lines. The Cyp17 cKO model allowed us to evaluate the role of SF-1 in Leydig cells, where we observed a reduced Leydig cell population, impaired spermatogenesis and dysregulated steroidogenic gene expression. The Cyp19 cKO mature males, where the SF-1 depletion was observed specifically in Sertoli cells, were comparable to control animals in most aspects, suggesting that SF-1 does not play an essential role in adult Sertoli cell function. Since both the Cyp17 cKO and Cyp19 cKO

males are fertile, we were surprised to note the occurrence of infertility in half of the dKO males, where both CYP17A1 and CYP19A1 positive cells expressed the Cre recombinase, while the other half showed reduced frequency of litters and the number of pups per litter. This indicated that SF-1 plays an important role in testicular cells positive for CYP19A1. As discussed in Chapter 4, it is possible that this occurs at an earlier stage, when progenitor Leydig cells were shown to temporally express CYP19A1 (376). A proportion of these fetal Leydig cells can dedifferentiate to form adult Leydig cells at puberty (378). The depletion of SF-1 at that early post-natal stage could therefore interfere with the proliferation of dedifferentiated fetal Leydig cell and thus reduce the number of adult Leydig cell population in later stages.

5.3.1 Role of SF-1 in CYP19A1 positive cells

CYP19A1 (aromatase) has been shown to be expressed in Leydig cells, though not in all nor with the same intensity as in cells of the interstitium (406). The same authors have demonstrated its presence in germ cells of the adult mouse testis. Sertoli cells, the main drivers of estrogen synthesis in prepubertal mice, showed weak CYP19A1 immunostaining in adults (407). Since there is no reported evidence of SF-1 expression in mouse testicular germ cells, and the analysis made in our study focused on adult animals, we assumed the recombination of SF-1 in Cyp19 cKO males was specific to Sertoli cells. This view was supported by the reduced expression of SF-1 in cells of the basal compartment of the seminiferous tubules, and continued strong expression in Leydig cells of the interstitium. Due to the well documented role of SF-1 in *Cyp19a1* regulation in mouse ovaries (231, 379, 408), we hypothesized that the absence of this nuclear receptor in mouse testicular cells would impair steroidogenesis, more specifically estrogen production, in the seminiferous tubules. In support of this concept, it has been shown by other

investigators that male mice deficient in aromatase develop normally and show normal fertility until the age of 5 months, after which failures in spermatogenesis appear, leading to infertility by the age of one year (409). As mentioned above, we did not detect infertility in the Cyp19 cKO males, some of which were kept in mating trials for over a year (data not shown), and *Cyp19a1* expression levels were comparable to controls, suggesting that estrogen production in these males was normal.

Be that as it may, the dKO males, where SF-1 depletion occurred in both CYP17A1 and CYP19A1 positive cells, showed important defects in seminiferous tubule formation and spermatogenesis, in addition to the reduced steroidogenesis and Leydig cell number. The adult Leydig cell population is derived from progenitor Leydig cells in infant testis that initiate proliferation in response to increased LH levels (377). Previous studies using the Cyp19a1-Cre transgenic mice to create a Neuregulin 1 cKO model showed that the Cre recombinase was expressed in Leydig cells of the infant testis at one week of age, but not in the adult testis (376). It is possible that the depletion of SF-1 at that early post-natal stage might have minimally impaired adult Leydig cell formation, insufficient to affect androgen production in the Cyp19 cKO males, but sufficient to induce a more drastic phenotype in the dKO males. This mechanism may explain the difference in reproductive capacity when compared to the Cyp17 cKO males. It would be interesting to verify this hypothesis by measuring SF-1 expression levels and Leydig cell numbers in Cyp19 cKO and dKO infant testes.

5.3.2 Role of SF-1 in testicular cell survival

While no direct physical contact exists between the Sertoli and Leydig cells of the testis, studies have shown that a reduction in Sertoli cell number due to damage or absence of proliferation

can cause abnormal function in adjacent Leydig cells, leading to a proportional decrease in the population of the latter (410). In male fetal gonads, an environment characterized by low levels of androgens, Sertoli cells, the first cell type to differentiate, enable the development of Leydig cells (411). At puberty, Sertoli cells respond to androgens produced by Leydig cells to support spermatogenesis and can also influence Leydig cell steroid production through complex cell–cell interactions (412). Indeed, mice deficient in androgen receptors specifically in Sertoli cells show reduced testosterone production, possibly due to the increased secretion of Amh from the Sertoli cells (413). In Chapter 4, we found that Sertoli cell SF-1 expression levels and Sertoli cell numbers were significantly lower than controls in seminiferous tubules of Cyp19 cKO males. Amh expression tended to be upregulated in the Cyp19 cKO Sertoli cells, but not in dKO. While SF-1 depletion in Sertoli cells did not noticeably impair spermatogenesis, it could be linked to the reduced proportion of SF-1-positive Leydig cells in the interstitial space of this model when compared to CON males.

Anamthathmakula et al. (323) demonstrated, using the Sertoli-specific Amh-cre mouse model, that the absence of SF-1 in fetal Sertoli cells post sex determination induced apoptosis. In their model, SF-1 depletion is Sertoli cells reduced Mouse double minute 2 homolog (*Mdm2)* expression, which caused an increase in Tumor protein P53 (TP53) levels. In Chapter 4, we observed increased apoptosis in the basal compartment of dKO seminiferous tubules, which could also explain the more drastic reproductive phenotype observed in the dKO male. We plan to measure the expression level of both *Mdm2* and *Tp53* in Cyp19 cKO and dKO testes to verify if SF-1 plays a similar role in regulating mature Sertoli cell cycle and survival.

No reduction in SF-1 expression was noted in Sertoli cells of Cyp17 cKO testes, yet a reduction in Sertoli cell number and reduced sperm count was observed. Moreover, markers of cell apoptosis, autophagy and cell-cycle arrest were upregulated in testes from Cyp17 cKO and dKO males. These results strongly indicate that the depletion of SF1 in Leydig cells affects spermatogenesis and Sertoli cell survival. This also suggests that the regulatory interactions between Sertoli and Leydig cells allow Leydig cells to influence Sertoli cell number. Future experiments using quantitative flow cytometry to measure Leydig and Sertoli cell numbers will allows us to validate these observations. Another idea would be to measure androgen receptor expression levels in isolated Sertoli cells from our models to determine whether 1-) reduced steroidogenesis in Leydig cells can affect androgen receptor expression in Sertoli cells (Cyp17 cKO) and 2-) SF-1 regulates androgen receptor expression in Sertoli cells (Cyp19 cKO).

5.3.3 Role of SF-1 in seminiferous tubule structure and spermatogenesis

Histological analysis of testes obtained from Cyp17 cKO, Cyp19 cKO and dKO males revealed seminiferous tubules with varying levels of abnormal structures, some with presence of nucleated cells in the lumen, others with absence of elongated spermatids. Some tubules, more prominently in dKO testes, showed no appearance of lumen, indicating an absence of seminiferous tubule fluid. This fluid is secreted by Sertoli cells and contains the necessary nutritional and hormonal factors to support spermatogenesis and transport mature spermatozoa into the rete testis and efferent ducts (414). Indeed, Sertoli cell-specific androgen receptor cKO male mice show impaired spermatogenesis and lack functional seminiferous tubule lumina (415). When efferent ducts were ligated in these cKO males, no increase in testis weight was observed, unlike their CON male counterparts, indicating that androgen receptor is required for Sertoli cell fluid secretion

(416). In addition to verifying that depletion of SF-1 in CYP17 or CYP19-positive cells affects testicular androgen receptor expression, testis weight after the ligation of the efferent ducts could be measured in our models to determine if SF-1 plays a role in Sertoli cell fluid secretion. Another future endeavor would be to verify the integrity of the blood-testis barrier (BTB) in our models, since a lumen cannot be formed in the seminiferous tubule when the BTB is disrupted (417). This would provide additional evidence to explain the abnormal seminiferous tubule morphology and impaired spermatogenesis we observed, mainly in the Cyp17 cKO and dKO males.

Studies have shown that meiosis can only be completed once Sertoli cell fluid secretion has been established, and functional maturation of Sertoli cells during postnatal development is required for spermatogenesis to occur (418). Absence of SF-1 during this period has been shown to negatively affect Sertoli cell function (366). Many seminiferous tubules in the cKO models described in Chapter 4 did not show presence of late stage elongated spermatids, evidence of impaired spermatogenesis. Closer evaluation showed that, in some tubules, only spermatogonia and first stage spermatocytes were present, while in others, spermatocytes and spermatids populated the lumen, indicating structural malformation and/or degeneration. In retrospect, the use of the periodic acid Schiff (PAS) technique for better visualization of acrosome formation, or toluidine blue staining for better chromatin structure assessment, would have given clearer view of DNA content to determine the germ cell differentiation level. We are currently measuring the expression of germ cell markers of specific stages such as *Dmc1* (expressed prior to the pachytene spermatocyte stage), *Sycp3* (expressed in spermatocytes up to and including the diplotene stage), *Clgn* (expressed in pachytene spermatocytes), *Spert* (expressed in round spermatids) and *Dbil5* (expressed in elongating spermatids and mature spermatozoa) to provide molecular evidence of this spermatogenic arrest and help us define the role of SF-1 in spermatogenesis.

5.4 Expectations, limitations and alternatives

The SF-1 cKO mouse models described in this thesis were originally designed to identify the role of SF-1 in different cell types of the developed mouse ovary. We expected the female mice from these models to show how the absence of SF-1 in granulosa cells (PR-Cre;SF-1$^{f/f}$ and Cyp19-Cre;SF-1$^{f/f}$ cKO) or in theca cells (Cyp17-Cre;SF-1$^{f/f}$ cKO) affects key ovulatory processes at different follicular stages, and that this impact is enhanced by the absence of this nuclear receptor in both ovarian cell types (dKO). While the depletion of SF-1 in granulosa cells of the peri-ovulatory follicle (PR-Cre;SF-1$^{f/f}$) showed a clear effect on female fertility, demonstrating novel roles for SF-1 in ovulation and luteinisation, the phenotype observed in the other three models was less convincing. We observed that Cyp17 cKO, Cyp19 cKO and dKO females were capable of forming corpora lutea and produced litter numbers and sizes comparable to controls, and were surprised to see that SF-1 presence in theca and granulosa cells of growing follicles was not essential for steroidogenesis and theca cell luteinisation, nor pregnancy maintenance. We believe this may have been due to the incomplete recombination of the SF-1 gene, which resulted in a less than 50% reduction of SF-1 expression in the targeted cells, which may have been sufficient to maintain normal steroidogenesis and SF-1 activity. In addition, an increased expression of the LRH-1 gene (*Nr5a2*) was observed in granulosa cells of the Cyp19-Cre;SF-1$^{f/f}$, indicating that this other orphan nuclear receptor of the same family may be able to maintain normal transcription of steroidogenic genes and compensate for SF-1 depletion. It would have been interesting to measure LRH-1 protein levels to further support this observation, and measure circulating and intra-organ hormone levels to confirm the absence of phenotype in these females. An alternative to these *in vivo* models would have been to culture theca and granulosa cells, separately, collected from SF-

$1^{f/f}$ females and reduced *Nr5a1* expression using a transmissible Cre recombinase. A more effective recombination of *Nr5a1* would allow us to better verify the role of SF-1 in these ovarian cell types.

We pursued a more in depth analysis of the male phenotype observed in these models when we realized that half the dKO males were infertile, and the other half were subfertile. This was unexpected since both the Cyp17-Cre;SF-$1^{f/f}$ and the Cyp19-Cre;SF-$1^{f/f}$ males presented normal fertility. Similar to the females, we believed the absence of phenotype in the males of those two models was due to an incomplete *Nr5a1* recombination in the targeted testicular cells. While we did isolate Leydig cells from Cyp17-Cre;SF-$1^{f/f}$ males to confirm the cell specific recombination level, it would have been interesting to do the same with Cyp19-Cre;SF-$1^{f/f}$ males. This would have been particularly interesting at an early post-natal stage, since our analysis demonstrated that the reduction in Leydig cell population was more drastic in dKO males. Our analysis also showed a higher proportion of malformed seminiferous tubules in the dKO males. It would have been interesting to collect testes at different stages of maturity to verify if these malformations are progressive and whether the phenotype we observed results from an early post-natal effect.

5.5 Role of SF-1 outside of the HPG axis

In addition to its well-known presence in organs of the HPG axis, SF-1 has been shown to regulate steroidogenesis in a variety of other tissues not involved in reproduction. Different mechanisms regulate the spatio-temporal expression of *Nr5a1*, such as tissue-specific exon usage and function and establishment of promoters and upstream regions of these exons (419). As mentioned in Chapter 1 of this thesis, the SF-1 KO mice do not develop adrenal glands and die within one week of birth due to adrenocortical insufficiency, revealing the essential role of this nuclear receptor in adrenal gland development as well as in corticosteroid synthesis (318).

Interestingly, a wide variation was observed in the expression of *Nr5a1* and other steroidogenic genes of the adrenal glands isolated from dKO males. This was unexpected since CYP17A1 is usually not expressed in mouse adrenal gland due to epigenetic silencing (420). Indeed, though estrogens and androgens can modulate corticosteroid production in the adrenal glands (421), they are not synthesized in this organ (422). Preliminary data did not link a reduced expression of SF-1 in adrenal gland to the infertility seen in some of the dKO males. Follow-up experiments to measure the expression level of enzymes involved in corticosterone synthesis (the principal steroid produced in rodent adrenal glands), such as CYP11B2 and CYP21, will confirm if the reduced testosterone levels in Cyp17 cKO and dKO males is affecting adrenal gland function (423).

Though modest when compared to steroidogenic tissues, *de novo* steroidogenesis from cholesterol occurs in mammalian skin cells as well, where progesterone has been shown to be synthesized but rapidly metabolized into deoxycorticosterone via the action of steroidogenic enzymes (424). SF-1 immunoreactivity has been detected in the epidermis, where it is believed to induce the expression of StAR for cholesterol transport (356). Interestingly, both CYP17A1 and CYP19A1 are also expressed in stromal cells of adipose tissue, where it allows the production of estrogens in both sexes (425). While it was demonstrated that several human skin diseases showed aberrant expression of StAR (426), the SF-1 cKO models presented in this thesis did not show visible occurrence of skin disorder. These mouse lines could provide relevant in future studies to determine if SF-1 is essential for steroidogenesis in epidermal cells, though skin-specific ablation remains to be verified.

Chapter 6. Final Conclusions

The results presented in this thesis contribute to our understanding of the role of SF-1 in the regulation of essential reproductive events in mature mouse gonads, adding to the preliminary information that exists to date (Figure 6.1). Although SF-1 action in post-natal ovarian function has generally been associated with regulating steroidogenesis, the first study presented in this thesis identified novel roles for SF-1 in cumulus expansion, ovulation and luteinization. Moreover, the data showed that, though SF-1 expression is reduced in mouse corpus luteum, its Cre-mediated depletion in luteal cells prevents successful progesterone production. Future studies should seek to 1-) uncover if SF-1 binds directly on genes involved in cumulus expansion and ovulation, 2-) identify which mechanisms are involved in SF-1 regulation of these important female reproductive events and 3-) detect possible compensation mechanisms that might be triggered by other transcription factors to allow partial cumulus expansion and ovulation in the PR-Cre;SF-$1^{f/f}$ cKO model.

The second study presented data demonstrating how SF-1 regulates mature mouse testicular function. The testis cord dysgenesis and impaired spermatogenesis caused by the depletion of SF-1 in both Leydig and Sertoli cells provides *in vivo* evidence that SF-1 plays an essential role in mature testicular function. It is clear that SF-1 regulates Leydig cell function and survival, and our results suggest that Sertoli cells can compensate for absence of SF-1 in Leydig cells and vice versa. Whether SF-1 is directly or indirectly involved in spermatogenesis and Sertoli cell survival remains to be elucidated. Evidence from female mice in this second study showed that theca cell specific depletion of SF-1 impairs steroidogenic gene expression without affecting fertility. Furthermore, the reduced expression of SF-1 in granulosa cells of antral follicles increased the expression of its homologue LRH-1, indicating a possible compensation in these ovarian cell type at this follicular stage.

Taken together, the results presented in this thesis provide strong evidence for a definitive role for SF-1 in the pituitary, ovary and testis. The mouse lines described here will serve as valuable new tools to better investigate the role of SF-1 in gonad function and present novel directions for the investigation of this nuclear receptor in other mammalian models.

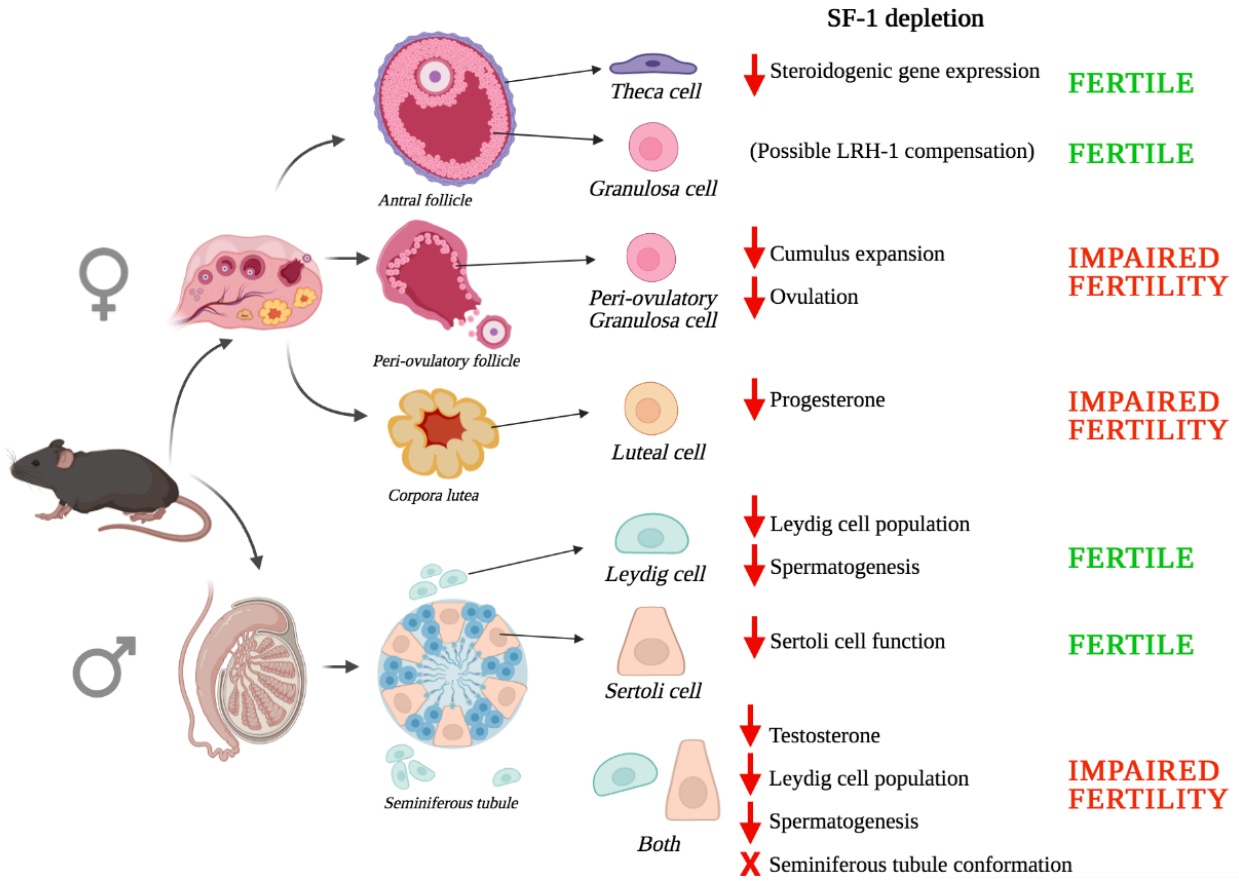

Figure 6.1 Effects of gonad specific SF-1 depletion on mouse reproductive function.

Schema created in Biorender.com

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