

**THE ROLE OF THE ISWI PROTEINS SNF2H AND SNF2L IN OVARIAN
FOLLICULOGENESIS**

By

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ABSTRACT

Folliculogenesis is a complex process which describes the maturation of the ovarian follicle, from the primordial stage all the way to the ovulation of the antral follicle, and its sequela, the formation of the corpus luteum (CL). Imitation switch (ISWI) proteins are a class of ATP-dependent chromatin remodelers which mobilize nucleosomes to regulate a number of cellular processes including transcription, replication, and DNA repair. The pattern of expression of the mammalian ISWI proteins SNF2H and SNF2L in the mouse ovary suggests a role in the coordination of the proliferation and differentiation of granulosa cells during folliculogenesis. Here, we report that SNF2H is associated with proliferating granulosa cells, while SNF2L expression is induced following the LH surge which triggers their terminal differentiation into luteal cells. Knockdown of *Snf2l* by siRNA is sufficient to downregulate the expression of StAR, an important steroidogenic enzyme, and marker of the CL. Furthermore, SNF2L is thought to directly regulate StAR expression by physically binding to its promoter as indicated by chromatin immunoprecipitation (ChIP). In order to identify additional targets regulated by SNF2L, an unbiased microarray screen was developed to look for genes induced by LH in a SNF2L-dependent manner. One of the candidates, *Fgl2* is strongly induced at 8h post hCG only in granulosa cells with intact SNF2L activity. Furthermore overexpression of SNF2L is sufficient to induce FGL2, and SNF2L is present on its promoter in the SIGC rat granulosa cell line. Some of the SNF2L binding partners that may be important in this regulation are PR-A and FLI-1, which have been found to interact with SNF2L by IP. Finally we describe here the phenotype of a *Snf2l* KO mouse which includes multiple reproductive defects, including resistance to superovulation, low secondary follicle counts, and a high incidence of abnormal antral follicles. Taken together

these data suggest an important role of ISWI proteins in folliculogenesis, particularly SNF2L, which may regulate multiple genes important for the terminal differentiation of granulosa cells into luteal cells following the LH surge.

DEDICATION

To my family Corinne, Marc and Lydia, and my fiancée Laura for their support and love.

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“I love fools’ experiments. I am always making them.”

Charles R. Darwin

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LIST OF ABBREVIATIONS

3 β -hsd: 3 beta- and steroid delta-isomerase
 ACF: ATP-utilizing chromatin assembly and remodeling factor
 ACTB: actin B
Adams1: a disintegrin-like and metallopeptidase (repolysin type) with thrombospondin type 1 motif, 1
 ADP: adenosine diphosphate
Akt: thymoma viral proto-oncogene 1
 AMH-R: anti-müllerian hormone receptor
 AMH: anti-müllerian hormone
Arrdc3: arrestin domain containing 3
 ATP: adenosine triphosphate
 BAF: BRG- or BRM-associated factor
 BAZ1A: bromodomain adjacent zinc finger-1A
Bmp15: bone morphogenetic protein 15
 bp: base pair
Bptf: bromodomain PHD finger transcription factor
Brg1: protein brahma homolog 1
Brm: brahma
 BSA: bovine serum albumin
Btg1: B-cell translocation gene 1
 cAMP: adenosine 3',5'-cyclic monophosphate
 CBP: CREB binding protein
 CD-1: cluster of differentiation 1 (lacking)
Cdkn1b: cyclin-dependent kinase inhibitor 1B
Cebp β : CCAAT/enhancer binding protein (C/EBP), beta
Cecr2: cat eye syndrome chromosome region, candidate 2 homolog
 CERF: CECR2-containing remodeling factor
 CHD: chromodomain helicase DNA-binding
 CHD1: chromodomain helicase DNA-binding protein 1
 ChIP: chromatin immunoprecipitation
 CHRAC: chromatin accessibility complex
 CIHR: Canadian Institute of Health Research
 CL: corpus luteum
 COC: cumulus-oocyte complex
 CpG: cytosine-phospho-guanine
 CRE: cAMP response element
 Cre: cyclization recombinase
 CREB: cAMP response element binding protein
 CREM: cAMP response element modulator
Ctsl: cathepsin L
Cx37: connexin 37
Cx43: connexin 43
Cxcl12: chemokine (C-X-C motif) ligand 12

Cxclr4: chemokine (C-X-C motif) receptor 4
Cyp11a1: cytochrome P450, family 11, subfamily A, polypeptide 1
Cyp17a1: cytochrome P450, family 17, subfamily a, polypeptide 1
Cyp19: cytochrome P450, family 19
d: day
dbcAMP: dibutyl adenosine 3',5'-cyclic monophosphate
Der1l: Der1-like domain family, member 1
DES: diethylstilbestrol
Dio2: deiodinase, iodothyronine, type II
DMEM: Dulbecco's modified Eagle's medium
DNA: deoxyribonucleic acid
Dnmt1: DNA methyltransferase (cytosine-5) 1
Dnmt3a: DNA methyltransferase (cytosine-5) 3A
Dnmt3b: DNA methyltransferase (cytosine-5) 3B
dpc: day post-coitum
DREF: DNA replication-related element factor
E2: 17 β -estradiol
Ecr: ecdysone receptor
Edn2: endothelin 2
EDTA: Ethylenediaminetetraacetic acid
EGF: epidermal growth factor
Egln3: egl nine homolog 3
Egr1: early growth response 1
EIF2S2: eukaryotic translation initiation factor 2, subunit 2 beta
EIF2S3: eukaryotic translation initiation factor 2, subunit 3 gamma
EIF5B: eukaryotic translation initiation factor 5B
ER- α : estrogen receptor alpha
ER- β : estrogen receptor beta
Erk1: extracellular signal-regulated kinase 1
Erk2: Extracellular signal-regulated kinase 2
Ex6DEL: exon 6 deleted
Fgf8: fibroblast growth factor 8
Fgl2: fibrinogen-like 2
Figla: folliculogenesis specific basic helix-loop-helix
FLI-I: flightless I
FLNA: filamin, alpha
Foxl2: Forkhead box L2
Foxo3a: forkhead box O3
FSH-R: follicle-stimulating hormone receptor
FSH: follicle-stimulating hormone
g: gram
GATA-1: GATA binding protein 1
GATA4: GATA binding protein 4
Gdf9: growth differentiation factor 9
GFP: green fluorescent protein

GO: gene ontology
 GST: glutathione S-transferase
 h: hour
 H1: histone linker 1
H1foo: H1 histone family, member O, oocyte-specific
 H2A-X: H2A histone family, member X
 H2A-Z: H2A histone family, member Z
 H2A: histone 2A
 H2B: histone
 H3: histone 3
 H4: histone 4
 hACF: human ATP-utilizing chromatin assembly and remodeling factor
Has2: hyaluronan synthase 2
 HBXAP: hepatitis B virus x associated protein
 hCG: human chorionic gonadotropin
 hCHRA: human chromatin accessibility complex
 HDAC1: histone deacetylase 1
 HDAC2: histone deacetylase 2
 HET: heterozygote
 hNURF: human nucleosome remodeling factor
 hRSF: human nucleosome remodeling and spacing factor
Hsd17b4: hydroxy-delta-5-steroid dehydrogenase, 17 beta- and steroid delta-isomerase 4
Hsd3b1: hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1
 HSP70: heat-shock protein 70
 i.p.: intra-peritoneal
 IGF: insulin-like growth factor
 IgG: immunoglobulin gamma
 IL8: interleukin 8
 IP: immunoprecipitation
 ISWI: imitation switch
 IU: international unit
 JAK/STAT: janus kinase/signal transducer and activator of transcription
 K: lysine
Kit: kit oncogene
Kitl: kit ligand
 KO: knockout
 L: liter
 LH-R: luteinizing hormone receptor
 LH: luteinizing hormone
 LIMMA: linear models for microarray data
 LoxP: locus of X-over P1
 LRPPRC: leucine-rich PPR-motif containing
 MA-10: MSV-transformed A10 cells
 MCP1: monocyte chemoattractant protein 1
 MDB3: methyl CpG binding domain 3
 Me: methyl

MeCP1: methyl-CpG binding protein 1
 MeCP2: methyl-CpG binding protein 2
 MEM: minimum essential media
 mg: milligram
 MI2: dermatomyositis-specific autoantigen Mi2beta (see CHD1)
 ml: milliliter
Mlh1: mutL homolog 1
 mm: millimeter
 mM: millimolar
Mmp2: matrix metalloproteinase 2
Mmp9: matrix metalloproteinase 9
 MMTV: mouse mammary tumour virus
 mRNA: messenger ribonucleic acid
 MS/MS: tandem mass spectrometry
 MTA1/2/3: metastasis-associated 1/2/3
 NCL: nucleolin
 NF-1: neurofibromatosis 1
 NF- κ B: nuclear factor of kappa light polypeptide gene enhancer in B-cells
Nlrp14: NLR family, pyrin domain containing 14
Nobox: newborn ovary homeobox gene
 NoRC: nucleolar remodeling complex
Nr5a1: nuclear receptor subfamily 5, group A, member 1
Nr5a2: nuclear receptor subfamily 5, group A, member 2
 NuRD: nucleosome remodeling and deacetylase
 NURF: nucleosome remodeling factor
 NURF301: nucleosome remodeling factor subunit 301
Oosp1: oocyte-secreted protein 1
P27kip1: cyclin-dependent kinase inhibitor p27 (see *Cdkn1b*)
 P4: pregn-4-ene-3,20-dione
 P450: cytochrome P450
P450sc: cytochrome p450 cholesterol side-chain cleavage enzyme
 PABPC1: poly(A) binding protein, cytoplasmic 1
Par-1: protease activated receptor 1
 PBS: phosphate buffered saline
 PCR: polymerase chain reaction
Pcsk5: proprotein convertase subtilisin/kexin type 5
Pde10a: phosphodiesterase 10A
Pde4b: phosphodiesterase 4B
 pEGFP: enhanced GFP plasmid
 PGC: primordial germ cell
Pgr: progesterone receptor
 pH: potential hydrogen
 PHD: plant homeodomain
 Pi3K: phosphatidylinositol 3 kinase
 PKA: protein kinase A

PMSF: phenylmethanesulfonylfluoride
 PMSG: pregnant mare serum gonadotropin
Pou5f1: POU domain, class 5, transcription factor 1
Ppia: peptidylprolyl isomerase A
 PR-A: progesterone receptor A
 PR-B: progesterone receptor B
 PRL: prolactin
 PRLR: prolactin receptor
 PRMT5: protein arginine methyltransferase 5
Pten: phosphatase and tensin homolog
Ptgs2: prostaglandin-endoperoxide synthase 2
 qPCR: quantitative polymerase chain reaction
Rad21: radiation sensitive isolate 21
 RAR: retinoic acid receptor
Ras: rat sarcoma
Rb: retinoblastoma
Rbap46: Retinoblastoma Associated protein p46
Rbap48: Retinoblastoma Associated protein p48
 rDNA: ribosomal deoxyribonucleic acid
Rgs2: regulator of G-protein signaling 2
 RIA: radioimmuno assay
 RIPA: radioimmunoprecipitation assay buffer
 RNA: ribonucleic acid
Rpl19: ribosomal protein L19
Rpl4: ribosomal protein L4
Rps9: ribosomal protein S9
 RSF: remodeling and spacing factor
 RT-PCR: reverse transcriptase polymerase chain reaction
 RU486: mifepristone
 SANT: SWI3, ADA2, N-CoR, TFIIB domain
 SARF: steroid receptor and activator remodeling factor
 SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis
 SF1: steroidogenic factor 1 (see *Nr5a1*)
Sfp4: secreted frizzled-related protein 4
 SIGC: spontaneously immortalized granulosa cells
 siRNA: small interfering ribonucleic acid
Sl: steel
 SMAD: mothers against decapentaplegic
Smad4: mothers against decapentaplegic homolog 4
 SNF2: sucrose nonfermenting 2
Snf2b: sucrose nonfermenting protein homolog 2
Snf2l: sucrose nonfermenting 2-like protein
Sohlh1: spermatogenesis- and oogenesis-specific basic helix-loop-helix-containing protein 1
Sohlh2: spermatogenesis- and oogenesis-specific basic helix-loop-helix-containing protein 2
 SP1: trans-acting transcription factor 1
 SPNA2: spectrin A2

SPNB2: spectrin B2
Spry2: sprouty homolog 2
 StAR: steroidogenic acute regulatory protein
Stat5a: signal transducer and activator of transcription 5A
Stat5b: signal transducer and activator of transcription 5B
Stra8: stimulated by retinoic acid gene 8
 SVOG-40: SV40 Tag-immortalized granulosa cells
 SWI/SNF: switch-type mating/ sucrose nonfermenting
 SWI2/SNF2: switch type mating 2 / sucrose nonfermenting 2
Taf4b: TATA box binding protein (TBP)-associated factor 4B
 TBST: Tris-buffered saline tween 20
 TFIID: transcription factor IID
 TGF- β : transforming growth factor beta
Timp1: tissue inhibitor of metalloproteinase 1
 TIP5: TIF-I interacting peptide 5
 TRF2: TATA box binding protein-related factor 2
 V/V: volume per volume equivalent
Vcan: versican
 Vol: volume
W: white
 WCRF: WSTF-related chromatin remodeling factor
 WICH: WSTF–ISWI chromatin remodeling complex
 WSTF: William syndrome transcription factor
 WT: wildtype
 Xq: long arm of X chromosome
Zp1: zona pellucida glycoprotein 1
Zp3: zona pellucida glycoprotein 3

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CHAPTER 1: INTRODUCTION

In addressing the fundamental problem of packaging large amounts of DNA within the relatively constrained environment of a eukaryotic nucleus, nature has evolved an elegant solution: chromatin. The most basic structural unit of chromatin consists of a segment of DNA wrapped around a histone protein core and is called the nucleosome. In addition to providing compact packaging, nucleosomes represent an ideal focal point for genome regulation because they can limit access to the underlying DNA sequence. Nucleosomes can be modified in a number of ways including changes in positioning, histone post-translational modifications, or methylation of the DNA sequence within it. Those alterations are stable enough to be inherited through cell divisions, yet labile enough to allow for rapid changes in gene expression in response to external stimuli. The manner in which such epigenetic modifications orchestrate the complex changes in gene expression associated with tissue proliferation and differentiation remains largely unknown. In this thesis we explore how a specific family of chromatin remodelers, ISWI, is involved in such regulation and its implications on both the physiology and pathology of the ovary.

1.1 The development of the ovary

The word “ovary” comes from the Latin word for egg, the production of which, as it implies, is its primary responsibility. The ovary is derived from an indifferent embryonic structure called the genital ridge which consists of mesenchymal cells and cells of mesonephric origin (Byskov 1986). In the mouse embryo, the pluripotent proximal epiblast gives rise to primordial germ cells (PGCs) around post-coitum day (dpc) 6.5-7 (Matsui & Okamura 2005). PGCs then migrate through the

primitive streak and into the hindgut (Molyneaux et al. 2001). From there they undergo a second bilateral migration to the genital ridges around dpc 10.5-11, following an extracellular matrix trail and chemoattractive signals such as Kit ligand (Farini et al. 2007). Once the PGCs reach the genital ridge they differentiate into mitotically active oogonia which are found clustered in nests (T'ingen et al. 2009). Those nests are broken down by both the death of a subset of the oogonia and the concurrent invasion of pre-granulosa cells to form sex cords (Byskov 1986). Eventually, the oogonia enter meiosis and become oocytes but promptly arrest at prophase I, while the surrounding somatic cells differentiate into proper granulosa cells and arrest at G0, giving rise to a large pool of dormant primordial follicles (Binelli & Murphy 2010).

Most primordial follicles undergo atresia before or shortly after birth (Morita & Tilly 1999) . In humans, nearly 6 million out of 7 million oocytes present in the foetal gonads are lost perinatally (Baker 1963). It is unclear why such a large proportion of oocytes are lost during development, given that oocytes cannot self-renew, but it could be a quality control mechanism or a means to limit the reproductive lifespan of some mammalian species. Recently, Johnson et al have suggested that germ cells may be renewed in adult ovaries from a bone-marrow derived progenitor cell (Johnson et al. 2004). This theory was mostly based on discrepancies between atresia rate and total follicle pool size, and circumstantial evidence of green fluorescent protein (GFP)-positive follicles within donor ovaries grafted in GFP recipient mice (Johnson et al. 2004; Faddy et al. 1976). However, these claims have been challenged by multiple groups which provided mathematical models accommodating observed atresia rate without de-novo oocyte formation, and experimental evidence of a lack of progenitor-derived eggs in multiple models of oocyte depletion (Eggan et al. 2006; John et al. 2007; Bristol-Gould et al. 2006; Byskov et al. 2005).

1.2 Folliculogenesis

Folliculogenesis is by definition the maturation program of an ovarian follicle, from its inception as a primordial follicle all the way to ovulation and its subsequent transformation into a corpus luteum (CL) and finally its degeneration. Folliculogenesis is also a reductive process by which a large pool of activated primordial follicles ultimately gives rise to only a few large antral follicles capable of ovulating. Therefore many factors come into play to balance follicle growth and follicle loss through atresia. Additionally the maturation of the follicle necessitates complex changes in morphology, including growth of the oocyte and its surrounding layers of granulosa cells, differentiation of granulosa cells and theca cells, formation of the antrum, cumulus cell expansion and ultimately ovulation and CL formation. Each of these steps is governed by a multitude of extrinsic and intrinsic factors, which will be briefly summarized here.

1.2.1 Primordial and primary follicles

After birth, cohorts of primordial follicles are recruited into the growing pool, to undergo a 3 week growth cycle. This process significantly alters the appearance of the primordial follicle which initially consists of an oocyte surrounded by a flattened layer of quiescent squamous granulosa cells and a basal lamina (Fig 1.1). Firstly, the oocyte grows in size and the layer of granulosa cells changes morphology by adopting a more cuboidal histology to form a primary follicle (Fig 1.1). While it is unclear how only subset of primordial follicles is selected to grow, many genes important in their transition to primary follicles have been identified. In particular, there are 3

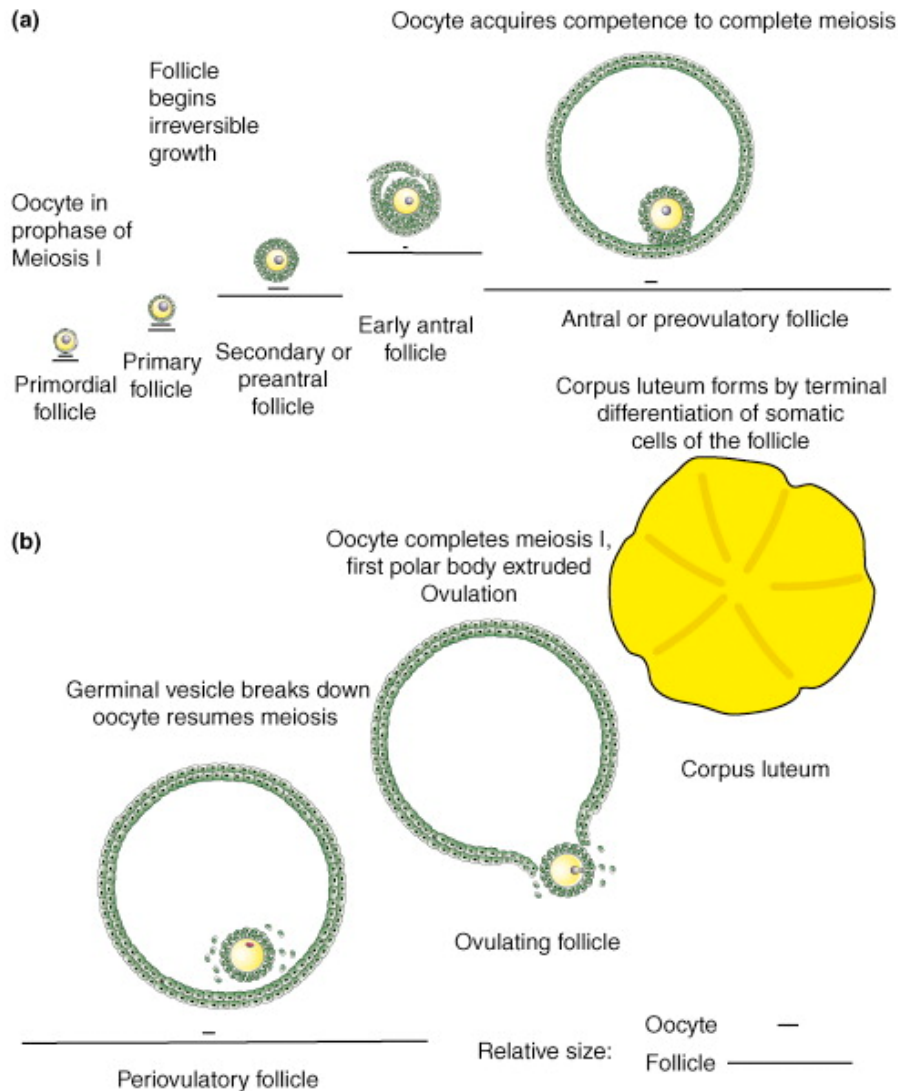


Figure 1.1 Changes in the oocyte and the somatic granulosa cells of the ovarian follicle through the course of maturation and differentiation. (a) Primordial follicles, formed before birth in most species, contain the oocyte arrested in the prophase of meiosis I and surrounded by pregranulosa cells. Primordial follicles are selected to initiate irreversible growth by unknown mechanisms. Growth of the oocyte is accompanied by granulosa-cell proliferation and subsequent formation of the fluid-filled antrum. The final stages occur with the acquisition of the oocyte competence to complete meiosis. Most follicles that initiate growth degenerate by atresia but a few reach the preovulatory state. (b) The preovulatory gonadotropin surge reinitiates meiosis and also induces multiple follicular changes that result in expulsion of the oocyte. The follicle collapses and the somatic cells undergo terminal differentiation to form the progesterone-secreting corpus luteum. The figure is not to scale, therefore the size of the oocyte relative to the follicle is indicated by the horizontal lines below each depiction. Reproduced with permission from Pépin et al (Pépin et al. 2007).

transcription factors which when knocked-out result in infertile mice with no follicles beyond the primordial stage. The newborn ovary homeobox (Nobox), the Spermatogenesis and oogenesis helix-loop-helix 1 (Sohlh1), and its paralog Sohlh2, are expressed in primordial and primary follicles (Rajkovic et al. 2004; Pangas et al. 2006a). The Sohlh family is also required for male fertility, suggesting common mechanisms between the transition of primordial follicles into primary follicles and the transition of type A spermatogonia into type B spermatogonia (Hao et al. 2008). In ovaries, Sohlh2 regulates oocyte-specific genes such as *Sohlh1*, *Nobox*, *Figla*, *Gdf9*, *Pou5f1*, *Zp1*, *Zp3*, *Kit*, *Oosp1*, *Nlhp14*, *H1foo*, and *Stra8*. Nobox also regulates genes known to be involved in folliculogenesis such as *Pou5f1* and *Gdf9* by directly interacting with Nobox elements found in their promoters (Choi et al. 2007; Choi & Rajkovic 2006). Of these, growth and differentiation factor 9, (*Gdf9*) seems to be particularly important for promoting the activation of primordial follicles, as immature female rats treated with recombinant GDF9 protein for 10 days have 29% decrease in primordial follicles with the corresponding 30% increase in primary follicles (Vitt et al. 2000). Together, these three transcription factors appear to be crucial for the transcriptional activation of genes necessary for primordial follicle recruitment. However it is unclear which signaling pathways and hormonal signals are responsible for their own activation although a few potential candidates exist.

One paracrine signaling system that may be critical for early folliculogenesis is based on the KIT tyrosine kinase receptor and its ligand (KITL). Standard genetics allowed for the identification of their respective loci, white (*W*) and steel (*S*), based on alteration in coat color (Mayer 1979). However mutation of those loci revealed a plethora of phenotypes, including female infertility and defects in PGC migration (Matsui et al. 1990). The analysis of specific *Kitl* mutants caused by X-

ray radiation, such as *S/panda*, also uncovered maturation arrests at the primary follicle stage (Huang et al. 1993). Similarly, treatment of immature mice with a KIT-blocking antibody prevents primordial follicles from developing into primary follicles, as well as impairs primary follicle growth and antrum fluid production, depending on the timing of the injection (Yoshida et al. 1997). Together these results suggest that KIT is important for the transition from primordial to primary follicle, yet its downstream signalling pathway in that system remains largely unknown. One possibility is that KIT phosphorylation activates AKT and inhibits FOXO3a resulting in the positive regulation of oocyte growth and the release of factors influencing granulosa cell differentiation (Reddy et al. 2005).

Foxo3a is an inhibitor of primordial follicle activation as evidenced by the knockout (KO) mice which have an accelerated depletion of primordial follicles leading to a premature ovarian failure (Castrillon et al. 2003). As expected, a transgenic mouse with a constitutively active FOXO3a expressed in the oocyte displays a severe retardation of follicle development with aberrant downregulation of BMP15, CX43, and CX37 (Liu et al. 2007). PTEN is a negative regulator of PI3K which, when dysregulated, suppresses FOXO3a (John et al. 2008). Therefore, as would be expected, the *Pten* KO is a phenocopy of the *Foxo3a* KO, with a premature activation of the primordial follicles (Reddy et al. 2008). These findings suggest that KIT and PTEN have opposing actions on the PI3K-AKT-FOXO3a signalling pathway. Ultimately this signalling cascade culminates with FOXO3a, a transcription factor, translocating into the nucleus and regulating *P27kip1*, an important cell cycle inhibitor (Dijkers et al. 2000).

Concurrently, in granulosa cells, another forkhead transcription factor FOXL2 may be important for the changes in cell morphology observed during the transition between primordial and primary

follicles. In *Foxl2* KO mice, granulosa cells fail to switch from a squamous to a cuboidal cytological phenotype and follicular development of secondary follicles is blocked (Uda et al. 2004). Another factor which may be involved in the activation of primordial follicles is the hormone AMH and its receptor AMHR2 which are produced by the granulosa cells. While *Amh* KO females appear to be fertile, young mice actually contain more pre-antral and small antral follicles, whereas mice over a year old are nearly completely depleted in primordial follicles (Durlinger et al. 1999). Thus it appears that the hormone which induces the degeneration of the Mullerian duct in males may also have a function as an inhibitor of early follicle development. Similarly, another paracrine factor which is able to inhibit the recruitment of primordial follicle is CXCL12 and its receptor CXCR4 which are produced by the oocyte. Supplementation of recombinant CXCL12 in an ex-vivo culture system of neonatal ovaries causes a significant accumulation of unactivated primordial follicles, whereas treatment with a CXCL12 inhibitor abolishes this effect (Holt et al. 2006). Interestingly, the same system may be involved in the migration of PGCs into the gonadal ridge (Molyneaux et al. 2003). Both the AMH-AMHR2 and CXCL12-CXCR4 systems may be important in the maintenance of a stable pool of primordial follicles by opposing both the activation of oocyte growth and the morphological differentiation of granulosa cells.

Therefore the recruitment of primordial follicles into the growing pool and the changes in oocyte size and granulosa cell morphology associated with the transition to a primary follicle may be dictated by a balance in the opposing actions of the paracrine systems of KIT-KITL, AMH-AMHR2 and CXCL12-CXCR4, the antagonistic signalling of PI3K and PTEN, the transcriptional

effects of NOBOX, SOHLH1, SOHLH2, FOXL2 and FOXO3a and the myriad of genes under their regulation.

1.2.2 Secondary follicles

The transition of primary follicles into secondary or pre-antral follicles is marked by further growth of the oocyte as well as proliferation of the granulosa cell layers surrounding the oocyte beyond two layers, and the differentiation of the outermost layer of stromal cells into theca cells. Secondary follicles start to appear in mice around 10-12d after birth and are characterised by 2 or more layers of granulosa cells surrounding the oocyte which may grow up to 9 layers of granulosa cells and one layer of theca cells before antrum formation begins (Fig 1.1). The growth of secondary follicles is governed by various paracrine factors but is independent of the follicle stimulating hormone (FSH). FSH receptor KO mice have normal early folliculogenesis but cannot get beyond the pre-antral stage and suffer from low estradiol levels, suggesting neither hormone is necessary for secondary follicle growth (Abel et al. 2000; Dierich et al. 1998).

There is however substantial experimental evidence suggesting that the growth of granulosa cells in secondary follicles is promoted by factors secreted from the oocyte. Elegant experiments in which follicles were oocyctomized, have shown that growth of granulosa cells in the absence of the oocyte is severely restricted (Vanderhyden et al. 1992). Furthermore, when oocytes from secondary follicles are transplanted back into primary follicles, they significantly increase the growth of the granulosa cells (Eppig et al. 2002). Some of the putative growth signals secreted by the oocyte have been isolated.

One of those signals is GDF9, a secreted factor that is part of the TGF- β superfamily, which is expressed by oocytes at all stages of follicle development, from primary oocytes to ova (McGrath et al. 1995). *Gdf9* KO ovaries contain only primordial and primary follicles rendering the females infertile, however the growth of the oocyte seems unimpaired, suggesting a decoupling of granulosa and oocyte growth (Dong et al. 1996).

The communication between oocyte and granulosa cells is not one-sided. In primary follicles of *Gdf9* KO ovaries, granulosa cells overexpress both KITL and inhibin α , suggesting they form a feedback loop with GDF9 which may help temper the growth of the oocyte to match the progression of the granulosa cells (Elvin et al. 1999). The primary follicles also fail to produce a theca cell layer, and eventually the oocyte degenerates. The remnant granulosa cells in the absence of the oocyte undergo a partial differentiation into a luteal-like state where they express both antral and CL markers such as, respectively, p450 aromatase and p450 side-chain cleavage (Elvin et al. 1999). This suggests that factors coming from the oocyte actively prevent the luteinisation of granulosa cells. Therefore a model emerges where KITL produced by the granulosa cells promotes the growth of the oocyte until it reaches a threshold size, at which point the level of GDF9 secreted by the oocyte is sufficient to act on granulosa cells and inhibit *Kitl* to stop oocyte growth while simultaneously stimulating the production of inhibin α which halts granulosa cell growth while keeping them in an unluteinized state. Such a negative feedback system would not only provide a way to synchronize oocyte and granulosa growth but also limit the growth of secondary follicles once they have reached the desired size.

One of the first events in the maturation of secondary follicles, is the formation of a theca cell layer. This is achieved by the differentiation of the stromal cells directly in contact with the

basement membrane at the edge of the outermost layer of granulosa cell into a theca interna, and a theca externa. The theca interna cells are important for later stages of folliculogenesis by providing granulosa cells with a source of androgens that can be converted to estradiol (Magoffin 2005), while the theca externa is comprised mostly of smooth muscle cells and fibroblasts which may be important at the time of ovulation (Magoffin 2005). The factors which control theca cell differentiation are largely unknown, although both the FSH receptor and luteinizing hormone (LH) receptor KO mice have secondary follicles with theca cells, suggesting it is not gonadotropin-dependent (Zhang et al. 2001; Dierich et al. 1998). Furthermore, as previously mentioned, *Gdf9* KO follicles fail to produce theca cells, indicating that GDF9 may play a positive role in their differentiation (Dong et al. 1996).

The only hint regarding the origins of the factors that promote the differentiation of the theca externa comes from a mouse model of hedgehog signalling. In a transgenic mouse where a constitutively activated smoothened, a seven trans-membrane domain receptor downstream of hedgehog ligands, is expressed specifically in granulosa cells under the *Amhr2* promoter, the theca externa fails to form as evidenced by the absence of smooth muscle actin staining (Ren et al. 2009). The lack of smooth muscle cells within the theca physically prevents ovulation from occurring, probably because their contraction is required for follicle rupture. It is likely that the signal for theca differentiation comes from the adjacent granulosa, in the form of sonic hedgehog or Indian hedgehog (Wijgerde et al. 2005).

1.2.3 Antral follicles

The development of the antral follicle is characterized by the formation of the antrum, a fluid-filled cavity within the follicle (fig 1.1). The antrum first begins to form in small pockets which

eventually coalesce into one large cavity. By doing so, it physically isolates two populations of granulosa cells. Firstly the outermost layer of granulosa cells that is in contact with the theca cell layer differentiates into mural granulosa cells which are responsible for estradiol production. Secondly, the granulosa cells which surround the oocyte become cumulus cells, which play a role in maintaining oocyte growth and promoting its competence. The antral follicle stage is particularly important because it involves a selective reduction of follicles by atresia, and it ultimately culminates in ovulation, the defining step of successful folliculogenesis.

Unsurprisingly, the factors that govern antral follicle maturation have been well studied. At this stage there is a switch from growth mechanisms involving primarily paracrine hormones to an environment modulated principally by endocrine hormones from the hypothalamic-pituitary-gonadal axis. They include first and foremost the gonadotropins FSH and LH and the sex steroids estradiol and progesterone. Their interaction lies at the heart of the two cell - two gonadotropin hypothesis which states that LH stimulates theca cells to produce androgens whereas FSH and LH induce aromatase in granulosa cells to convert the androgens into estradiol (Hillier et al. 1994). The feedback loop between gonadotropins and sex steroids is bimodal with low estradiol levels during early folliculogenesis being suppressive of LH, whereas the acute rise of estradiol at pro-oestrus positively feedbacks on the hypothalamic-pituitary axis and triggers the gonadotropin surge (Lindzey et al. 2006). Progesterone may also assist estradiol in stimulating the LH surge as evidenced by the muted response when rats are treated with the progesterone receptor antagonist RU486 (Mahesh & Brann 1998). Both FSH and estradiol are thought to regulate antral follicle growth by directly acting on the granulosa cell cycle, specifically through cyclin D2 and p27kip1 (Robker & Richards 1998).

FSH is an essential factor for the survival of the antral follicle by inhibiting apoptosis and follicular atresia (Kumar et al. 1997). Furthermore, FSH is required in mural granulosa cells to stimulate the production of estradiol, which is important for the growth of the antral follicle. FSH KO and FSH receptor KO mice have normal amounts of follicles from earlier stages, however they are completely devoid of antral follicles or CLs suggesting the gonadotropin is necessary for their formation and maintenance (Kumar et al. 1997; Dierich et al. 1998).

The aromatase KO is also very informative on the role of estradiol in antral follicle growth and survival since the enzyme produced by the *Cyp19* gene is required for the production of estradiol. In those mice antral follicles form but are unable to ovulate and their ovaries contain a large number of atretic follicles (Fisher et al. 1998). When the KOs are aged, their ovaries become cystic and develop many haemorrhagic follicles (Britt et al. 2000). Similarly both estrogen receptor (*Er*) α KOs and *Er* β KOs have antral follicle defects and reduced ovulation rates, particularly the *Er* α KO which also suffers from cystic ovaries and haemorrhagic follicles (Dupont et al. 2000). Taken together these results suggest that FSH is required for antral follicle formation whereas estradiol is needed for the later growth and survival of the antral follicle as well as its subsequent ovulation. Importantly, the level of estradiol being produced by the follicle directly relates to the amount of FSH receptor expressed which may be key to ensuring the survival of dominant follicles. While the exact mechanism of dominant follicle selection remains poorly understood, experimental evidence in bovine systems delineated a role for FSH, IGF and estradiol in protecting the follicle cells against apoptosis in the face of declining levels of FSH due to negative feedback from the hypothalamic-pituitary axis to secretions of inhibin and estradiol (Rivera & Fortune 2003). IGF may potentiate FSH signalling through the PI3K signalling pathway to

maximise the amount of estradiol being produced. This role appears to be conserved in the mouse since *Igf1* KOs' follicles arrest at the preantral stage, and have markedly reduced levels of aromatase and FSH receptor (Baker et al. 1996; Zhou et al. 1997).

Another factor of importance in antral follicles is TAF4B, which may also help potentiate the induction of FSH target genes. TAF4B is a subunit of the TFIID complex of TATA binding proteins and associated factors whose expression is restricted to granulosa cells. *Taf4b* KO mice are resistant to both FSH and estradiol-dependent signals and respond with less growth and more apoptosis of granulosa cells (Voronina et al. 2007).

1.2.4 Ovulation

The final step in the maturation of the antral follicle is ovulation triggered by the LH surge. For ovulation to occur many events need to take place, including cumulus-oocyte expansion, enzymatic digestion of the follicular wall, and rupture of the follicle to allow the expulsion of the ovum. At this stage few antral follicles remain, most of them having been lost by atresia, while those that are left are exquisitely sensitive to FSH and LH because of high receptor expression. The dominant follicles which are primed to ovulate will have undergone a rapid increase in size in response to the high local concentration in estradiol and develop large antral cavities (fig1.1).

The expansion of cumulus cells, another gonadotropin-dependent event is important for both the ovulation process and ultimately the proper interaction between the ovum and the sperm. Both EGF and FSH are potent inducers of cumulus cell expansion. However, they fail to induce expansion in oocyctomized complexes, suggesting there are factors secreted by the oocyte in response to those hormones which act locally on the cumulus cells (Buccione et al. 1990). Direct

physical interaction between the oocyte and the cumulus cell is not required since the addition of denuded-oocyte conditioned culture medium is sufficient to induce expansion (Buccione et al. 1990).

Two important factors secreted in response to EGF and FSH have been isolated which play an essential role in cumulus expansion: hyaluronan synthase 2 (HAS2) and prostaglandin synthase 2 (PGS2) (Diaz et al. 2006). The cumulus-oocyte complex (COC) is stabilized by a network of extracellular matrix consisting primarily of hyaluronic acid, and the HAS2 enzyme produced by the cumulus cells plays a crucial role in the formation of this matrix. PGS2 is responsible for the production of prostaglandins, which in turn stimulate hyaluronic acid synthesis (Eppig 1981). Both HAS2 and PGS2 expression seem to be induced by the GDF9 and BMP15 soluble factors produced by the oocyte confirming the important role of oocyte communication in coordinating this process (Gui & Joyce 2005; Su et al. 2004; Dragovic et al. 2005). Furthermore, denuded oocytes isolated from *Gdf9*^{+/-} (HET) / *Bmp15* KO double transgenic mice treated with FSH fail to induce cumulus expansion when co-cultured with normal wildtype (WT) oocyctomized follicles suggesting they act synergistically to regulate HAS2 and PGS2 (Su et al. 2004).

Once the COC complex has expanded, it is ready to be ovulated. In most inbred mouse strains ovulation usually happens around 12h after the LH surge (Masters & Wheeler 1996). Ovulation is often referred to as an inflammatory process because many of the same factors are at play, such as the previous example of prostaglandins. Sex steroids are also involved in this process, but there is a shift from estradiol to progesterone. The progesterone receptor gene (*Pgr*) produces two isoforms PR-A and PR-B with distinct roles in reproduction. PR-A specific mutant mice fail to ovulate in response to the LH surge and accumulate large preovulatory antral follicles with

expanded COCs but do not produce CLs (Mulac-Jericevic et al. 2000a). The phenotype suggests progesterone is involved in regulating the transcription of genes essential for follicular rupture. Two of those putative genes are the *Adamts1* and *CathepsinL* proteases which are found to be dysregulated in the *Pgr* KO. Of those, *Adamts1* appears essential for the weakening of the follicular wall since knocking it out is sufficient to recapitulate the anovulatory phenotype (Shindo et al. 2000).

A number of other transcriptional regulators appear to be involved in the ovulatory process although their targets are less well defined. Of those, the ubiquitous transcriptional activator CEBP/ β and the orphan nuclear receptors NR5A1 and NR5A2 are highly induced by LH, and their conditional deletion results in anovulatory mice (Sterneck et al. 1997; Pelusi et al. 2008; Duggavathi et al. 2008). A fourth regulator, and the subject of this thesis, SNF2L, is also induced by LH and interacts with PR-A, and will be discussed in greater detail in the later sections.

Once the follicular wall is weakened, the next and final step in ovulation is the contraction of the smooth muscle cells of the theca externa. While little is known about how this process is regulated, one particular factor appears to be able to trigger those contractions. Endothelin 2 (END2) is a member of the vasoactive endothelin family, better known for their stimulation of contraction of smooth muscle cells in blood vessel walls to help regulate blood pressure (Giannessi et al. 2001). Like the above mentioned proteases, *End2* appears to be a *Pgr*-regulated gene, suggesting progesterone is critical for the transcription of genes involved in multiple processes associated with ovulation (Palanisamy et al. 2006). Furthermore addition of END2 is sufficient to induce follicular contractions, while treatment with the endothelin receptor antagonist tezosentan at the time of ovulation significantly reduces superovulation yield (Ko et al. 2006).

1.2.4 Corpus luteum formation

After ovulation the remnant follicle undergoes drastic changes in both form and function to become a CL. The main function of the CL is the transient production of the sex steroid progesterone which is required to maintain pregnancy should the ova be fertilized. To produce the high levels of progesterone required to stimulate the uterus, mural granulosa cells exit the cell cycle and differentiate into an entirely new cell type: the luteal cell. The main triggers for this differentiation are the LH surge and the loss of inhibitory effects of the oocyte.

Just as FSH may promote cell cycle progression in granulosa cells by increasing the ratio of cyclin D2 to P27KIP1, the exit of the cell cycle brought upon by the terminal differentiation of granulosa cells into luteal cells may be due to LH decreasing the ratio of cyclin D2 to P27KIP1 (Robker & Richards 1998).

The hormone prolactin (PRL) and its receptor (PRLR) may be crucial for the differentiation of the CL in rodents. The *Prlr* KO mice are infertile because their uterus cannot support implantation (Ormandy et al. 1997). This is linked to the fact that their CLs are abnormal as a result of increased apoptosis, poor angiogenesis, and a downregulation of LHR and the steroidogenic enzymes associated with the production of progesterone (Grosdemouge et al. 2003). Insights into how PRLR signalling influences differentiation are gained by examining mutants of its downstream effectors of the JAK/STAT pathway. *Stat5a* and *Stat5b* KOs phenocopy the *Prlr* KO and exhibit dysregulated cell cycle modulators such as increased levels of the cell cycle inhibitor CDKN1B (Teglund et al. 1998).

As previously mentioned, the oocyte inhibits luteinisation, since its removal causes the mural granulosa cells to undergo spontaneous luteinisation (Vanderhyden et al. 1992). Also, many of the oocyte-secreted factors which are responsible for the regulation of the juxtaposed granulosa cells are part of the TGF- β superfamily. Therefore it is tempting to speculate that the inhibitors of luteinisation are TGF- β family members such as GDF9. TGF- β signalling culminates with the transcription factors of the SMAD family. This hypothesis is strengthened by a study showing that the conditional *Smad4* KO mice are subfertile because of the premature luteinisation of the follicles (Pangas et al. 2006b). Furthermore the phenotype includes a range of defects such as reduced superovulation yield, an increase in atresia combined with a decrease of antral follicle numbers, as well as a number of abnormal follicles such as oocytes trapped in CLs and poor antrum formation (Pangas et al. 2006b). Additionally, the steroidogenic enzymes CYP11a1, HSD3b1, HSD17b4 and StAR are significantly upregulated in the KO (Pangas et al. 2006b).

1.3 Epigenetics

Epigenetics is defined as the study of heritable changes in gene expression that are independent of the underlying DNA sequence. These changes can be stably inherited through one or multiple cellular divisions, and even across one or multiple generations. Development provides a good example of epigenetics in action since many changes in gene expression occur during the progression from a single totipotent cell to the vast array of differentiated cells which comprises an organism, yet each of these cells contains identical DNA sequence. It follows that there must exist a system which is stable enough for a cell to remember its identity as it replicates, yet flexible enough to allow for the drastic changes that accompany differentiation. The mechanisms which inscribe and propagate epigenetic information in a cell are diverse and include non-coding RNAs,

DNA methylation, histone post-translational modifications and nucleosome positioning. There is a significant amount of overlap and cross-talk between each mechanism, which we are only beginning to unravel. Here we will concentrate on epigenetic factors which directly modify nucleosomes albeit not necessarily for heritable changes.

1.3.1 DNA methylation

DNA methylation generally refers to the covalent modification of cytosine by the addition of a methyl group at CpG dinucleotides. It satisfies the requirement of an epigenetic mark since it is a stable modification and is passed down during replication. Methylation is usually associated with condensed parts of the genome such as telomeres, centromeres and repeat elements. Methylation is also associated with gene repression, and CpG islands are a common feature of gene promoters, although they are generally unmethylated (Saxonov et al. 2006). The repressive nature of methylated CpG stems from the fact that they promote changes in chromatin conformation favouring heterochromatin formation, probably by recruitment of repressive proteins with methyl cytosine binding ability such as MeCP1 and MeCP2 (Nan et al. 1998).

Methylation is established and maintained by a class of enzymes called DNA methyltransferases. DNMT1 is responsible for the maintenance of methylation after replication by targeting hemi-methylated CpG sites and methylating the contralateral cytosine of the newly synthesized daughter strand (Miranda & Jones 2007). DNMT3a and DNMT3b are de-novo methyltransferases responsible for the establishment of new methylation marks, particularly during cellular differentiation (Miranda & Jones 2007). The loss of methylation is generally thought to be a passive process where methylated CpG through replication becomes hemi-methylated, with each subsequent cellular division further diluting the methyl marks.

Of all of the epigenetic marks DNA methylation is the most stable and difficult to remove, which makes it ill-suited for relatively rapid changes of expression. However it is ideal for the transgenerational transmission of epigenetic information. Imprinting is a good example in eutherian mammals, where methyl marks put in place during male gametogenesis can influence foetal growth (Willison 1991).

1.3.2 Chromatin organization

The nucleosome consists of about 146bp of DNA wrapped around a histone octamer made up of two units each of H2A, H2B, H3 and H4. While the nucleosome is the most basic unit of chromatin, it can form higher order structures by folding upon itself. It can form an 11nm fibre by coiling itself and a 30nm with a coiled coil structure stabilized by the histone linker H1 (van Holde & Zlatanova 1996). The 30nm fibre itself can form complex three-dimensional structures such as loops, rosettes and ultimately metaphase chromosomes which in somatic cells is the most condensed form of chromatin (Odenheimer et al. 2009).

The core histones share a similar structure with a basic globular domain and an unstructured tail which, as we will explore, can be the subject of modifications. In addition to the core histones there exist many variants which can be incorporated into the nucleosome. One such variant, H2AX, can replace H2A in genomic regions with DNA lesions and, when phosphorylated, can recruit the DNA repair machinery (Yuan et al. 2010). H2AZ is another H2A variant which may mark promoters of quiescent genes and by modifying the interaction of the nucleosome with chromatin remodelers such as ISWI may keep the chromatin configuration stable and the gene primed for activation (Li et al. 2005; Goldman et al. 2010). There exist many other histone variants

encoded by multiple intron-less genes, H3 alone is encoded by over a dozen genes, although little is known about how they functionally differ (Loyola & Almouzni 2007).

Chromatin is generally classified into two categories: euchromatin and heterochromatin. Euchromatin is usually defined as uncondensed, gene-rich, transcriptionally active and early replicating. By contrast, heterochromatin is highly condensed, relatively gene-poor, transcriptionally silent and late replicating. Heterochromatin is further divided into facultative heterochromatin, which under certain circumstances can revert to euchromatin, and constitutive heterochromatin which is associated with chromosomal structures such as telomeres and centromeres and remains condensed throughout much of the cell cycle (Fedorova & Zink 2008). This dichotomy in types of chromatin is likely an oversimplification, and as more large scale ChIP sequencing becomes more readily available, it is likely to be further subdivided such as the black, green, blue, red and yellow chromatin of *D. melanogaster* (Schübeler 2010).

The type of chromatin environment in proximity to a gene can have a profound influence on its expression. For example when transgenes are juxtaposed to constitutive heterochromatin they can take on a variegated pattern of expression because of heterochromatin spread. This property proved to be a useful tool for the identification of many chromatin-modifying genes as either enhancers of variegation or suppressors of variegation (Grigliatti 1991).

1.3.3 Histone modifications

Histone tails can be modified by covalent linkages of various chemical groups such as phospho, methyl, acetyl, ubiquitin, ADP-ribosyl, and citrullin. Those modifications can stack since many residues of H3 and H4 tail are subject to modification, and in some instances, one residue can

have multiple groups, such as for example mono-, di- or tri-methylation of lysines. The amount of permutations and combinations gives rise to an almost irreducibly complex histone code (Wang et al. 2004).

Some general rules about the histone code have started to emerge. For example dimethylation of lysine 4 of H3 (H3K4me2), H3K4me3, and H3/H4 acetylation are features of euchromatin while H3K27me3, H3K9me3 and H4K20me are common in heterochromatin (Kouzarides 2007). It is unclear, however, if these modifications are the cause or the consequence of the chromatin state, and how they integrate with DNA methylation.

One property of histone tails which is of particular interest is the ability of modifications to restrict which chromatin remodelling complexes can interact with the nucleosomal substrate while presumably actively recruiting others. For example acetylation of the H4 tail prevents ISWI-based chromatin remodelling complexes from interacting with the nucleosome (Schwanbeck et al. 2004). Meanwhile H4K4me3 facilitates the interaction of the nucleosome with the ISWI-containing NURF complex thanks to a zinc finger motif called PHD in the NURF protein (Li et al. 2006). Therefore it is tempting to speculate that the histone code can dictate nucleosomal positioning and the formation of various types of chromatin structures via these types of interactions.

1.3.4 ATP-dependent chromatin remodelers

The defining structures of ATP-dependent chromatin remodelers are the presence of a conserved SNF2 helicase-like domain and an ATPase domain. Targeted mutations of the ATP binding domain are sufficient to abrogate the chromatin remodelling activities of this superfamily of proteins (Pazin & Kadonaga 1997). The best studied and prototypical families include SWI/SNF,

ISWI, and CHD, each of which may contain multiple paralogs which take part in various complexes. ATP-dependent chromatin remodelers usually act as catalytic motors, often as dimers, within larger complexes (Vignali et al. 2000). The other subunits found in such complexes are thought to control both the type of nucleosomal substrate and the kind of remodelling being performed, since they often bear domains which interact with histone tail modifications such as bromodomains (acetyl), PHD fingers (methyl), chromodomains (methyl), or SANT domains. For example, the latter is present in the ISWI family, and is known to mediate interactions with unacetylated H4 tails (Yu et al. 2003).

The mechanism by which ATP-dependent chromatin remodelers displace nucleosomes differs between each family. For example SWI/SNF slide the nucleosome by inducing torsion and bending DNA (Lorch et al. 2005), while ISWI complexes create DNA loops and propagate them around the nucleosome to slide it in a stepwise fashion (Lia et al. 2008). SWI/SNF remodelers may also be able to displace whole histones octamer or dimers in cis or in trans (Narlikar et al. 2002). The displacement of nucleosomes can serve many purposes since it can either cover or expose specific DNA sequence and, as such, plays a role in transcription, repression, DNA damage repair, and DNA replication.

The SWI/SNF family, originally identified in yeast on the basis of mating type switching (SWI) and sucrose non-fermenting (SNF) screens, is conserved in eukaryotes. In mammals there are two paralogs, *Brg1* and *Brm* which can both form part of the BAF complex. However, they do not appear to be redundant since deletion of BRG1 is embryonic lethal (Bultman et al. 2000). Deletion of *Brm* however gives rise to a muted phenotype, with pups only slightly larger than normal, suggesting it may negatively regulate cell growth during foetal development (Reyes et al. 1998).

The prototypical CHD remodeler, MI2, contains both chromodomains and PHD fingers and takes part in the NURD complex. The NURD complex is unique in that it contains both ATP-dependent chromatin remodelers, and histone modifying proteins, namely HDAC1 and HDAC2. The NURD complex has been shown to be crucial to early mouse development, as deletion of its *P66α* subunit is embryonic lethal, possibly because of DNA methylation defects (Marino & Nüsse 2007).

Finally the ISWI family is also represented by two paralogs in mammals, *Snf2b* and *Snf2l*. *Snf2l* produces two alternatively spliced transcripts, one of which contains an additional exon which renders the protein catalytically inactive, yet still able to incorporate into complexes (Barak et al. 2004). As we will explore in the next sections, *Snf2b* and *Snf2l* take part in a variety of complexes with distinct functions.

1.4 ISWI complexes and function

Expression of the ISWI proteins SNF2H and SNF2L during mouse development suggests SNF2H is associated with proliferating tissues while SNF2L is present in differentiating cell types (Lazzaro & Picketts 2001). Interestingly, SNF2L expression was enriched in the ovary and closer examination by in-situ hybridization showed it was localized to follicles, particularly in differentiating granulosa cells from mice treated with hCG (Fig 1.2). SNF2H expression is more ubiquitous, and in the ovary seems to be associated with actively proliferating granulosa cells (Lazzaro & Picketts 2001). It is tempting to speculate that the two paralogs have evolved separate and non-overlapping functions, including in the ovary (Fig 1.3). A clue about that specialization comes from the fact that they form distinct complexes (Table 1.1). SNF2H is present in the

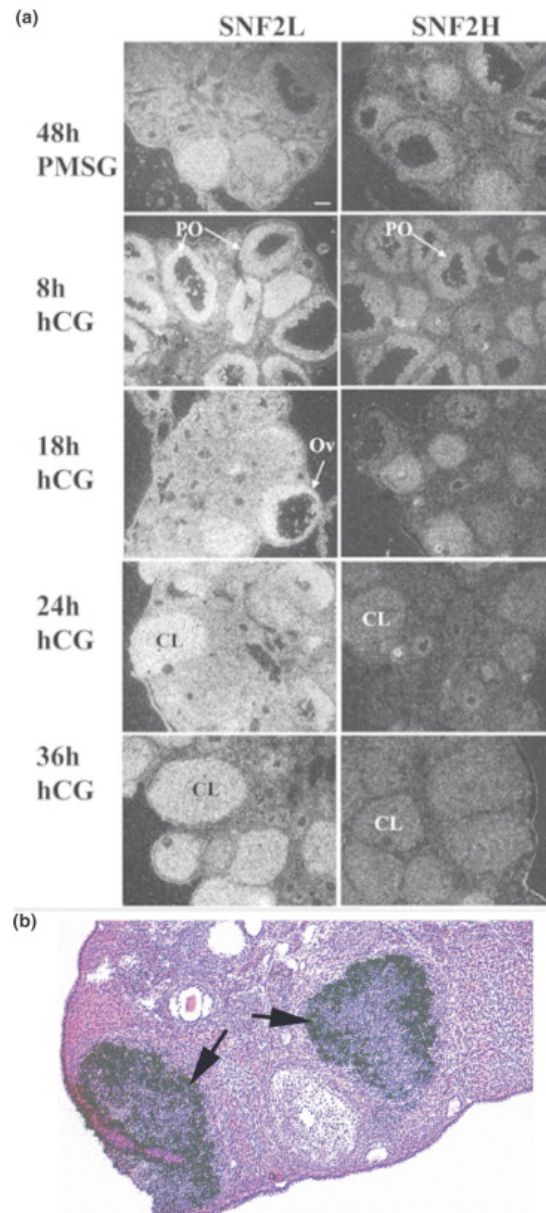


Figure 1.2 Localization of the ISWI ATPases, *Snf2h* and *Snf2l*, in the mouse ovary through ovulation, formation of the corpus luteum and the luteal phase of the ovarian cycle. (a) Treatment of mice for 48 h with pregnant mare serum gonadotropin (PMSG) stimulates the formation of preovulatory follicles (PO) with strong expression of *Snf2h* and *Snf2l* in granulosa cells, as indicated by the brighter signal in this darkfield micrograph. Treatment with the LH-like hormone, human chorionic gonadotropin (hCG), provokes ovulation (Ov) and continued strong expression of *Snf2l* beginning as early as 8 h after hCG treatment (8h) and continuing through ovulation, as shown by the postovulatory 18 h hCG micrograph. The expression is prominent in the newly formed corpora lutea (CL) 24 and 36 h after hCG treatment and persists through diestrus, as indicated by the dark blue signal in corpora lutea (arrows) in (b). Reproduced with permission from Pépin et al (Pépin et al. 2007).

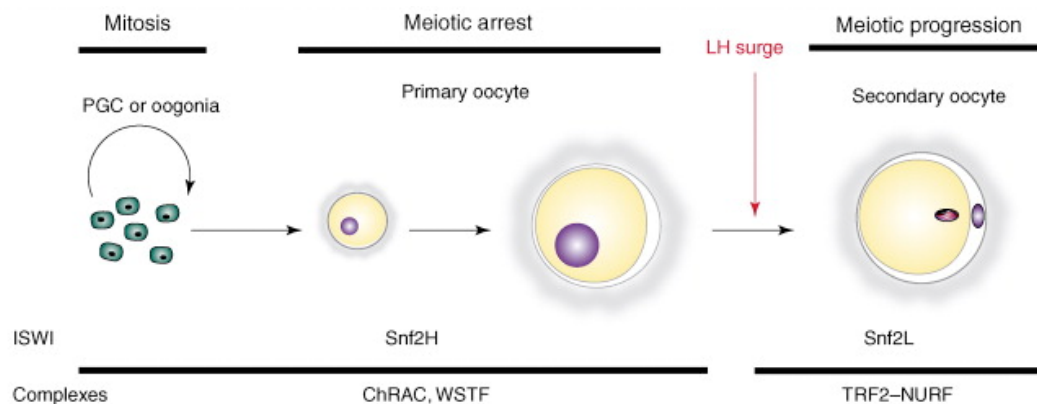


Figure 1.3 A model of the ISWI proteins and complexes involved in the progression of mammalian oogenesis. Oogenesis is a process of growth and maturation, which begins with primordial germ-cell (PGC) formation and culminates with the meiotically competent egg. PGCs undergo self-renewal and proliferation before colonizing the genital ridge (incipient gonad) during fetal development. This self-renewal relies on ISWI in *Drosophila*. Once established in the fetal ovary, the PGCs (now oogonia) cease mitosis and enter the initial stages of meiosis. The meiotic progression arrests at prophase I and the primary oocytes lie dormant in the ovary until they are recruited to grow. The epigenetic integrity of the oocyte genome and its meiotic arrest are maintained actively by Snf2h-containing complexes, such as ChRAC and WSTF. Following ovulation, triggered by the luteinizing hormone (LH) surge, the oocyte undergoes a series of changes, including downregulation of Snf2h and de-repression of meiosis-promoting genes. The resumption of meiosis and differentiation into a mature secondary oocyte might be dependent on a switch to Snf2l-containing complexes, such as NURF. Reproduced with permission from Pépin et al (Pépin et al. 2007).

Table 1.1 Characteristics of mammalian ISWI-based chromatin remodeling complexes.

ATPase	Complex	Other subunits	Function	Drosophila ortholog	References
SNF2H	WCRF/hACF	WCRF180 (BAZ1A)	Chromatin assembly, pericentric heterochromatin maintenance	ACF	(Collins et al. 2002; Ito et al. 1997)
	hRSF	HBXAP	Chromatin assembly	N/A	(LeRoy et al. 1998)
	hCHRAC	WCRF180 CHRAC15 CHRAC17	Chromatin assembly, pericentric heterochromatin maintenance	CHRAC	(Zheng et al. 2004; Varga-Weisz et al. 1997)
	WICH	WSTF	Chromatin assembly, pericentric heterochromatin maintenance	N/A	(Bozhenok et al. 2002; Poot et al. 2004)
	NuRD/cohesin	Rad21 MI-2 RbAP46/48 HDAC1/2 MTA1/2/3 P66 MDB3	Cohesin loading onto alu elements	NuRD	(Hakimi et al. 2002)
	NoRC	TIP5	Repression of rDNA transcription	N/A	(Strohner et al. 2001; Li et al. 2005)
SNF2L	hNURF	BPTF RbAP46 RbAP48	Regulator of engrailed	NURF	(Tsukiyama et al. 1995; Lazzaro & Picketts 2001; Barak et al. 2003; Badenhorst et al. 2002)
	Not yet isolated	N/A	N/A	ISWI-TRF2	(Martianov et al. 2002)
	CERF	CECR2	Regulates neurulation	N/A	(Banting et al. 2005)
	Snf2l-PR	PR-A (NURF?)	Luteinization of granulosa cells?	NURF-EcR	(Lazzaro et al. 2006; Badenhorst et al. 2005)

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ChRAC, WICH, RSF, ACF and NURD/cohesin complexes whose functions generally gravitate towards nucleosome spacing during replication or chromosome condensation, whereas SNF2L is present in the NURF and CERF complexes which regulate specific gene expression during development (Pépin et al. 2007). Notable exceptions are the NoRC and B-WICH complexes which contain SNF2H and respectively either repress or promote rDNA transcription by changing the chromatin structure and interacting with RNA polymerase I (Percipalle & Farrants 2006).

1.4.1 NURF

The NURF complex was originally isolated in *D. melanogaster* embryo extracts by standard chromatography, and was shown to be able to remodel the HSP70 promoter in-vitro in an ATP-dependent manner (Tsukiyama & Wu 1995). Furthermore, it was proposed to be a transcription coactivator on the basis of its interaction with TRF2 and DREF and its ability to activate transcription in a subset of DREF-responsive promoters (Hochheimer et al. 2002).

The NURF complex is particularly interesting because its function in development has been well characterized in several model organisms. Mutants of the NURF subunit NURF301 in *D. melanogaster* have a peculiar phenotype where the expression of two homeotic genes ultrabithorax and engrailed is dysregulated (Badenhorst et al. 2002). Furthermore they have impaired transcription of heat shock proteins, puffy X chromosomes, and form melanotic tumours. The latter are caused by the transformation of larval blood cells, and are a direct consequence of the dysregulation of JAK/STAT signalling, suggesting NURF may antagonize that pathway (Badenhorst et al. 2002). Another informative phenotype of the NURF301 mutant is the failure in larval to pupal metamorphosis (Badenhorst et al. 2005). This block in the initiation of

metamorphosis is a direct consequence of the loss of ecdysone hormonal signalling, and as expected NURF directly interacts with the ecdysone receptor (Badenhorst et al. 2005). These findings suggest the NURF complex may be important for nuclear receptor-dependent transcription. Furthermore this hypothesis has been corroborated by evidence that ISWI, probably as part of NURF, also facilitates PR-dependent signalling on MMTV minichromosomes (Di Croce et al. 1999), and RAR transcription in reconstituted chromatin template in-vitro (Dilworth et al. 2000).

In *C. elegans* both ISWI and NURF mutations are synthetic enhancers of the multi-vulva phenotype, suggesting both proteins also function as determinants of cell fate in vulval cells by antagonizing RAS-like pathways and promoting RB-like differentiation and cell cycle exit (Andersen et al. 2006).

NURF was the first SNF2L-containing complex isolated in mammals. It was found to be enriched in brains where it may be potentiating neurite outgrowth and, mirroring previous observations in drosophila, also regulating engrailed expression (Barak et al. 2003). The *Bptf* KO, whose gene encodes the mouse ortholog of NURF301, is embryonic lethal at the post-implantation stage dpc 8.5 (Landry et al. 2008). Embryos implant but fail to undergo normal germ layer differentiation, and do not develop visceral endoderm. Furthermore, microarray experiments revealed dysregulation of many genes important for the establishment of the ectoderm, mesoderm and endoderm lineages, including homeobox transcriptions factors and TGF- β targets such as *Fgf8*, *Gsc* and *T*. This finding strongly suggests that NURF may play a role in facilitating SMAD transcription.

Taken together, these reports suggest a role for NURF during folliculogenesis, perhaps by facilitating nuclear receptor transcription, promoting RB-like pathways or interacting with TRF2-like core transcriptional machinery (fig 1.4).

1.4.2 CERF

CERF is the only other SNF2L-containing complex in mammals identified to date. It is comprised of SNF2L and CECR2, a transcription factor involved in neurulation (Banting et al. 2005). CECR2 is predominantly expressed in the neural ectoderm in embryos, and the inactivation of the gene by gene-trapping causes exencephaly (Banting et al. 2005). CECR2 was originally cloned after sequencing a region of the human chromosome 22 looking for potential causes for the cat eye syndrome (Footz et al. 2001). Cat eye syndrome is characterised by developmental defects of the eye, kidney, anus and heart, sometimes accompanied by mild mental retardation (Rosias et al. 2001).

1.5 Rationale and specific aims.

Chromatin remodelling proteins are emerging as important regulators of cellular proliferation and differentiation. Yet their role in reproduction remains largely unexplored. Recently, it was discovered that members of the ISWI family are expressed differentially in reproductive tissues. It is therefore of interest to characterize the role of both ISWI paralogs *Snf2b* and *Snf2l* in the ovary and determine if they have a function in folliculogenesis. We hypothesize that SNF2H complexes play a role in the proliferation of granulosa cells during the growth of secondary and antral follicles in response to FSH and estradiol. Furthermore we hypothesize that SNF2L complexes play a role in the differentiation of granulosa cells by specifically regulating the expression of genes important

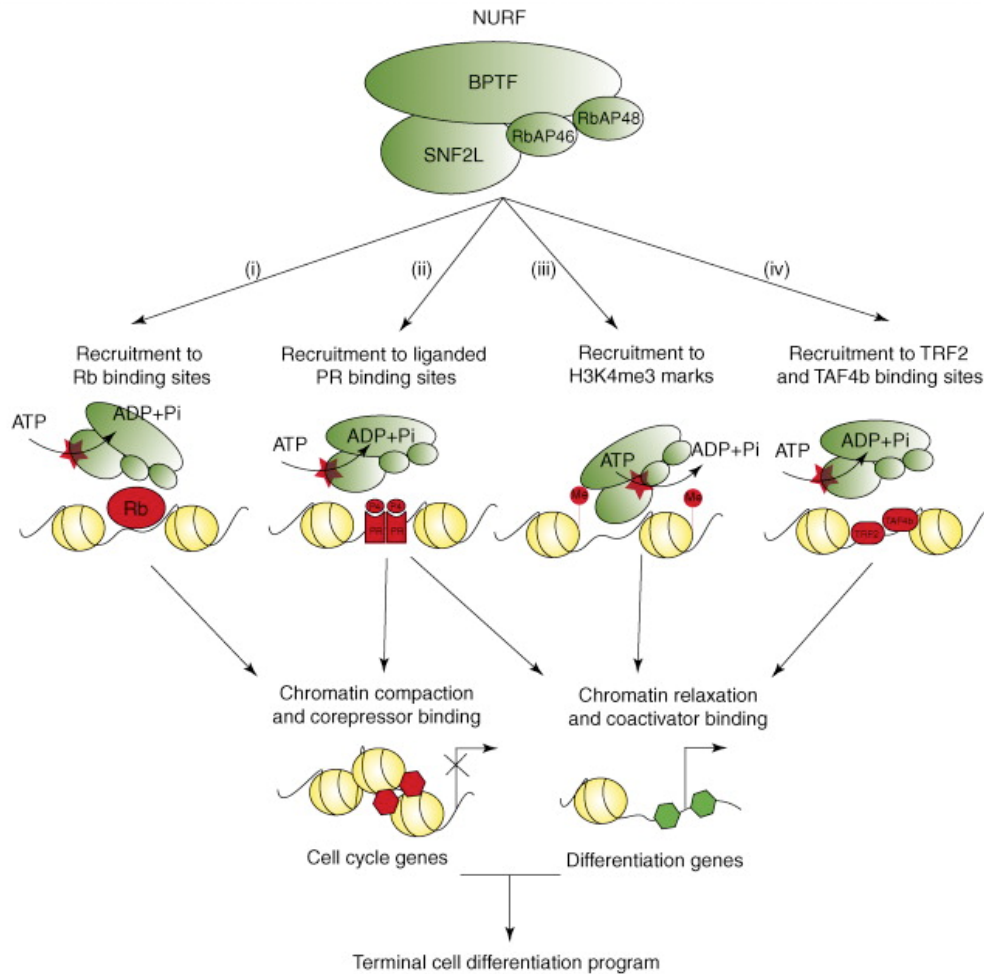


Figure 1.4 Representation of the potential mechanisms for NURF regulation of the terminal differentiation of follicular somatic cells.

NURF might regulate the differentiation program of granulosa cells by four different interactions with chromatin templates. These interactions act in concert to terminate the cell cycle and initiate terminal differentiation of granulosa cells through the facilitated binding of co-activators (green hexagons) or co-repressors (red hexagons). (i) NURF might interact with Rb downstream targets through its shared association with RbAP46/48 to antagonize the expression of cell cycle genes. (ii) NURF can be recruited by liganded progesterone receptors (PR) to activate or repress progesterone-responsive genes through a direct interaction with the LXXLL motif of Snf2L. (iii) NURF is recruited to transcriptionally active euchromatic islands by an interaction between the BPTF PHD fingers and histone H3 trimethylated at lysine 4 (H3K4me3), interaction represented as a red disk, enabling the epigenetically stable expression of luteal genes. (iv) NURF might facilitate TRF2- and/or TAF4b-dependent transcriptional initiation on the chromatin templates. In each case, ATP hydrolysis (red star) repositions the nucleosome, enabling the consequent transcription of differentiation genes to occur. Reproduced with permission from Pépin et al (Pépin et al. 2007).

for luteinisation in response to LH and progesterone. With the opportunity to gain insight into SNF2L's role in reproduction using newly-developed mice with a deletion of exon 6 which renders the protein inactive, we hypothesize that folliculogenesis will be impaired by the loss of SNF2L function. This thesis addresses the role of SNF2L in the ovary, and particularly its function in granulosa cells. To address these questions we have the following aims:

Aim 1 – To characterise the spatio-temporal expression of *Snf2b* and *Snf2l* in the ovary.

Aim 2 – To identify genes in granulosa cells whose expression is regulated by SNF2L.

Aim 3 – To characterize the reproductive phenotype of *Snf2l* KO mice.

Aim 4 – To identify SNF2L binding partners in granulosa cells.

The results from these experiments will provide valuable insight into the role of the ISWI chromatin remodelling proteins in regulating proliferation and differentiation using the well defined process of ovarian folliculogenesis. Further understanding of the role of chromatin remodelling factors in fertility could lead to a better comprehension of the causes of infertility in humans and may offer novel therapeutic targets.

CHAPTER 2: THE ISWI PROTEIN SNF2L REGULATES STEROIDOGENIC ACUTE
REGULATORY PROTEIN EXPRESSION DURING THE TERMINAL
DIFFERENTIATION OF OVARIAN GRANULOSA CELLS

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2.1 Abstract

Luteinization is a complex process, stimulated by gonadotropins, that promotes ovulation and development of the corpus luteum (CL) through terminal differentiation of granulosa cells. The pronounced expression of the mammalian ISWI genes, SNF2H and SNF2L in adult ovaries prompted us to investigate the role of these chromatin remodeling proteins during follicular development and luteinization. SNF2H expression is highest during growth of preovulatory follicles and becomes less prevalent during luteinization. In contrast, both SNF2L transcript and SNF2L protein levels are rapidly increased in granulosa cells of the mouse ovary 8 hour after human chorionic gonadotropin (hCG) treatment, and continue to be expressed 36 h later within the functional CL. We demonstrate a physical interaction between SNF2L and the progesterone receptor (PR)-A isoform, which regulates PR-responsive genes required for ovulation. Moreover, chromatin immunoprecipitation demonstrated that, following gonadotropin stimulation, SNF2L is associated with the proximal promoter of the steroidogenic acute regulatory protein (StAR) gene, a classic marker of luteinization in granulosa cells. Interaction of SNF2L with the StAR promoter is required for StAR expression, as siRNA knockdown of SNF2L prevents the activation of the StAR gene. Our results provide the first indication that ISWI chromatin remodeling proteins are responsive to the luteinizing hormone (LH) surge and that this response is required for the activation of the StAR gene and the overall development of a functional luteal cell.

2.2 Introduction

The terminal stage of development of the mammalian ovarian follicle occurs when it is transformed into the corpus luteum (CL). This complex process, known as luteinization, is essential to the success of early gestation, principally because it brings about the secretion of progesterone, thereby facilitating implantation and survival of the embryo. Luteinization is initiated by the preovulatory luteinizing hormone (LH) surge, and, in most species, comprises differentiation of ovarian granulosa and theca cells into their luteal counterparts (Murphy 2000). In recent years, many laboratories have sought to identify the genes that are induced by the LH surge as a means to identify markers and regulators of both ovulation and luteinization (reviewed in (Murphy 2000; Espey & Richards 2002; Robker et al. 2000b). For example, the progesterone receptor (PR) and the steroidogenic acute regulatory protein (StAR) represent genes involved in ovulation and luteinization, respectively. PR is a member of the nuclear receptor transcription factor superfamily, consisting of two isoforms, A and B, that are derived from the use of alternative promoters within the same gene (Kraus et al. 1993; Kastner et al. 1990; Evans 1988). Gonadotropin treatment results in a rapid increase in expression of both PR isoforms that is specific to granulosa cells (Shao et al. 2003). Generation of targeted mutation of PR, or the PR-A isoform alone, demonstrated that PR upregulation is essential for ovulation, while its absence does not interfere with the terminal differentiation of granulosa cells into a CL (Conneely & Lydon 2000; Mulac-Jericevic et al. 2000b; Lydon et al. 1995). On the other hand, StAR is essential for steroidogenesis, it first appears in granulosa cells following the gonadotropin signal that provokes ovulation (Espey & Richards 2002) and its expression peaks following terminal differentiation, when the CL is synthesizing substantial amounts of progesterone (Ronen-Fuhrmann et al. 1998;

Orly & Stocco 1999; Pescador et al. 1999). This expression pattern renders StAR an important marker of the luteinization process.

Despite advances in the identification of genes involved in the luteinization process, the precise mechanisms underlying their regulation remains poorly understood. Further, our understanding of epigenetic regulation of these genes during ovarian cell differentiation is confined to a few investigations of the modification of histone tails by phosphorylation and acetylation (Salvador et al. 2001) and consequent association with the StAR promoter (Christenson et al. 2001; Gevry et al. 2003; Hiroi et al. 2004a; Hiroi et al. 2004b).

Conformational and post-translational changes of chromatin are important mediators of differentiation as they promote the changes in expression (both activation and repression) of genes that characterize the differentiated phenotype. In the case of luteinization, the extensive tissue remodeling involves renewed expression of some genes, particularly those associated with steroidogenesis, and silencing of others, specifically, those related to the cell cycle (Murphy 2003). In other cell models, it has been shown that the mobilization of nucleosomes is catalyzed by the superfamily of ATP-dependent chromatin remodeling complexes, multi-protein machines that use the energy from ATP hydrolysis to mobilize nucleosomes to bring about regulation of specific genes (Lusser & Kadonaga 2003). These complexes are diverse, both in composition and in function, with the common feature being the presence of a SNF2 (Sucrose Non-Fermenting 2 gene) domain within one subunit (Eisen et al. 1995). The SNF2 domains fall within three categories, the SWI2/SNF2 family, the Imitation SWI (ISWI) family, and the Mi-2 family that is distinguished by additional chromatin motifs (Lusser & Kadonaga 2003).

The ISWI protein was originally identified in *Drosophila* and was shown to participate in three distinct complexes, ACF (ATP-utilizing chromatin assembly and remodeling factor), CHRAC (chromatin-accessibility complex), and NURF (nucleosome remodeling factor) (Tsukiyama & Wu 1995; Ito et al. 1997; Varga-Weisz et al. 1997; Tsukiyama et al. 1995). Both ACF and CHRAC function to assemble and spatially distribute nucleosomes, whereas NURF was shown to be involved in the specific regulation of target genes (reviewed in (Corona & Tamkun 2004)). There are two mammalian ISWI homologs, SNF2H and SNF2L (Okabe et al. 1992; Lazzaro & Picketts 2001; Aihara et al. 1998). SNF2H was found to be prominent in the mammalian equivalents of the ACF and CHRAC complexes and is believed to play a role in nucleosome assembly (Poot et al. 2000; Bochar et al. 2000). In contrast, the SNF2L protein is a component of a mammalian NURF complex that is prevalent in the brain where it promotes the *in vitro* terminal differentiation of neurons (Barak et al. 2003).

Our recent investigation of the murine orthologues, *SNF2H*¹ and *SNF2L* demonstrated expression in a number of tissues in a pattern suggestive of a role for SNF2H in proliferating cell populations and SNF2L in the regulation or maintenance of a differentiated phenotype (Lazzaro & Picketts 2001). Both genes were highly expressed in the adult mouse ovary and transcripts for both were abundant in the granulosa cells of preovulatory follicles. The *SNF2L* signal increased markedly in the developing corpus luteum at a time when *SNF2H* was reduced. Given the remarkable distribution of these gene products in ovarian tissue, we were interested in their relation to the processes of ovulation and its sequel, the formation of the CL. Here we

¹ For simplicity we use upper case text to refer to the mammalian ISWI genes and proteins, even in instances where we refer to the mouse, rat, and porcine orthologues.

demonstrate that human chorionic gonadotropin (hCG; a LH analog) induction of ovulatory changes provokes SNF2L expression and that the SNF2L protein physically interacts with the PR and with the StAR promoter, suggesting that chromatin remodeling initiated by the mammalian ISWI proteins contributes to both ovulation and luteinization.

2.3 Results

Expression of *SNF2L* in the mouse ovary

To explore the differential expression pattern of the two ISWI homologs in the ovary, we examined follicular development from juvenile mice at postnatal days 12, 14 and 16 using *in situ* hybridization. At this time mice are prepubertal and ovarian follicles are undergoing growth to the pre-antral and antral stages. During this period of development, *SNF2L* expression was low and constant in granulosa, theca and interstitial cells throughout the ovary (Fig. 2.1A). Although expression of *SNF2H* appeared to be ubiquitous at postnatal day 12, by days 14 and 16, the localization of *SNF2H* mRNA became more pronounced and restricted to the highly proliferative granulosa cells of developing follicles (Fig. 2.1A). The intensity of the signal was greater in the smaller, rapidly proliferating, pre-antral follicles than in the larger antral follicles indicating that antral follicles, while expressing *SNF2H*, have relatively fewer proliferating granulosa cells. However, given that *in situ* hybridization is not easily quantifiable, confirmation of such an observation would require other methods. Sense probes for both *SNF2H* and *SNF2L* were used as controls and showed very little background hybridization (Fig. 2.1A and data not shown). This suggests that *SNF2H*, but not *SNF2L* expression may be required in the response of granulosa cells to signals that promote follicle cell proliferation and follicle growth.

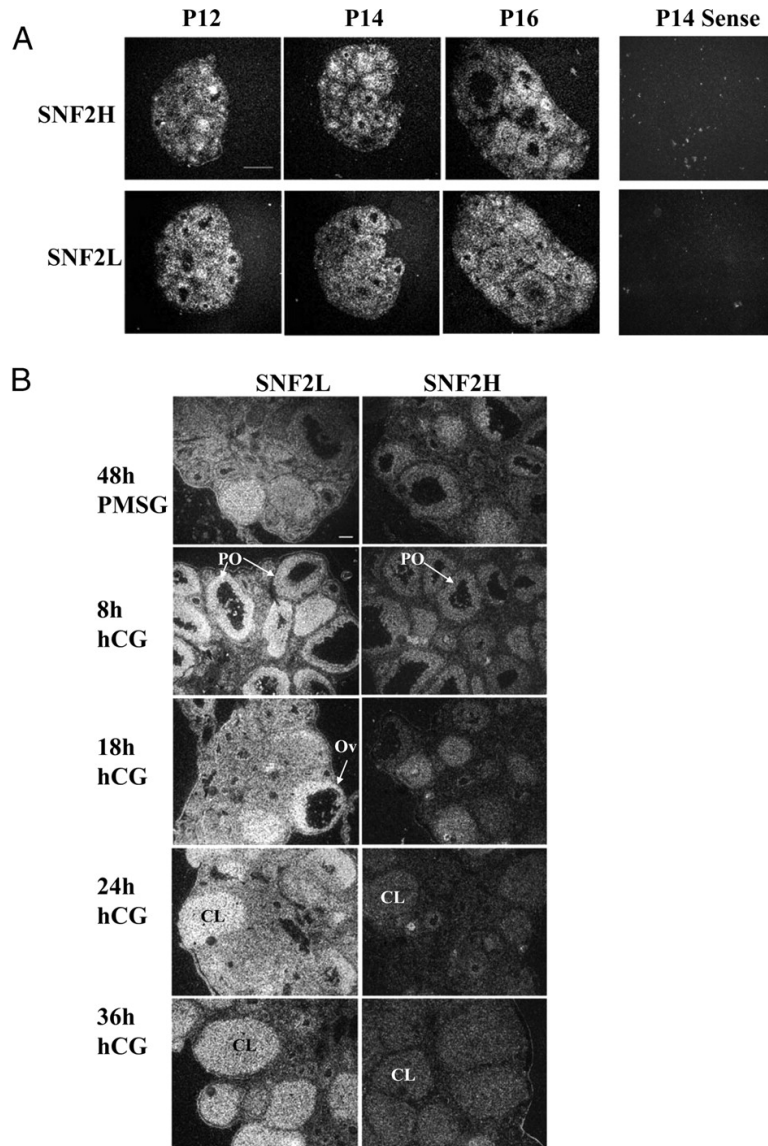


Figure 2.1 In situ hybridization analysis of SNF2L and SNF2H during ovary development and following gonadotropin stimulation. (A) Mouse ovaries were harvested from juvenile mice during the period of follicular development at postnatal days 12, 14, and 16. Bright field images of ovary sections hybridized to SNF2H (top) or SNF2L (bottom) are presented. Sense probes for SNF2H and SNF2L were used as negative controls and showed low background levels in bright field images at all developmental time points as depicted by the P14 image in the far right panel. (B) Ovary sections were analyzed following 48h treatment with PMSG to stimulate synchronous follicle growth to the antral stage, or 8, 18, 24, or 36 h after gonadotropin treatment to induce ovulation and luteinization. Hybridization to SNF2L probes are shown on the left, while adjacent sections hybridized to SNF2H are on the right. Sense probes gave similar results to those shown in panel A (data not shown). PO, preovulatory follicle; OV, ovulatory follicle; CL, corpus luteum.

Our previous analysis of adult mouse ovaries demonstrated a high level of *SNF2L* mRNA in the CL and concomitantly, association with the granulosa-derived luteal cells, supporting a role for *SNF2L* protein in luteal differentiation (Lazzaro & Picketts 2001). We therefore examined the expression of *SNF2L* mRNA in mouse ovaries prior to, and following induction of ovulation (Fig. 1B). In ovaries from mice treated with PMSG for 48 h to induce synchronous follicular growth to the antral stage, there was little evidence of *SNF2L* transcripts in the granulosa cells of preovulatory follicles (Fig. 2.1B). However, treatment with hCG to induce ovulation, resulted in a dramatic increase in *SNF2L* RNA by 8 h after administration, such that all preovulatory follicles displayed high levels of the signal (Fig. 2.1B). Examination of ovaries taken at 8 h after hCG by Northern blot confirmed the increased expression of the *SNF2L* transcript (Fig. 2.2A). The quantitative increase of approximately 1.5-fold (normalized to actin) was less robust than the apparent increase in preovulatory follicles (Fig. 2.1B), due to the use of whole ovaries in the Northern analysis. By 18 h after hCG treatment, which corresponds to approximately 6 h after ovulation, expression of *SNF2L* remained high in the differentiating granulosa cells of ovulated follicles and the incipient CL (Fig. 2.1B). The strong signal in the CL persisted through at least 24 and 36 h after hCG treatment (Fig 2.1B).

SNF2H showed a very different pattern of response to the ovulatory stimulus. Consistent with our developmental analysis, *SNF2H* was more strongly expressed in the granulosa layer of antral follicles at 48 h after PMSG treatment. There was no apparent change in the expression of *SNF2H* mRNA in preovulatory follicles 8 h after hCG injection (Fig. 2.1B), however Northern blots indicated that there was a reduction of approximately 1.5-fold in whole ovaries (Fig. 2.2A). At 6 h following ovulation (18 h after hCG), expression of *SNF2H* remained prominent in the developing CL, while it underwent a clear decline at 24 and 36 h (Fig 2.1B).

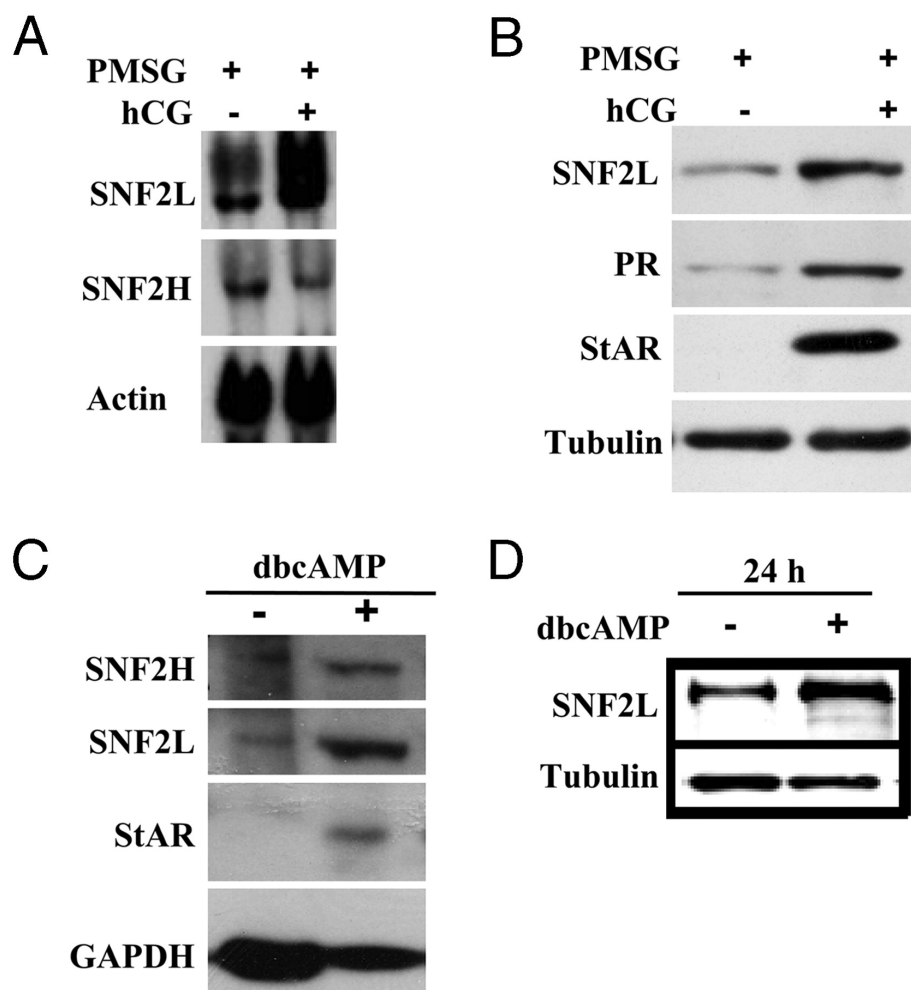


Figure 2.2 SNF2L expression increases upon luteinization.(A) Northern blot analysis of RNA isolated from mouse ovaries stimulated to promote follicular growth or after an 8 h hCG treatment to induce luteinization. SNF2L but not SNF2H expression is enhanced by hCG treatment. (B) Protein extracts generated from granulosa cells harvested from mouse ovaries. SNF2L, PR and StAR expression increased following hCG treatment compared to tubulin. (C) Analysis of protein expression in primary granulosa cells isolated from rat showed induction of StAR and SNF2L protein levels after treatment with 1mM dbcAMP for 3 h. (D) Similar analysis of porcine granulosa cells stimulated to undergo luteinization with dbcAMP showed a marked increase in SNF2L expression after 24 h.

Treatment with hCG induces SNF2L protein levels

To determine whether the SNF2L protein was expressed in a pattern resembling its cognate mRNA in granulosa cells following hCG treatment, we generated antibodies that specifically detected SNF2L (Supplementary data). Western blot analysis using this antiserum revealed that there was a three-fold increase in the relative abundance of SNF2L protein in granulosa cells harvested from ovaries from PMSG treated mice at 8 h following the ovulatory stimulus (Fig. 2.2B). We also examined the abundance of StAR and PR, markers of granulosa cell differentiation in the rodent ovary (Espey & Richards 2002). Both showed concurrent increases in expression following gonadotropin treatment (Fig. 2.2B), indicating that the upregulation of the SNF2L protein occurred in cells that were committed to the differentiation program.

We then investigated whether *SNF2L* regulation relative to PR and StAR expression was recapitulated in the process of cell differentiation in primary cultures of rat or porcine granulosa cells, or a human ovarian granulosa cell line. In primary cultures of rat granulosa cells, addition of the cAMP analogue, dibutyryl cAMP (dbcAMP) induced a rapid (<3 hour) induction in StAR protein levels with a concomitant induction of SNF2L (Fig. 2.2C). This result is in agreement with observations of granulosa cells cultured from porcine ovaries (Fig. 2.2D) in which dbcAMP stimulation also caused a significant increase in SNF2L protein levels.

To assess whether cells expressing increased levels of SNF2L protein were also the cells that expressed StAR, we examined the primary rat granulosa cells for StAR and SNF2L protein expression by co-immunofluorescence. SNF2L protein was present in the nucleus in freshly isolated cells in a relatively uniform pattern with some cells containing nuclear speckles whereas StAR protein was not detectable, as expected (Fig. 2.3A). Upon FSH and hCG stimulation for 24 h, SNF2L protein expression in the nucleus became more prominent, with a distinctive and

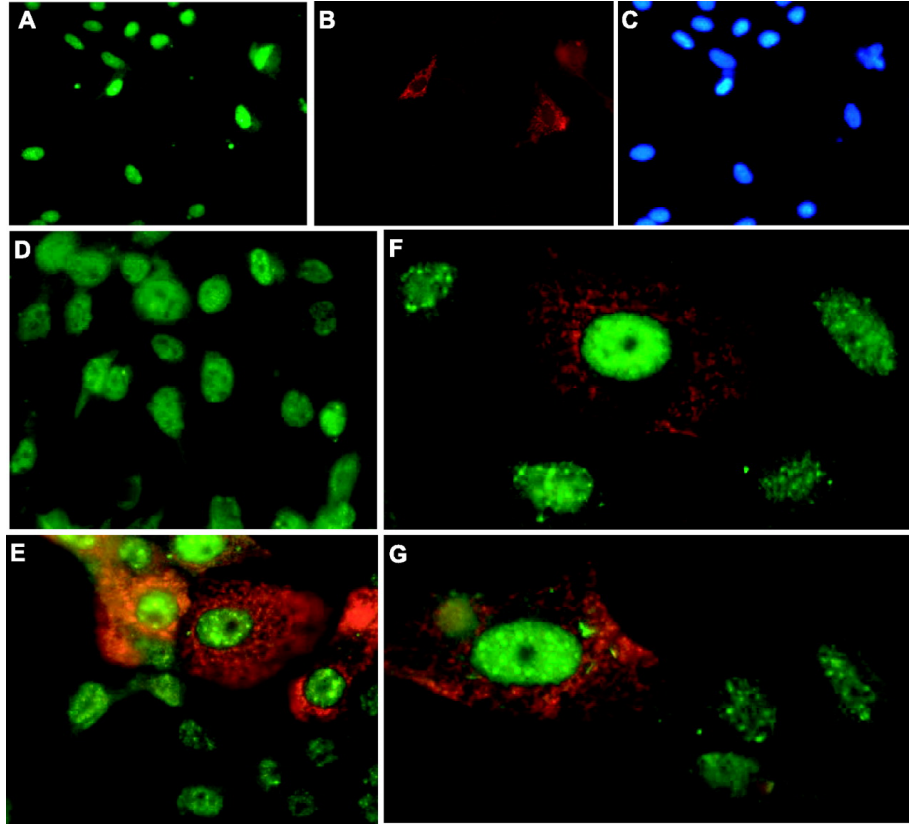


Figure 2.3 SNF2L and StAR are co-expressed in rat granulosa cells undergoing luteinization. Primary rat granulosa cells grown on coverslips were stained with SNF2L and StAR antibodies either before (A) or after 24 h treatment with FSH and hCG (B-D). Following hormone treatment SNF2L staining (green) becomes more intense in the nucleus, forming conspicuous speckles. The cells with the most intense SNF2L signal are also the cells positive for StAR protein (red). All images were taken with a 100 x objective with panels C and D were increased a further 2-fold using a digital zoom.

intense punctate pattern in most cells, conspicuously in cells that also expressed the StAR protein (Fig. 2.3B-D). These findings are consistent with the idea that SNF2L protein regulates StAR gene expression.

Finally, *SNF2L* expression was examined in SVOG-4o cells, derived from human granulosa cells immortalized with the SV40 early genes. Treatment with AMP analogs has been shown to promote differentiation and progesterone synthesis in this line (Lie et al. 1996). A low level of StAR could be detected in untreated cells, which was followed by an approximately ten-fold induction of StAR after 24 h of dbcAMP treatment (Fig. 2.4A). This increase was accompanied by accumulation of progesterone in the media (data not shown), suggesting that the cells had attained luteal potential *in vitro*. Moreover, a slight increase in SNF2L protein was evident by 3 h and SNF2L protein levels continued to increase up to 24 h (Fig. 2.4A). Taken together, the data gathered using granulosa cells derived from several species indicate that *SNF2L* expression is induced by gonadotropin stimulation and during granulosa cell differentiation.

SNF2L physically interacts with PR

We observed a rapid increase in expression of *SNF2L* following gonadotropin stimulation and continued high levels of *SNF2L* transcripts within CL. These two observations suggest that SNF2L protein function may be important for transcriptional regulation of both ovulation and terminal differentiation of granulosa cells into a CL. It is well known that PR upregulation is essential for ovulation, while its absence does not interfere with the terminal differentiation of granulosa cells into a CL (Conneely & Lydon 2000; Mulac-Jericevic et al. 2000b; Lydon et al. 1995). Moreover, the SNF2L protein contains several LXXLL nuclear receptor binding motifs

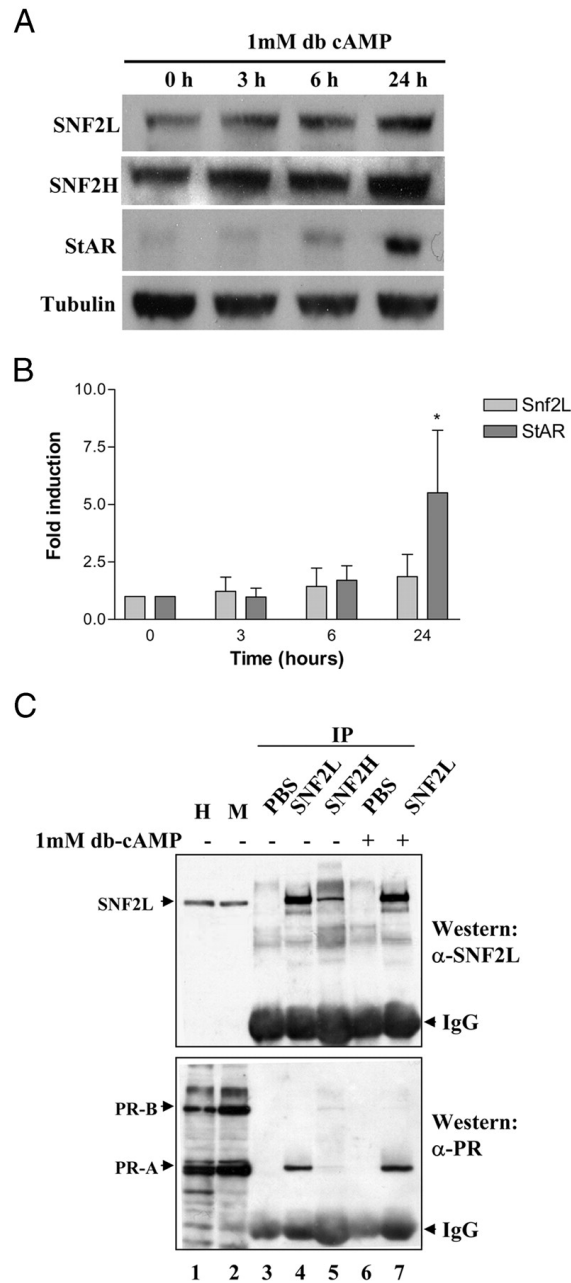


Figure 2.4 SNF2L interacts with PR-A in SVOG-4o human granulosa cell line. (A) Human granulosa cells, SVOG-4o, were treated with dbcAMP for 0, 3, 6, or 24 h and then analyzed for protein expression of SNF2L, SNF2H, and StAR in comparison to tubulin. StAR expression was rapidly increased by 24 h, whereas a modest increase in SNF2L was apparent by 24 h. (B) Granulosa cells were cultured for 24 h +/- treatment with dbcAMP prior to immunoprecipitation with anti-SNF2L (lanes 4 and 7), anti-SNF2H antibodies (lanes 5), or pre-bleed serum (PBS; lanes 3 and 6). Following IP, the proteins were analyzed by Western blot for either SNF2L (upper panel) or PR (lower panel). H and M correspond to control protein extracts from human or mouse granulosa cells. Bands corresponding to SNF2L, PR-B, PR-A, or IgG are identified by arrowheads.

that could mediate such an interaction. We therefore investigated whether SNF2L may interact with PR in granulosa cells by immunoprecipitation of protein extracts from SVOG-4o cells with antisera specific for SNF2L, SNF2H, or pre-immune serum. Consequent immunoblotting demonstrated that PR-A co-immunoprecipitated with SNF2L in both control SVOG-4o cultures and in cells that were treated with dbcAMP to induce differentiation (Fig. 2.4B, lanes 4 and 7). No significant amount of PR-A nor PR-B was detected when the pre-bleed serum or the SNF2H antibody was used for immunoprecipitation (Fig. 2.4B, lanes 3, 5 and 6). These results indicate that, in human granulosa cells, there is an interaction between SNF2L and PR-A that occurs in both differentiated and undifferentiated granulosa cells. It remains to be determined whether this interaction promotes gene repression or activation at specific target loci in response to hormone treatment. Moreover, whether such an interaction is necessary for ovulation must await the generation of SNF2L knockout mice.

SNF2L regulates expression of StAR

Since our previous studies have suggested that SNF2L protein plays an important role in terminal differentiation of neurons, we next asked whether SNF2L protein regulated terminal differentiation of granulosa cells. The best characterized gene of a terminally differentiated granulosa cell is that encoding StAR in which there is pronounced upregulation as luteinization ensues. The promoter and regulatory elements of this gene have been well documented rendering it a good candidate for chromatin immunoprecipitation (ChIP) assay for involvement of chromatin remodeling directed by the SNF2L protein.

SNF2L ChIP assays were performed using primary cultures of pig granulosa cells in order to obtain sufficient quantities of cell extracts. Extracts were immunoprecipitated with the SNF2L

antibody or with sheep IgG as a negative control. Following immunoprecipitation, either a 400 bp region of the StAR proximal promoter or a 500 bp fragment within the StAR ORF was amplified by PCR. The results of these experiments (Fig. 2.5A) show that SNF2L specifically associates with the proximal promoter of the StAR gene but not at a region further downstream corresponding to a coding sequence. Moreover, binding of SNF2L to the promoter was increased in cells after treatment with cAMP for 24 h (Fig. 2.5A). The increased occupancy of SNF2L on the StAR promoter suggests that ISWI chromatin remodeling is a step in the activation of the StAR gene by the cAMP/protein kinase pathway.

To determine if SNF2L interaction with the StAR promoter is required for activation of the StAR gene we used siRNA to knock-down SNF2L expression prior to treatment with dbcAMP. SVOG-4o cells treated with siRNA to SNF2L showed a significant decrease (~85-95%) in SNF2L protein (Fig. 2.5B, compare lanes 1 to 2) that was specific since siRNA GFP oligonucleotides had no effect on either SNF2L or StAR expression. Treatment with dbcAMP for 3 and 6 h resulted in a modest 2-fold increase in StAR protein levels (Fig. 2.5B, compare lanes 1 to 3 and 5). However, StAR protein expression was compromised when induced with dbcAMP in the presence of SNF2L siRNA oligonucleotides (Fig. 2.5B, compare lanes 3 to 4 and lanes 5 to 6). Taken together, these results suggest that gonadotropin stimulation increases binding of SNF2L at the StAR promoter and that this interaction is required for proper activation of this gene, most likely for nucleosome remodeling towards a chromatin environment conducive to transcription.

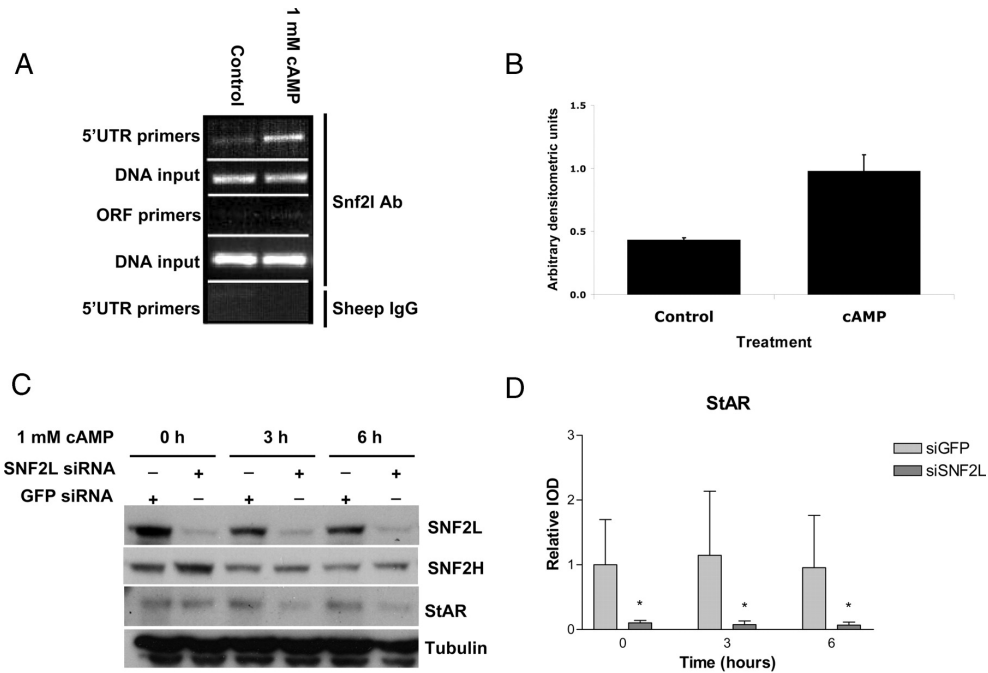


Figure 2.5 SNF2L regulates StAR gene expression. (A) Primary porcine granulosa cells either untreated or treated with cAMP were used for chromatin immunoprecipitation (ChIP) assay using a sheep anti-SNF2L antibody or sheep IgG. After ChIP a 400 bp region of the StAR proximal promoter was substantially elevated from the cells pre-treated with cAMP relative to control. (B) SVOG-4o cells were transfected with siRNA oligonucleotides specific to SNF2L or GFP (negative control) for 48 h and then stimulated to luteinize with dbcAMP for 0, 3, or 6 h. Proteins were isolated for Western blot analysis with antibodies to SNF2L, SNF2H, StAR, or the control protein tubulin. SNF2L expression was dramatically reduced in the presence of siRNA oligonucleotides. StAR protein expression was upregulated in an SNF2L-dependent manner.

2.4 Discussion

The gonadotropin surge promotes maturation of preovulatory follicles through the subsequent transcription of a large number of genes that ultimately provoke ovulation and initiate luteinization of granulosa cells into the corpus luteum (Murphy 2000). Although many different signaling pathways are invoked, all most certainly impinge upon the chromatin structure of the specific target genes that are activated during these processes. Here, we provide the first evidence for an *in vivo* and *in vitro* role for the ISWI chromatin remodeling protein SNF2L in this process and, more specifically, in the regulation of StAR gene expression, a well-defined marker of luteinization (Murphy 2000; Espey & Richards 2002). The sum of observations from multiple models indicates that SNF2L expression is induced by the LH surge, acting through cAMP. In consequence, there is a rapid association of SNF2L with the StAR promoter. In support of this view are the observations that reducing SNF2L protein levels by siRNA prevented the induction of StAR expression, demonstrating a requirement for chromatin remodeling to activate this target gene.

This study was prompted by our earlier findings that *SNF2L* transcripts were prominent in the CL (Lazzaro & Picketts 2001). By means of a more thorough spatial and temporal expression study we have now extended this work by demonstrating that both SNF2L protein and RNA expression increased during follicular maturation, ovulation, and development of the mouse CL. This change was rapid, occurring ~8 h after hormone treatment, timing that concurs with terminal differentiation (Richards 1994). Moreover, preliminary microarray studies monitoring rapid changes in gene expression during porcine luteinization have similarly shown an early increase in SNF2L expression (John Gadsby, personal communication). In contrast, SNF2H levels were

highest during early stages of follicular growth. This finding provides support for the view that SNF2H and SNF2L may have diverged sufficiently to perform distinct ISWI functions (Lazzaro & Picketts 2001). SNF2H has been identified as a member of several protein complexes with remodeling activity and it appears to play an important role in DNA replication through highly condensed chromatin, nucleosome assembly and spacing, and in chromatin condensation (Corona & Tamkun 2004). Moreover, *SNF2H* knockout mice die during the peri-implantation stage of embryogenesis due to an inability of early blastocyst-derived cells to proliferate (Stopka & Skoultschi 2003). Our observations are consistent with a role for the SNF2H protein in regulating proliferation as *SNF2H* transcripts were most prevalent in the granulosa cells of small pre-antral follicles, which have recently been shown to be actively proliferating upon staining with phosphohistone H3 antibodies (Ruiz-Cortes et al. 2005).

In contrast, *SNF2L* maintains a distinct expression pattern from *SNF2H* throughout mouse development and has been suggested to be an important regulator of transcription associated with cell differentiation and/or maturation (Lazzaro & Picketts 2001). Indeed, we have recently shown a role for the SNF2L-containing complex, NURF, in the regulation of engrailed genes during neuronal differentiation (Barak et al. 2003). In addition, ectopic expression of SNF2L in proliferating neuroblasts induces their differentiation (Barak et al. 2003). It may be that the ratio of SNF2H to SNF2L contributes to growth and differentiation of particular cell types, as we observed that SNF2H levels slightly increased in granulosa cells when SNF2L was reduced by siRNA knockdown (Fig. 2.5B).

Following *in vivo* hCG treatment, we observed a rapid increase (within 8 h) in SNF2L expression, suggesting that it is a regulatory target of the hormone signal. It is well known that the LH

receptor transduces early intracellular signals via G-protein mediated synthesis of cAMP and the subsequent activation of PKA (Richards 1994). PKA modulates transcriptional activity through the phosphorylation of transcription factors (Sassone-Corsi 1995); and histones (Salvador et al. 2001). Major targets include the cAMP response element binding protein (CREB) and the cAMP response element modulators (CREM;(Sassone-Corsi 1994)). Interestingly, the SNF2L proximal promoter contains two well-conserved CRE consensus sites, suggesting that cAMP mediated SNF2L activation may be induced by the preovulatory LH surge. Indeed, examination of SNF2L expression during differentiation of granulosa cells *in vitro* among a number of culture systems following 24 h of hormone treatment was sufficient to observe a consistent increase in protein levels in mouse, rat, and porcine primary cultures and a human Svog-4o cell line. Nonetheless, there were differences in the timing and the intensity of the response, suggesting that other signaling pathways may mediate the response. Alternatively, the differences may arise endogenously from the morphologic and temporal variation known to exist in the process of luteinization among species (Murphy 2000).

The ChIP assay demonstrated that SNF2L interacts with the region of the StAR proximal promoter that contains conserved consensus binding sites for a number of factors previously shown to transactivate the StAR gene. Among these can be found C/EBP β and SF-1, both of which have been shown to be important for StAR transcription in a number of species (Christenson et al. 1999; Sugawara et al. 1996; Reinhart et al. 1999). In addition, other studies have demonstrated a requirement for CBP, SP1, GATA-4, and CREB in the activation of the StAR gene (reviewed in (Hiroi et al. 2004a; Stocco et al. 2001)). Our results suggest that chromatin remodeling also plays an important role in the regulation of the StAR gene because, when SNF2L

expression is compromised by siRNA inactivation, StAR gene expression is attenuated. What role might SNF2L have at the StAR promoter? Studies using MA-10 Leydig cells have shown that, in response to dbcAMP treatment, there is a rapid increase in SF-1 and C/EBP β binding observed at the StAR promoter (Hiroi et al. 2004a; Hiroi et al. 2004b). Moreover, cAMP induces modifications of the N-terminal tail of H3, including rapid hyperacetylation at K9 and K14, dimethylation of K4 and loss of K9 dimethylation (Christenson et al. 2001; Hiroi et al. 2004b). As depicted in Figure 2.6, the LH surge may activate SNF2L through PKA, thereby inducing one or more of several sequelae. The SNF2L protein may interact with CBP or CREB to facilitate H3 modifications through mobilization of nucleosomes. The protein may also use its chromatin remodeling function to enhance binding of transcription factors (eg. C/EBP, CREB and SF-1) that are essential transactivators of the StAR promoter. Further studies are required to delineate the precise function of SNF2L at this and other promoters in the ovarian context.

In addition to regulating the StAR promoter, SNF2L may also be involved in regulation of PR responsive genes through the direct physical interaction with PR-A. Indeed, SNF2L contains four putative LXXLL nuclear receptor binding motifs that could mediate such an interaction with PR-A. Other studies suggest functional significance of this interaction. In the mouse mammary tumor virus (MMTV) promoter, PR can interact with *Drosophila* NURF, specifically with the ISWI and NURF-38 subunits, to promote chromatin remodeling and to enhance binding of NF-1 in a model of synergistic activation (Di Croce et al. 1999). It may therefore be possible that SNF2L and PR-A may interact to activate or repress PR-responsive genes including those that promote ovulation (Conneely & Lydon 2000; Mulac-Jericevic et al. 2000b; Lydon et al. 1995). Whether SNF2L is crucial for both ovulation and/or CL formation must await the generation of mice

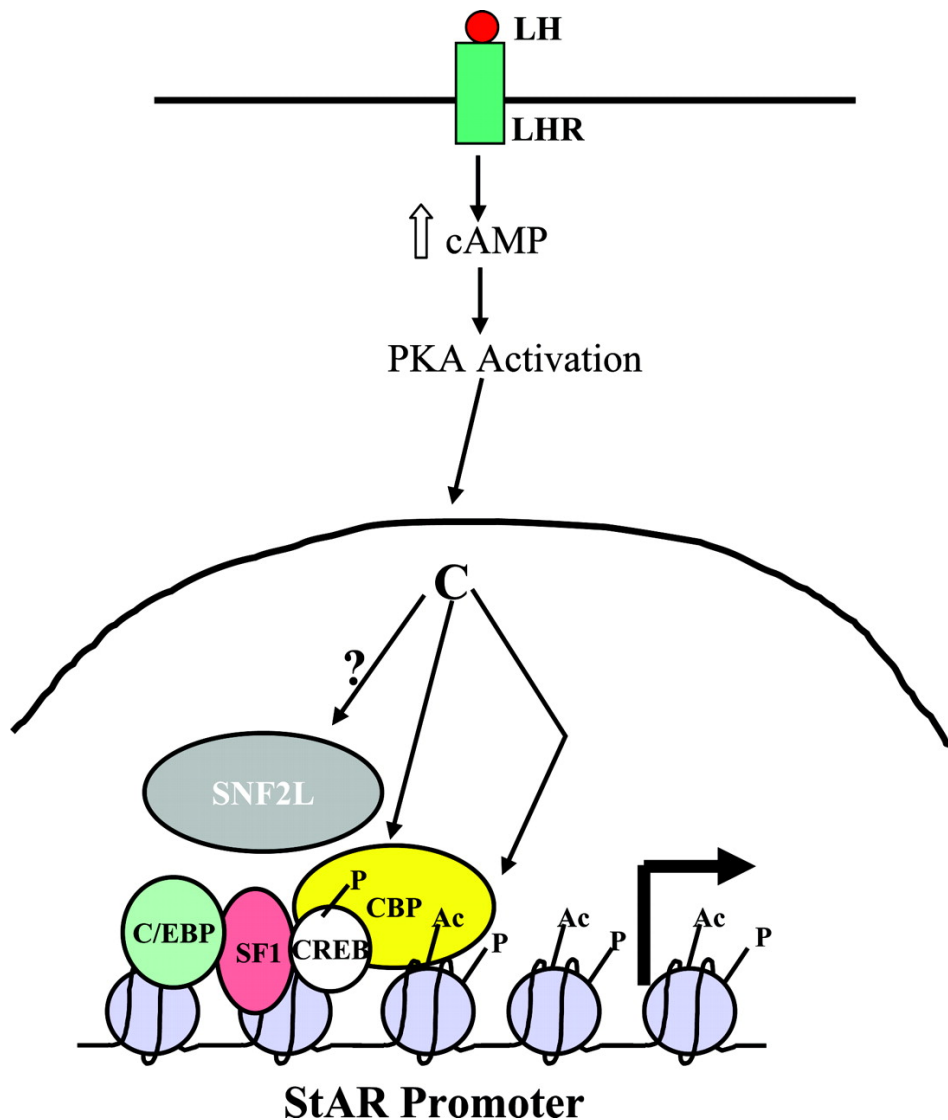


Figure 2.6 Proposed model of StAR activation by SNF2L. LH stimulates cAMP levels and subsequent activation of the catalytic subunit of PKA (C). The catalytic subunit of PKA promotes phosphorylation of histone H3 (P on nucleosomes) and CREB (denoted by P). It remains a possibility that SNF2L may also be activated by PKA phosphorylation (arrow with ?). Phosphorylated CREB recruits histone acetyltransferases, including CBP (and possibly P/CAF) and may also recruit SNF2L to the StAR promoter to facilitate histone acetylation and activation of StAR transcription. Promoter recruitment of SNF2L may also involve other transcription factors such as C/EBP and SF1. Alternatively, SNF2L may facilitate promoter occupation of these same transcription factors.

bearing cell-specific targeted mutation of the SNF2L gene, although work from *Drosophila* suggests that both ISWI and NURF are required early during ovary development (Badenhorst et al. 2002; Deuring et al. 2000).

In summary, our study provides the first evidence of regulation of ISWI expression and ISWI-dependent regulation of target genes necessary for ovarian function. Further, it relates the expression of SNF2L to the processes of terminal differentiation that represent the ultimate fate of the ovarian follicle.

2.5 Materials and Methods

Animals and Hormone Treatments

Female CD-1 mice (Charles River Laboratories) were injected intraperitoneally (ip) at 24-26 days of age with 5 IU pregnant mare serum gonadotropin (PMSG–Folligon; Intervet) to stimulate follicle growth. After 48 h, mice received a single ip injection of 5 IU hCG (Sigma) to induce ovulation and luteinization. Ovaries were dissected at selected times after hormone treatments and fixed for *in situ* hybridization analyses as described (Lazzaro & Picketts 2001), or subjected to extraction of RNA (Chomczynski & Sacchi 1987), or protein. Ovaries were also isolated from female mice at 12, 14 and 16 days after birth for *in situ* hybridization analyses. All animal studies were approved by the University of Ottawa Animal Care Committee, accredited by the Canadian Council on Animal Care.

Granulosa Cell Cultures

Immature female Sprague-Dawley rats were injected subcutaneously for 3 days, from 19-21 days of age, with 1 mg/day diethylstilbesterol (Sigma) to stimulate follicle growth. Granulosa cells

were harvested from dissected ovaries by follicle puncture using a 25-gauge needle and washed twice in DMEM/F12 culture medium containing antibiotics. Cells were plated at a density of 8×10^5 cells/ml on 60-mm plates in DMEM/F12 containing 2% FBS and antibiotics. Cells were incubated in a humidified 95% air/5% CO₂ incubator at 37°C. Once cells adhered to plates (~3-4 h) follicle stimulating hormone (FSH) (275 mIU/ml; Sigma) was added for 24-48 h, followed by treatment with 1 mM dbcAMP (Roche) or hCG (10 IU/ml; Sigma) for selected times.

The cell line SVOG-4o, derived from human ovarian granulosa cells immortalized with SV40 early genes, was the generous gift of Dr. N. Auersperg (University of British Columbia) and was cultured in MCDB105:199 medium with 10% fetal calf serum, 2mM glutamate, and 400 µg/ml hydroxycortisone and penicillin/streptomycin. SVOG-4o cells were induced to differentiate in the presence of 1mM cAMP (Roche).

For siRNA experiments, cells were grown to confluence then transfected with SNF2L-specific siRNA oligonucleotide as described previously (Barak et al. 2003) or a GFP siRNA oligonucleotide (Dharmacon; p-002102-01-20) using Oligofectamine (Invitrogen). After transfection cells were cultured in medium alone for 4 h and then supplemented with 10% fetal calf serum for 48 h prior to treatment with 1 mM cAMP for 0, 3, 6, or 24 h.

Porcine granulosa cells were aspirated from medium-sized (3-5 mm) follicles from prepubertal pig ovaries and cultured as previously described (Pescador et al. 1997). Cells were pooled ($6-8 \times 10^6$ cells/ml) in minimum essential medium (MEM; Invitrogen) containing 1 mg/L insulin (Sigma), 0.1 mM nonessential amino acids (Invitrogen), 5×10^4 IU/L penicillin (Invitrogen), 50 mg/L streptomycin (Invitrogen), 0.5 mg/L fungizone (Invitrogen) and 10% (vol/vol) fetal bovine serum (FBS; Invitrogen). Incubations were carried at 37°C in 95% humidified air with 5% CO₂. At initiation of culture, some cultures were treated with 1 µM 8-bromo-cAMP (Sigma), while control

cultures received medium alone. Cultures were terminated at intervals through 48 h for Western and for 24 h for chromatin immunoprecipitation (ChIP) analyses.

Protein Analysis

Protein extracts were prepared from freshly isolated mouse granulosa cells or from cultures of rat and human granulosa cells by resuspending washed cellular pellets in appropriate volumes of RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris pH 8) containing a protease inhibitor cocktail and 0.5 mM phenylmethylsulfonyl fluoride (PMSF, Sigma). Lysates were incubated for 30 minutes on ice and centrifuged for 10 minutes at 10,000 x g. Western blots were prepared from protein samples fractionated on SDS-PAGE gels and transferred to Immobilon-P membranes (Millipore). All blots were blocked with 5% milk in Tris-buffered saline with .05% Tween 20 (TBST). Commercially available primary antibodies included anti-StAR (1mg/ml; Affinity Bioreagents), anti-progesterone receptor (1:50; Novocastra Laboratories Ltd) and anti- β -tubulin (1:100; Developmental Studies Hybridoma Bank). Primary antibodies for SNF2L (25 ng/ml) and SNF2H (1:3000) were raised in sheep by Affinity Biologicals Inc. The antigens were purified GST fusion proteins to fragments corresponding to amino acids 1-82 of the SNF2LA isoform (Okabe et al. 1992) or amino acids 1- 237 of human SNF2H (Aihara et al. 1998). Secondary antibodies were horseradish peroxidase-conjugated anti-sheep IgG, anti-rabbit IgG, anti-mouse, and biotinylated anti-mouse IgG used with a streptavidin-horseradish peroxidase label. Proteins were detected on blots using chemiluminescence.

For immunoprecipitation, 500 mg of SVOG-4o human granulosa cell protein extract was combined with either pre-bleed serum, SNF2L or SNF2H antibodies and Protein G-Sepharose in RIPA buffer containing protease inhibitors and 0.5 mM PMSF, and mixed overnight at 4°C on a

rotating mixer. Antibody-protein complexes bound to Protein G-Sepharose beads were washed extensively in RIPA buffer with protease inhibitors at 4°C and eluted from the beads with the addition of SDS-PAGE sample loading buffer and heating. Samples were separated on SDS-PAGE gels and transferred to Immobilon P for Western blot analysis. Quantification of Western and Northern blots was performed using NIH Image (Version 1.63).

For immunofluorescence, coverslips containing rat granulosa cells were washed three times with cold phosphate buffered saline (PBS), then, incubated for 5 minutes on ice in a 3:1 ethanol:methanol solution, followed by four more washes with cold PBS. After fixation, cells on coverslips were blocked for 10 min in 2% bovine serum albumin (BSA) in PBS for 1 hour at room temperature then incubated with the anti-SNF2L (described above) or rabbit anti-StAR antibodies (1:100; gift of Dr. Douglas M. Stocco, Texas Tech) diluted in 2% BSA in PBS for 1 hour at room temperature. Cells were then washed with PBS three times followed by 1 hour incubation with an appropriate secondary antibody diluted at 1:1500 (anti-rabbit or sheep IgG alexa 488 or 594) with 2% BSA in PBS at room temperature in the dark. Cover slips were mounted on slides with Vectashield Mounting Medium for Fluorescence (Vector Laboratories Inc.). We examined slides using a Zeiss Axiophot photomicroscope using the 100X objective lens.

Chromatin immunoprecipitation (ChIP) assays

ChIP assay followed the method of Kuo and Allis (Kuo & Allis 1999), with minor modifications. DNA and cell proteins in granulosa cell cultures were cross-linked for 10 min at room temperature by addition of formaldehyde to a final concentration of 1%. Cells were washed and scraped in ice-cold phosphate-buffered saline (PBS) containing protease inhibitors (1 mM PMSF, 1 µg/ml aprotinin and 1 µg/ml pepstatin A; all reagents from Sigma), collected by centrifugation and

resuspended in 200 μ l of CHIP lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1 and protease inhibitors). Cells were incubated 10 min on ice and disrupted by sonication, and centrifuged (10 min, 20,000 x g at 4°C). The supernatant was then diluted 10 fold in CHIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris, pH 8.1, 167 mM NaCl and protease inhibitors, Sigma). An aliquot of 2 μ l of lysate was used for purification of total DNA. Each sample was precleared by incubating with 80 μ l salmon sperm DNA/protein A-agarose 50% gel slurry (Upstate Biotechnology Inc., Lake Placid, NY) for 60 min at 4°C to reduce nonspecific background. One sample (2 ml) was divided, and each 1 ml subsample incubated with 5 μ g of antibody and treated overnight at 4°C with agitation. The antibody used in this experiment was ovine anti-SNF2L. Control precipitation was performed with an equivalent dilution of sheep IgG (Upstate Biotechnology Inc.). Immunocomplexes were collected with 60 μ l of salmon sperm DNA/protein A-agarose for 2 h at 4°C with rotation and were washed once with each of the following buffers in sequence: low salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl); high salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl); LiCl wash buffer (0.25 M LiCl, 1% NP40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1); TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Immunocomplex elution was achieved by adding 250 μ l of elution buffer (1% SDS, 0.1 M NaHCO₃). The DNA-protein cross-linking was reversed by incubation at 65°C for 6 h followed by proteinase K treatment. DNA was recovered by purification with the Qiaquick PCR purification column (Qiagen). A 0.5 kb fragment from the proximal promoter region of the steroidogenic acute regulatory protein (StAR) was amplified by PCR in total total DNA and immunoprecipitated DNA. The sense primer employed was 5'-CCATCCCCTTGCACCACAAC-3' and antisense primer was 5'-

TTTCCTGGTAGCGGAGGCAGGCC-3'. PCR products were resolved on agarose gels and visualized by means of an Alpha-Imager gel documentation system.

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CHAPTER 3: THE ISWI PROTEIN SNF2L IS REQUIRED FOR
SUPEROVULATION AND REGULATES FGL2 IN DIFFERENTIATING
GRANULOSA CELLS.

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3.1 Abstract

ISWI proteins are catalytic subunits of chromatin remodeling complexes that alter nucleosome positioning by hydrolyzing ATP to regulate access to DNA. In mice there are two paralogs, Snf2h and Snf2l, which participate in different complexes and have contrasting patterns of expression. Here we investigate the role of Snf2l in ovaries by characterizing a mouse bearing a deletion of exon6 disrupting the ATPase domain. Snf2l knockout (KO) mice produce significantly fewer eggs than control mice when superovulated. Gonadotropin stimulation leads to a significant deficit in secondary follicles and an increase in abnormal antral follicles. KOs also fail to induce Fgl2 in response to hCG, while overexpression of Snf2l is sufficient to drive its expression in granulosa cells. Snf2l is also shown to directly interact with the Fgl2 promoter as demonstrated by chromatin immunoprecipitation, and with the nuclear receptor co-activator Fli-I as shown by IP. These results begin to establish a role for Snf2l in the precise coordination of gene expression in granulosa cells during folliculogenesis, and its broader implications in fertility.

Keywords: ISWI; Snf2l; Fgl2; Fli-I; folliculogenesis; superovulation; knockout.

3.2 Introduction

Regulation of chromatin structure underlies many critical cellular processes such as replication, silencing and transcription. This is achieved via various epigenetic means such as DNA methylation, histone post-translational modifications and nucleosome positioning. The latter is critical to expose an area of DNA, which would otherwise be inaccessible to

transcription factors. There exist three main types of chromatin remodeling complexes based on the identity of their ATP-dependent catalytic subunits: MI2, SWI/SNF and ISWI (Narlikar et al. 2002). The best-characterized family is the SWI/SNF which is conserved across eukaryotes, and represented by two orthologs in mammals: *Brg1* and *Brm*. While they participate in similar complexes, *Brg1* is an essential gene while *Brm* is not. *Brg1* Knockouts (KOs) die at the peri-implantation stage (Bultman et al. 2000) while *Brm* KOs are viable albeit 15% heavier than control littermates (Reyes et al. 1998). Similarly, the ISWI family is conserved in eukaryotes and represented by two orthologs in mammals: *Snf2h* and *Snf2l* (Lazzaro & Picketts 2001). *Snf2h* KOs embryos also die during the peri-implantation stage, demonstrating that *Snf2h* is required for the proliferation of both the inner cell mass and the trophoctoderm (Stopka & Skoultschi 2003). Contrastingly, SNF2L expression appears to be restricted to differentiated cell types and adults tissues, particularly the brain and reproductive organs (Lazzaro & Picketts 2001). In the brain, SNF2L regulates the expression of Engrailed 1 and 2 and promotes neurite outgrowth as part of the NURF complex (Barak et al. 2003). In the ovary SNF2L is expressed in granulosa cells and its expression coincides with the maturation of follicles and their differentiation into luteal cells (Lazzaro et al. 2006). Folliculogenesis is a complex process that begins with the recruitment of a cohort of primordial follicles transitioning into primary follicles and undergoing both growth to become secondary follicles and tissue remodeling to form the characteristic antrum of pre-ovulatory follicles under the influence of the follicle-stimulating hormone (FSH) (reviewed in Edson et al. 2009). Antral follicles later ovulate in response to the luteinizing hormone (LH) surge and subsequently begin their differentiation into a corpus luteum (CL). The transition between each stage is a reductive process dependent on the integration of many hormonal signals and

the precise coordination of gene expression, which allows for only a few follicles from the initial growing pool to ovulate. This study examines the role played by SNF2L in this process by characterizing the reproductive phenotype of a *Snf2l* KO mouse.

3.3 Materials and methods

Transgenic mouse lines

The *Snf2l* KO transgenic mouse line and the appropriate littermate WT control line were generously provided by David J. Picketts (Ottawa Hospital Research Institute, Ottawa, Ontario, Canada). Briefly, the mice are a hybrid strain resulting from the cross between the ubiquitously expressing *Gata-1 Cre* transgenic line in a CD-1 background provided by Dr. S. Orkin (Howard Hughes Medical Institute, Chevy Chase MD, USA) and the conditional KO line Ex6DEL *Snf2l* developed by Dr. D.J. Picketts in a 129Sv background. The Ex6DEL *Snf2l* line was generated using a linear fragment of genomic DNA spanning Exon 6 of *Snf2L* flanked with LoxP sites which was introduced by electroporation into J1ES cells. Positive recombinant clones were injected into female 129Sv blastocysts to produce chimeric animals, which were subsequently bred to homozygosity. All animal studies were approved by the University of Ottawa Animal Care Committee, accredited by the Canadian Council on Animal Care.

In-vivo hormonal treatments

Female mice were injected ip at 24–26 d of age with 5 IU PMSG (Folligon; Intervet, Boxmeer, The Netherlands) to stimulate follicle growth. After 48 h, the mice received a single ip injection of 5 IU hCG (Sigma-Aldrich, St. Louis, MO) to induce ovulation and

luteinization. Alternatively, females of 22-25 d of age were injected sc with 1 mg/d of diethylstilbesterol (DES) (Sigma, St. Louis, MO) for 3 days to stimulate follicle growth. Ovaries were dissected at selected times after hormone treatments and fixed for histology or subjected to extraction of granulosa cells.

Cell culture

Granulosa cells from *Snf2l*/WT and KO mice were mechanically isolated from ovaries of mice treated with either PMSG for 48h or with diethylstilbesterol for 3d as indicated. Briefly granulosa cells are harvested by puncturing individual follicles using a 25G needle and applying light pressure with tweezers. Granulosa cells are grown at a density of 1×10^6 cells/ml on 60-mm plates in DMEM/F12 containing 2% fetal bovine serum, 10nM E2 and antibiotics.

The SIGC cell line, derived from rat ovarian granulosa cells spontaneously immortalized (Stein et al. 1991), was the generous gift of Dr. J.J. Peluso (University of Connecticut Health Center, Farmington, USA) and was cultured in DMEM:F12 containing 5% serum and antibiotics. Cells were incubated in a humidified 95% air/5% CO₂ incubator at 37 C.

Plasmids and transfections

SIGC cells were stably transfected with a previously described (Barak et al. 2003) pcDNA3 vector containing a Flag-tagged *Snf2l*, or a pEGFP N1 (Clontech, Palo Alto, CA, USA) control plasmid using Lipofectamine Reagent (Invitrogen, Rockville, MD, USA) according to manufacturer's instruction and selected with geneticin (400µg/ml).

Tissue preparation and histological analysis.

Ovaries and blood samples were collected at various timepoints from hormonally-treated mice at necropsy. Plasma samples were sent to Ligand Assay & Analysis Core Laboratory (Chalottesville, VA, USA), which performed both 17β -estradiol (E2) and progesterone (P4) radioimmunoassays (RIAs). Ovaries were fixed in 10% neutral-buffered formalin overnight before being transferred to 70% ethanol, and embedded into paraffin. For histological analyses, entire ovaries were sectioned throughout using 5 μ m thick sections, staining one out of every 10 sections with hematoxylin and eosin. Tissue sections were scanned using the Aperio ScanScope, and images were captured using the Aperio ImageScope program (Aperio Technologies, Inc., Vista, CA). For follicle counts, the methodology of Pederson and Peters was employed (Pedersen & Peters 1968). Briefly, follicles are categorized as primordial if a partial or complete layer of squamous granulosa cells surrounds the oocyte. Follicles are classified as primary if a single layer of cuboidal granulosa cells surrounds the oocyte, and secondary if there are two or more layers granulosa cells with no visible antrum. The antral follicle category included all follicles containing an antrum, from early antral follicles to large pre-ovulatory follicles. Follicles in each section were counted only if the nucleus of the oocyte was visible. The total amount was estimated by multiplying the total number of follicles by 10. Abnormal follicle structures were also scored, even if the oocyte was degenerated, and were represented as occurrence/slide. The experiments include the ovaries of at least 3 independently treated mice.

Oocyte counts

To determine the superovulation yield, ova were collected from females induced by PMSG and hCG as described above. 16-18 h after treatment with hCG, oviducts were dissected and placed into DMEM medium and emptied of their contents. To separate the ova from the cumulus cells, 0.3mg/ml hyaluronidase (Sigma-Aldrich) was added to the media. After 5-10 min incubation, the ova were separated from the cumulus cells by gentle pipetting and counted. The experiment was repeated 4 times with 3 animals per group.

Assessment of fertility

To determine litter size and frequency, *Snf2l* KO females were joined with *Snf2l* KO males and compared to pairs of littermate control *Snf2L* WT female mated with *Snf2L* WT males. Each experiment compared 4 breeding pairs of each genotype. Each breeding pair consisted of mice of 6 weeks of age, which were kept together for the duration of the experiment (5 months). Pups were weaned before the birth of the next litter, and litter size, sex of the pups and dates of birth were recorded.

Western blotting

Granulosa cells retrieved from hormonally treated animals or from SIGC in culture were lysed in ProteoJET™ (Fermentas, Burlington, ON, Canada), containing protease inhibitor cocktail (Sigma- Aldrich) and 1mM PMSF. Lysates were run on precast precast NuPAGE 4-12% Bis-Tris gel (Invitrogen). The primary antibody used for SNF2L has been previously described (Lazzaro et al. 2006), while anti-FGL2 was used at 1:2500 (Santa Cruz Biotechnology, Santa-Cruz, CA, USA; sc-100276), and anti-FLI-I was used at 1:500 (Abcam, Cambridge, UK; ab28089).

Immunoprecipitation

Total protein were extracted from 1×10^7 SIGC-Snf2l or their parental line (SIGC control) using 1 ml of mammalian lysis buffer [50 mM Tris-HCl pH 7.6, 100 mM NaCl, 5 mM EDTA, 0.4% (v/v) NP-40, 10% (v/v) glycerol] containing protease inhibitor cocktail (Sigma-Aldrich) and 1 mM PMSF. Cells lysates were incubated overnight at 4°C with rotation with 50 µl of ANTI-FLAG M2 Affinity Gel 50% gel slurry (Sigma-Aldrich). The beads were washed 5 times with 1 ml of ice cold wash buffer [50 mM Tris-HCl pH 7.6, 100 mM NaCl, 10% (v/v) glycerol] followed by centrifugation at 500 x g for 2 min at 4°C. The immunocomplexes were eluted from the beads using 100 µl of 100 µg/ml of 3X FLAG peptide (Sigma-Aldrich, St. Louis, MO, USA) in Tris-buffered saline [50 mM Tris-HCl pH 7.5, 150 mM NaCl]. The immunoprecipitation procedure was done in duplicate.

Mass spectrometry identification

Samples were resolved on a NuPAGE 4-12% Bis-Tris gel (Invitrogen). The gel was fixed overnight in 50% (v/v) methanol followed and stained for 1h with Bio-Safe Coomassie (Bio-Rad, Hercules, CA, USA) and destained for 30 min in ddH₂O. The proteins found to be differentially immunoprecipitated between the SIGC-Snf2l and the SIGC control were manually excised from the gels and sent to a mass spectrometry facility for further processing and identification (Centre Génomique du Québec, Sainte-Foy, Canada). All of the procedures for sample preparation, tryptic digestion, mass spectrometry and database searches by the Centre Génomique du Québec are described in detail by Novak et al (Novak et al. 2009). The Scaffold software (Proteome Software Inc., Portland, OR) was used to validate MS/MS based

peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm (Keller et al. 2002). Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least two unique identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al. 2003). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Only the most abundant proteins present in every band (i.e. containing at least 20 unique peptides) are reported in table 4. Protein #2 was lost during tryptic digestion and was therefore not identified.

ChIP

The ChIP assay was performed using the EZ-ChIP Chromatin Immunoprecipitation kit (Millipore, Billerica, MA, USA) with the following modifications. Briefly, SIGC-Snf2l and SIGC control were cultured for 8h with 500 nM of progesterone. An aliquot equivalent to 1×10^6 cells cross-linked and sonicated was incubated overnight at 4°C with rotation with 100 µl of anti-flag M2 Magnetic beads 50% gel slurry (Sigma-Aldrich). The beads were washed with Low Salt Immune Complex Wash Buffer, High Salt Immune Complex Wash Buffer and LiCl Immune Complex Wash Buffer and TE. The DNA-protein crosslinking was reversed by incubation at 65°C for 6h with NaCl at a final concentration of 200 mM, followed by RNase A treatment at 37°C for 30 min and proteinase K treatment at 45°C for 2h. DNA purification was performed with the Qiagen PCR purification kit according to the manufacturer's instruction. Fragments from the proximal promoter region of the Fgl2 gene

were amplified by real-time PCR using the primers described in supplemental methods Table1.

Microarray

RNA was extracted from granulosa cells isolated from either *Snf2l* WT or KO mice treated with PMSG followed by hCG at 0h and 4h using the RNeasy kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Each experiment includes 4 datapoints (WT-0h, WT-4h, KO-0h, KO-4h) using granulosa cells pooled from 5 mice for each, and was repeated 3 times and analyzed independently by microarray. The platform used was the Affymetrix Genome 430 v2.0 chips which includes over 39,000 transcripts, and the data was analyzed using ArrayAssist (Stratagene, La Jolla, CA, USA). Signal intensities were normalized by variance stabilization (Huber et al. 2002) and probe sets differentially expressed between treatment (0h and 4h) and conditions (WT and KO) were detected with limma (Smyth 2004). The probe sets which significantly differed ($p < 0.05$) by treatment in WT but not KO were further compared. Gene function was inferred using the Gene Ontology (GO) terms annotations from the GO Consortium (www.geneontology.org).

Q-PCR

Quantitative RT-PCR was used to validate some of the targets identified by microarray such as *Arrdc3*, *Btg1*, *Dio2*, *Egln3*, *Fgl2*, *Rps9*, *Dio2*, and *Mlh1*. Granulosa cells were extracted following treatment with PMSG and hCG as described above and mRNA was purified using the RNeasy Kit (Qiagen, Valencia, CA, USA). cDNA was prepared using superscript III First-Strand Synthesis System (Invitrogen) according to the manufacturers' protocol. Relative expression was determined using the geometric mean of *Rpl19* and *Ppia* which was identified

as the most stable and reliable endogenous control in our cells using the GeNorm (<http://medgen.ugent.be/~jvdesomp/genorm/>) algorithm (Vandesompele et al. 2002). Q-PCR reactions were ran on the Applied Biosystems 7500 Fast RT-PCR system using SYBR green Fast reaction settings (ABI, Carlsbad, California) with default cycling conditions. Primer sequence information is shown in supplemental methods table 1.

Statistical analyses

Figures were plotted using GraphPad Prism 4.0 software (Graphpad Software, San Diego, CA, USA) and paired observations were analyzed by t-test. Continuous variables such as gene expression by Q-PCR were compared by two-way ANOVA followed by Tuckey's post-hoc test. Statistical significance was inferred at $p < 0.05$.

3.4 Results

***Snf2l* KO mice reproduce normally but have a decreased superovulatory response.**

To investigate the role of SNF2L in fertility, a mouse was used in which the gene was inactivated by deleting exon6 which encodes for the P-loop of the ATP-binding domain. In contrast to the *Snf2b* KO mice (Stopka & Skoultschi 2003), *Snf2l* KO mice are viable into adulthood and are able to reproduce stably over several generations. Having previously reported that *Snf2l* expression is mostly restricted to reproductive tissues (Lazzaro & Picketts 2001; Lazzaro et al. 2006), it was of interest to examine both the male and female gonads in greater detail. Surprisingly, both ovaries and testes from *Snf2l* KO mice had normal gross morphology and were of similar mass to those of WT mice (Fig 3.1A). Furthermore, when 4

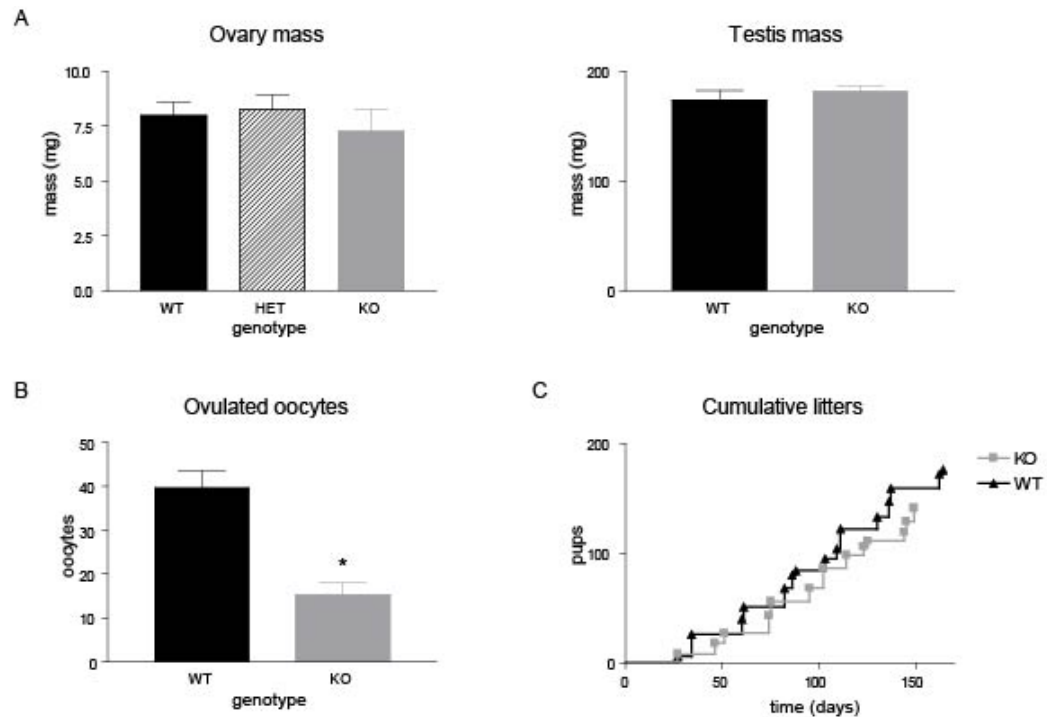


Figure 3.1 Characterization of the fertility of *Snf2l* KO mice as assessed by gonad size, superovulation yield and mating productivity. A) Gonads from female *Snf2l* KO (N=8), HET (N=35), and WT (N=11) and male KO (N=24) and WT (N=21) mature mice (1 month old) were harvested and weighed. B) Immature females (d24-26) from *Snf2l* KO (N=12) and WT (N=12) lines were treated with 5IU of PMSG and 48h later with 5IU hCG for 18h. Eggs were retrieved from the oviduct and counted. C) Cumulative litter counts from 4 pairs of KO males and females or WT male and females over 5 months. Data is representative of 3 experiments. The results are shown as the mean of 3 or more experiments $\pm$ SEM, and significance is inferred by one-way ANOVA with post test or by t-test if $p < 0.05$ (as indicated by *).

mating pairs of *Snf2L* KO mice were compared to 4 age-matched litter-mate WT controls, there was no overt reproductive handicap as they both produced litters of similar size and frequency over the course of 5 months (Fig 3.1B). When allowed to mate freely for up to a year or until they no longer reproduce, the average litter size of *Snf2L* KO mating pairs (N=8) was 7.99 ± 2.12 pups/litter while WT mating pairs (N=12) had on average 6.99 ± 1.92 pups/litter giving a fertility index of 0.28 ± 0.11 and 0.23 ± 0.11 pups/female/day respectively. However, when superovulated, *Snf2L* KO mice yield significantly fewer eggs than control WT mice (Fig 3.1C), suggesting that either folliculogenesis is impaired, or that hCG is unable to trigger ovulation of all the mature follicles in the *Snf2L* KO mice under high gonadotropin conditions.

***Snf2L* KO mice treated with exogenous gonadotropins have fewer secondary follicles and more abnormal antral follicles.**

To gain further insight into the cause of the superovulation deficit of *Snf2L* KO mice, follicle numbers were counted in ovarian sections of mice treated with either PMSG alone for 48h or PMSG for 48h followed by hCG for 18h. As expected, treatment with PMSG alone strongly induces folliculogenesis in WT mice while there is a strong trend for fewer secondary follicles in the KO mice ($p=0.051$) (Fig 3.2). Furthermore, there is a trend for increased numbers of primary follicles suggesting delays or defects in granulosa cell growth (Fig 3.2). However, these deficits do not translate into differences in antral follicle numbers between WT and KO animals suggesting they do not contribute to the superovulation deficit. To look for defects occurring in the peri-ovulatory period, ovaries were collected 18h after treatment with hCG, or just after ovulation.

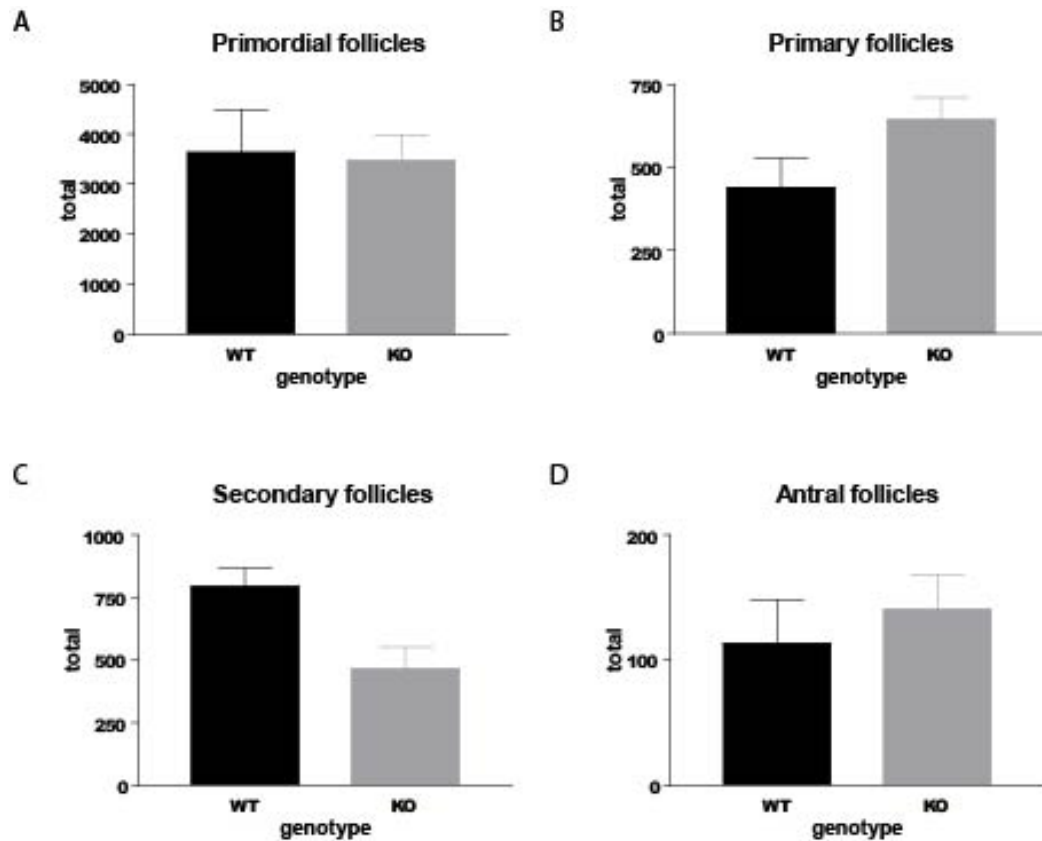


Figure 3.2 Follicle counts in serial sections of stained ovaries from PMSG-treated Snf2l KO and WT mice. Immature female mice (d24-26) from Snf2l KO (N=3) or Snf2l WT (N=3) lines were treated with 5IU of PMSG and sacrificed 48h later. Ovaries were serially sectioned (5 μ m) and stained and every tenth section was examined. Follicles were counted if the nucleus of the oocyte was visible and classified as A) primordial, B) primary, C) secondary or D) antral. Total count was estimated by multiplying the result by 10. The results are shown as a mean of 3 experiments $\pm$ SEM, and significance was inferred by t-test at $p < 0.05$ (as denoted by *).

Interestingly, the same trend for increased primary follicles and reduced numbers of secondary follicles is present, this time reaching statistical significance (Fig 3.3). Few intact antral follicles are left at that time suggesting most have ovulated, however there is a trend for an increased amount of abnormal follicles, such as haemorrhagic follicles, oocytes trapped in the corpus luteum, oocytes with an expanded cumulus trapped inside the antrum and finally oocytes devoid of cumulus cells within degenerating abnormal antral follicles (Fig 3.4). The latter abnormal antral follicles are significantly more abundant in *Snf2L* KO mice and may account for the deficit in superovulated ova (Fig 3.4).

***Snf2L* KO mice have abnormal levels of estradiol and progesterone and treatment with diethylsilylbestrol restores secondary follicle growth.**

Sex steroids play an integral part in the regulation of the growth, survival and maturation of follicles as underscored by the severity of the ovarian phenotypes of the various steroid receptor KO mice (Robker et al. 2000a; Dupont et al. 2000; Hu et al. 2004). To investigate whether serum levels of sex steroids were affected in the *Snf2L* KO mice, estradiol and progesterone concentrations were measured in the blood of superovulated mice by RIA. Estradiol is one of the main drivers of proliferation of granulosa cells and its levels are quickly depressed following treatment with hCG (reviewed by Su et al. 2006). In mice treated with PMSG alone, both WT and *Snf2L* KO mice have equivalent levels of estradiol, however following treatment with hCG, the estradiol concentration decreases significantly faster in the KO (Fig 3.5A). Conversely, hCG is known to increase the production of progesterone by luteal cells, whose concentration stabilizes by 48h (Wade et al. 2002). Interestingly, the serum progesterone concentration is significantly higher in *Snf2L* KO mice at the 48h timepoint (Fig 3.5B). It is unclear whether the differences in serum levels of estradiol and progesterone are

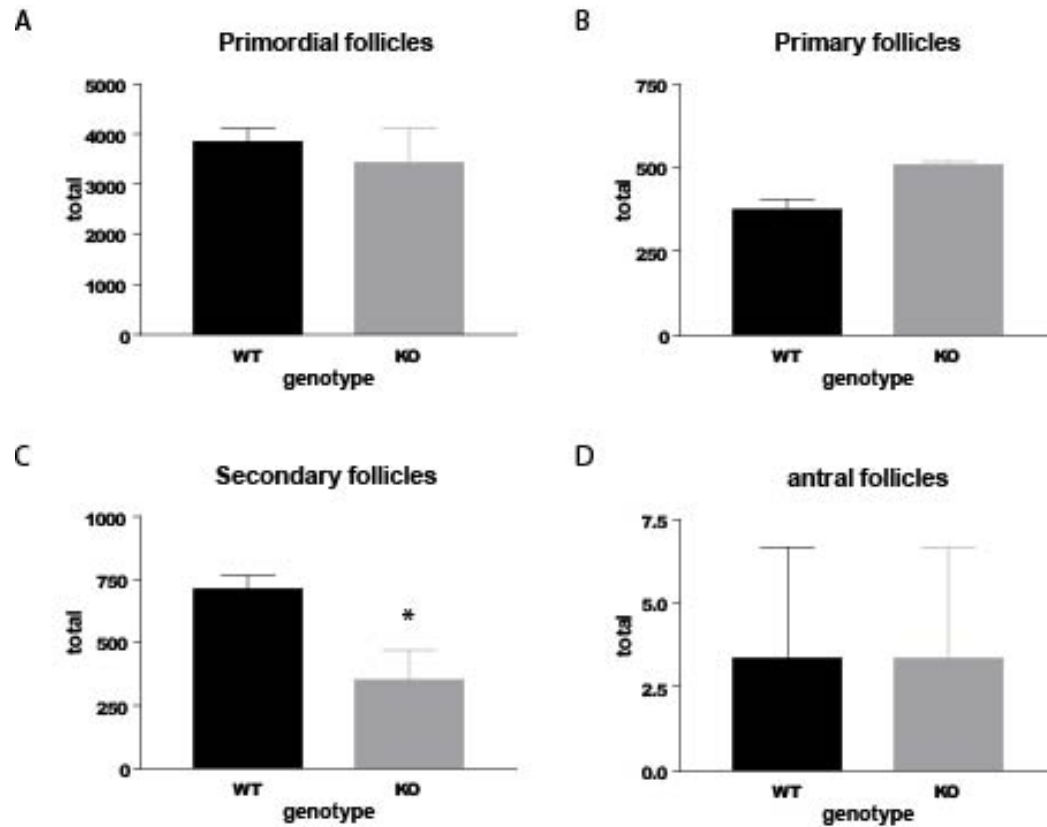


Figure 3.3 Follicle counts in serial sections of stained ovaries from PMSG and hCG-treated *Snf2l* KO and WT mice. Immature female mice (d24-26) from *Snf2l* KO (N=3) or *Snf2l* WT (N=3) lines were treated with 5IU of PMSG, 48h later with 5IU hCG and then sacrificed 18h later. Ovaries were serially sectioned (5 μ m) and stained and every tenth section was examined. Follicles were counted if the nucleus of the oocyte was visible and classified as A) primordial, B) primary, C) secondary or D) antral. Total count was estimated by multiplying the result by 10. The results are shown as a mean of 3 experiments $\pm$ SEM, and significance was inferred by t-test at $p < 0.05$ (as denoted by *).

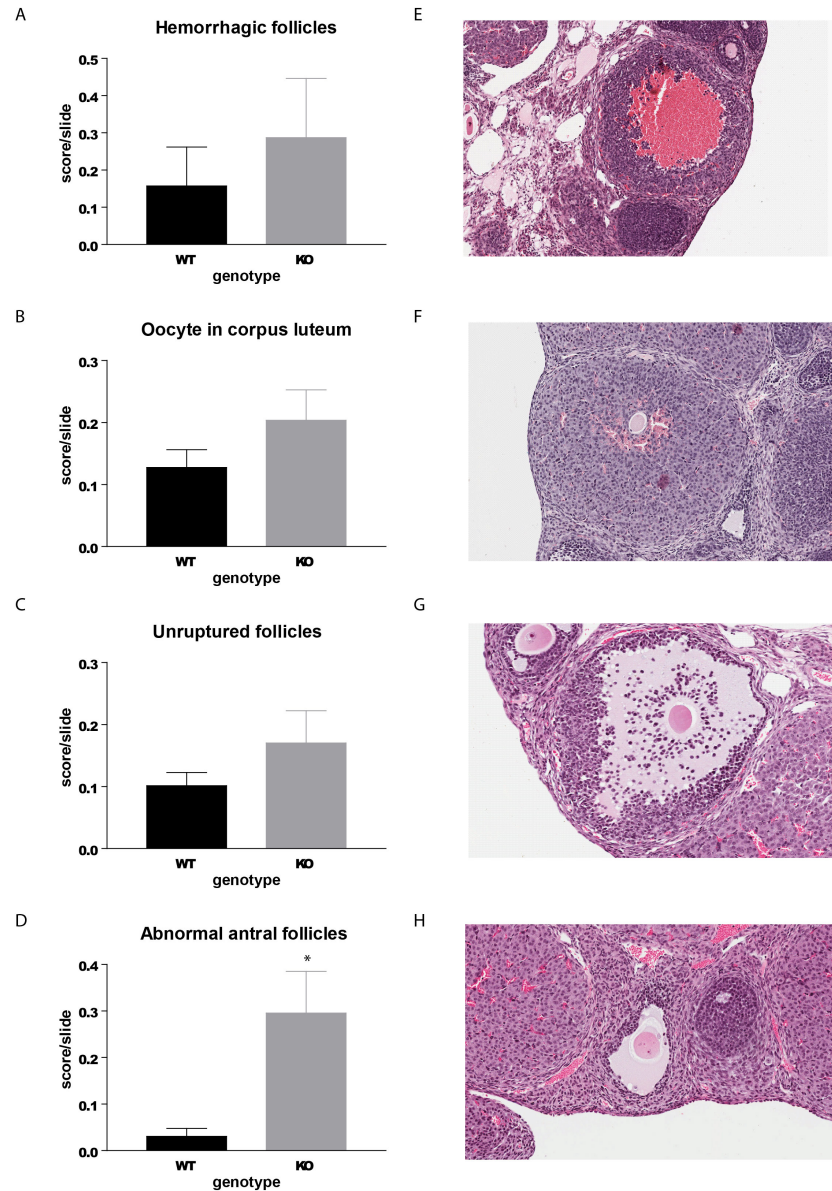


Figure 3.4 Abnormal follicles in serial sections of post-ovulatory ovaries. Immature female mice (d24-26) from *Snf2l* KO (N=3) or *Snf2l* WT (N=3) lines were treated with 5IU of PMSG, 48h later with 5IU hCG and then sacrificed 18h later. Ovaries were serially sectioned (5 μ m) and stained and every tenth section was examined. Abnormal structures were counted such as A) hemorrhagic follicles, B) oocytes in CLs, C) unruptured antral follicles or D) abnormal antral follicles. Structures that were considered abnormal when: E) antrums were completely filled with blood, F) CLs had an oocyte, often degenerated, G) oocytes, often with a degenerate zona pellucida, and expanded cumulus cells in otherwise normal graafian follicles which had failed to ovulate, and H) small degenerate antral follicles, often with only 2 layers of granulosa cells and oocytes which were devoid of cumulus cells. The results are shown as a mean of 3 experiments $\pm$ SEM, and significance was inferred by t-test at $p < 0.05$ (as denoted by *).

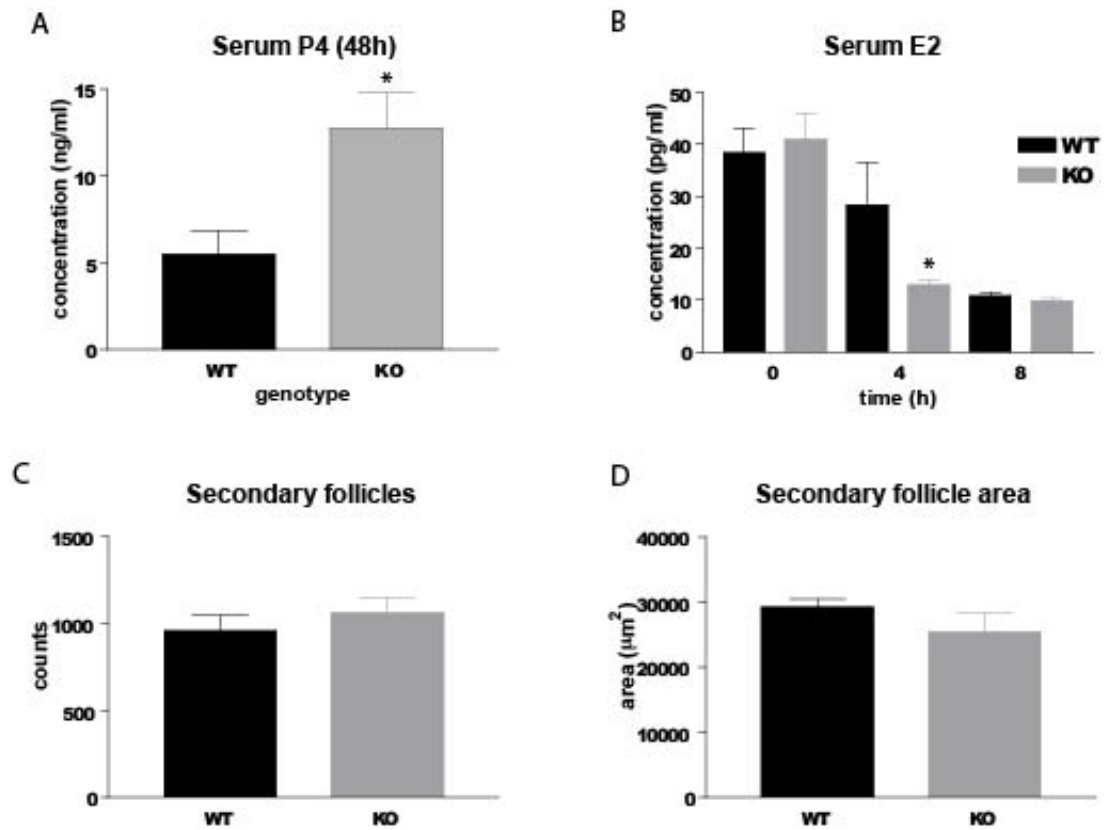


Figure 3.5 Sex steroid levels in superovulated Snf2L KO mice and the effects of DES treatment. Serum samples from immature female mice (d24-26) that were treated with 5IU of PMSG and 48h later with 5IU of hCG were analyzed by RIA for the levels of A) E2 (N=37) and B) P4 (N=16). Immature mice (N=8, d25) were treated with 0.1mg of DES for 3 consecutive days and their ovaries were fixed, serially sectioned (5μm) and stained with every tenth section examined. C) Secondary follicles were counted if the nucleus of the oocyte was visible D) and the cross-sectional area was traced and measured. The results are shown as a mean of 3 experiments $\pm$ SEM, and significance was inferred by one-way ANOVA or t-test if $p < 0.05$ (as denoted by *).

causative or consequences of the phenotypes observed. However treatment of immature mice with the strongly estrogenic compound diethylstilbestrol (DES) is sufficient to recruit equivalent numbers of secondary follicles, of similar sizes in both WT and KO mice (Fig 3.5C), suggesting estrogen responsiveness is not deficient.

Identification of hCG-responsive genes that are dependent on SNF2L.

To better understand the role of *Snf2l* in granulosa cells at the time of the LH surge, a microarray screen was devised to specifically look for genes whose expression changes in response to hCG treatment in a *Snf2l*-dependent fashion (Fig 3.6A). The 4h post-hCG time point was chosen to enrich for genes that are directly induced by LH, and because it coincides with the induction of SNF2L (Lazzaro et al. 2006). When comparing gene expression in granulosa cells of *Snf2l* KO and WT mice treated with PMSG for 48h (Table 3.1), or PMSG and hCG for 4h (Table 3.2) we find that many genes are altered in the KO. By further contrasting gene expression between the 0h and 4h hCG treatment timepoints we find that the expression of 232 genes is significantly changed in both the WT and KO granulosa cells (Fig 3.6B). However, of more interest are the 85 genes whose expression significantly changes in WT animals but do not in *Snf2l* KO mice, following treatment with hCG (Fig 3.6B). Within that group of genes, there are several gene ontology profiles which are significantly overrepresented such as developmental process (n=16 p<0.003), anatomical structure development (n=12 p<0.042), negative regulation of cell growth (n=2 p<0.042), cellular developmental process (n=11 p<0.042), cell differentiation (n=11 p<0.042). Of those, we chose to further investigate 8 genes (Table 3.3), whose combined expression shows a trend of attenuated induction peaking at 4h when validated by qPCR (Fig 3.6C)(supplemental Fig 3.1).

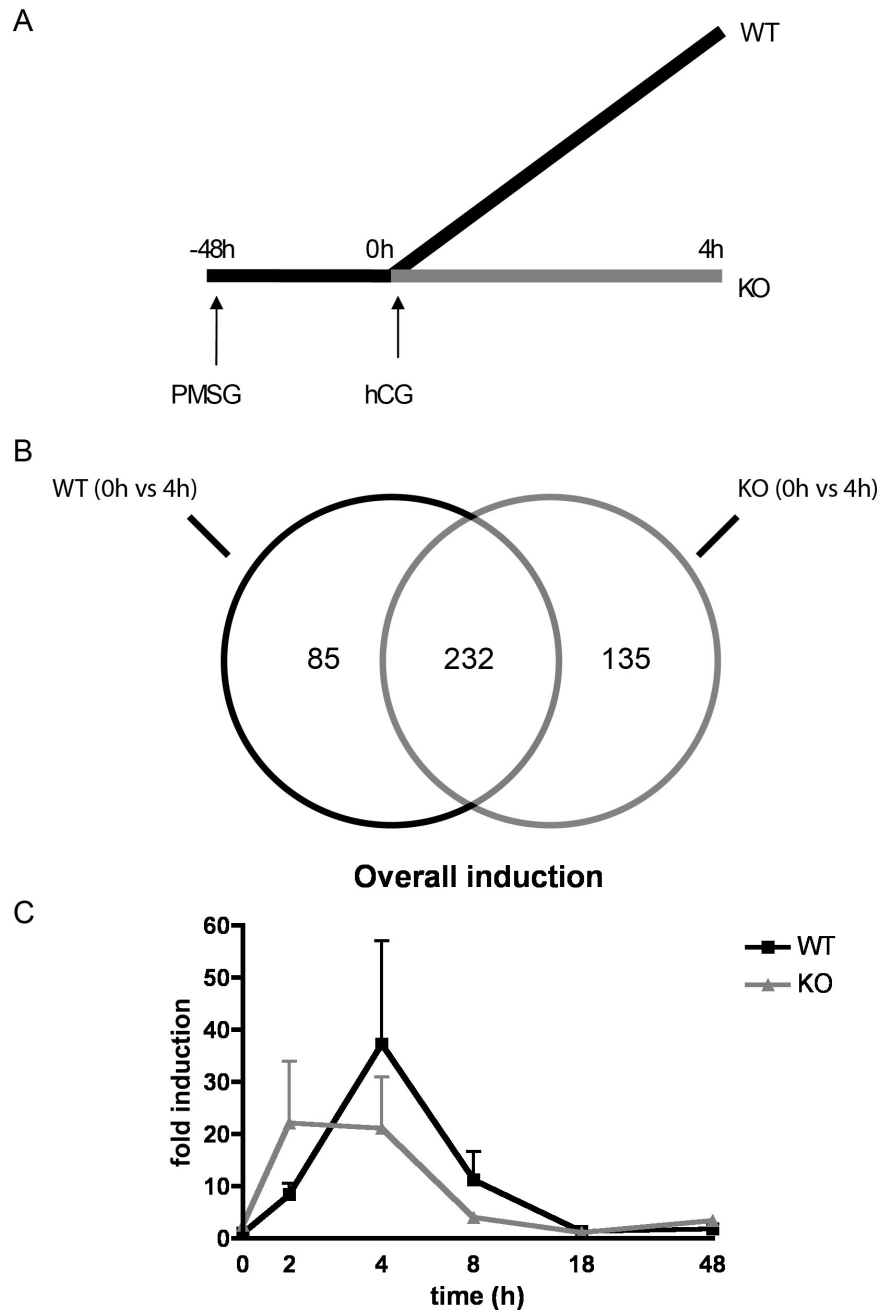


Figure 3.6. Microarray screen for *Snf2l* targets and Q-PCR validation. A) A screen was devised to compare granulosa cells from superovulated WT and KO mice in 3 independent experiments involving 5 mice per timepoint where treatment (hCG 0h vs 4h) was contrasted. B) Venn diagram representing gene sets which were significantly changed by treatment for each genotype. C) 8 genes which significantly changed in WT but not in KO were validated by qPCR over a broader range of timepoints (0h, 2h, 4h, 8h, 18h, 48h) and their mean induction profile is shown.

Table 3.1 List of genes with the greatest fold difference between *Snf2L* KO and WT in PMSG treated mice.

Gene	Full name	Gene ontology	LogFC	pfp
Muc10	mucin 10	Extracellular space	11.36	0.02
Hnf1b	Hnf1 homeobox B	Regulation of transcription	11.06	0.0213
Nr2e3	nuclear receptor subfamily 2, group E, member 3	Regulation of transcription	10.39	0.0171
Zfand5	zinc finger, AN1-type domain 5	Nucleic acid binding	9.58	0.03
Gsc	Goosecoid homeobox	Regulation of transcription	9.41	0.01
B3galt2	UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 2	galactosyltransferase activity	-10.70	0.005
Cts8	Cathepsin 8	cysteine-type endopeptidase activity	-10.29	0.0114
Fut 9	fucosyltransferase 9	fucosyltransferase activity	-9.48	0.0238
4933423N03	RIKEN cDNA 4933423N03 gene	unknown	-9.20	0.0391
Fastkd3	FAST kinase domains 3	Protein kinase activity	-8.70	0.008

LogFC indicates log fold change of the gene in the *Snf2L* KO. Pfp is the percentage of false prediction.

Table 3.2 List of genes with the greatest fold difference between Snf2L KO and WT in PMSG + hCG treated mice.

Gene	Full name	Gene ontology	LogFC	pfp
Peg3	Paternally expressed 3	Nucleic acid binding	11.15	0
Speer3	spermatogenesis associated glutamate (E)-rich protein 3	Biological process	10.88	0.0033
Vav1	Vav1 oncogene	Rho guanyl-nucleotide exchange factor activity	10.65	0.0233
Dydc1	DPY30 domain containing 1	Biological process	10.38	0.0214
Metap1	methionine aminopeptidase-like 1	Peptidase activity	9.89	0.028
Adck2	aarF domain containing kinase 2	Protein kinase activity	-10.96	0.03
Aoah	Acyloxyacyl hydrolase	Lipase activity	-10.70	0.02
Mgat5b	mannoside acetylglucosaminyltransferase 5, isoenzyme B	Transferase activity	-10.70	0.01
Nox4	NADPH oxidase 4	Oxidoreductase activity	-10.48	0.0117
B3galt1	UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 1	galactosyltransferase activity	-10.29	0.0138

LogFC indicates log fold change of the gene in the Snf2L KO. Pfp is the percentage of false prediction.

Table 3.3 List of candidate genes with the greatest difference in induction WT when compared to KO.

Gene	Full name	Gene ontology	LogFC	P-value
MPP7	membrane protein, palmitoylated 7 (MAGUK p55 subfamily member 7)	Tight Junction	4.75	0.0133
MLH1	mutL homolog 1	DNA mismatch repair	4.49	0.0004
DIO2	deiodinase, iodothyronine, type II	thyroxine 5'-deiodinase activity	4.01	0.0069
RPS9	ribosomal protein S9	structural constituent of ribosome	3.74	0.0018
FGL2	fibrinogen-like protein 2	Peptidase activity	3.22	0.0599
BTG1	B-cell translocation gene 1, anti-proliferative	transcription cofactor activity	3.21	0.0108
EGLN3	EGL nine homolog 3	Oxidoreductase activity	3.16	0.0087
ARRDC3	Arrestin domain containing 3	Biological process	3.15	0.0165

LogFC indicates log fold change of the gene in the Snf2I KO.

***Fgl2* expression is regulated by SNF2L, which is bound to its promoter, and found in a complex with the nuclear receptor co-activator FLI-I.**

Fgl2 was one of the targets identified by microarray whose induction was largely abrogated in the *Snf2l* KO as confirmed by qPCR (Fig 3.7A). Furthermore FGL2 was of particular interest given its previously identified role in reproduction (Clark et al. 2004), and its dependence on PRA for expression. FGL2 protein levels were found to be lower in *Snf2l* KO granulosa cells 8h after treatment with hCG (Fig 3.7B). Additionally, when the SIGC rat granulosa cell line was transfected with a construct driving the expression of hSNF2L, it was sufficient to induce the expression of *Fgl2*, which was further increased by treatment with progesterone (Fig 3.7C). These data suggested that *Fgl2* might be a *bona-fide* target of SNF2L chromatin remodeling activity. To investigate if SNF2L directly interacts with the *Fgl2* promoter, a chromatin immunoprecipitation (ChIP) experiment was performed, taking advantage of the FLAG tag on the expression vector. Immunoprecipitation (IP) using beads with FLAG antibody significantly enriched portions of the proximal *Fgl2* promoter in SIGC transfected with hSNF2L (SIGC-Snf2l) when compared to control cells (SIGC-GFP) (Fig 3.8). Finally, having previously reported that SNF2L physically interacts with PRA (Lazzaro et al. 2006), we sought to identify other binding partners, which may play a role in the regulation of Snf2l targets. IP using FLAG antibodies led to the enrichment of several protein bands in the SIGC-Snf2l (Fig 3.9A), which were identified by mass spectrometry (Table 3.4). Many of the identified components play a role in chromatin structure (FLNA, SPNa2, SPNb2, ACTb) (Dingová et al. 2009), in translation (EIF5b, EIF2s3, EIF2s2, PABPC1) (Sato & Maquat 2009; Pestova et al. 2001), or are known to interact with chromatin remodeling complexes

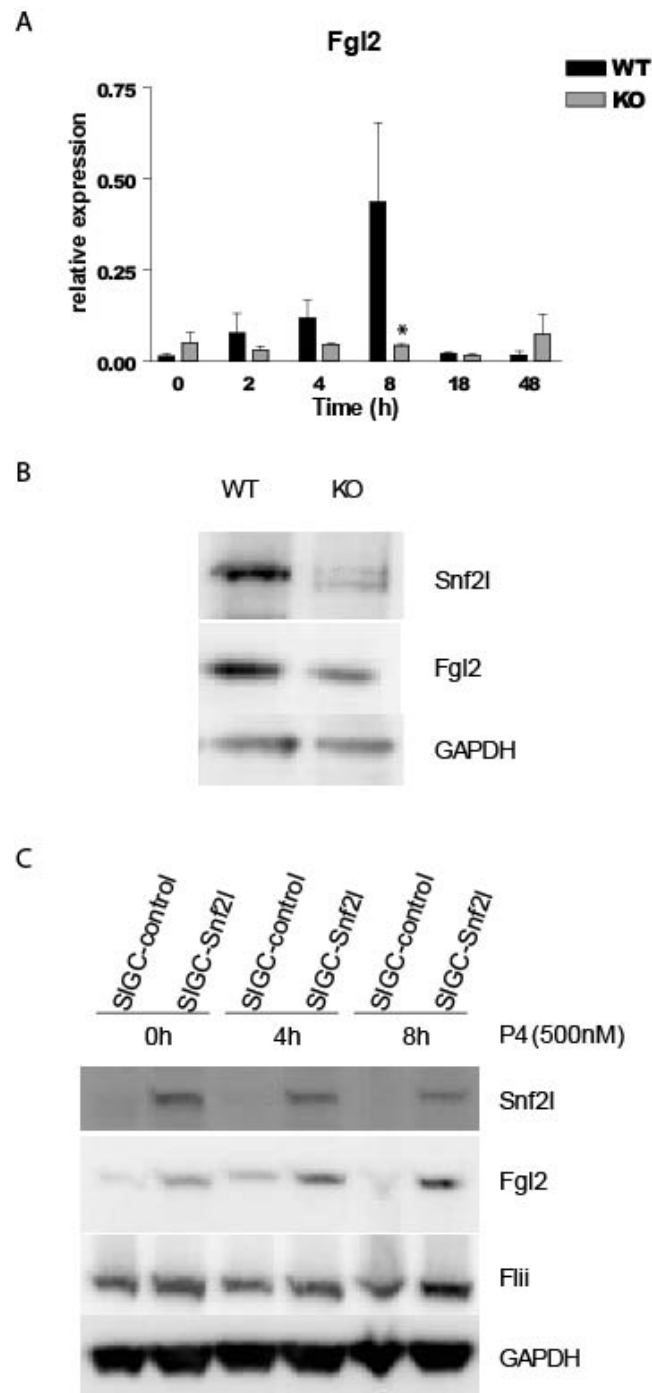


Figure 3.7 Fgl2 is not induced in Snf2l KO mice, and Snf2l overexpression causes expression Fgl2. A) Q-PCR validation of Fgl2 in 3 superovulation experiments with granulosa cells extracted over a broader range of timepoints (0h, 2h, 4h, 8h, 18h, 48h). B) Representative western blot analysis of Fgl2 expression in WT and KO mice after 8h with hCG. C) SiGC cells stably transfected with Snf2l or a control vector were treated with 500nM of P4 for 0h, 4h, and 8h and analysed by western blot. The results are shown as a mean of 3 experiments +/- SEM, and significance was inferred by two-way ANOVA with post-test if $p < 0.05$ (as denoted by *).

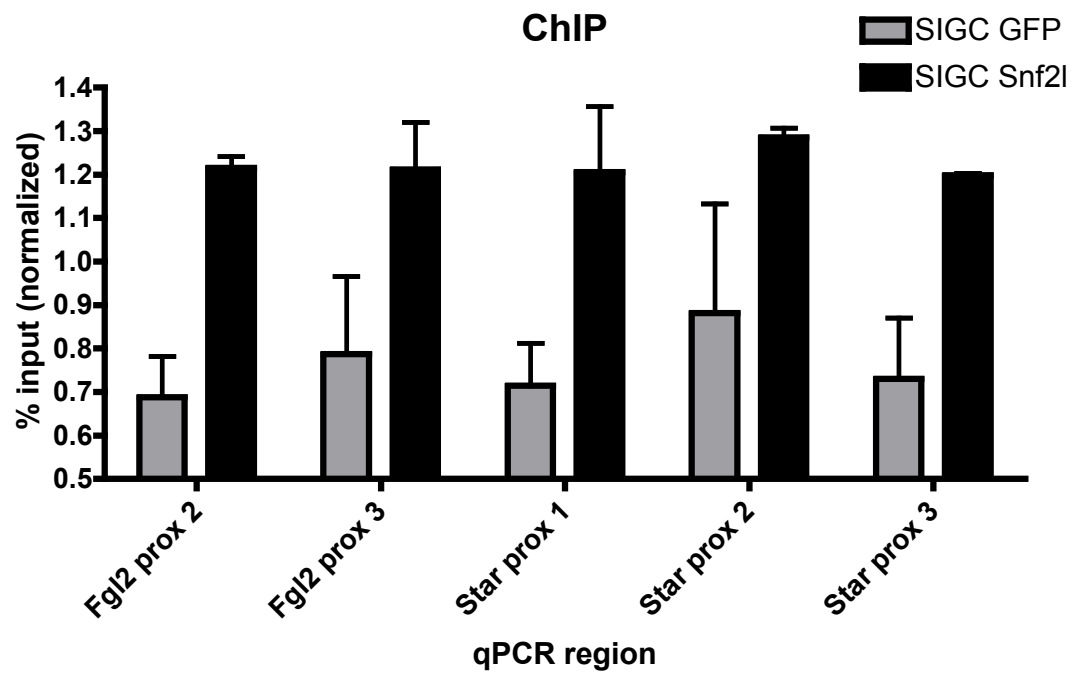


Figure 3.8 ChIP assay of Snf2l on the Fgl2 promoter.

SIGC cells transfected with Snf2l or a control plasmid were treated with P4 for 8h then cross-linked and immunoprecipitated using anti-FLAG magnetic beads. Isolated DNA was amplified by Q-PCR using primers specific to the proximal promoter of Fgl2, StAR and expression was normalized to input.

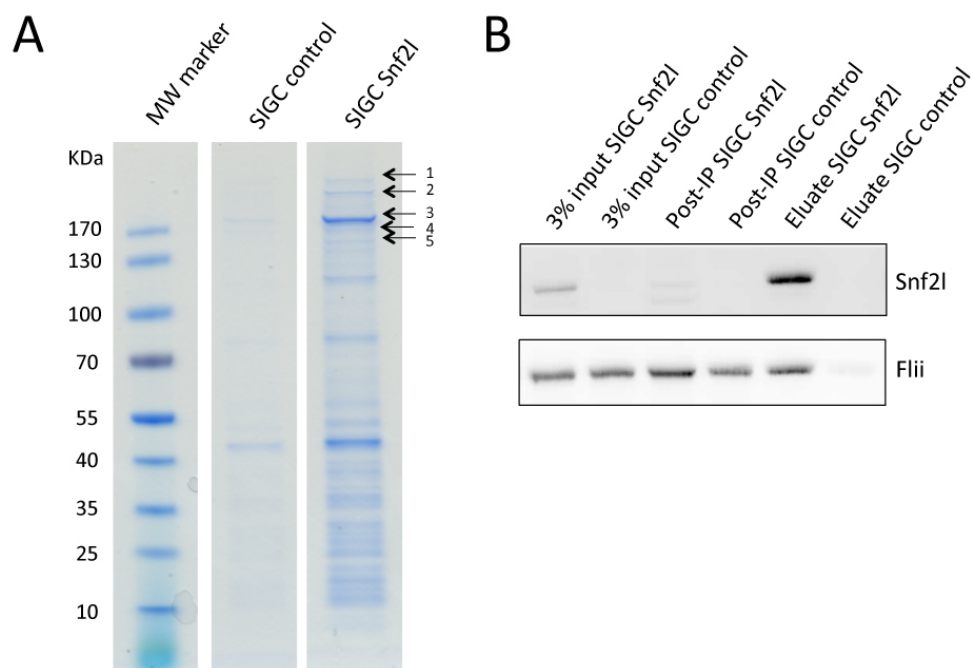


Figure 3.9 Immunoprecipitation of Snf2I binding partners.

Protein lysates from SIGC cells transfected with Snf2I or a control plasmid were immunoprecipitated using anti-FLAG magnetic beads. A) Enriched proteins were run on an acrylamide gel and the indicated bands were excised for mass spectrometry analysis. B) Putative binding partner Fli-I was validated by running the eluates from the IP and analyzing them by western blot.

Table 3.4 List of candidate Snf2l-binding partners identified in SIGC by mass spectroscopy.

Spot #	Protein name (symbol)	Accession #	Observed MW (kDa)	Theoretical MW (kDa)	# of unique peptide (% coverage)
1	Filamin α (Fina)	C0JPT7	300	280	86 (41)
	Spectrin $\alpha 2$ (Spna2)	Q6IRK8	300	282	92 (41)
	Spectrin $\beta 2$ (Spnb2)	UPI0000507D18	300	274	58 (29)
3	Eukaryotic translation initiation factor 5B (Eif5b)	Q8N5A0	180	139	37 (28)
4	Leucine rich PPR motif-containing protein (Lrpprc)	Q5SGE0	170	157	52 (36)
	Flightless I (Flii)	Q5RKI5	170	145	36 (26)
5	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 1 (Smarca1)	B1AUP9	145	121	41 (30)
6	Nucleolin (Ncl)	P13383	120	77	33 (40)
7	Protein arginine methyltransferase 5 (Prmt5)	UPI00001D0E97	90	73	33 (50)
	Heat shock 70-KD protein 8 (Hspa8)	P63017	90	71	24 (44)
	Polyadenylate-binding protein 1 (Pabpc1)	Q9EPH8	90	71	20 (37)
8	Eukaryotic translation initiation factor 2 subunit 3 (Eif2s3)	P81795	60	51	20 (46)
9	Eukaryotic translation initiation factor 2 subunit 2 (Eif2s2)	Q6P685	55	38	20 (53)
	Ribosomal protein L4 (Rpl4)	Q6P3V9	55	47	24 (48)
10	Actin β (Actb)	P48975	50	42	24 (73)

(NCL, LRPPRC, PRMT5, FLI-I) (Liu et al. 2002; Pal et al. 2003; Angelov et al. 2006; Jeong et al. 2009). Of particular interest is the nuclear receptor co-activator FLI-I, which has been shown to regulate ER α -dependent genes as a part of a complex with the SWI/SNF protein BRG1, and whose association with SNF2L was validated by western blot (Fig 3.9B).

3.5 Discussion

Studies of model organisms such as *D. melanogaster* and *C. elegans* have revealed pleiotropic roles for the evolutionarily conserved ISWI family of ATP-dependent chromatin remodelers, including but not limited to the precise control of gene expression during development. In mammals there exist two ISWI paralogs, namely *Snf2b* and *Snf2l*, whose expression profile suggests non-overlapping functions (Lazzaro & Picketts 2001). More precisely, *Snf2b* appears to be ubiquitously expressed and involved in a variety of essential structural functions as evidenced by the early embryonic lethality of the KO (Stopka & Skoultschi 2003). Conversely, *Snf2l* expression appears to be mainly restricted to the reproductive tissues and brain (Lazzaro & Picketts 2001), where it may function as a master regulator of gene expression (Barak et al. 2003; Lazzaro et al. 2006). The most definitive approach to investigate the role of SNF2L in those tissues is the characterization of a KO model. In contrast to the *Snf2b* KO, ablation of *Snf2l* activity as described herein does not affect survival, thus demonstrating it is not an essential gene. Furthermore, fertility is only modestly impaired, with abnormalities in follicle maturation, particularly at the primary/secondary stage and during the peri-ovulatory period, which translate into a poor response to superovulation. Treatment of mice with estrogenic compounds such as DES is sufficient to rescue secondary follicle numbers in the KO,

although it is unlikely that the deficit in secondary follicles limits superovulation rate, since antral follicle numbers are normal in PMSG-treated *Snf2l* KO mice. A more probable explanation is the abnormal development of a portion of the antral follicles, as evidenced by the oocytes within those structures left behind after ovulation. Defects in the rupture of antral follicle is a common phenotype of the various steroid receptor KO transgenics (Dupont et al. 2000; Hu et al. 2004), and particularly that of the PR-A or its targets ADAMTS1 and CTSL (Robker et al. 2000a).

Interestingly, we have previously reported that SNF2L physically interacts with PR-A (Lazzaro et al. 2006), but it remains unclear which genes may be regulated in that manner. This study identifies many potential candidates, including *Fgl2*, a tissue prothrombinase implicated in abortion pathology of the maternal decidua and fetal trophoblast (Clark et al. 2004). Remarkably, the presence of the *Fgl2* transcript has also been reported in the rat ovary (Rychlik et al. 2003), and its expression in primary mouse granulosa cell is dependent on PgrA (Sriraman et al. 2010). It is therefore tempting to speculate that PR-A and SNF2L act in concert to regulate the expression of *Fgl2*. In agreement with this hypothesis, the overexpression of *Snf2l* in the SIGC granulosa cell line is sufficient to induce the expression of *Fgl2*, particularly in the presence of progesterone. Additionally, *Snf2l* directly interacts with the proximal promoter of *Fgl2* in those cells as evidenced by ChIP. It remains uncertain how the dysregulation of *Fgl2* may contribute to the phenotype observed in the *Snf2l* KO ovaries, and further studies will be needed to decipher its role, particularly in the tissue remodeling of antral follicles.

Very few complexes containing SNF2L have been identified to date, namely NURF (Landry et al. 2008),

and CERF (Banting et al. 2005), and neither has been reported to be present in ovaries. It was therefore of interest to identify which complex components may be present in granulosa cells, which was accomplished by combining IP with mass spectrometry. Surprisingly no core components of either complexes were identified in this manner. However, several candidates may warrant further investigation: LRPPRC is a mitochondrial matrix protein, which interacts with CECR2, one of the main components of CERF (Liu et al. 2002); NCL is a histone chaperone that enhances the chromatin remodeling activity of ACF, a SNF2H-containing complex (Angelov et al. 2006); PRMT5 is an arginine methyltransferase which has been suggested to bridge chromatin remodeling and histone post-translational modification via its interaction with the SNF2H-containing NuRD complex (Le Guezennec et al. 2006); FLI-I is a nuclear receptor co-activator which has been shown to mediate the interaction between SWI/SNF complexes and ER α to activate estrogen-responsive genes (Jeong et al. 2009). The latter binding partner is of particular interest given the known interaction between SNF2L and PR-A, and was subsequently validated by western blot. Remarkably, both SNF2L (Lazzaro et al. 2006) and FLI-I (Jeong et al. 2009) proteins contain LXXLL motifs, which facilitate their interaction with nuclear receptors such as PR-A.

Taken together, these results suggest that SNF2L plays a role in reproduction by coordinating the expression of genes such as *Fg/2* in granulosa cells. Furthermore, a better understanding of the role of SNF2L in superovulation may open up diagnostic or therapeutic avenues for the common clinical problem of poor response to gonadotropins in fertility patients (Serafini et al. 1988; Mitwally & Casper 2002).

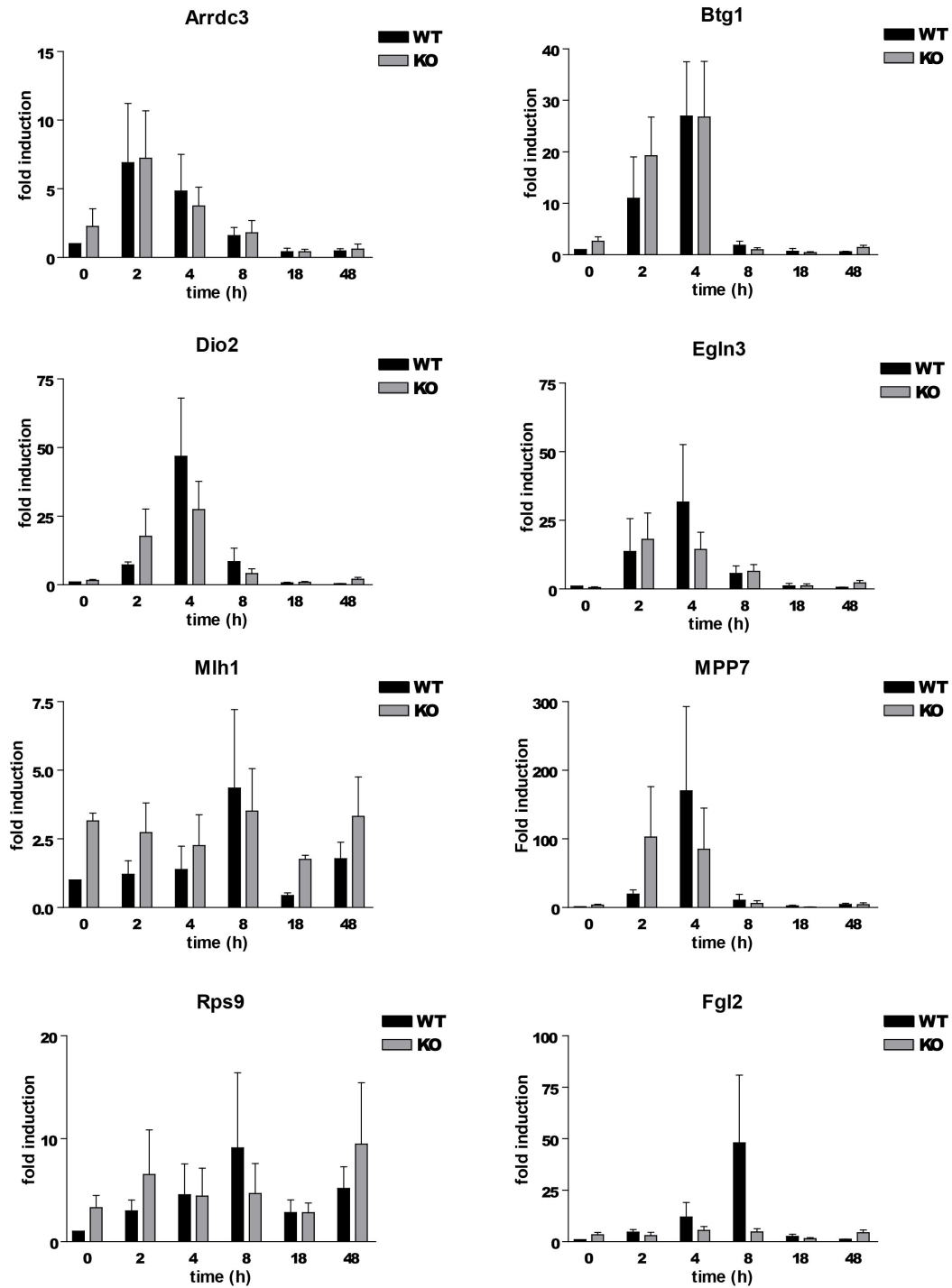
3.6 Acknowledgements

We thank Dr. R.N. Freiman for his assistance with RIA measurements of steroid hormone concentration. This work was supported in part by a scholarship from the Ontario Graduate Scholarship in Science and Technology.

3.7 Article Précis

Transgenic *Snf2l* KO mice have an impaired response to superovulation caused by defects in follicle maturation and a dysregulated expression of FGL2 in granulosa cells.

3.8 Supplementary figures and tables



Supplemental figure 3.1 Validation of 8 Snf2l-dependent gene targets by Q-PCR.

Q-PCR validation of 8 putative targets in 3 superovulation experiments with granulosa cells extracted from Snf2l KO and WT mice over a broader range of timepoints (0h, 2h, 4h, 8h, 18h, 48h) following superovulation treatment.

Supplemental table 3.1: List of primers.

Gene	Forward primer	Reverse primer
Arrdc3	ATCCAGGAGTTTCGGTTCCT	CTCTGCAAACGTGTCTCCAA
Btg1	GAAGCGCCTATTCAAACCTGC	GGGAATGGAGGTGAGAGTCA
Dio2	ATGTAACCAGCACCGGAAAG	ATGCAGAAAGGCAGACTCGT
Egln3	TGACTGCAACTGGCTGGTAG	GGGAAGTAGCAGCAGAAACG
Fgl2	GGATGGCAAGTGTTCCAAGT	CCATGGTCTCCATGTCACAG
Mlh1	CACAGCACCAAGACCAAGCTA	AGGACTGTCTAAGGCCAGCA
Mpp7	GAGCAGTATGACACGGCAGA	TGCTGTGCATCACTCATCAA
Rps9	GCTAGACGAGAAGGATCCCC	CGGGCATGGTGAATAGATTT
Rpl19	GAAATCGCCAATGCCAACTC	TCTTAGACCTGCGAGCCTCA
Ppia	AGGGTGGTGACTTTACACGC	GATGCCAGGACCTGTATGCT
Fgl2 prox1	TTCAGAGTCTTCTTTTGTAGT	CTTTCATGCTCACACTCC
Fgl2 prox2	AGTCAGGTCCTGTGGTAATGCCT	AGCCCATGGTGCTTTTCATGCTCA
Fgl2 prox3	TGCGAGCAAATGTGAGGCCCT	ACGTTTGTCCCCCTCTACTCTCCC
Star prox1	TGCCTAGCCCAGTGTGTTGGAGA	GGTGCAGGGGCAGCACACATG
Star prox2	TGGAGACAGGGACTCATGCAGGC	AGATTAACATGGCCGGTGCAGGG
Star prox3	TGGGTGGTACTGCAAGTTCATGCA	GCACACACCAAGAGTTCAGGTTCCCT
Gapdh prox	CAAACCCCGGGGACCCCCTA	TCCAGTTGAGAGCATGGGCTGC

3.9 Supplement to chapter 3: A pilot study on the use of SNF2L and FGL2 as biomarkers of superovulation response in a 17 patient cohort undergoing fertility treatment.

Introduction

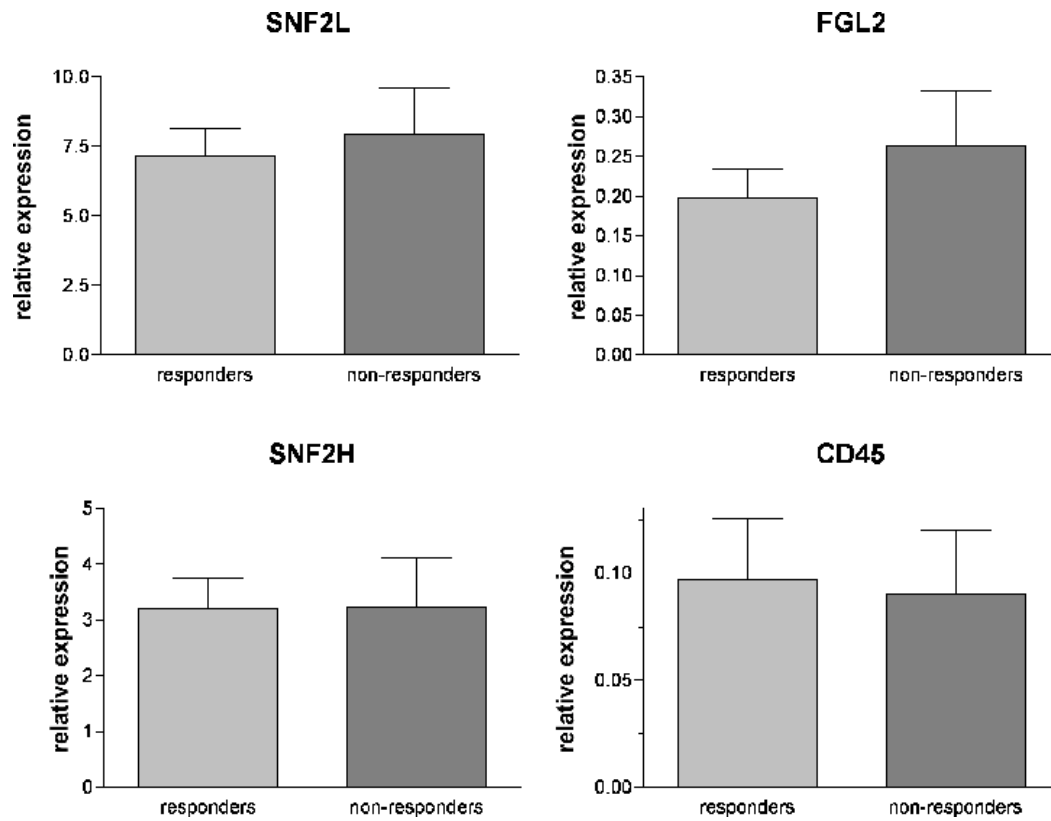
A major hurdle in the treatment of infertility in women is the access to a supply of healthy eggs for in-vitro fertilization (IVF). In order to achieve this, patients are artificially stimulated with gonadotropins, usually FSH, until they present a pool of follicles that have grown to a sufficient size, at which time the maturation is completed by the administration of hCG. The eggs are then collected using ultrasound-guided transvaginal aspiration. Patients undergoing ovarian stimulation risk both poor and excessive response to the hormonal treatment (Freiesleben et al. 2010). The latter can cause ovarian hyperstimulation syndrome, which in rare instances can lead to severe life-threatening complications (Zivi et al. 2010). Meanwhile, poor response to superovulation, while not life threatening, is a common reason for IVF failure and affects between 9 and 24% of patients (Venetis et al. 2010). Therefore, it is of great interest to both predict the amplitude of the response to ovarian stimulation and isolate the root causes of poor response. While many epidemiological studies have identified contributing factors, such as age, basal gonadotropin levels, and basal sex steroid levels, they cumulatively have only small predictive value on the response rate (Venetis et al. 2010). In this retrospective study, we sought to investigate the use of SNF2L and FGL2 expression in granulosa cells as biomarkers for the prediction of the response of patients to superovulation.

Materials and Methods

This study was conducted in collaboration with the Ottawa Fertility Clinic and was approved by the Research Ethics Board. The 18 patients who were recruited for this study consented to both the Adverse Effect and Risk form and the Use of Reproductive Material form. Patients ranged in age from 25 to 44 years with a median age of 38. They were given FSH (103-475 IU) daily for 8-12 consecutive days, at which point they were given either 5000 or 10000 IU of hCG prior to oocyte retrieval. Both follicle size during treatment and estradiol levels at trigger were monitored. Following retrieval of the oocytes from the aspirated follicular fluid, remaining granulosa cells were allowed to passively sediment and were subsequently washed 4 times with saline, pelleted, and flash frozen in liquid nitrogen. Blinded granulosa cell samples were used for RNA extraction, while 2 patients were excluded because of low granulosa cell numbers and poor RNA yield. Remaining samples were used to make cDNA and analyzed by qPCR for their expression levels of SNF2L, FGL2, SNF2H and CD45. PCR primers were as follows SNF2L: “FWD-GGGCCGTCCACCTCTCAGGA” “REV-TCITTTGCTCGGTCGGCTTTTCA”; FGL2: “FWD-GGCAAATGTTCAAAGTGTCCTCCAGCC” “REV-AGCACTGTCCAGCCTCCCCC”; SNF2H: “FWD-GCACCCAAGGCTCCTCGACC” “REV-GTGCCTGTGCTGCGTTAGGC”; and CD45: “FWD-GCTCCGCCGCCAATGCAAAA”. Patients were classified as poor responders (N=8) if they produced 4 or fewer eggs, and normal (N=10) if they produced 5 or more eggs following superovulation treatment.

Results

When patient are segregated as poor responders and normal responders and their granulosa cells are compared for their expression of SNF2L, FGL2, and SNF2H by qPCR we see no significant differences in expression (supplemental figure 3.2). The expression of the white blood cell marker



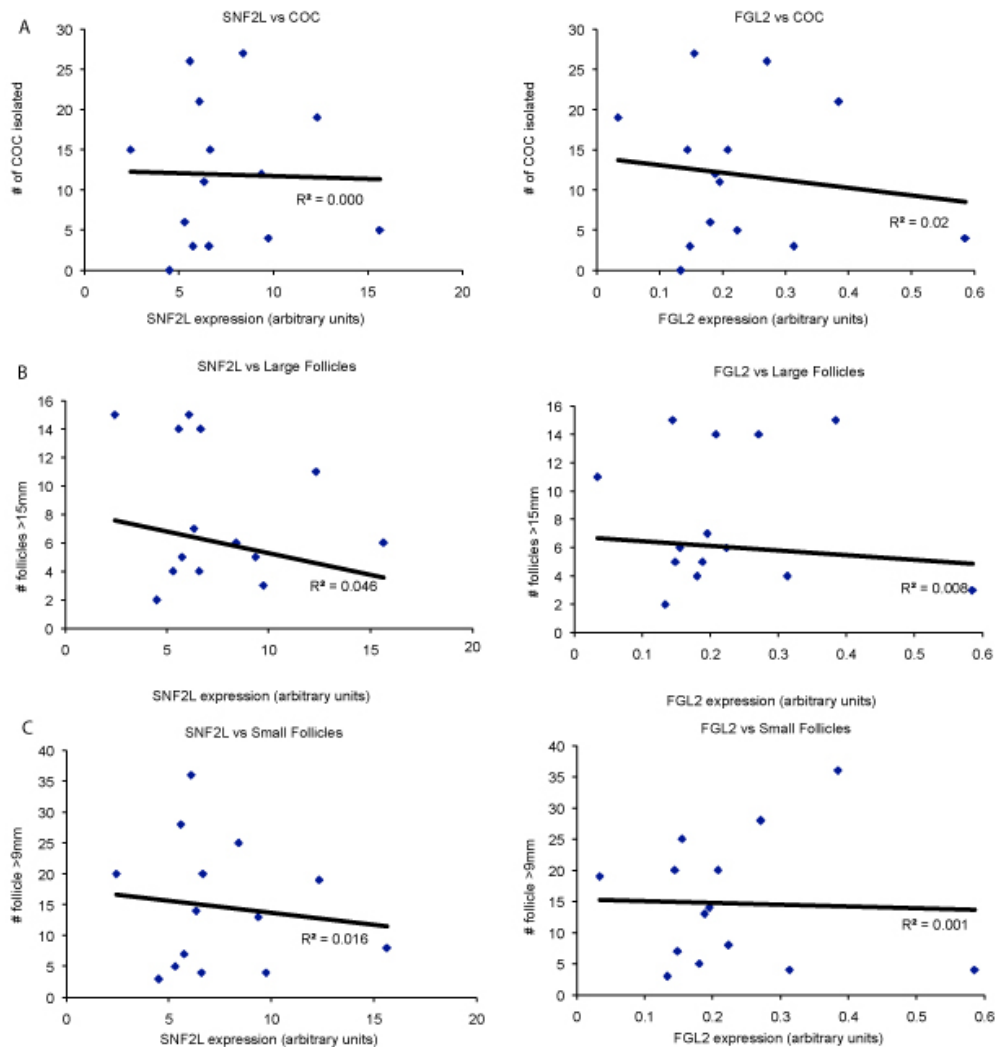
Supplemental Figure 3.2 Expression of SNF2L, FGL2, SNF2H and CD45 in isolated granulosa cells of patients classified as normal or poor responders.

Expression levels of SNF2L, FGL2, SNF2H and CD45 were analyzed by qPCR in granulosa cells collected from IVF patients at the time of oocyte retrieval. Patients were classified as poor responders if they produced 4 or fewer oocytes. Results are shown as mean $\pm$ SEM.

CD45 was also quantified to ensure that the level of contamination by blood cells is equivalent in both groups, since some aspirates may contain blood. Similarly when we compare either SNF2L or FGL2 expression to clinically relevant markers of superovulation response such as the number of cumulus-oocyte complexes (COC) retrieved for IVF, the number of follicles >9mm diameter and the number of follicles >15mm diameter we see no correlation between these parameters and the expression of SNF2L or FGL2 (supplemental figure 3.3). The levels of estradiol at the time of trigger do not correlate with expression of SNF2L or FGL2 (data not shown). The same trends were observed for SNF2H and CD45.

Discussion

Experiments in mice (unpublished results, chapter 3) suggest that low activity or reduced levels of expression of *Snf2L* and *Fgl2* may underlie a poor response to superovulation. Furthermore SNF2L in granulosa cells has recently been identified as a marker of competent follicles in humans (Hamel et al. 2008), while SNF2H may be a marker of competent bovine oocytes (Torner et al. 2008). However in our study, it does not appear that low levels of SNF2L or FGL2 in granulosa cells of retrieved follicles correlate with a poor response of the patient to superovulation. The levels of SNF2L, FGL2 or SNF2H do not predict the magnitude of the response to superovulation. The poor predictive power of these putative biomarkers in humans may be due to species-specific differences or may be limited by the timing of the collection of the COCs, which may not correspond to an informative timepoint. Alternatively the sampling may be biased in favor of responsive follicles with high SNF2L or FGL2 expression since small follicles with poor growth in unresponsive patients were not isolated. It may be interesting to sequence the SNF2L



Supplemental Figure 3.3 Correlation of clinical parameters related to superovulation with expression of SNF2L and FGL2 in granulosa cells by qPCR.

Expression levels of SNF2L and FGL2 were analyzed by qPCR in granulosa cells collected from IVF patients at the time of oocyte retrieval and correlated to clinical parameters such as A) the number of COC harvested per patient, B) the number of large follicles (>15mm) imaged by ultrasound at the time of oocyte retrieval, and the number of medium-sized (>9mm) follicles. Each datapoint shown on the scatter plot represents one patient, the solid line represents the slope of the distribution along with its R-squared value.

gene in patients with poor response, especially in families with low fertility, since the genic region (Xq25) has previously been associated with premature ovarian failure (Prueitt et al. 2000).

CHAPTER 4: GENERAL DISCUSSION

The focus of this thesis has been to investigate the roles of the ISWI proteins SNF2H and particularly SNF2L in ovarian function. Folliculogenesis is a well studied ovarian phenomenon which relies on the coordinated response of the oocyte, the granulosa cells and the theca cells to the endocrine signals of gonadotropins and sex steroids. Much emphasis has also been placed on the role of paracrine signalling between the oocyte and the granulosa cells in regulating both growth and differentiation. While some of the hormone-responsive transcription factors responsible for regulating genes involved in the growth and differentiation of those cell types have been identified, few studies have looked at the necessary underlying changes in chromatin. SNF2H and SNF2L are expressed in the ovary, and preliminary evidence suggested they had opposing roles on proliferation and differentiation making them ideal candidates for the chromatin remodelling activities which accompany folliculogenesis. Here we will discuss how the expression of *Snf2h* and *Snf2l* correlates with the progression of folliculogenesis and its implications on granulosa cell biology. We will address the significance of the regulation of key genes by SNF2L on the differentiation of granulosa cells. And finally we will contrast and compare the ovarian phenotype of the *Snf2l* KO mouse with other KOs with impaired folliculogenesis.

4.1 Summary of findings

The initial finding on which these studies are based was the cloning and characterization of the murine ISWI genes *Snf2h* and *Snf2l*. To build upon the observation that both paralogs were expressed in the ovary, in the first study we looked at their expression profile by in-situ

hybridization in PMSG and hCG treated ovaries. We found that both *Snf2l* and *Snf2b* were expressed in granulosa cells of follicles, with *Snf2l* being induced by 8h after treatment with hCG and maintaining high expression in the CL whereas *Snf2b* seemed to be downregulated following hCG treatment. We found that the SNF2L protein, but not SNF2H, was induced by treatment of animals with hCG or primary cells with dbcAMP in mouse, rat, and porcine granulosa cells. SNF2L was also induced by dbcAMP treatment in the SVOG-40 human immortalized granulosa cell line, where it was found to physically interact with PR-A. Finally we established that StAR, a marker of luteinisation, was significantly reduced when SVOG-40 cells were treated with an siRNA directed against SNF2L. The regulation of StAR by SNF2L is likely by direct remodelling of the promoter since ChIP in porcine granulosa cells showed physical binding which is enhanced by dbcAMP.

In the second study we sought to further investigate the role of SNF2L in ovaries by characterising the reproductive phenotype of a *Snf2l* KO mouse. While the mice were fertile they had a significantly reduced yield of ova following superovulation. To find the cause of such deficit, ovaries were analysed following treatment with PMSG or PMSG and hCG and were found to have significantly fewer secondary follicles. Secondary follicle growth could be rescued by treating the mice with diethylstilbestrol, suggesting that they were able to respond to estrogen, and that an estrogen deficit may be contributing to the inefficient transition to secondary follicle stage. Ovaries collected following ovulation revealed that the reduced superovulation yield may be due to abnormalities in antral follicle formation, particularly lack of cumulus cells and poor mural granulosa growth. To find candidate genes regulated by SNF2L following the LH surge, a microarray screen was devised comparing gene induction

4h after treatment with hCG in WT and KO mice. One of the identified candidates, *Fgl2*, failed to be induced in the KO, while overexpression of *Snf2l* in SIGC rat granulosa cell line was sufficient to induce its expression. Furthermore we confirmed by ChIP that SNF2L is present on the *Fgl2* promoter following treatment with progesterone. Finally we sought to identify prospective SNF2L complexes which would be responsible for the observed biological activity. One of the proteins identified on the basis of its binding to SNF2L is FLN-1, a steroid receptor co-activator.

4.2 General discussion

4.2.1 SNF2L regulates StAR

One of the key findings of our studies is that *Snf2l* is induced by LH. However the signalling pathway leading to its activation has not yet been unravelled. Recently it was found that the *Snf2l* promoter contains both cAMP response elements (CRE) and SP1 binding sites (Xia et al. 2008). It is likely that the LH receptor upregulates *Snf2l* via the cAMP/PKA pathway leading to the phosphorylation of CREB and its translocation to the *Snf2l* promoter. What is less clear is how SNF2L facilitates LH-dependent activation of StAR. One of the main pathways activated by the LH receptor is comprised of the ERK1/2 signalling molecules and their downstream effector CEBP/ β , which have been shown to be essential for terminal differentiation of granulosa cells (Fan et al. 2009). The spatio-temporal regulation of the StAR promoter has been well defined and includes the binding of transcription factors such as NR5A1 and CEBP/ β and a multitude of changes in histone modifications including the acetylation of histone H3 at lysines 9 and 14, dimethylation at lysine 4 and demethylation of

lysine 9. SNF2L may be facilitating the binding of NR5A1 or CEBP/ β , remodelling nucleosomes to enable modification of the histones or may help to permanently remodel nucleosomes following binding of transcription factors or modifications of the histones. Loss of either NR5A1 or CEBP/ β leads to infertility (Fan et al. 2009; Pelusi et al. 2008), with some features resembling the milder Snf2l KO phenotype. Furthermore, both SNF2L and NR5A1 proteins contain LXXLL motifs which could mediate their interaction (Suzuki et al. 2003). Alternatively, some clues may come from the new SNF2L binding factors identified by mass spectrometry. PRMT5 was found to interact with SNF2L in granulosa cells and is known to methylate H3 and H4 (Majumder et al. 2010), suggesting SNF2L could be involved in the methylation of H3 at lysine 4 on the StAR promoter.

4.2.2 SNF2L regulates *Fgl2*

The transcriptional regulation of another identified target, *Fgl2*, is likely via a different mechanism. *Fgl2* was found to be specifically regulated by PR-A in a primary granulosa cell screen comparing cells infected with an adenovirus encoding for *Pgr-A* and treated with either a receptor agonist or antagonist (Sriraman et al. 2010). Thus it is likely that *Fgl2* is not directly induced by LH and its associated pathways, but by the secondary induction of progesterone and the PR-A receptor. Furthermore, we presented evidence that SNF2L interacts with both PR-A and the steroid receptor coactivator FLN-I, probably via the multiple shared LXXLL nuclear receptor motifs. Therefore we would like to suggest the existence of a novel complex, which we will call Steroid receptor Activating and Remodelling Factor (SARF). This complex would include a steroid receptor, in this case PR-A, the nuclear receptor co-activator FLN-I and the chromatin remodeler SNF2L (Fig 4.1). Many of the observed phenotypes of the

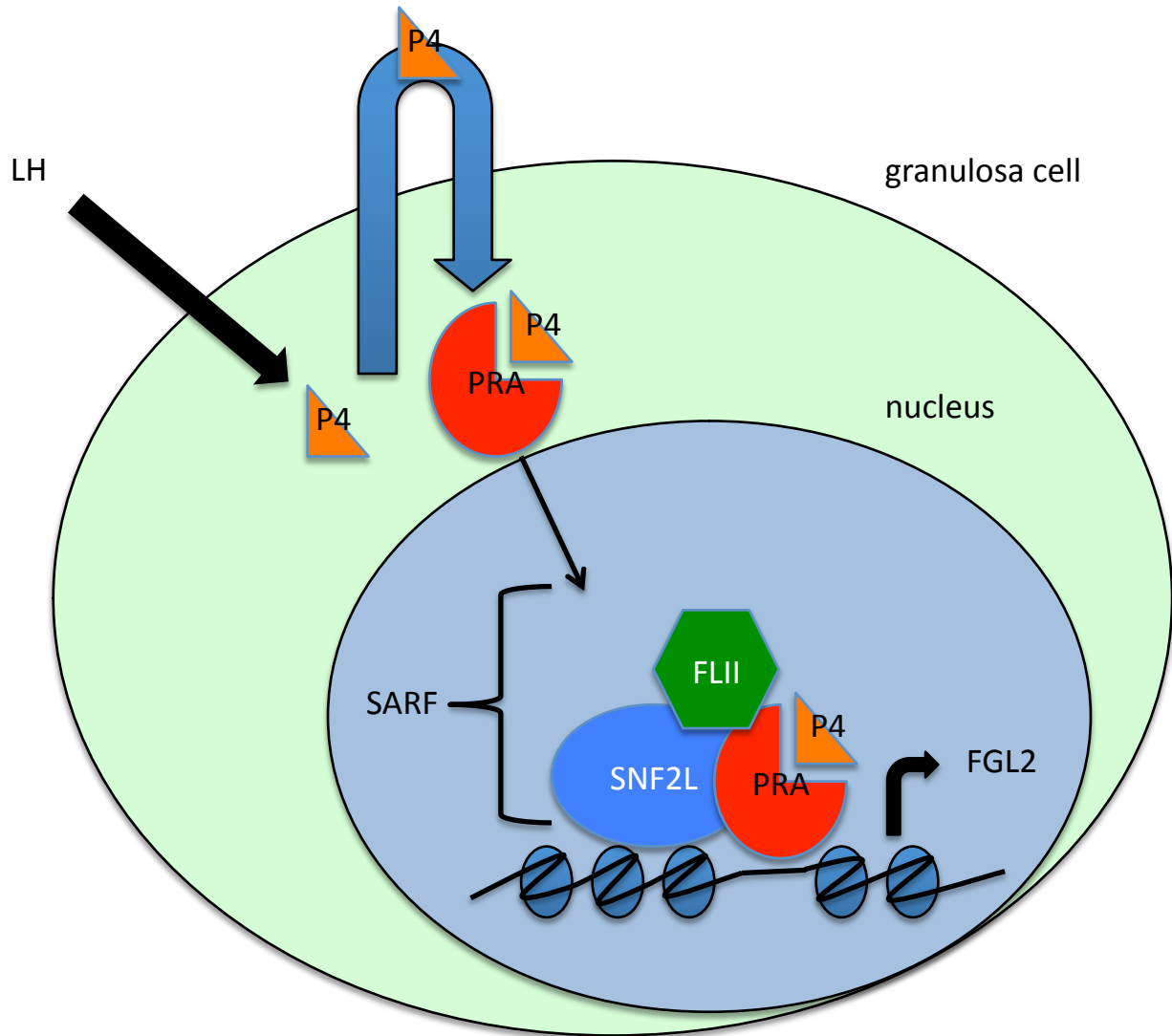


Figure 4.1 Proposed model of *Fgl2* activation by the putative SARF complex. LH triggers the luteinization of granulosa cells and induces the production of P4. P4 acts in an autocrine manner to activate PRA. PRA interacts with both SNF2L and FLII to induce remodeling of the *Fgl2* promoter and facilitate transcriptional activation. The PRA/SNF2L/FLII interaction may underlie a novel putative complex termed SARF, which may be important for the activation of PRA-responsive genes such as *Fgl2*.

Snf2L KO could be explained by depressed steroid hormone signalling. The impaired ovulation because of deficient tissue remodelling observed in the PR-A KO is similar to the reduced superovulation yield, and trapped oocytes found in the *Snf2L* KO (Robker et al. 2000a).

ADAMTS-1 and CTSL are important for tissue remodelling in the antral follicle following the LH surge (Robker et al. 2000a), and our data suggest that FGL2 could be another peptidase whose expression is dependent on PR-A. Further research will be needed to establish the function of FGL2 in the ovary, either in its membrane-bound or soluble state. One possibility is that it may use its pro-thrombinase activity to induce a hypercoagulable state in the follicular fluid at the time of ovulation, which could protect the ovary against haemorrhage following follicular rupture. The observed trend of increased haemorrhagic follicles in the *Snf2L* KO mouse, albeit not statistically significant, may be a consequence of low *Fgl2* expression. Another function of FGL2 could be to activate thrombin not for its pro-coagulant properties but rather for its ability to induce PAR-1 signalling in the granulosa cells (Osuga et al. 2008). PAR-1 is emerging as an important player in antral follicle maturation and CL formation. PAR-1 induces gelatinase activity in the antrum, by promoting both MMP9 and MMP2 (Hirota et al. 2003). These matrix metalloproteases in turn are important in cleaving type IV collagen fibres and remodelling the pre-ovulatory follicles, which could also have implications on steroidogenesis (Ke et al. 2004). A second function of PAR-1 is the stimulation of the chemoattractive molecules IL8 and MCP1, by inducing NF- κ B transcription (Hirota et al. 2003). These chemokines have been speculated to play a role in setting up the inflammatory state associated with ovulation, and regulating both luteogenesis

and luteolysis (Hirota et al. 2003). Because most of these effectors have not been mutated in mice it is difficult to know whether the resulting phenotype would resemble our observations in *Snf2L* KO mice. It does, however, raise the possibility that some of the defects could be attributed to loss of FGL2 activity.

4.2.3 Putative targets of SNF2L

In addition to the targets mentioned above we have identified a total of 85 potential genes that fail to be induced by hCG in the *Snf2L* KO when compared to WT mice. Here we will discuss those which have been reported to have a function in granulosa cells and warrant further investigation: *Pcsk5*, *Sfrp4*, *Vcan*, *Spry2* and *Egr1*.

PCSK5 is a member of the proprotein convertase subtilisin/kexin family, whose protease activity is required for the activation of certain proteins such as MMPs and proTGF- β .

Interestingly, *Pcsk5* has been found to be transiently induced in granulosa cells and theca cells of rats stimulated with PMSG and hCG at around 3-6h after hCG, corresponding well with the 4h activation window of our screen (Bae et al. 2008). Treatment with the progesterone receptor antagonist RU486 an hour before the hCG injection abolishes the induction of *Pcsk5* suggesting that, like *Fgl2*, it is regulated by the secondary induction of progesterone (Bae et al. 2008). This previous study identified many factors important for antral follicle maturation and predicted to be cleaved by PCSK5 including GDF9, BMP15, inhibin, and members of the MMP and ADAMTS families.

SFRP4 is a secreted frizzled-related protein which is selectively expressed in cumulus granulosa cells but not mural granulosa cells. Its expression is induced 4h following treatment

with hCG and remains elevated up to 24h later (Hernandez-Gonzalez et al. 2006). While *Sfp4* KO mice have normal ovulation, they are infertile because of a failure to produce a normal CL, instead having altered luteal cell morphology and poor vascularization, a phenotype closely resembling the *Prhr* KO (Hsieh et al. 2005).

VCAN is an important proteoglycan that binds hyaluronan during cumulus expansion, prior to ovulation, and is found near the neovasculature in the CL, suggesting that it may play a role in tissue remodelling (Russell et al. 2003b). *Vcan* is induced 10-fold following treatment by hCG, reaching a maximum at 4h, but is not induced by FSH or progesterone (Russell et al. 2003b). VCAN is also a preferred substrate of the protease ADAMTS1, and they colocalize at the time of ovulation, suggesting VCAN digestion may be an important factor in follicular rupture (Richards 2005).

Spry2 encodes a protein of the sprouty family of tyrosine kinase inhibitors which appears to be important for both cumulus granulosa cells and the CL. *Spry2* is transiently induced in granulosa cells by hCG, peaking at 3h, and this effect appears to be a consequence of EGF-like peptides, whereas oocyte-secreted TGF- β factors BMP15 and GDF9 have inhibitory effects on *Spry2* expression (Sugiura et al. 2009). Thus it appears that the oocyte can modulate EGF signalling in granulosa cells via the effects of TGF- β secreted factors on *Spry2* expression. *Spry2* appears to be expressed in human CLs (Sugiura et al. 2009), and was also identified as a marker of oocyte competence in the bovine ovary (Robert et al. 2001).

Egr1 is another gene which was identified in both our screen and also in the study by Robert et al. looking for markers of oocyte competence in the bovine ovary (Robert et al. 2001). *Egr1*

is especially interesting because it is expressed in a bimodal manner, first in the small growing secondary follicles, and then again in an LH-dependent manner in pre-ovulatory follicles where it peaks at 4h after hCG (Espey et al. 2000; Russell et al. 2003a). It appears that EGR1 acts as a transcription factor and induces the expression of the LH-R in luteinizing granulosa cells (Yoshino et al. 2002).

In addition to the aforementioned genes, many other potential candidates were identified that are known to be expressed in differentiating granulosa cells but whose function is less clearly defined, including: *Derl1*, *Rgs2*, *Pde10a*, *Pde4b*, and *Timp1*. Future investigations of the contributions of SNF2L to follicular development and ovulation should include a consideration of these putative SNF2L target genes.

4.2.4 Binding partners of SNF2L

By using an immortalized rat granulosa cell line and overexpressing a flag-tagged Snf2l we were able to immunoprecipitate a variety of interacting proteins which were then sequenced by mass spectrometry. Importantly we were able to pull down and sequence the bait itself, SNF2L, from the precipitate, demonstrating that the flag antibody was specific. In addition to the previously discussed FLI-I, many of the interacting partners have an established role in chromatin remodelling. LRPPRC for example is known to interact with CECR2, the main component of the SNF2L-containing complex CERF (Liu et al. 2002). Although CECR2 itself is restricted to neuronal tissues, it is possible that LRPPRC may participate in other SNF2L-containing complexes. The immunoprecipitation also pulled down PRMT5, a protein arginine methyltransferase, although this interaction is unlikely to be specific as it is a frequent contaminant when using FLAG antibodies (personal communication, J. W. Whetstone). A

number of cytoskeletal proteins were also identified including spectrins, actins and filamins. It is unclear whether those interactions are specific, since we used whole-cell lysates; however, all three components have well established roles in transcription and chromatin remodelling in the nucleus (Castano et al. 2010; Zheng et al. 2009; Yue et al. 2009; Young & Kothary 2005). Another candidate, nucleolin is a nucleolar protein that is involved in ribosomal maturation, which may also play a role as a histone chaperone facilitating ACF-dependent remodelling (Angelov et al. 2006).

A number of putative binding partners are involved in translation, including EIF5b, EIF2s3, EIF2s2, RPL4 and PABPC1. While this association may seem counter-intuitive, there is increasing evidence that some translation may occur in the nucleus, and much of the machinery, such as ribosomes and translation initiation factors, have been detected in the nucleus (Dahlberg et al. 2003). One possible role of nuclear translation may be nonsense-mediated decay, a process by which nascent transcripts may be scanned by ribosomes in search of premature stop codons, which could trigger transcript degradation (Iborra et al. 2004). To achieve this type of scanning, both the translation machinery and the transcription machinery must intimately interact, a process that could be facilitated by chromatin remodeling. While chromatin remodelers are not known to participate in nonsense-mediated decay it may be an exciting area of investigation.

4.2.5 Folliculogenesis in *Snf2l* KO mice.

One of the most intriguing phenotypes of the *Snf2l* KO mice is the deficit in secondary follicles and the trend for increased primary follicles. This peculiarity is compounded by the fact that antral follicle numbers are unaffected, suggesting that enough secondary follicles

make it to the antral stage to generate a normal pool. Because we differentiate primary and secondary follicles based on the number of granulosa cell layers, such observation could be the result of a growth defect of granulosa cells. As we have discussed, secondary follicle growth does not require sex steroids or gonadotropins but is dependent on growth signals originating from the oocyte, particularly members of the TGF- β superfamily such as GDF9 and BMP15. It is possible that the response to GDF9 or BMP15 may be muted in the granulosa cells of *Snf2l* KO mice, and further research will be needed to reveal whether the putative targets *Spry2* or *Pcsk5* may play a role in this process. It appears however that secondary follicles are still able to grow under the stimulation of the estrogenic compound DES, suggesting the growth deficit is not a result of their inability to replicate, but more likely a defect in the TGF- β response. The downstream effectors of TGF- β signalling is the SMAD family of transcription factors. Interestingly, the SNF2L-containing NURF complex is known to interact with SMAD proteins, and co-regulate some of the targets of this pathway in mouse embryonic stem cell lines (Landry et al. 2008). Additionally, the abnormal antral follicles we observe with poor mural granulosa growth and no cumulus cells are identical to the phenotype observed in *Smad4* KO mice (Pangas et al. 2006b). Together these observations suggest that SNF2L may play a role in co-regulating SMAD4 targets in granulosa cells.

Another pathway which may be facilitated by SNF2L and/or NURF is the JAK/STAT signalling pathway. As previously mentioned STAT5a and STAT5b are required for proper differentiation of granulosa cells and CL formation, and they negatively regulate the cell cycle inhibitor P27kip1 (Teglund et al. 1998). NURF is known to regulate STAT targets in testis somatic and germ cells (Cherry & Matunis 2010), and NURF mutants in drosophila develop

melanotic tumours as a result of JAK/STAT dysregulation in blood cells (Badenhorst et al. 2002). However, CL formation appears to be normal in *Snf2l* KO mice, which in fact produce significantly more progesterone than control mice. It is unclear why *Snf2l* KO mice have significantly higher circulating progesterone levels at 48h after hCG, considering luteal cells from *Snf2l* KO mice have equivalent levels of P450_{scc}, 3 β -HSD and more CYP17 than control mice (data not shown).

Surprisingly, the *Snf2l* KO does not resemble the *Bptf* KO, which is embryonic lethal at dpc 8.5 (Landry et al. 2008). This raises a number of questions about the role of SNF2L in the NURF complex. One possibility is that in the absence of SNF2L, SNF2H can take part in the NURF complex and compensate functionally. It is known for example that a small proportion of ACF and ChRAC complexes contain SNF2L instead of SNF2H (Bozhenok et al. 2002). Another explanation may be that SNF2L's catalytic activity is not required for all of NURF's functions. There is evidence that cells can produce a catalytically inactive splice variant of SNF2L, although its function is unclear (Barak et al. 2004). Either possibility may explain why the *Snf2l* KO phenotype is relatively benign.

4.3 Conclusion

Taken together, the experimental data we have collected suggests that SNF2L may play a role in folliculogenesis. While additional investigation will be needed to confirm some of the putative targets identified by the microarray screen, we have collected convincing evidence that two important genes in granulosa cell function, StAR and FGL2, are regulated by SNF2L. Furthermore we have identified two novel interacting partners, PR-A and FLI-I, which together with SNF2L may be involved in the regulation of progesterone-responsive

genes as part of a putative complex: SARF. Reverse immunoprecipitation experiments will be needed to confirm the existence of such a complex. While we have described a phenotype of gonadotropin resistance in the context of superovulation in mice, it would be of interest to determine if Snf2l is also involved in the poor response that some patients experience during ovarian stimulation (Kim 1995). Furthermore, the chromosomal locus where hSNF2L is located, Xq25-26.1, has been identified as a critical region for both premature ovarian failure and ovarian cancer (Choi et al. 1997; Mumm et al. 2001). Recent studies have found that targeting SNF2L may be a viable approach to treating cancer cells, and leads to DNA damage and apoptosis (Ye et al. 2009). Therefore SNF2L represents a unique target which may be important in the treatment of infertility and ovarian cancer.

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CONTRIBUTIONS OF COLLABORATORS

All the studies included in this thesis were done under the supervision of Dr. Barbara Vanderhyden and/or Dr. David Picketts. Unless otherwise specified, experiments were conducted by David Pépin. Unless otherwise specified, manuscripts in this thesis were written by David Pépin and revised by Dr. Barbara Vanderhyden.

Chapter 2

Title: The imitation switch protein SNF2L regulates steroidogenic acute regulatory protein expression during terminal differentiation of ovarian granulosa cells.

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Authors: Maribeth A. Lazzaro, David Pepin, Nazario Pescador, Bruce D. Murphy, Barbara C. Vanderhyden, and David J. Picketts.

This paper lists both David Pépin and Maribeth A Lazzaro as equally contributing first authors. Maribeth Lazzaro was responsible for the in-situ hybridization assessment of SNF2H and SNF2L, The immunofluorescence microscopy and the immunoprecipitation experiments. Nazario Pescador performed the western blot and ChIP in porcine granulosa cells.

Chapter 3

Title: The ISWI ATPase Snf2L is required for superovulation and regulates Fgl2 in differentiating granulosa cells

Journal: Unpublished manuscript (prepared for submission to Molecular Endocrinology)

Authors: David Pépin, Francois Paradis, Carolina Perez-Iratxeta, David J. Picketts, Barbara C. Vanderhyden.

Dr. François Paradis performed the immunoprecipitation experiments. Dr. Carolina Perez-Iratxeta was responsible for the analysis of the microarray data.

APPENDIX

A.1 Kallikreins 5, 6 and 10 differentially alter pathophysiology and overall survival in an ovarian cancer xenograft model.

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Abstract

Human tissue kallikreins (KLKs) are members of a multigene family of serine proteases aberrantly expressed in many cancer types. In ovarian cancer, 12 KLKs are upregulated, and of those KLK5, 6 and 10 have been the focus of investigations into new diagnostic and prognostic biomarkers. Despite promising results as biomarkers, little is known about the contributions of KLK5, 6 and 10 to ovarian cancer pathophysiology.

In this study, a panel of 13 human ovarian cancer cell lines was screened by ELISA for secretion of KLK5, 6, 8, 10, 13, and 14. The ES-2 cell line, devoid of these kallikreins, was transfected with expression vectors of KLK5, 6 and 10 individually or in pairs. Co-expression of KLK5, 6 and 10 was correlated with decreased aggressivity of ovarian cancer cell lines *in-vitro*. ES-2 clones overexpressing KLK5, 10/5, 10/6, 5/6 made significantly fewer colonies in soft agar. Furthermore, survival of mice injected with ES-2 clones overexpressing KLK10, 10/5, 10/6, 5/6 was significantly longer, while KLK6 was shorter, than survival of control mice. All groups displaying a survival advantage had reduced ascites incidence and an absence of cellular aggregates within those ascites. The survival advantage conferred by KLK10 overexpression could be recapitulated with the exogenous administration of a recombinant KLK10. In conclusion, these findings indicate that KLK5, 6 and 10 may modulate the progression of ovarian cancer, and interact together to alter tumour pathophysiology. Furthermore, results support the putative role of KLK10 as a tumour suppressor and suggest it may hold therapeutic potential in ovarian cancer.

Keywords: Ovarian cancer; Kallikrein; Ascites; KLK5, KLK6, KLK10.

Introduction

The recently discovered tissue kallikreins are a family of secreted serine proteases encompassing 15 members (KLK1-15) whose genes (KLK1-15) are clustered in tandem on a 300kb region on chromosome 19q13.4 [1]. KLK proteins are detected in many biological fluids including blood, seminal plasma, sweat, saliva, cerebrospinal fluid, milk, and interstitial spaces where they can be activated and/or inactivated by enzymatic cleavage [2]. KLKs cleave a broad range of substrates including extracellular matrix (ECM) proteins, insulin-like growth factor binding proteins, protease-activated receptors (PAR), other kallikreins and even themselves [2]. Moreover, KLKs are often expressed in groups, such as KLK3, 4, 5, 6, 8, 10, 13 and 14 in the breast or KLK2, 3, 4, 5, 11, and 15 in the prostate [2]. These observations have led to the hypothesis that kallikreins can act in a cascade to mediate their biological effect, also known as the KLK activome [3]. Preliminary evidence suggests that KLK5 may be an initiator of KLK cascades, such as in the case of the activation of pro-KLK2, 3, 6, 7, 11, 12, 14, resulting in the degradation of ECM components of semen, and liquefaction [4].

Kallikreins have been implicated in a number of diseases such as Alzheimer's and multiple sclerosis [5;6], inflammatory bowel disease [7], arthritis [8], sepsis [9], diabetes [10], skin diseases [11] and cancer [12]. Because KLKs are secreted and readily detectable in biological fluids, they have emerged as potentially valuable diagnostic biomarkers, particularly in cancer, where KLK3 (PSA) has proven to be invaluable for prostate cancer screening. Most KLK are expressed under hormonal control, and the responsiveness of KLK2 and 3 to androgens in prostate cancer cell lines [13], and KLK6 and 10 to estrogens in breast cancer cell lines is well documented [14;15]. The pattern of expression of KLKs, as well as their hormonal regulation, suggests they may be

involved in endocrine-related adenocarcinomas of the reproductive tract such as prostate, testis, breast, cervical, and ovarian cancers.

Accumulating evidence suggests that at least 12 of the 15 kallikreins are upregulated in ovarian cancer. Of those, KLK4, 5, 6, 7, 10, and 15 are associated with unfavorable prognosis while expression of KLK8, 9, 11, 13, and 14 is associated with favorable prognosis [12]. This study focuses on KLK5, 6 and 10 which are frequently overexpressed in ovarian cancer and found in elevated levels in the ascites and serum of patients [16;17;18]. Notably, KLK6 and KLK10 in the serum are indicators of poor prognosis [19;20], and KLK6 is a predictor of disease recurrence [21]. High levels of KLK10 in the serum is associated with advanced stage serous tumours with large residual disease and poor response to chemotherapy [22], while low levels of KLK10 in the tumour predicts poor overall survival [23].

While little is known about the biological basis of the contribution of KLK5, 6 and 10 to ovarian cancer, the ability of KLK5 and 6 to cleave ECM proteins [4;24], and activate PAR signaling [25], suggest that they are directly implicated in various aspects of carcinogenesis. Degradation of ECM components may facilitate the detachment of malignant cells from the tumour and the invasion of normal tissues, while some of the released ECM peptides may have both pro and anti-angiogenic qualities [24;26]. Moreover, PAR signaling has important roles in vasoregulation, cell growth and inflammation [25;27;28]. KLK10 was identified as a putative tumour suppressor in breast [29] and gastric cancers [30], and is often silenced in ovarian cancer cell lines and tumours [31], despite its expression in the serum being an unfavorable prognostic marker. This apparent paradox exemplifies the dichotomy of kallikreins as both positive and negative regulators of processes involved in carcinogenesis such as angiogenesis, growth, invasion, and metastasis [32].

While evidence of aberrant expression of multiple kallikreins in ovarian cancer is mounting, little is known about their contribution to the pathophysiology of the disease. Herein we report the first attempt to unravel the distinct contribution of KLK5, 6 and 10 in the progression of ovarian cancer, and the first use of a recombinant KLK10 for the treatment of a xenograft model of ovarian cancer.

Materials and methods

Cell culture

The origin of the ovarian cancer cell lines Caov-3, OVCAR-3, OVCAR-4, OV2008, C13, OVCA433, SKOV-3, OVCA429, Hey, ES-2, OCC-1, A2780cp, and A2780s used in this study and their culture conditions are described in a previous publication [33]. The cell lines HT1080 and NIH3T3 used as controls were procured from ATCC (Manassas, VA, USA) and cultured according to their recommendations.

Construction of stably transfected ES-2 cell lines over-expressing kallikreins

The plasmids pcDNA3.1D/V5-His/lacZ (Invitrogen, Mississauga, ON, Canada) with geneticin resistance, and pIRESpuro-2 (Clontech, VWR, Mississauga, ON, Canada) with puromycin resistance were used as backbones and stably transfected into the ES-2 cell line to provide vector controls. In short, multiple clones stably transfected with pIRESpuro-2 were used as single vector controls, and multiple clones successively transfected by pcDNA3.1D/V5-His/lacZ and pIRESpuro-2 were used as double vector control. The cDNAs for KLK5, KLK6 and KLK10, as well as the pcDNA-KLK5 expression construct on a pcDNA3.1D/V5-His-TOPO backbone, were kindly provided by Dr. E.P. Diamandis (Toronto, ON, Canada). The

KLK10 expression vector in pCMV-neo was provided by Goyal et al. and has been previously described [34]. Briefly, PCR amplification, restriction digestion and ligation of DNA fragments representing the cDNAs of KLK5, 6, and 10 into the expression vectors pIRESpuro-2 were performed, and the resulting constructs were stably transfected into ES-2 cells. A minimum of 3 clones of each were picked and one was randomly chosen to derive the respective cell lines ES-2-KLK5, ES-2-KLK6, and ES-2-KLK10 for *in vivo* experiments. For double transfectants, a minimum of 3 independent clones of pCMV-neo expressing KLK10 were further transfected with the pIRES-puro-2 expressing KLK5 or KLK6 and one of each was randomly chosen to generate respectively the ES-2-KLK5/10 and ES-2-KLK6/10 cell lines. The cell line ES-2-KLK5/6 was generated from one of the 3 clones by stably transfecting the ES-2-KLK6 cell line with the pcDNA-KLK5 construct. Transfection of ES-2 cells was carried out using LipofectomineTM 2000 (Invitrogen, Mississauga, ON, Canada) according to the protocol provided by the manufacturer. The clones described above were selected and maintained in DMEM media (Thermo Scientific, Waltham MA, USA) containing geneticin (400µg/ml) and/or puromycin (10µg/ml) (Gibco BRL, Carlsbad, CA, USA).

Cell Proliferation Assay

To evaluate proliferation, cell growth was analyzed in the parental ES-2 cells lines and 3 or more clones stably transfected with constructs for KLK5, KLK6, KLK10, KLK5/6, KLK5/10, KLK6/10 or Vector control using 12-well plates with initial plating densities of 10,000 cells/well. After 96 hours, cells were trypsinized and subsequently counted with a Coulter Counter (Beckman Coulter Inc., Fullerton CA, USA).

Anchorage independent growth

The protocol used has previously been described by M. Pace et al [35]. Briefly, 5×10^3 cells were suspended in 3 ml complete medium containing 3.5% low melting-point agarose and poured on top of the bottom layer of 7% agarose in the same medium in wells of a 6-well plate. Medium (0.5ml) was added to each well and changed every 2-3 days. A solution of *p*-iodonitrotetrazolium violet (1ml) was added to each well at day 7 and colonies were stained for 24 hours, counted, and photographed.

Invasion assay

The *in-vitro* invasive potential of the ovarian cancer cell lines was evaluated using BD BioCoat™ Tumour Invasion System (BD Biosciences, Bedford, MA) and the assay was performed according to the manufacturer's instructions. Briefly, after the interior of the insert was re-hydrated with PBS buffer (Gibco BRL) for two hours, 750µl of medium containing 5% FBS was added to the bottom well. Cells (5×10^4) suspended in 500µl serum-free medium were then added to the top chamber and the plate was incubated at 37°C in 5% CO₂ atmosphere for 21 hours. The invasive cells which appeared at the bottom of the insert were stained with 4µg/mL Calcein AM in Hanks buffered salt solution for 1 hour at 37°C and the fluorescence was recorded using CytoFlour™ II fluorescence plate reader (PerSeptive Biosystems, Framingham, MA).

For the invasion assay on the kallikrein overexpressing clones, we used the HTS transwell 96 system® (Corning, Lowell, MA). Briefly, The transwells were coated with basement membrane extracts as instructed by the manufacturer, and cells (5×10^4) in 50µl serum-free medium were then added to the top chamber, while 150µl of media with 10% serum was added to the reservoir. The plate was incubated at 37°C in 5% CO₂ atmosphere for 24 hours. The cells which migrated to

the bottom of the insert were stained with hematoxylin according to the manufacturer's protocol, and the membranes were mounted on slides, scanned using the ScanScope (Aperio, Vista, CA), and the amount of blue pixels was quantified using the Aperio software (Aperio, Vista, CA).

Xenograft

All animal experiments performed in this study were in compliance with the *Guidelines for the Care and Use of Animals* established by the Canadian Council on Animal Care, and were approved by the Animal Care Committee at the University of Ottawa. Female CD-1 nu/nu mice (Charles River Laboratory, Wilmington, MA, USA) aged 5-6 weeks were housed with food and water *ad libitum*, on a 12h daylight cycle. The tumour cell IP xenograft method was described previously [33]. Briefly, after one week of acclimatization, the mice were injected intraperitoneally (IP) with 10^7 ES-2 cells, or one of its derivative clones chosen at random from the cell lines stably transfected with KLK5, KLK6, KLK10, KLK5/6, KLK5/10, KLK6/10, Vector single control, or Vector double control, resuspended in 0.8ml of phosphate-buffered saline. Groups were then blinded until the end of the experiment at day 56. Disease progression was monitored daily, based on general wellness and overall health, while body mass was recorded twice a week until a predetermined endpoint was reached. The time at which symptoms of the disease first appeared, such as mild abdominal distension, or small palpable mass was recorded. Endpoints included: dehydration and/or weight loss of over 15% despite fluid therapy, any evidence of respiratory distress, body weight increase of over 5g from the average body weight of control mice at the same age in the same population, presence of a palpable abdominal mass that impairs mobility or affects wellness and finally presence of abdominal distension that impairs mobility or affects wellness or causes significant discoloration evident on the dorsal or ventral skin.

Upon necropsy, tumour samples were weighed and divided to be either immediately flash-frozen in liquid nitrogen and stored at -20°C , or fixed in 10% buffered formalin (VWR, Mississauga, ON, Canada) for 24 hours and stored in 70% ethanol prior to processing into paraffin-embedded blocks, which were cut into $5\mu\text{m}$ sections for hematoxylin and eosin (H&E) staining. Ascites volume was measured, and the samples were assessed microscopically to determine the presence of cellular aggregates. Samples were then spun at $2500 \times g$ for 10 minutes to collect the supernatant for storage at -20°C for subsequent measurements of KLK levels by ELISA.

Blood sampling

For the survival experiment, blood samples were taken one week prior to injection and weekly according to a predefined schedule until endpoint was reached or the schedule ended. Blood was also taken at endpoint prior to necropsy when possible. For the recombinant KLK10 therapy experiment, each dosage group (0, 0.2, 1, and 5mg) had one animal per timepoint (-1h, 1h, 2h, 4h, 6h, 8h, 12h, and 24h). In both experiments, 100-200 μl of blood were recovered by saphenous vein puncture with a 25G5/8 gauge needle (BD, Franklin Lakes, NJ, USA) and collected into microvettes® CB300LH (Sarstedt, Germany) coated with heparin, centrifuged 5 min at 2000g, and plasma was separated to be stored at -20°C until ELISA were performed.

ELISA of kallikreins

For the panel of ovarian cancer cell lines, cells were cultured in 24-well plates with 5×10^4 cells and 1 ml of medium per well. Media samples were collected after incubation at 37°C for 3 days. ELISAs for KLK5 [36], KLK6 [37], KLK8 [38], KLK10 [39], KLK13 [40], and KLK14 [41]

were performed according to the protocols published previously. For the ES-2 clones secreting KLKs, a sample of media, after overnight culture, was collected on the day of the xenograft for ELISA analysis of KLK5, 6 and 10. Similarly, ELISA were performed on both human ascites samples and mouse serum and ascites samples diluted from 5-fold to 8000-fold, depending on the KLK concentration, in a dilution buffer (50mM Tris-Cl pH7.8, with 60mg/ml of BSA and 0.5mg/ml of sodium azide). Samples of ascites from women with ovarian cancer were obtained from the Ottawa Ovarian Cancer Tissue Bank with appropriate patient consent and approval from the Research Ethics Board of the Ottawa Hospital.

Recombinant KLK10 production

KLK10 cDNA was amplified by PCR using oligos KLK10FP (TATACGTAGCGCTGCTCCCCAAAACGACAC) and KLK10RP (GTCCTAGGATCGATTGGAGCGTATGAC) [34] from a pCMV-neo vector carrying KLK10 cDNA. After double digestion with *Sna*BI and *Avr*II, the amplified DNA fragment was inserted into pPIC-9, pre-digested with *Sna*BI and *Avr*II. The resulting plasmid, pPIC-KLK10, was then transformed into the *Pichia pastoris* host strain KM71 by electroporation (*Pichia* Expression kit, Invitrogen life technologies).

Fermentation of 15-litres of recombinant KLK10 was conducted using a BIOSTAT® ED fermenter (B.Braun Biotech International, Allentown, PA, USA) and a process based on *Pichia* fermentation Process Guidelines from Invitrogen. Briefly, fermenter was inoculated with an inoculum prepared in a 2800 ml shaker-flask for a starting OD₆₀₀ of ~0.3. After a 20-hour glycerol batch phase, a 4-hour glycerol feed phase was followed. Induction was initiated by starting glycerol

feeding and lasted for about 40 hours. Cells were removed by centrifugation and supernatant was collected.

Purification of KLK10 from the supernatant was carried out using a CM-sepharose column (Amersham Biosciences, ON, Canada) as described previously [39].

Treatment with recombinant KLK10

For the blood clearance experiment, we first tested a single bolus dose IP of recombinant KLK10 (0, 0.2, 1, and 5 mg in 1 ml) with 5 nu/nu mice per dose and sampled the blood at various time points as described above. The animals were closely monitored for the first 12h, and then periodically for 15 days before being sacrificed.

For the toxicity experiment we tested doses of 0, 50, 200, and 800µg in 1ml of KLK10, administered daily IP in 3 animals per group for 7 days, followed by 7 days of daily monitoring with no treatment before being sacrificed. At necropsy, the liver, lung, heart and kidney were removed and divided to be either immediately flash-frozen and stored at -20°C or fixed in 10% buffered formalin (VWR, Mississauga, ON, Canada) for 24 hours and stored in 70% ethanol prior to processing into paraffin-embedded blocks, which were cut into 5µm sections for H&E staining. Sections were analyzed for signs of inflammation and damage.

For the therapeutic experiment, the nude mice were randomly divided into one control and 2 treatment groups (8 animals/group). Treatment duration was 14 days and the study ended at 8 weeks post-xenograft. Animals still alive at the end of the study were sacrificed. On day 1, animals were injected IP with 0.2ml of PBS buffer, or 0.2ml of PBS containing 5mg of KLK10 followed immediately by an injection of 10^7 ES-2 cells resuspended in 0.8mL PBS buffer. From then on,

1ml of PBS buffer or 1 ml of PBS containing 5mg of KLK10 were injected IP to each animal either daily or twice daily (as indicated) from day 2 to day 14.

For the *in-vitro* treatment experiment, 10^5 ES-2 cells per well were seeded in a 12-well plate containing either serum-free DMEM or DMEM with 10% fetal calf serum, and supplemented with 4 doses of recombinant KLK10 (0, 300ng/ml, 3000ng/ml and 30000ng/ml) for 96h. Cell viability was determined by trypan blue exclusion using a ViCell Counter (Beckman Coulter, Fullerton, CA).

Survival curves and statistical analyses

Kaplan-Meier survival curves were plotted using GraphPad Prism 4.0 software (Graphpad Software, San Diego, CA, USA) and compared using a logrank test. Pathophysiological parameters such as ascites volume and tumour burden and results from *in-vitro* experiments were compared by one-way ANOVA followed by Tukey's post test. Proportions such as incidence of aggregates or ascites were compared by CHI square. Statistical significance was inferred at $p < 0.05$.

Results

Secretion of kallikreins 5, 6 and 10 correlates with reduced aggressiveness in a panel of ovarian cancer cell lines, yet is detectable in the ascites of ovarian cancer patients.

Expression of the kallikrein cluster including KLK4 to KLK14 has previously been reported in ovarian cancer [32]. However it has also been reported that different kallikreins can have diametrically opposed effects on patient prognosis in a variety of cancers [32]. To verify that kallikrein expression is recapitulated in ovarian cancer cell lines, a panel of thirteen ovarian cancer cell lines (CAOV-3, OVCAR-3, OVCAR-4, OV2008, C13, OVCA433, SKOV-3, OVCA429, Hey,

ES-2, OCC-1, A2780cp, A2780s) was screened for secretion of KLK 5, 6, 8, 10, 13 and 14 into the culture media by ELISA (supplemental Table 1). On the basis of kallikrein expression, the cell lines could be segregated into non-expressors (SKOV-3, OVCA429, Hey, ES-2, OCC-1, A2780cp, A2780s) and expressors (CAOV-3, OVCAR-3, OVCAR-4, OV2008, C13, OVCA433). In the latter group, all shared common expression of KLK5/6, and 5 of 6 expressed KLK10, 4 of 6 KLK8, 3 of 6 KLK13 and none expressed KLK14. To investigate any link between kallikrein expression and aggressiveness of the cell lines, these two groups were compared for their ability to invade into matrigel, form colonies in soft agar and develop tumours intraperitoneally in nude mice (supplemental figure A.1). In contrast to the non-expressors, the cells expressing kallikreins did not invade matrigel, did not form colonies in soft agar, and as previously reported by us [33], were very poor at forming tumours in nude mice (Table A.1). A panel of 11 ascites samples from ovarian cancer patients was screened by ELISA to determine the incidence rate of this KLK5, 6 and 10 co-expression pattern (supplemental figure A.2). The panel of ascites samples included patients with primary ovarian tumours characterized as: endometrioid carcinoma (1), clear cell carcinoma (2), serous carcinomas (4), or poorly differentiated carcinoma (4). All samples were post-chemotherapy including cisplatin, carboplatin, taxol and/or topotecan. No trends or correlations were seen with kallikrein expression levels and treatment or histological subtype. All samples contained KLK6 and 10, while only 64% of the samples contained detectable levels of KLK5. Of note, the concentration of KLK6 (412.9 ± 82.6 ng/ml) was, on average, much higher than KLK5 (42.4 ± 27.7 ng/ml) and KLK10 (80.7 ± 39 ng/ml).

Table 1. Kallikrein expression profile and tumourigenicity of a panel of 13 ovarian cancer cell lines.

Cell Lines	KLK Expression (in media)	In vitro Invasion (matrigel)	Anchorage Independence (soft agar)	Xenograft (in mice) [27]	
				Tumour incidence	Median survival (days)
CAOV-3	5,6,8,10	-	-	unknown	N/A ^b
OVCAR-3	5,6,8,10	-	-	0/6	N/A
OVCAR-4	5,6,8,10,13	-	-	unknown	N/A
OV2008	5,6,8,10,13	-	-	4/5	66
C13	5,6,10,13	-	-	0/5	N/A
OVCA433	5,6	-	-	0/3	N/A
SKOV-3	ND ^a	+/-	+/-	2/7	105
OVCA429	ND	+	+	3/3	62
HEY	ND	+	+	2/3	24
ES-2	ND	+	+	5/5	16
OCC-1	ND	-	+	3/3	15
A2780cp	ND	-	+	3/3	24
A2780s	ND	-	+	3/3	46

^a ND=not detectable; ^b N/A=not applicable

Stable overexpression of KLK 5, 6 and 10, alone or in pairs, in clones of the kallikrein-deficient ES-2 cell line, results in altered anchorage-independent growth but does not affect cellular proliferation or invasive potential.

KLK5, 6 and 10 were the most commonly expressed kallikreins in the less aggressive ovarian cancer cell lines suggesting a correlation between the expression of those kallikreins and tumourigenic potential. To tease apart the roles of each kallikrein and their interactions on tumourigenicity, ES-2 cells which do not express any of the tested kallikreins (Table A.1) were stably transfected with expression vectors for KLK5, 6, 10 alone or in pairs. 3 or more clones of KLK5, 6, 10, 5/6, 5/10, 6/10 along with empty plasmid control and unmodified ES-2 cells were compared for anchorage-independent growth, proliferation and invasion (Fig A.1). Expression of KLK5, 5/6, 5/10, and 6/10 was sufficient to significantly reduce the ability of ES-2 cells to form colonies in soft agar when compared to vector-only control, but did not alter the rate of proliferation over 96h or modulate the ability of the clones to invade in a transwell assay.

Stable overexpression of KLK 5, 6 and 10, alone or in pairs, in clones of the kallikrein-deficient ES-2 cell line, results in altered survival of a mouse xenograft model.

To investigate whether differential kallikrein expression could regulate the aggressiveness of ovarian cancer cells *in-vivo*, an intra-peritoneal (IP) xenograft model was employed. For this purpose the ES-2 ovarian cancer cell line is ideal, since it does not express kallikreins 5, 6 and 10 (Table A.1) and readily forms rapidly-progressing tumours IP in nude mice that are accompanied by ascites, thus mimicking disease progression in humans [33]. Clones derived from this cell line, stably secreting KLK5, 6, 10, 5/6, 5/10 and 6/10 in the culture media along with the appropriate empty vector controls (Table A.2) were injected IP in immunodeficient nu/nu mice. The mice were injected with 10^7 cells of each clone per animal, in groups of 8, which were then blinded, and closely monitored for endpoints. Survival of the group expressing KLK5 did not differ from the

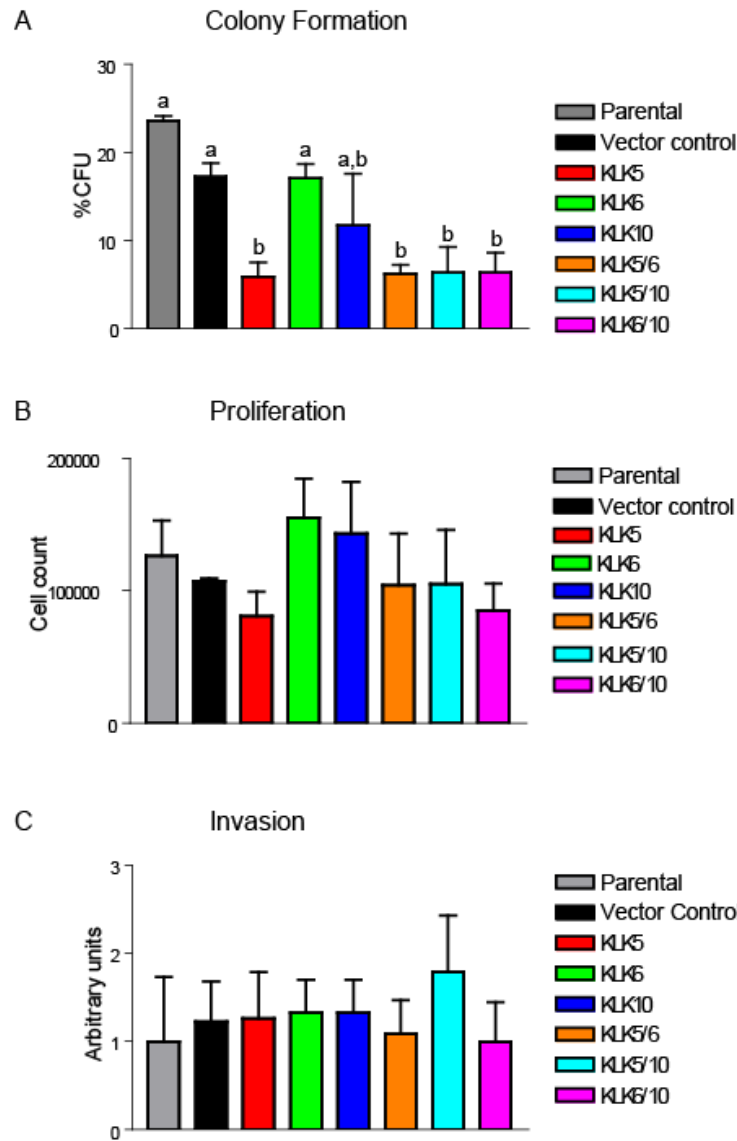


Figure A.1 Clones overexpressing KLK5, 6 and 10, alone or in pairs, display differential anchorage-independent growth but do not differ in proliferation or invasive capacity. Three or more clones of the ES-2 cell line overexpressing KLK5, 6 or 10 or pairs of KLK5/6, KLK5/10 and KLK6/10 were compared to parental ES-2 cells or vector-transfected controls *in-vitro* for their tumourigenic potential. A) Clones were grown in soft agar and the number of colonies was counted and is represented as percentage of the cells which formed colonies. B) Clones were grown for 96h in serum-containing media and cell numbers were counted. C) Clonal cells resuspended in serum-free media were deposited in an insert coated with basement membrane extract and allowed to invade the transwell bathing in media with 10% serum for 24h and migrating cells were quantified. The results are shown as the mean of 3 or more clones $\pm$ SEM, and significance is inferred by one-way ANOVA with post test if $p < 0.05$ and is indicated by different letters above each bar. In C, the data are normalized to the parental control.

Table 2. Stable overexpression of KLK5, 6 and 10, alone or in pairs, in clones of the ES-2 cell line results in secretion of kallikrein into the cell culture media.

ES-2 clone	Kallikrein level in media (ng/ml)		
	hK5	hK6	hK10
Parental	<0.2	<0.2	<0.5
Single vector control	<0.2	<0.2	<0.5
Double vector control	<0.2	<0.2	<0.5
KLK5	227	<0.2	<0.5
KLK6	<0.2	352	<0.5
KLK10	<0.2	<0.2	316
KLK5/6	58	202	<0.5
KLK5/10	74	<0.2	40
KLK6/10	<0.2	260	1400

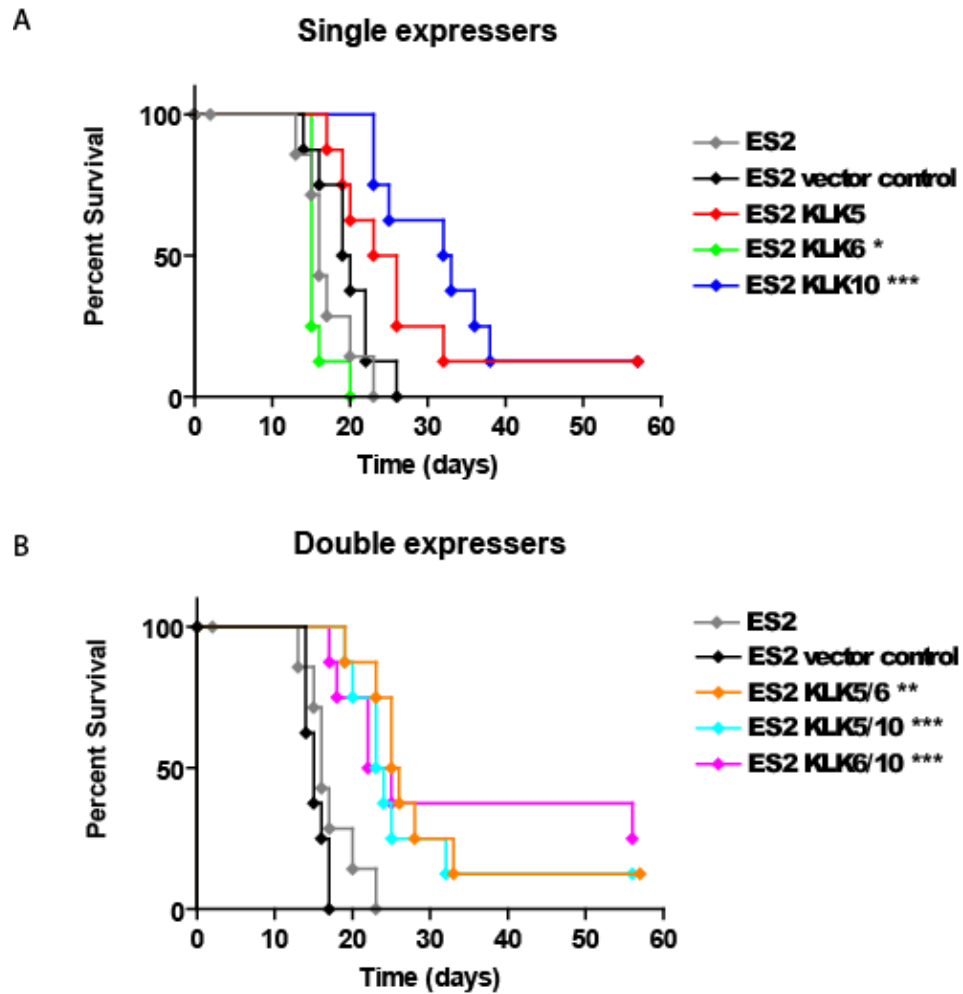


Figure A.2 Clones overexpressing of KLK5, 6 and 10, alone or in pairs, differentially affect survival times when xenografted into nude mice.

A) Clones of the ES-2 cell line overexpressing KLK5, 6 or 10 or B) pairs of KLK5/6, KLK5/10 and KLK6/10 were injected IP in nude mice and survival was compared to control mice xenografted with the appropriate vector backbone clones. The results are shown as a Kaplan-Meier plot, and significance using a logrank test versus appropriate control was inferred at $p < 0.05$. * denotes $p < 0.05$, ** denotes $p < 0.01$, and *** $p < 0.001$.

control group (Fig A.2) while survival of the groups expressing KLK10 ($p<0.001$), KLK5/6 ($p<0.01$), KLK5/10 ($p<0.0001$), and KLK6/10 ($p<0.0001$) was significantly longer than their appropriate controls by logrank test (Fig A.2). Survival of the group overexpressing KLK6 alone was significantly shorter ($p<0.05$) than the control cell line but not the parental line. The groups KLK5, KLK10, KLK5/6 and KLK5/10 each had one disease-free surviving mouse, while group KLK6/10 had two, upon study termination at day 57, whereas all the mice in the control groups developed disease.

Mice xenografted with kallikrein-secreting tumours display changes in pathophysiology.

To clarify the link between KLK secretion and survival, several disease-related metrics were compared across all groups upon necropsy (Table A.3). The most prevalent endpoint was abdominal distension (82%) resulting from ascites accumulation, followed by respiratory distress (8%) caused by pleural effusions, dehydration and weight loss (7%), and finally impaired mobility (3%). Some animals were not endpointed because of the absence of disease upon study termination ($N=8$), or because they died of the disease prior to being endpointed ($N=6$). Tumour histology, spread and sites of metastases were similar amongst groups, with a preference for the omentum, peritoneal membrane, diaphragm, reproductive organs, liver and intestines. Both tumour burden and ascites volume were recorded in animals who reached endpoint, and non-zero values were used to calculate the mean (Table A.3). Mean ascites volume did not differ between groups with the notable exception of control-double which progressed past their distension endpoint before being sacrificed because of their rapid rate of disease progression. A statistically significant lowered incidence of ascites at necropsy was observed in animals of groups KLK5/10 ($p<0.01$) and KLK6/10 ($p<0.01$) with only 37.5% occurrence rate when compared to control

Table 3. Nude mice xenografted with ES-2 derived clones overexpressing KLK5, 6 and 10, alone or in pairs, develop different pathophysiologies.

	N	Ascites (%)	Mean ascites Volume (ml)	Aggregates (%)	Mean kallikrein concentration in ascites (ng/ml)	Mean tumour burden (g)	Median kallikrein onset (days)	Median symptom onset (days)
Parental	7	100	3.97 ±0.52	85.7	ND ^a	0.72 ±0.16	N/A ^b	13
Single vector control	8	87.5	2.57 ±0.79	85.7	ND	1.34 ±0.19	N/A	16.5
KLK5	8	75	3.70 ±0.58	33.3	905 ±352	1.65 ±0.17	12	21
KLK6	8	87.5	3.97 ±0.51	71.4	522	1.15 ±0.18	5	14
KLK10	8	87.5	3.86 ±0.40	0*	3680 ± 850	1.53 ±0.16	19.5	24.5
Double vector control	8	100	8.23 ±0.38	100	ND	1.40 ± 0.15	N/A	13
KLK5/6	8	75	4.25 ±0.73	0*	KLK5 1890 ±836 KLK6 1290 ±513	1.26 ±0.16	8	20
KLK5/10	8	37.5*	3.52 ±0.87	0*	KLK5 393 ±112 KLK10 464 ±301	2.34 ±0.23	14	21
KLK6/10	8	37.5*	4.00 ±1.58	0*	KLK6 1612 ±684 KLK10 16618 ±1230	3.97 ±0.42 *	7	16.5

^a ND=not detectable; ^b N/A=not applicable; * denotes statistical significance relative to appropriate control at p<0.05 by CHI-square or one-way ANOVA. Mean values are indicated ± SEM.

groups which all developed ascites. Paradoxically, the KLK6/10 group also had on average a higher tumour burden ($p < 0.01$), likely because of the longer ascites-free survival. Amongst the animals that did develop ascites within the groups of KLK5, KLK10, KLK5/6, KLK5/10, KLK6/10, a significantly lower incidence of multicellular free-floating aggregates in the ascites was recorded (Table A.3). The aggregates present in the ascites were compact spheres of cells of uniform size ($\sim 1\text{mm}^3$) visible to the naked eye. The kallikrein concentrations measured by ELISA in the ascites showed levels of KLK6 (Table A.3) to be comparable to levels seen in patient ascites, while levels of both KLK10 and KLK5 were elevated in comparison to the patient samples, especially in the combination groups.

The survival time of each group of mice can be divided into a period prior to onset of symptoms, followed by a symptomatic period which culminates at endpoint. Variability between groups is already present when looking at the onset of symptoms (Table A.3), suggesting kallikreins may affect early disease progression. To follow the early disease progression, plasma kallikrein levels were measured by ELISA in each animal weekly and upon necropsy, to serve as a surrogate marker of tumour burden. Kallikreins were detectable in the plasma well before the onset of the first symptoms in all mice, suggesting that asymptomatic trace disease is detectable by measuring circulating kallikreins. In all groups, serum concentration quickly rises and culminates with endpoint as seen in the last three measurements (Fig A.3). Additionally, the surviving tumour-free animals of groups KLK5/6, KLK5/10, and KLK6/10 (Fig A.2B) did not display any detectable levels of kallikreins in the plasma for the duration of the study.

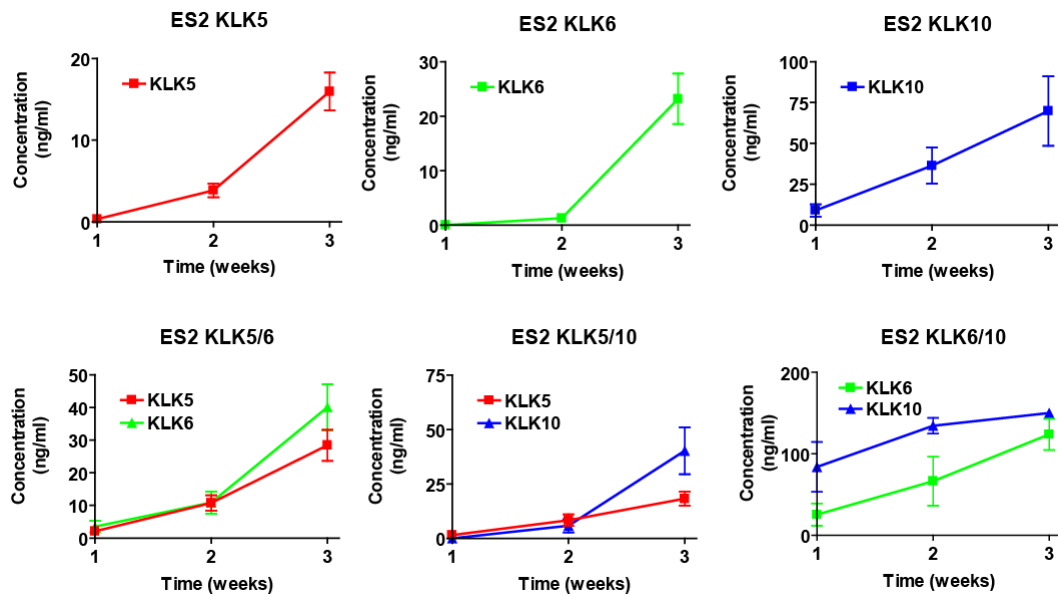


Figure A.3 Plasma kallikrein levels reflect the progression of the disease in xenografted mice. Plasma kallikrein levels were recorded weekly by ELISA in the mice xenografted with ES-2 clones overexpressing of KLK5, 6 and 10, alone or in pairs. The last three weekly measurements before endpoint of individual mice were plotted as the mean concentration of the group $\pm$ SEM.

Intraperitoneal administration of recombinant KLK10 recapitulates increased survival in an ES-2 xenograft model.

To further confirm that the observed anti-tumourigenic effects of kallikreins were specific, a survival experiment using recombinant KLK10 was performed since, as a single agent, it showed the most promise (Fig A.2A). A pilot study was first conducted to ensure the recombinant KLK10 had no side effects in healthy mice before testing it in tumour-bearing animals. A single bolus IP dose (0, 0.2, 1, 5mg) of KLK10 or daily IP injections (0, 0.05, 0.2, 0.8mg) for 14 consecutive days were both well tolerated with no changes in body mass or general wellness, and no visible toxicity upon review of tissue sections of the liver, lung, heart and kidney (data not shown). The drug was judged safe and suitable for treatment of tumour-bearing animals, with doses of up to 5mg being completely cleared of the blood by 12h (Fig A.4A).

To test the efficacy of the recombinant KLK10, IP doses of 5mg were administered once or twice daily for 14 days and compared to PBS injected control in the ES-2 xenograft model. Statistically significant increases in survival were observed in animals treated with recombinant KLK10 at 5mg once daily ($p < 0.05$), and twice daily ($p < 0.01$), and one complete responder was found to be disease-free at the end of the study (Fig A.4B).

The recombinant KLK10 doses injected IP lead to plasma concentrations orders of magnitude higher at one hour (Fig A.4A) than the highest doses recorded in KLK10 tumour-bearing mice (Table 3), albeit transiently. To investigate if such doses could be cytotoxic, ES-2 cells were treated *in-vitro* with increasing concentrations of recombinant KLK10 for 96h in the presence or absence of serum. Recombinant KLK10 caused significant cell death when compared to PBS-treated control, although this effect was completely inhibited by adding 10% serum to the culture media (Fig A.4C).

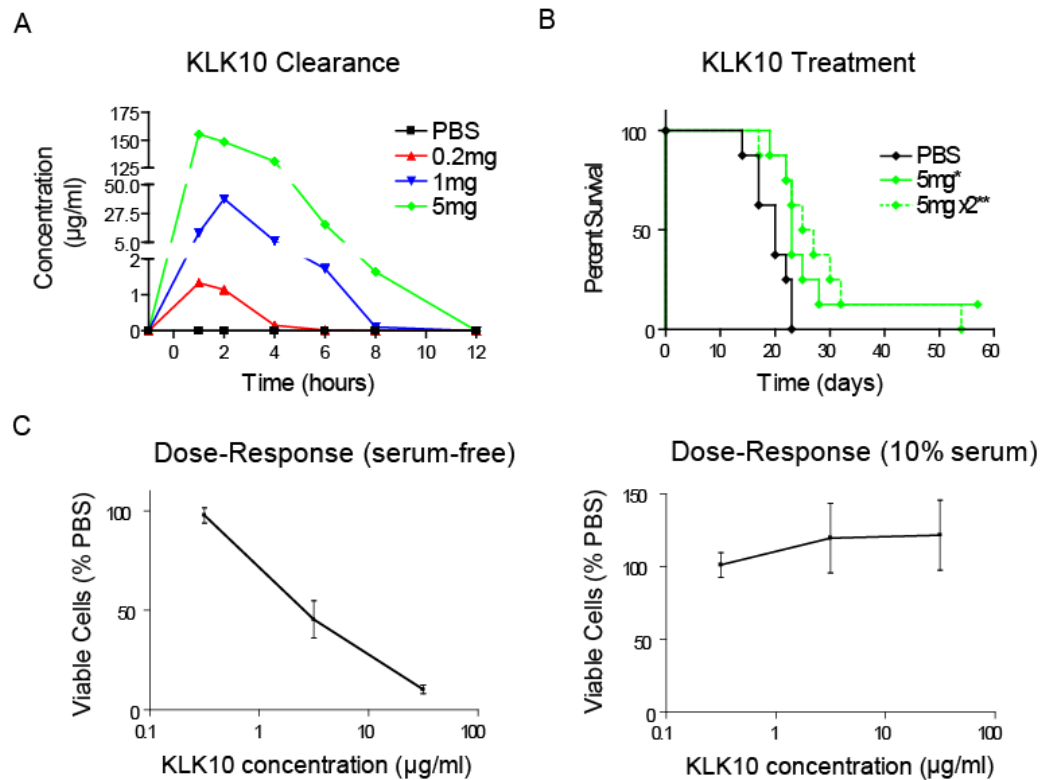


Figure A.4 Mice xenografted with ES-2 cells were treated with various IP doses of recombinant KLK10. A) Mice were injected with a bolus of recombinant KLK10 IP and blood samples were taken at different time intervals to measure plasma concentrations of KLK10 by ELISA. B) Mice were injected IP with either PBS or recombinant KLK10 daily or twice daily for 14 days post xenograft with ES-2 in a survival experiment. C) ES-2 cells were treated with various doses of recombinant KLK10 (0, 0.3, 3, 30 µg/ml) for 96h in serum-free or serum-containing media and cell viability was determined by trypan blue exclusion. * denotes $p < 0.05$, ** denotes $p < 0.01$, and *** $p < 0.001$.

Discussion

This study revealed for the first time a correlation between expression of multiple kallikreins (KLK5, 6, 8, 10, 13 and 14) and reduced aggressivity in a panel of 13 ovarian cancer cell lines. Of the kallikreins tested, KLK5, 6 and 10, were the most consistently expressed in cell lines with a less aggressive phenotype, which were incapable of forming colonies in soft agar, invading matrigel or forming tumours in nude mice. Paradoxically, KLK5, 6 and 10 were found to be expressed in a high proportion of ascites of ovarian cancer patients in agreement with other published reports [42], and have previously been associated with poor patient prognosis in ovarian cancer [32]. The role of kallikreins in ovarian cancer progression has been scarcely studied outside of prognostic and diagnostic applications, and their effects in other cancers have been contradictory, in large part due to their pleiotropic and sometimes opposing effects on cell viability and apoptosis, metastasis, angiogenesis, tissue remodeling and EMT [32]. Because kallikreins often act in a cascade and at least 12 kallikreins are concomitantly upregulated in ovarian cancer it is difficult to tease out the individual contribution of each kallikrein to the pathophysiology of this disease.

To systematically investigate the contributions of KLK5, 6 and 10 to ovarian cancer development, the ES-2 cell line was used, since it did not express any of the kallikreins tested, to generate clones overexpressing KLK5, 6 and 10 alone or in pairs. The resulting clones displayed altered anchorage-independent growth *in-vitro*, as well as varying aggressivity *in-vivo*. Cells overexpressing KLK5, 5/6, 5/10, and 6/10 produced significantly fewer colonies in soft agar than vector-transfected controls. Similarly, mice xenografted with cells overexpressing KLK10, 5/6, 5/10, 6/10 had a significant survival advantage over their respective control mice, while mice with KLK6-secreting tumours had significantly decreased survival. The increased survival of the

KLK10 group was reminiscent of the decreased tumourigenicity of the MDA-MB-231 breast cancer cell line overexpressing KLK10 observed by Goyal et al [34]. This observation further supports the hypothesis of KLK10 as a putative tumour suppressor, silenced in prostate, testicular, and breast cancer as well as in acute lymphoblastic leukemia. Furthermore, it may be that the ES-2 cell line is exquisitely sensitive to overexpression of KLK10 since, in these cells, the KLK10 locus is hypermethylated, suggesting that silencing contributed to its transformation [31]. In contrast to the KLK10 group, the mice xenografted with cells overexpressing KLK6 died significantly earlier than the control mice. The increased aggressiveness of the KLK6 clone was not unexpected as KLK6 overexpression is thought to be an early phenomenon in ovarian carcinoma development [43]. KLK6 has been associated with increased invasiveness, growth and angiogenesis, by virtue of its ability to degrade ECM components such as denatured type I collagen, fibronectin, vitronectin and laminin [44], or activate PAR-2 signaling [45] which has been implicated in mediating cellular proliferation in colon cancer cells [46].

The importance of the specific mix of kallikreins present and their relative abundance on the activome is underscored by the drastic difference within our clones, and with other published reports such as the findings of Prezas et al. [47] who have shown that the OV-MZ-6 ovarian cancer cell line engineered to co-express KLK4/5/6/7 displayed an increased tumourigenicity. Furthermore, the data suggests that some kallikreins may have dominant or inactivating/activating effects over other kallikreins, suggesting for example that the drastically different behaviour of KLK6 versus KLK5/6 clones, could be due to the ability of KLK5 to inactivate other kallikreins *in-vitro* [48]. Conversely animals with tumours expressing a combination of KLK6/10 have an intermediate median survival time compared to either KLK6 or 10 alone, suggesting that the effects in this case can oppose each other in an additive manner. The basis of this interaction may

rely on the ability of KLK6 to cleave and activate itself [24], while KLK10's function may be independent of enzymatic activity since it appears to be catalytically inactive *in-vivo* [42]. Taken together these results suggest that kallikreins 5, 6 and 10 can mediate effects important for tumourigenicity, and their interactions may be complex and dependent on the kallikreins' activome.

To understand the mechanisms underlying the survival differences, it was possible to exploit the fact that the implanted tumours secreted kallikreins into the blood and ascites, thus providing us with a means to track tumour burden. The use of kallikreins to track tumour burden has previously been documented in the clinic, most notably with KLK3 (PSA) in prostate cancer [49-51]. It has also been suggested that both KLK6 and KLK10 could be useful diagnostic biomarkers, which, combined with CA-125 can increase the sensitivity of the screening test [22]. Similarly we detected kallikreins 5, 6, and 10 in the circulation well before the onset of any symptoms, and their levels increased as disease progressed, only to peak at necropsy. Interestingly, the disease-free surviving mice of groups KLK5, KLK5/6, KLK5/10 and KLK6/10, never displayed any detectable levels of kallikreins, suggesting a failure to implant or to grow to a detectable size. From these combined data, it is possible to infer that some of the survival effects of kallikreins 5, 6 and 10 are mediated by an inhibition of tumour implantation, possibly because of inhibition of anchorage-independent growth.

The finding that the disease phenotype also varied qualitatively amongst groups gives insights into the contribution of kallikreins to the pathophysiology of ovarian cancer. The most common endpoint in the survival experiment was distension as a result of ascites, therefore a reduction in the incidence of ascites can have a large influence on survival. As such, groups KLK5/10 and KLK6/10 had a marked reduction in the incidence of ascites and a corresponding

longer survival. While the mechanisms by which KLK5, 6 and 10 influence ascites formation has not yet been established, kallikreins are known to mediate processes such as inflammation, oedema, angiogenesis and blood pressure [2], all of which are relevant to ascites accumulation [46;46;52;53]. Furthermore all groups which had a survival advantage had a marked reduction in the incidence of aggregates in the ascites, possibly because the effect of kallikreins on anchorage-independent growth. Aggregates in the ascites of ovarian cancer patients have previously been identified, and may contribute to the spreading of the disease [54]. Taken together, these results raise the possibility that KLK5, 6 and 10 play an inhibitory role in the formation of ascites and the cellular aggregates within it, which consequently reduces the morbidity and mortality of the mice. Paradoxically, all three kallikreins tested are already known to be elevated in ascites of patients [36;42;42], particularly KLK6, albeit at lower levels than what was recorded in the ascites of mice in this study. It is tempting to speculate that patients with high levels of circulating KLK10, particularly in combination with low or null amounts of KLK6, may be less prone to ascites accumulation and those ascites may be less likely to contain cellular aggregates.

The positive and dominant effects of KLK10 on overall survival make it an attractive putative therapeutic agent for ovarian cancer. To test this prospect, a recombinant protein was generated, which was found to be devoid of proteolytic activity, in accordance with earlier published studies [22]. The recombinant KLK10 protein was injected into the peritoneum to maximize the exposure of peritoneal tumours and detached cellular aggregates to the drug. The recombinant KLK10 was found to be well tolerated at up to 5mg, although only a fraction of the drug crossed into the circulation, and it was quickly cleared from the blood. Remarkably, the recombinant KLK10, despite being present only intermittently, was sufficient to significantly increase survival of treated mice at doses of 5mg once or twice daily, and completely cured one of

the mice. While we do not know whether the cells failed to implant in the presence of KLK10 at the time of injection, or the tumours regressed later during the treatment, the substrate-dependence of the clones suggest the former.

The molecular pathway by which the catalytically inactive KLK10 exerts its biological effects remains elusive, despite the accumulating evidence of its tumour-suppressing qualities. *In-vitro* results suggest KLK10 may be cytotoxic to cancer cells at high concentrations and that a component of fetal calf serum can inhibit this toxicity. Taken together these results indicate that the effects observed with the KLK10 secreting clones on survival and on the pathophysiology are specific to KLK10 and could be partially recapitulated with a recombinant protein, suggesting it may have therapeutic value. Finally these findings support the hypothesis that KLK10 is a tumour suppressor and further underline the involvement of KLK5, 6 and 10 in ovarian pathophysiology.

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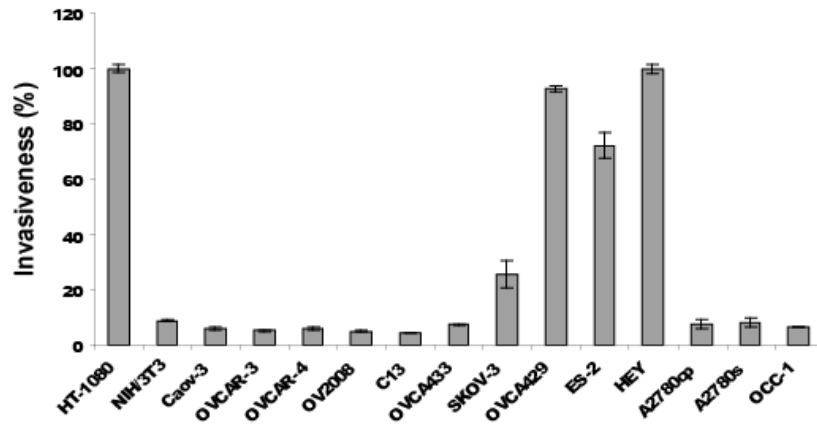
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Article Précis

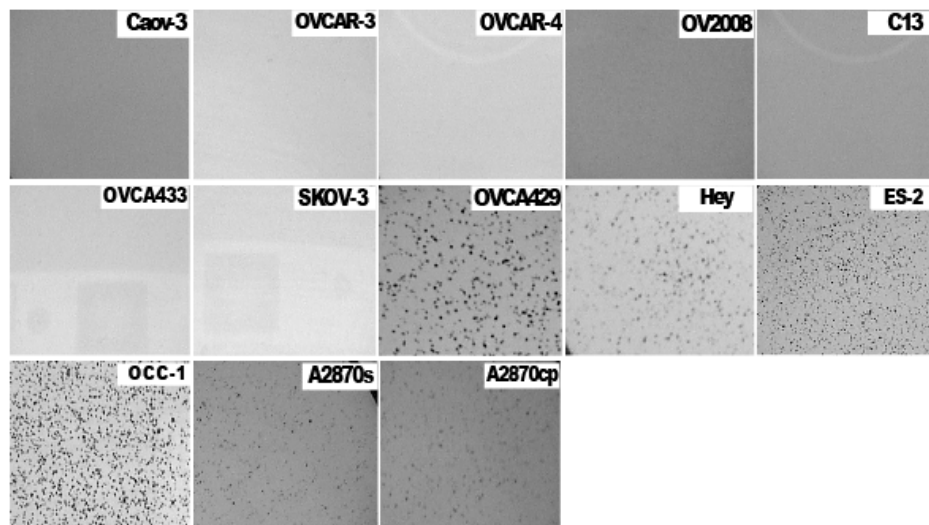
In a xenograft model of ovarian cancer, kallikrein 10 expression can increase survival and lower the incidence of ascites, suggesting a novel therapeutic approach.

Supplemental figures and tables

A



B



Supplemental figure A.1 Transwell invasion and colony formation in soft agar in a panel of 13 ovarian cancer cell lines. A) The ovarian cancer cell lines CAOV-3, OVCAR-3, OVCAR-4, OV2008, C13, OVCA433, SKOV-3, OVCA429, HEY, ES-2, OCC-1, A2780cp, A2780s were compared for their abilities to invade matrigel. Invading cells were quantified using a fluorescent plate reader and compared to a positive control HT-1080 and a negative control NIH3T3. Values represent the mean of 3 experiments +/- SEM. B) The formation of colonies in soft agar was also assayed and representative photographs are shown.

Supplemental Table 1. KLK 5, 6, 8, 10, 13 and 14 concentrations, in the media at 72h, in a panel of 13 ovarian cancer cell lines.

Cell lines	Kallikrein concentration in culture media (ng/ml)					
	KLK5	KLK6	KLK8	KLK10	KLK13	KLK14
CAOV-3	138 ± 32	6.0 ± 2.3	0.4 ± 0.5	4.3 ± 4.0	ND	ND
OVCAR-3	4.2 ± 0.7	2.1 ± 0.5	2.2 ± 0.5	1.0 ± 0.9	ND	ND
OVCAR-4	0.4 ± 0.2	3.5 ± 1.7	0.5 ± 0.2	3.9 ± 1.3	0.1 ± 0	ND
OV2008	18.4 ± 2.6	3.4 ± 0.3	4.5 ± 1.1	27.9 ± 18.9	7.5 ± 2.8	ND
C13	6.6 ± 1.0	6.7 ± 5.4	ND	29.5 ± 8.4	1.4 ± 0.3	ND
OVCA433	15.1 ± 2.1	24.3 ± 5	ND	ND	ND	ND
SKOV-3	ND ^a	ND	ND	ND	ND	ND
OVCA429	ND	ND	ND	ND	ND	ND
ES-2	ND	ND	ND	ND	ND	ND
HEY	ND	ND	ND	ND	ND	ND
A2780cp	ND	ND	ND	ND	ND	ND
A2780s	ND	ND	ND	ND	ND	ND
OCC-1	ND	ND	ND	ND	ND	ND

^a ND=not detectable. Values represent the mean concentration from 3 ELISA experiments +/- SEM.

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January 2007 – May 2007: Lecturer in 3rd year human anatomy & physiology course (PHS3240). University of Ottawa.

May 2007- August 2007: Visiting scientist at Hirosaki University, Japan.
Projects : Endostatin in ovarian cancer, and photodynamic therapy for disseminated peritoneal metastases.

September 2006 - September 2008: Supervised 2 honors student, 2 summer students and 1 COOP student.

May 2004 – May 2005: Elected Vice-President of Communications of the Graduate students' Association (GSAÉD), University of Ottawa.

May 2002 – August 2002 & May 2003 – August 2003: Summer student, Ottawa Regional Cancer Center (ORCC), Ottawa, ON.
Project: The role of chromatin remodeling epigenetics in ovarian cancer tumorigenesis and chemoresistance.

September 2002- Decembre 2002: Teaching Assitant (TA), Cellular Biology Lab (BIO3153 & 3553), University of Ottawa.

May 2001 – August 2001: Summer student, Adherex Technologies, Ottawa, ON.
Project: Cloning VE-cadherin and developing tools to study it as a target of angiolytic therapy.

January 2001 – May 2001: (Volunteer) Eastern Cereal and Oilseed Research Centre, Ottawa, ON
Project: Genotyping and histological tests for transgenic canola crossing.

Scholarships & Awards

2004-2008: Ontario Graduate scholarship in science and technology (OGSST), OSAP. (\$15 000/year).

2004-2008: Entrance scholarship, Faculty of Graduate and Post-doctoral Studies, University of Ottawa. (Tuition waiver).

2008 : Larry Ewing Memorial Trainee Travel Fund (LEMTTF), SSR. (\$275).

2006: Best poster award, 3rd National Conference on Ovarian Cancer Research, ORCA. (\$500).

2004: Best abstract, 7th Annual Interdisciplinary Conference, University of Ottawa. (\$500).

2002: Dean's list award, University of Ottawa. (\$500).

2002: Ottawa Hospital Research Institute (OHRI) excellence award, OHRI. (\$1500).

2001: Industry summer NSERC award, NSERC. (\$5000).

2001: Biotechnology director's award for excellence, University of Ottawa. (\$1000).

Volunteering

2007: Volunteer & coordinator. 2nd Sino-Canada Bilateral Workshop. Ottawa, Canada.

2005-2007: Judge at the Aventis Biotechnology Challenge. Ottawa, Canada.

2002-2007: Volunteer-partner. Lets Talk Science (LTS), a program promoting scientific interest and literacy among primary and secondary school students. Ottawa, Canada.

2003-2004: Elected member of Graduate Students' Association (GSAÉD) Board of Directors, University of Ottawa.

2004 : Coordinator & Moderator. 7th Annual Interdisciplinary Conference. Ottawa, Canada.

2002, 2004 : Volunteer. 1st and 2nd Canadian Conference on Ovarian Cancer Research. Ottawa, Canada.

Presentations

Pépin D, Picketts DJ. Vanderhyden BC. The role of the ISWI protein Snf2L in mammalian reproduction. 5th meeting on chromatin structure and function. November 2009, Costa-Rica.

Pépin D, Picketts DJ. Vanderhyden BC. The ISWI protein Snf2L regulates multiple genes involved in the steroid biosynthesis pathway. 41st Annual SSR Meeting (27-30 May 2008), Kona, Hawaii, USA.

Pépin D, Vanderhyden BC. Overexpression of kallikreins 5, 6 and 10 in an ovarian xenograft model differentially alters pathophysiology and overall survival. Second Sino-Canada bilateral Workshop. November 2007, Ottawa; First Canada-Japan bilateral Workshop. July 2007, Japan; Third Canadian Conference on Ovarian Cancer Research. May 2006, Vancouver.

Pépin D, Vanderhyden BC. The role of the ISWI protein SNF2L in granulosa cell luteinization. 25th Annual Ottawa Reproductive Biology Workshop. May 2007, Ottawa; 24th Annual Ottawa Reproductive Biology Workshop. May 2006, Ottawa; 40th meeting of the SSR, June 2006 Quebec City; 23rd Annual Ottawa Reproductive Biology Workshop. May 2005, Ottawa.

Pépin D., Lazzaro M., Picketts D., Vanderhyden B. SWItching from proliferation to differentiation: a tale of two genes. 2nd Canadian Conference on Ovarian Cancer, Ottawa, ON. May 2004.

Pépin D., Lazzaro M., Picketts D., Vanderhyden B. SWItching from proliferation to differentiation: a tale of two genes. 22nd Canadian Workshop on Human Reproduction and Reproductive Biology, Ottawa. May 2004.

Pépin D. Public Library of Science (PLOS): A Case for Open Access Publishing. 7th Annual interdisciplinary Conference, GSAÉD. Ottawa, February 2004.

Pépin D., Lazzaro M., Picketts D., Vanderhyden B. The role of hISWI genes SNF2H and SNF2L in ovarian development and cancer. 21st Canadian Workshop on Human Reproduction and Reproductive Biology. Ottawa, May 2003.

Pépin D. Vanderhyden B. The role of hISWI genes SNF2H and SNF2L in ovarian development and cancer. 1st Canadian Conference on Ovarian Cancer. Ottawa, May 2002.

Journal Articles

Maribeth A. Lazzaro, David Pepin, Nazario Pescador, Bruce D. Murphy, Barbara C. Vanderhyden, and David J. Picketts. The ISWI protein SNF2L regulates steroidogenic acute regulatory protein (StAR) expression during terminal differentiation of ovarian granulosa cells. *Mol Endocrinol*. 2006 Oct; 20(10): 2406-17.

Pépin D, Vanderhyden BC, Picketts DJ, Murphy BD. ISWI chromatin remodeling in ovarian somatic and germ cells: revenge of the NURFs. *Trends Endocrinol Metab*. 2007 Jul; 18(5): 215-24.

David Pépin, Genevieve Huppe, Andrea Wakefield, Quan Yang, Chee-Wui Chu, Zhong-Qi Shao, Barbara C. Vanderhyden. Overexpression of kallikreins 5, 6 and 10 in an ovarian xenograft model differentially alters pathophysiology and overall survival. (manuscript submitted to PLOS ONE).

Pépin D, Vanderhyden BC. The ISWI protein SNF2L is required for superovulation and regulates FGL2 in differentiating granulosa cells. (Manuscript submitted to *Molecular Endocrinology*).

**Professional
Affiliation**

Member of the Society for the Study of Reproduction.