The Mammalian Ovary from Genesis to Revelation

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Two major functions of the mammalian ovary are the production of germ cells (oocytes), which allow continuation of the species, and the generation of bioactive molecules, primarily steroids (mainly estrogens and progestins) and peptide growth factors, which are critical for ovarian function, regulation of the hypothalamic-pituitary-ovarian axis, and development of secondary sex characteristics. The female germline is created during embryogenesis when the precursors of primordial germ cells differentiate from somatic lineages of the embryo and take a unique route to reach the urogenital ridge. This undifferentiated gonad will differentiate along a female pathway, and the newly formed oocytes will proliferate and subsequently enter meiosis. At this point, the oocyte has two alternative fates: die, a common destiny of millions of oocytes, or be fertilized, a fate of at most approximately 100 oocytes, depending on the species. At every step from germline development and ovary formation to oogenesis and ovarian development and differentiation, there are coordinated interactions of hundreds of proteins and small RNAs. These studies have helped reproductive biologists to understand not only the normal functioning of the ovary but also the pathophysiology and genetics of diseases such as infertility and ovarian cancer. Over the last two decades, parallel progress has been made in the assisted reproductive technology clinic including better hormonal preparations, prenatal genetic testing, and optimal oocyte and embryo analysis and cryopreservation. Clearly, we have learned much about the mammalian ovary and manipulating its most important cargo, the oocyte, since the birth of Louise Brown over 30 yr ago. (***Endocrine Reviews* 30: 624–712, 2009**)

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I. Introduction

The word "ovary" is derived from the Latin word "ovum," meaning egg. The mammalian ovary is not only the female gonad, containing the supply of germ cells to produce the next generation, but also the female reproductive gland, controlling many aspects of female development and physiology. After the union of an oocyte and a spermatozoon to become a zygote, all cells up to the eight-cell stage of embryogenesis appear to have similar totipotency (potential to become any lineage), because these cells all appear morphologically identical. However, with the formation of a 16-cell morula, the cells begin the process of differentiation with cells being allocated to ei-

ther the inside or outside of the embryo. This process is exaggerated further at the blastocyst stage in which three lineages are defined: trophectoderm (future placenta), epiblast (future embryo), and primitive endoderm (future yolk sac). After implantation and further differentiation, cells within the epiblast eventually form the precursors of the primordial germ cells (PGCs), the first cells of the future ovary to be defined. The PGCs enter the indifferent gonad, and eventually the ovary forms and permits the PGCs to differentiate into oocytes, which enter meiosis and subsequently arrest; this differentiation step and entry into meiosis suggest that the last of the oocyte "stem cells" (*i.e.*, the PGCs) likely disappear at this stage of fetal life. The meiotically arrested oocytes eventually become surrounded by pre-granulosa cells and form individual primordial follicles, the resting pool of oocytes that have the potential to be recruited into the growing follicle pool in the postpubertal mammal, to be fertilized, and to contribute to the next generation (Fig. 1).

Through various types of developmental, genetic, physiological, and hormonal analyses, the above steps in the reproductive cycle of a mammalian female have begun to be understood in great detail. Studies in mice have proven invaluable for identifying genes critical to normal ovarian development and function. Mutations in many of the human homologs of these genes often contribute to infertility in women (Table 1). In the process of deconstructing the female reproductive life cycle, techniques for manipulation of the human (and nonhuman) oocyte have been developed to more effectively create "test tube" babies. In addition, we have begun to understand conditions in which these well-orchestrated events of female reproductive development and physiology go awry, leading to diseases that range from psychologically distressing, such as infertility, to life-threatening, such as ovarian cancer. In this review, we describe the development, physiology, and pathology of the mammalian ovary from its formation to all the wondrous details that have been discovered about it *in vivo*, in the test tube, and in the clinical reproductive setting.

II. Ovarian Development and Differentiation

Future components of the mammalian ovary develop long before a distinct ovary-like organ can be discerned. In this section, we describe how the germ cells arise and reach the undifferentiated gonad, the factors involved in formation of the ovary, and the early steps that distinguish the female germline from the male germline.

A. Primordial germ cell formation and migration

Despite the early descriptions of mouse PGCs over 50 yr ago (1), the last decade has continued to see dramatic advances in our understanding of the molecular mechanisms of PGC formation and migration (current knowledge summarized in Table 2). Chiquoine (1) had initially shown that the putative PGC stained strongly for alkaline phosphatase as early as embryonic day (E) 8.5. Consistent with these findings, these alkaline phosphatase-positive

Abbreviations: AC, Adenylyl cyclase; ADAMTS1, a disintegrin-like and metallopeptidase with thrombospondin type 1 motif, 1; AdCre, adenovirus expressing Cre recombinase; AHR, aryl hydrocarbon receptor; AKAP, A-kinase anchoring protein; AKR1C18, aldo-keto reductase family 1, member C18; ALPL, alkaline phosphatase, liver/bone/kidney; AMH, anti-Müllerian hormone; AMHR2, AMH type II receptor; APC/C, anaphase-promoting complex/cyclosome; AR, androgen receptor; *Areg*, amphiregulin; ART, assisted reproductive technology; BCL, B cell lymphoma/leukemia; BDNF, brain-derived neurotrophic factor; bHLH, basic helix-loop-helix; BMP, bone morphogenetic protein; BPES, blepharophimosis/ptosis/epicanthus inversus syndrome; *BRCA1*, breast cancer 1; *Btc*, betacellulin; CBX2, chromobox homolog 2; CDK, cyclin-dependent kinase; C/EBPβ, CCAAT/enhancer-binding protein β; CEEF, cumulus expansion-enabling factor; cKO, conditional knockout; CL, corpus luteum; CSF, cytostatic factor; *CSF1*, colony stimulating factor 1; CSPG2, chondroitin sulfate proteoglycan 2; CTNNB1, β-catenin; CX43, connexin 43; CYP11A1, cytochrome P450 side-chain cleavage enzyme; CYP17A1, cytochrome P450 17α-hydroxylase/17,20-lyase; CYP19A1, cytochrome P450 aromatase; CYP26B1, cytochrome P450 26B1; DAZL, deleted in azoospermia-like; DHH, desert hedgehog; DKK1, dickkopf homolog 1; DND1, dead end homolog 1; dpc, days post-coitus; DPPA3, developmental pluripotency-associated 3; E, embryonic day; EGF, epidermal growth factor; EGFR, EGF tyrosine kinase receptor; EHMT2, euchromatic histone lysine N-methyltransferase 2; EMT, epithelial-to-mesenchymal transition; EMX2, empty spiracles homolog 2; ER, estrogen receptor; *Ereg*, epiregulin; $\alpha\beta$ERKO, ERα and ERβ knockout; FBXO43, F-box protein 43; FF-MAS, follicular fluid-meiosis-activating sterol; FGF, fibroblast growth factor; FGFR2, FGF receptor 2; FIGLA, factor in the germline α; *FMR1*, fragile X mental retardation 1; FOG2, friend of GATA2; FOX, forkhead box; FSHR, FSH receptor; FST, follistatin; FZD, FRIZZLED; *Gct*, granulosa cell tumor; GDF9, growth differentiation factor 9; *GREM1*, gremlin 1; GS, germline stem (cells); GV, germinal vesicle; GVBD, GV breakdown; HAS2, hyaluronan synthase 2; hCG, human chorionic gonadotropin; H3K9me2, dimethylated histone 3 lysine 9; H3K27me3, trimethylated histone 3 lysine 27; HMG, high-mobility group; HMGA2, HMG AT-hook 2; HPG, hypothalamic-pituitary-gonadal; *Hsd3b1*, 3β-hydroxysteroid dehydrogenase; IBMX, 3-isobutyl-1-methylxanthine; ICSI, intracytoplasmic sperm injection; IFITM3, interferon-induced transmembrane protein 3; IHH, Indian hedgehog; IKKβ, inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta; Ial, inter-α-trypsin inhibitor; IOSE, immortalized OSE; iPS, induced pluripotent stem (cells); IVF, *in vitro* fertilization; KTS, lysine-threonine-serine; *Lfng*, lunatic fringe; LHCGR, LH/choriogonadotropin receptor; LHX9, LIM homeobox protein 9; LIF, leukemia inhibitory factor; LRH1, liver receptor homolog 1; LRP6, low-density lipoprotein receptor-related protein 6; *Mapk1/3* dKO, *Mapk1 Mapk3* double mutant mice; MII, meiosis II; miRNA, microRNA; MOS, Moloney sarcoma oncogene; MPF, maturation-promoting factor; MT1, metallothionein 1; mTOR, mammalian target of rapamycin; NF-κB, nuclear factor-κB; NGF, nerve growth factor; NLRP, leucine-rich repeat and pyrin domain containing; NOBOX, newborn ovary homeobox; NOS3, nitric oxide synthase 3; NR5A, nuclear receptor subfamily 5, group A; *Nr2c2*, nuclear receptor subfamily 2, group C, member 2; NRIP1, nuclear receptor interacting protein 1; NTF5, neurotrophin 5; NTRK, neurotrophic tyrosine kinase receptor; OOX, oocytectomized; OSE, ovarian surface epithelium; P, postnatal day; PAH, polycyclic aromatic hydrocarbon; PDE, phosphodiesterase; PDGF, platelet-derived growth factor; PGC, primordial germ cell; PI3K, phosphatidylinositol 3-kinase; PIP2, phosphatidylinositol 4,5-bisphosphate; piRNA, Piwi-interacting RNA; PKA, protein kinase A; PMSG, pregnant mare serum gonadotropin; POF, premature ovarian failure; PPARγ, peroxisome proliferator-activated receptor γ; PR, progesterone receptor; PRDM, PRDI-BF1-RIZ domain containing 1; PRL, prolactin; PRLR, PRL receptor; PRMT5, protein arginine N-methyltransferase 5; PTCH, patched; PTEN, phosphatase and tensin homolog; PTGER2, prostaglandin E receptor 2, subtype EP2; PTGS2, prostaglandin synthase 2; PTX3, pentraxin 3; *Rangap1*, Ran GTPase activating protein 1; RISC, RNA-induced silencing complex; RNAi, RNA interference; RSPO1, R-spondin homolog 1; SF1, steroidogenic factor 1; shRNA, short hairpin RNA; siRNA, small interfering RNA; SMO, Smoothened; SNP, single nucleotide polymorphism; SOHLH1, spermatogenesis and oogenesis helix-loop-helix 1; SOX9, SRY-box containing gene 9; SRY, sex-determining region of chromosome Y; STAR, steroidogenic acute regulatory protein; STAT, signal transducer and activation of transcription; *Stra8*, stimulated by retinoic acid gene 8; SV40, simian virus 40; SYCP3, synaptonemal complex protein 3; TAF, TATA-binding protein-associated factor; TAg, T antigen; TEX14, testis-expressed gene 14; TIMP1, tissue inhibitor of metalloproteinase 1; TNFAIP6, TNFα-induced protein 6; TR4, testicular orphan nuclear receptor 4; UTR, untranslated region; WEE2, WEE1 homolog 2; WT1, Wilms tumor 1 homolog.

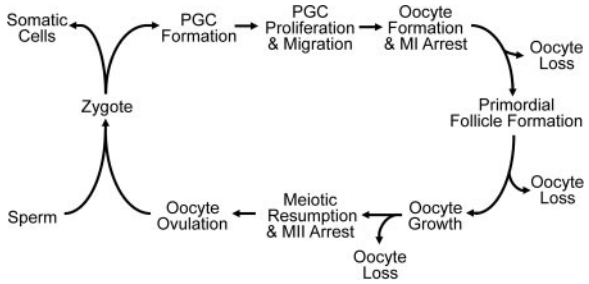

FIG. 1. Reproductive life cycle of a mammalian female. An oocyte and a spermatozoon will fuse to form a zygote and undergo multiple steps in embryogenesis. At about E6.5 in mouse, the PGC will be allocated and go through multiple steps to reach the genital ridge. In an XX mammal, the germ cell will form an oocyte that arrests at meiosis I (MI). During prenatal life in women, and in the perinatal period in mice, the oocyte will be encased in somatic cells to become primordial follicles. Upon recruitment into the growing pool, the oocyte increases in size during folliculogenesis. The LH surge will induce resumption of meiosis, release of the first body, arrest at MII, and subsequent ovulation of the oocyte into the fallopian tube. Fertilization with a spermatozoon will induce the completion of meiosis and release of the second polar body. The cycle continues in the next generation of females. During the reproductive cycle, there are multiple steps where significant oocyte loss is observed.

cells were shown to be depleted in the classic *white spotting* (KIT) and *steel* (KIT ligand) mouse mutants that are known to lack germ cells in their gonads (2, 3). By 1967, Ozdzenski (4) was able to identify these putative PGCs at the base of the allantois as early as E8.0. Additional microscopic studies in the 1970s (5, 6) were extremely helpful in characterizing these cells and their migration (see below). However, it was not until 1990 that additional experimental proof confirmed that these alkaline phosphatase-positive cells were in fact PGCs. First, at E7.25, a cluster of cells were observed containing a "spot" in their cytoplasm that stained intensely for alkaline phosphatase activity; these cells were present at the base of the yolk sac before formation of the allantois (7). Second, follow-up studies confirmed that these cells were in fact the only PGCs because ablation of the cells resulted in embryos without germ cells whereas transplantation of these cells leads to their proliferation. Thus, using alkaline phosphatase as a marker, the female and male (mouse) germline was thought to be specified by at least E7.25.

One enigma was that alkaline phosphatase was not required for this process; mutation of ALPL (alkaline phosphatase, liver/bone/kidney) does not alter the number of PGCs or their migration (8). This indicated that ALPL marked these cells but was not involved in either the formation or function of PGCs.

What then are the factors involved in formation of PGC precursors and their specification, and when do these factors act? Knockout models have helped greatly to define members of the bone morphogenetic protein (BMP) family as major extrinsic factors that are key to the early development of PGC precursors (reviewed in Refs. 9–11 and summarized in Table 3). BMP4 and BMP8B, secreted from the extraembryonic ectoderm (12, 13), and BMP2, secreted from the visceral endoderm (14), are required for the early discrimination of PGC precursors from the somatic cells of the embryo. These BMPs signal in a dosage-dependent manner to the epiblast cells through a BMP receptor cascade that involves phosphorylation of the BMP SMADs, SMAD1 and SMAD5, both of which, along with their common SMAD partner, SMAD4, have been shown to function in this pathway (15–18). In contrast, the other BMP-signaling SMAD, SMAD8, is dispensable for this process (19). It is believed that BMPs begin to act on the pluripotent proximal epiblast cells between E5.5 and E6.0 to allow them to be "competent" to become a PGC precursor. Signals (some of which are likely BMPs) from the extraembryonic ectoderm and the visceral endoderm result in restriction and formation of PGC precursors only from epiblast cells in the posterior of the embryo (20). One of the earliest genes induced by BMPs is *Ifitm3* (interferon-induced transmembrane protein 3; Fragilis), an excellent early marker for the competence step as well as the further differentiation of the PGC (21). However, like ALPL, absence of IFITM3 and its related family members does not alter PGC formation (22), making it a functionally dispensable but key marker protein.

At approximately E6.25, six of the IFITM3-positive epiblast cells adjacent to the extraembryonic ectoderm express the protein PRDM1 (PRDI- BF1- RIZ domain containing 1; BLIMP1); these cells are the first PGC precursors and the first cells of the mammalian embryo for which their fate is committed (23). PRDM1 is a transcriptional repressor that contains a PRDI-BF1-RIZ domain and five Krüppel-like C_2H_2 zinc finger (DNA binding) domains. PRDM1 was first identified in a screen for gene products differentially expressed at E7.5 in founder PGC but not adjacent somatic cells (21). Within 1 d, there are 20–28 PRDM1-positive tightly clustered cells, and by E7.5, 40 PRDM1-positive cells are also positive for alkaline phosphatase and show nearly 100% concordance with DPPA3 (developmental pluripotency-associated 3; STELLA), another nonessential marker of PGCs (24, 25). Lineage tracing experiments confirmed that the PRDM1-positive cells were the germline-restricted PGC progenitors, and that by E7.5, these 40 cells were the founder population of PGCs.

In parallel with the expression and lineage-tracing studies, confirmation that PRDM1 was essential for PGC specification came from several additional mouse knockout studies (23). First, *Bmp4* null mice that lack PGCs also lack PRDM1-positive cells. Second, null mutations of *Prdm1* show that heterozygotes have a reduction in the number of

TABLE 1. Mutations associated with infertility in women

Gene	Phenotype	OMIM gene [OMIM infertility]
Bone morphogenetic protein 15 (*BMP15*)	Hypergonadotropic ovarian failure (POF4)	300247 [300510]
Bone morphogenetic protein receptor 1B (*BMPR1B*)	Ovarian dysfunction, hypergonadotropic hypogonadism and acromesomelic chondrodysplasia	603248
Chromobox homolog 2, *Drosophila* polycomb class (*CBX2*; M33)	Autosomal 46,XY, male-to-female sex reversal (phenotypically perfect females)	602770 (67)
Chromodomain helicase DNA-binding protein 7 (*CHD7*)	CHARGE syndrome and Kallmann syndrome (KAL5)	608892 [612370]
Diaphanous homolog 2 (*DIAPH2*)	Hypergonadotropic, premature ovarian failure (POF2A)	300108 [300511]
Fibroblast growth factor 8 (*FGF8*)	Normosmic hypogonadotropic hypogonadism and Kallmann syndrome (KAL6)	600483 [612702]
Fibroblast growth factor receptor 1 (*FGFR1*)	Kallmann syndrome (KAL2)	136350 [147950]
FSH receptor (*FSHR*)	Hypergonadotropic hypogonadism and ovarian hyperstimulation syndrome	136435
FSH β (*FSHB*)	Deficiency of FSH, primary amenorrhea and infertility	136530 [229070]
Forkhead box L2 (*FOXL2*)	Isolated POF (POF3) associated with BPES type I; *FOXL2* 402C→G mutations associated with human granulosa cell tumors	605597 [608996]
Fragile X mental retardation 1 (*FMR1*)	Premature ovarian failure (POF1) associated with premutations	309550 [311360]
GnRH receptor (*GNRHR*)	Hypogonadotropic hypogonadism	138850
GnRH 1 (*GNRH1*)	Normosmic hypogonadotropic hypogonadism	152760 (769, 770)
Kallmann syndrome 1 (*KAL1*)	Hypogonadotropic hypogonadism and anosmia, X-linked Kallmann syndrome (KAL1)	308700
KISS1 receptor (*KISS1R*; GPR54)	Hypogonadotropic hypogonadism	604161
LH β (*LHB*)	*LHB* G102S mutations associated with infertility	152780
LH/choriogonadotropin receptor (*LHCGR*)	Hypergonadotropic hypogonadism (LH resistance)	152790
Nuclear receptor subfamily 0, group B, member 1 (*NROB1*; DAX1)	X-linked congenital adrenal hypoplasia with hypogonadotropic hypogonadism; dosage-sensitive male-to-female sex reversal	300473 [300200; 300018]
Nuclear receptor subfamily 5, group A, member 1 (*NR5A1*; SF1)	46,XY male-to-female sex reversal and streak gonads and congenital lipoid adrenal hyperplasia; 46,XX gonadal dysgenesis and 46,XX primary ovarian insufficiency	184757 (771)
Premature ovarian failure 1B (*POF1B*)	Hypergonadotropic, primary amenorrhea (POF2B)	300603 [300604]
Prokineticin 2 (*PROK2*)	Normosmic hypogonadotropic hypogonadism and Kallmann syndrome (KAL4)	607002 [610628]
Prokineticin receptor 2 (*PROKR2*)	Kallmann syndrome (KAL3)	607123 [244200]
R-spondin family, member 1 (*RSPO1*)	46,XX, female-to-male sex reversal (individuals contain testes)	609595
Sex-determining region Y (*SRY*)	Mutations lead to 46,XY females; translocations lead to 46,XX males	480000
SRY-related HMG-box gene 9 (*SOX9*)	Autosomal 46,XY male-to-female sex reversal (campomelic dysplasia)	608160
Tachykinin 3 (*TAC3*)	Normosmic hypogonadotropic hypogonadism	162330
Tachykinin receptor 3 (*TACR3*)	Normosmic hypogonadotropic hypogonadism	162332

Because of space limitations, most cases associated with female-to-male sex reversal due to steroidogenesis defects, syndromes, and chromosomal abnormalities are excluded from the table. The primary reference (in *parentheses*) is included for work not yet described in OMIM.

PGCs at E7.5, whereas homozygous mutants have near zero PGCs (23, 26). The few "PGC-like" cells that were observed at the base of the allantois in the null mutants failed to increase in number, and none of these cells migrated normally. High levels of PRDM1 expression are also required for PGC specification as determined by the reduction in PGCs in heterozygous mutants and the absence of germ cells in mice carrying a hypomorphic PRDM1-green fluorescent protein reporter allele (27).

Although ALPL, DPPA3, and IFITM3 are important nonessential markers for the lineage restricted PGC, PRDM14 was identified as a relative of PRDM1 that is not only expressed but required in PGCs (28, 29). PRDM14 is expressed at least as early as PRDM1 (*i.e.*, ~E6.5) in PGC precursors, and similar to PRDM1, expression of PRDM14 is dependent on BMP4 signaling through at least SMAD1 (*i.e.*, no PRDM14-positive cells are observed in mice null for these genes). Phenotypically, absence of PRDM14 results in few PRDM1-positive cells, with only a few of these cells observed to migrate. However, unlike *Prdm1* null mice, which die during embryogenesis (23, 26), *Prdm14* null female and male mice are viable but infertile. Thus, *Prdm14* is the second identified gene that is essential for specification of the mammalian germline.

TABLE 2. PGC events and pathways in the mouse

PGC event	Timepoint	Major pathways and genes (nonessential markers)
Induction/competence	E6.0–6.5	BMP2/4/8 signaling through SMAD1/5 (IFITM3)
Early specification	E6.5	PRDM1 and PRDM14 induced; LIN28 induction suppresses let-7 maturation, allowing PRDM1 protein to be expressed; HOXB1 suppressed and restricted at somatic lineage
Late specification	E7.5	PRDM1 and PRDM14 mark all future PGCs; pluripotency markers POU5F1, SOX2, and NANOG turned on (DPPA3, ALPL)
Chromatin reprogramming (similar to ES cells)	E8.5	H3K27me3 induced; H3K9me2 erased; H2/H4 RMC2 induced
Migration and entry into genital ridge	E8.5–11.5	KIT ligand/KIT; NANOS3; DND1
Loss of imprinting and reversal of chromatin reprogramming	E10.5–12.5	PRDM1-PRMT5 translocation out of nucleus
Meiosis competent state	E11.5	DAZL
Meiotic entry	E12.5	CYP26B1 down-regulated in XX germ cells

Because the germline fails to develop in the absence of PRDM1 and PRDM14, what is the relationship of these two PRDI- BF1- RIZ domain-containing proteins? Based on analysis of *Prdm1* null embryos and *Prdm14* null embryos, it is clear that PRDM1 continues to be expressed in *Prdm14* null embryos and vice versa, indicating that these proteins function in independent pathways to specify the germline (29). However, continued PRDM14 expression requires PRDM1 and vice versa. Although the direct regulators of PRDM14 and PRDM1 are unknown, let-7 family microRNAs are important modulators of PRDM1 expression, and LIN28 is also required in PGC specification. During an *in vitro* screen for genes involved in PGC specification, small interfering RNA (siRNA) knockdown of *Lin28* was found to reduce the number of PGC-positive colonies (30). LIN28 is known to suppress the maturation of let-7 microRNAs (31–35). Furthermore, the *Prdm1* 3′ untranslated region (UTR) contains an important let-7 binding site. Thus, LIN28 induction suppresses the levels of let-7 microRNAs, which relieves the inhibition of PRDM1 synthesis and allows PGC specification to proceed.

How do PRDM1 and PRDM14 function to establish the mammalian germline? To address this question, the Surani and Saitou laboratories (21, 23, 29) have performed detailed single cell quantitative analysis of gene expression using cells from wild-type, PRDM1 mutant, and PRDM14 mutant embryos. Analysis of wild-type embryos had first identified PRDM1 and PRDM14. It also became clear that major (parallel) events in PGC specification are repression of somatic cell gene expression, induction of PGC-enriched gene expression, and reexpression of pluripotency genes. In particular, the *Hoxa1* and *Hoxb1* genes, which are highly expressed in the somatic epiblast cells in the posterior portion of the developing embryo at E7.25-E7.5, are never synthesized in the founder PGCs. Likewise, other mesodermal genes [*e.g.*,

TABLE 3. Phenotypes of mice with mutations in PGC markers and pathway components (order based on expression and/or function)

Gene (pseudonym)	Phenotype/findings	Ref.
Bmp2	Embryonic lethal; reduced PGCs	14, 772
Bmp4	Embryonic lethal; no PGCs	12, 773
Bmp8b	Viable; male infertility; reduced PGCs	13, 774
Smad1	Embryonic lethal; reduced PGCs	16, 17, 775
Smad5	Embryonic lethal; reduced PGCs	15, 776, 777
Smad4	Embryonic lethal; absent PGCs	18, 778, 779
Ifitm3 (Fragilis)	Not essential for PGC function	22
Prdm1 (Blimp1)	Embryonic lethal; PGC specification defect	23
Prdm14	Infertility; PGC specification defect	29
Ehmt1 (Glp)	Unknown PGC function	
Ehmt2 (G9a)	Unknown PGC function	
Dppa3 (Stella)	Not essential for PGC function	24, 25
Alpl (Alkaline phosphatase)	Not essential for PGC function	8
Pou5f1 (Oct4)	Pluripotency marker	780, 781
Nanog	Pluripotency marker	781–783
Sox2	Pluripotency marker	781, 784
Nanos3	Infertile; PGC migration defect	44
Dnd1 (Ter)	Infertile; PGC migration defect	45
Kitl	Variable phenotypes depending on mutation; PGC migration defect	785
Kit	Variable phenotypes depending on mutation; PGC migration defect	785
Tgfbr1 (Alk5)	Embryonic lethal; enhanced PGC migration	42, 786

brachyury (*T*), *Fgf8*, and *Snai1*] are also suppressed. However, in the absence of PRDM1, the majority of PGC-like cells are positive for *Hoxa1* and/or *Hoxb1*. This somatic cell repression program is still intact in the absence of PRDM14. Absence of PRDM14, but not PRDM1, results in failed induction of the lineage restricted DPPA3, whereas absence of PRDM1, but not PRDM14, leads to absence of the PGC-specific gene *Nanos3* (see below). PGCs also show induction of several pluripotency "master regulatory" genes including *Sox2*, *Pou5f1* (*Oct4*), and *Nanog*. Absence of PRDM14 leads to repression of *Sox2*, whereas *Prdm1* null cells show variable expression of *Sox2*. Thus, the somatic cell repression function is unique to PRDM1, whereas both PRDM14 and PRDM1 regulate some PGC-specific transcripts as well as the pluripotency-associated protein SOX2.

Since PRDM1 was identified almost two decades ago, much more data have been accumulated on it than PRDM14, which was only presented in publications 2 yr ago. As mentioned earlier, PRDM1 functions in multiple tissues, and its absence leads to embryonic lethality, whereas PRDM14 is only required in the mammalian germline. In addition to their roles in PGC specification, both PRDM1 and PRDM14 have been implicated in cancer. As implied by its pseudonym (BLIMP1, B-lymphocyte-induced maturation protein 1), PRDM1 is a master regulator of the terminal differentiation of B cells into Ig-producing plasma cells through its ability to act as a transcriptional repressor, blocking the transcription of a diverse set of genes such as *Myc* and *p53*. Loss of function mutations in human chromosome 6q21, the location where *PRDM1* maps, are implicated to cause B and T cell lymphomas, whereas other studies have demonstrated key tumor suppressor roles of PRDM1 in these lineages. Alternatively, gene amplification of 8q13, where *PRDM14* maps, are observed in multiple cancers including breast cancer, which demonstrates increased expression of *Prdm14* mRNA and protein (36). Increased expression of PRDM14 in breast cancer cells stimulates growth, whereas knockdown induces apoptosis. Retroviral insertion into the *Prdm14* locus in mice results in its overexpression and consequent B cell lymphomas (37). These studies suggest that PRDM14 functions as an oncogene. In human ES cells, PRDM14 maintains cell renewal (38). This information on their tumor suppressor *vs*. oncogenic roles confirms that PRDM1 and PRDM14 appear to exert their effects in different manners.

At E7.5, a time in which PGC specification has occurred, PGCs express markers of pluripotency including POU5F1, SOX2, NANOG, as well as DPPA3 and ALPL. At this time point, the methylation pattern of a PGC is predominantly dimethylated at histone 3 lysine 9 (H3K9me2), whereas there are low levels of trimethylated histone 3 lysine 27 (H3K27me3). As their symbols imply, both PRDM1 and PRDM14 have PRDI-BF1-RIZ domains (also called SET domains) that have structural similarity to histone methyltransferases. In B cells, where PRDM1 is a master regulator, PRDM1 interacts with euchromatic histone lysine N-methyltransferase 2 (EHMT2) (39), which performs dimethylation mainly at histone 3 lysine 9. Between E7.5 and E8.5, PGCs demonstrate major chromatin changes, increasing the levels of H3K27me3 and erasing the H3K9me2 methylation marks (40, 41), patterns that resemble the chromatin patterns of pluripotent stem cells. Similar to ES cells, the H3K27me3 marks also appear to be involved in the repression of the "somatic cell" gene expression program. H3K9me2 erasure occurs despite the presence of EHMT2, likely because euchromatic histone methyltransferase 1 (EHMT1), which complexes with EHMT2 for methylation of H3K9me2, is downregulated by E7.25 (28). Although it is unclear how PRDM1 and PRDM14 directly influence these lysine methylation changes, chromatin changes in H3K27me3 and H3K9me2 from E7.5 to E8.5 do occur. Furthermore, additional data are evident on the roles of PRDM1 in arginine methylation. PRDM1 complexes with protein arginine N-methyltransferase 5 (PRMT5) to dimethylate histone 2A and histone 4 at arginine 3 by E8.5. Along with these changes, the PGCs arrest at the G_2 stage of the cell cycle and transiently become transcriptionally silent as they migrate from the base of the yolk sac along the hindgut to the genital ridge (40).

The exact trigger that initiates PGC migration to the genital ridge and the chemoattractants that are required for directional movement toward the genital ridge are slowly beginning to be understood. The trigger(s) could be the expression of a key receptor on the PGC and/or the expression of the secreted chemoattractant from the genital ridge. An extracellular matrix gradient along the path of migration is important, and if too much matrix is laid down, PGCs show reduced migration. For example, suppression of TGFβ signaling by knocking out *Tgfbr1* (Alk5) leads to enhanced migration due to reduction in the levels of TGFβ-induced collagen type 1 in the extracellular matrix (42). One of the best candidates to function as a chemoattractant for PGCs is KIT ligand. Using PGCs obtained from E10.5 and E11.5 embryos, *in vitro* migration assays demonstrated that KIT ligand could function as an effective chemoattractant for the PGCs and that the phosphatidylinositol 3-kinase (PI3K)/AKT and SRC kinase pathways were involved downstream of KIT in the PGC (43). Although it is not clear whether KIT ligand/KIT signaling is involved in the earliest steps of activation and migration, these data support a late role for this pathway in PGC migration into the genital ridge.

Although not involved in PGC migration, several additional gene products are necessary for PGC survival during their migration. For example, two RNA binding proteins, NANOS3 and DND1 (dead end homolog 1; TER), are expressed in PGCs from E7.5 onward and protect the PGCs from apoptosis; mutations in either *Nanos3* or *Dnd1* lead to few germ cells in the genital ridge and germ cell deficiency (44, 45). Consistent with the pleiotropic roles of the KIT ligand/KIT pathway, these proteins not only function in PGC migration but also aid in PGC survival and proliferation.

During the migration stage, the histone marks and the expression of the major pluripotency genes are maintained. However, beginning at approximately E10.5-E12.5 and coinciding with entry of the PGCs into the gonadal ridge, chromatin and gene expression changes are observed. At approximately E11.5, the PRDM1-PRMT5 complex, which is observed in the nucleus from the specification stage through PGC migration, translocates to the cytoplasm. In parallel, the pluripotency-associated genes also begin to be down-regulated, whereas the RNA helicase DHX38 [DEAH (Asp-Glu-Ala-His) box polypeptide 38], which is normally repressed through arginine methylation of histones by the PRDM1-PRMT5 complex, begins to be expressed (46). The E8.5-E11.5 period, when the PGCs show highest expression of the pluripotency-associated genes, is also the only window for production of embryonic germ cells from the PGC. It is postulated that secreted molecules and proteins from the somatic cells in the genital ridge directly influence these major reprogramming events. Among the changes that are observed are genome-wide demethylation, erasure of imprinting, and reactivation of the inactive X chromosome in females. Recent transgene studies have helped to explain X chromosome reactivation and the role of the female gonadal environment in this process (47). The erasure of histone 2A/4 arginine 3 methylation is consistent with the aforementioned translocation of PRDM1-PRMT5 complex out of the nucleus. Lastly, once PGCs enter the genital ridge, they appear to lose their ability to migrate.

B. Formation of the bipotential gonad

Similar to the PGC, the sex of the gonadal ridge initially is irrelevant; PGCs are attracted equally to an XX or an XY gonadal ridge. The undifferentiated or bipotential gonadal ridge arises at approximately E10.5 between the coelomic epithelium and the mesonephros, the two distinct tissues along with the PGCs that contribute most of the cells of the future ovary or testis during the subsequent sexual differentiation stages. The initial steps in the process appear to be a thickening of the coelomic epithelium. Several factors have been identified to play key roles in formation of the genital ridge with so-called bipotential

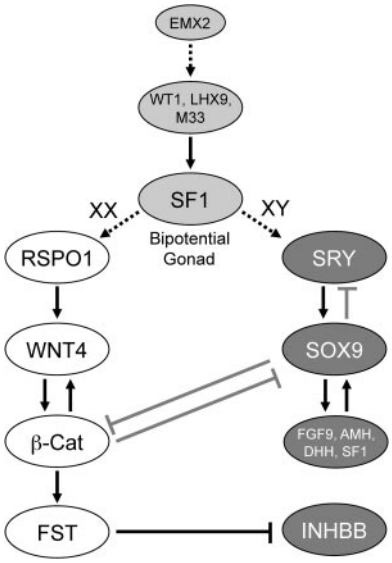

FIG. 2. Gonad function and sexual differentiation. As shown, several major gene products influence the formation of the bipotential gonad (*light gray*), the development of the ovary (*white*), and the development of the testis (*dark gray*). SF1 is a central player in the bipotential gonad, being regulated by WT1, LHX9, and M33 (CBX2), and at other steps in gonadal differentiation. In XY gonads, SRY functions in a short window in pre-Sertoli cells to up-regulate the transcription of SOX9 that is already expressed at low levels through the action of SF1. This higher SOX9 expression then suppresses SRY in a negative feedback loop and also up-regulates itself through the combined actions of SF1 and SOX9 on the SOX9 promoter. SOX9 also up-regulates FGF9 that signals back through FGFR2 to maintain/increase SOX9 expression. The ovarian differentiation pathway involves RSPO1 increasing the signaling of WNT4, which up-regulates β-catenin. β-catenin acts to up-regulate WNT4 and other proteins such as FST. The testis pathway appears to mainly antagonize this pathway through decreasing β-catenin levels. Likewise, β-catenin antagonizes the testis pathway by destabilizing SOX9.

possibilities (*i.e.*, the potential to develop into a testis or an ovary depending on the genetic makeup of the somatic cells in and surrounding the genital ridge; see *Sections II.C and II.D*).

Several of the major "bipotential gonad" gene products also set the stage for the upcoming differentiation into either a testis or an ovary (Fig. 2 and Table 4). Probably the first key gene in the development of the bipotential gonad is the homeobox gene empty spiracles homolog 2 (*Emx2*). In the absence of EMX2, which is expressed in the epithelium, the thickening of the coelomic epithelium is not obvious, resulting in sex-independent absence of the gonads as well as absence of the Müllerian duct and Wolffian duct derivatives (48). Thus, EMX2 appears to be a transcriptional regulator of subsequent events leading to gonad and urogenital system formation.

Wilms tumor 1 homolog (*Wt1*) is the second gene important for formation of the bipotential gonad. *WT1* mutations or deletions (*e.g.*, 11p13) in patients are associated with several human syndromes that include genitourinary abnormalities. 46,XY male-to-female sex reversal is ob-

TABLE 4. Mouse mutants with defects in the formation of the gonad

Gene	Phenotype	Ref.
Emx2	Midgestation embryonic lethality; absence of gonads due to defects in the coelomic epithelium	48
Wt1	Midgestation embryonic lethality; lack of gonads by E14	51
Wt1 (−KTS splice variant)	Streak gonads in males and females	53
Wt1 (+KTS splice variant)	Male-to-female sex reversal	53
Lhx9	Viable mice; male-to-female sex reversal	56
Cbx2 (M33)	Lethality (60%) between birth and 21 d; male-to-female sex reversal	65
Pod1	Neonatal lethality; disorganized gonads	68
Nr5a1 (Sf1)	Early postnatal lethality; absence of both gonads and Müllerian duct derivatives	59

served typically in Frasier syndrome (49), occasionally in Denys-Drash syndrome (50), and in one patient with WAGR syndrome (50). WT1 is a transcription factor with four zinc finger domains. Although knockout of *Wt1* in mice leads to midgestation embryonic lethality, it was noted that male and female knockout mice lacked gonads by E14, indicating that WT1 is required for early formation of the bipotential gonad (51). WT1 is expressed in the coelomic epithelium during gonad formation, and in the Sertoli cells and granulosa cells during their formation. Consistent with its expression during gonadal development, analysis of the *Wt1* mutants at E11 and E12 showed that the thickening of the coelomic epithelium was markedly reduced compared with wild-type embryos but that PGC migration into these genital ridges still occurred. These findings indicate that WT1 may not be required for the initial step in gonad formation but becomes essential soon after, at least for further development and/or maintenance of these cells.

WT1 acts at several points in the pathways of bipotential gonad formation and sex determination. There are two alternative splice variants of WT1 that include or exclude the lysine-threonine-serine (KTS) amino acids between the third and fourth zinc fingers; functionally, the presence of the KTS amino acids prevents the fourth zinc finger from binding to DNA, resulting in lower transcriptional activity (52). To study the relative significance of these two isoforms, mutations were made to disrupt one or the other form (53). The absence of the WT1(−KTS) isoform results in streak gonads in both male and female gonads secondary to increased cell death during gonad formation. These results are consistent with data demonstrating that the WT1(−KTS) isoform functions to regulate the expression of steroidogenic factor 1 (SF1; NR5A1, nuclear receptor subfamily 5, group A, member 1) (54). Alternatively, absence of the WT1(+KTS) form of WT1 (mimicking the mutation seen in Frasier syndrome in which there is less of the +KTS variant) leads to male-to-female sex reversal and reduced SRY (sex-determining region of chromosome Y) and SOX9 (SRY-box containing gene 9) expression, consistent with a later role of WT1 in sex determination (see *Section II.C*). In addition, WT1 and GATA4 transcriptionally cooperate on the mouse, pig, and human *SRY* promoters, and the synergy is strongest with the WT1(+KTS) isoform (55), which is also consistent with the above *in vivo* data.

LIM homeobox protein 9 (LHX9) is another key regulator that functions in the development of the bipotential gonad. Absence of LHX9 does not alter viability; however, all postnatal mice are phenotypically female with no gonads (56). *Lhx9* is expressed in the coelomic epithelium of the genital ridge at E9.5, and by E11.5, *Lhx9* is expressed highly in the coelomic epithelium and at lower levels in the developing gonad. Analysis of the *Lhx9* null gonads at earlier embryonic stages demonstrates no morphological differences from wild type at E11.5, with normal migration of the PGC into the genital ridge, but no further development thereafter, and a complete loss of any defined gonad-like structures by E13.5. The absence of any gonad results in a lack of testosterone and anti-Müllerian hormone (AMH) synthesis in XY embryos, leading to no development of the Wolffian duct and failure of the Müllerian duct to regress, respectively. This situation phenocopies the experiments that Alfred Jost performed over 50 yr earlier in which removal of embryonic rabbit gonads results in a ductal system that resembles a female (57).

Because the embryonic phenotype of the *Lhx9* null mouse resembles the *Wt1* null mouse, what is the pathway relationship of these two transcription factors and what regulates *Lhx9*? Although transcriptional regulators of *Lhx9* in the genital ridge have not been reported, another study has demonstrated that *Lhx9* is regulated in the developing heart by a transcriptional complex of GATA4 and FOG2 (friend of GATA2; ZFPM2, zinc finger protein, multitype 2) (58). This is important because the GATA4/FOG2 complex is also involved in the regulation of SRY (see *Section II.C*). LHX9 subsequently functions along with WT1 in the regulation of SF1, which is first expressed in the coelomic epithelium and also in the daughter cells that migrate into the urogenital ridge to become either Sertoli cells or granulosa cells. Analysis of the $Lhx9^{-/-}$ urogenital ridge at E11.5 shows very low levels of SF1, whereas $Sf1^{-/-}$ urogenital ridges have normal levels of LHX9 (56). Likewise, SF1 is not expressed in the $Wt1^{-/-}$

urogenital ridges (54). Furthermore, in addition to the four binding sites for WT1 in the Sf1 promoter, there also is one binding site for LHX9, and both LHX9 and WT1(−KTS) synergize to regulate transcription of Sf1 (54). Consistent with these findings and the function of SF1 as the key gene downstream of WT1 and LHX9, absence of SF1 leads to failure of development of the bipotential gonad and absence of gonads at birth (59), phenocopying the WT1 and LHX9 knockout mice. These findings are recapitulated in human patients that have SF1 (*NR5A1*) mutations, resulting in XY male-to-female sex reversal (Table 1) (60–64).

Further evidence for the central role of SF1 in bipotential gonad formation comes from analysis of additional knockout mice. Mice lacking CBX2 (chromobox homolog 2; M33) also display XY male-to-female sex reversal and defects in ovarian development (65). CBX2 is a polycomb gene homolog that likely functions through effects on chromatin structure. Absence of CBX2 leads to variable gonadal phenotypes, including many cases of XY null mice in which the gonad appeared to be an ovary and where external genitalia were feminized. *Cbx2*$^{-/-}$ XX offspring were all sterile, ovaries were always smaller, and in two of 16 cases they were absent. Examination of E13.5 null males revealed an absence of testis cords. Many of these findings are consistent with CBX2 playing a role upstream of SF1, a finding consistent with nearly identical defects that are observed in the spleens and adrenal glands of *Cbx2* and Sf1 knockout mice (66). SF1 was decreased at the mRNA and protein levels in the CBX2 knockout spleen and adrenal gland, and at least in Y1 mouse adrenocortical cells, chromatin immunoprecipitation studies showed that CBX2 binds to the Sf1 locus. Consistent with these findings, a mutation in human *CBX2* was discovered to play an important role in sex determination (67). Because of maternal age, prenatal karyotype analysis was performed, and the fetus was shown to be 46,XY. However, the child that was born was phenotypically female with normal ovaries and a female reproductive tract. After analysis of several sex determination genes, compound heterozygous mutations (inherited from each parent) were found in the *CBX2* alleles, resulting in P98L and R443P alterations in each of the CBX2 proteins. These mutations were at evolutionarily conserved amino acid positions. Whereas transfection of a wild-type *CBX2* construct into H295R cells resulted in induction of the endogenous SF1 gene or SF1-luciferase constructs, the two *CBX2* mutant constructs showed minimal induction of SF1 or luciferase. Thus, CBX2 positively regulates SF1 in the bipotential gonad to influence downstream expression of SRY, SOX9, or both in the sex determination cascade. Mutations in *CBX2* and other genes that affect sex determination and female fertility are presented in Table 1.

Whereas absence of the above factors leads to suppressed levels of SF1, a different scenario is observed in mice null for Pod1 (also known as *Tcf21*, transcription factor 21) (68). POD1 is a basic helix-loop-helix (bHLH) transcription factor and is expressed at E11.5 in the coelomic epithelium and the region between the gonad and mesonephros with persistence of expression in both glands at E12.5 and through birth. In the absence of POD1, external genitalia are feminized and the gonads of both sexes are disorganized. Beginning at E11.0, the knockout gonads were shorter, and by E12.5, testes resembled ovaries with absence of testis cords and lack of formation of the male-specific coelomic vessel, whereas the ovary lacked a mesenchymal zone. In both cases, the gonads remained close to the adrenal glands. Similar to the above mutants, PGCs migrate normally into the genital ridges. Although SRY and SOX9 were expressed in presumptive pre-Sertoli cells and differentiated Sertoli cells, levels of SOX9 were suppressed initially and disappeared by E18.5. The major cause of the gonadal defects appears to originate in the steroidogenic interstitial cell population. Normally, wild-type embryonic ovaries do not express CYP11A1 (cytochrome P450 side-chain cleavage enzyme), a downstream target of SF1, and in the male gonad CYP11A1 is not expressed in Leydig cells until after E12.5. However, CYP11A1 is expressed at earlier timepoints (E11.5) in POD1-deficient gonads of both sexes, and there is a delay in expression of SF1. At E12.5, SF1 is expressed in the developing testis, but not the coelomic epithelium, and POD1 and SF1 do not colocalize. However, in the Pod1 knockout, the same cells in the coelomic epithelium and gonad that normally express POD1 (marked by a *lacZ* reporter) are now SF1-positive. Other studies suggest that POD1 acts indirectly to repress Sf1 at the transcriptional level. Thus, POD1 appears to act in an interstitial cell progenitor to suppress SF1, and in the absence of POD1, promiscuous expression of SF1 and its downstream target, CYP11A1, disrupt testis and ovary development. Therefore, not only does absence of SF1 result in defects in the bipotential gonad and sex determination, but ectopic production of SF1 is also detrimental to ovarian and testicular differentiation programs.

C. The XX gonad is not an innocent bystander in sex determination

In mammals, the sex chromosomes, in particular the presence of a Y chromosome, determine whether the undifferentiated gonad will differentiate into a testis (in the case of an XY mammal such as a man) or an ovary (in the case of an XX mammal such as a woman) (69). One fewer X chromosome (*i.e.*, XO observed in Turner's syndrome, in which the

woman has streak ovaries) or two or more copies of the X chromosome in the presence of a Y chromosome (*e.g.*, XXY as observed in Klinefelter's syndrome, in which the man has testes) does not alter the sex differentiation of the bipotential gonad (70, 71). These studies, along with genetic analysis of sex-reversed patients (XX males or XY females), in which a portion of the Y chromosome was either translocated to another chromosome or was deleted from the Y chromosome, respectively, helped to identify the *SRY* gene in mice and men (72, 73). Because this review centers on the ovary, we will focus our discussion on how SRY prevents the development of an ovary and directs the bipotential gonad to form a testis. More detailed discussions of the history of SRY and its role in sex determination are reviewed by leading groups in this field (74–76).

Based on all of the above studies, it was believed that the presence of SRY actively caused testis development to occur and that in the absence of SRY, the ovary passively developed (*i.e.*, the so-called "default" pathway). The *Sry* gene encodes a high-mobility group (HMG) box motif that is responsible for its DNA binding characteristics and its ability to bind DNA. The HMG box, which is the most conserved SRY sequence between mammals, is most prone to mutations that cause male-to-female sex reversal in patients. Based on studies in the mouse, the SRY protein is expressed in each pre-Sertoli cell during a narrow window of several hours in the period of gonadal differentiation (E10.5-E12.5), resulting in up-regulation of *Sox9*, the major (if not the only) gene transcriptionally downstream of SRY (77). In addition to their roles in the bipotential gonad, several of the same transcription factors act on the major genes involved in sex determination. SF1, WT1, and the GATA4/FOG2 complex are required for the transcription of *Sry* (55). Furthermore, the initial low level of expression of *Sox9* in the bipotential gonad requires SF1 (59), and the subsequent high level induction of *Sox9* that is required for testis formation is regulated first by both SRY and SF1 and then by SF1 and SOX9 as part of a feedback loop (78). Because only 10% of cases of male-to-female sex reversal are due to mutations in SRY, many genetic studies were helpful in identifying mutations of gene products involved in sex determination that are downstream of SRY, such as SOX9. For example, loss of function mutations in *SOX9* in humans causes the severe bone disease, campomelic dysplasia, in which XY male-to-female sex reversal is also observed (79, 80), whereas duplication of the region encoding *SOX9 (i.e.*, gain of function mutation) has been shown to cause XX female-to-male sex reversal (81). SOX9 not only results in a positive feedback on itself but also up-regulates SF1 to positively regulate *Sox9* (82) and also induces fibroblast growth factor 9 (FGF9) that signals back through FGF receptor 2 (FGFR2) to increase *Sox9* levels (83–87). These studies continued to support the idea that induction of the "male" genes *Sry* or *Sox9* positively causes the undifferentiated gonad to develop into a testis.

In 1993, both McElreavey *et al.* (88) and Goodfellow and Lovell-Badge (89) proposed the "Z" model for sex determination. In this model, the XX gonad produces a factor "Z" that actively stimulates the ovarian differentiation cascade, and SRY or some downstream target of SRY inhibited this cascade. As shown in Fig. 2, there is an interplay of gene products in the testis and ovarian differentiation cascades that functions to suppress key proteins in the opposing pathway. A significant amount of data have begun to accumulate in support of the Z model. Along with the identification of the genes mutated or duplicated in cases of XX female-to-male and XY male-to-female sex reversal, several mouse models have been created to understand the ovarian differentiation pathway (Table 5). Loss of function mutations in R-spondin homolog 1 (RSPO1) were identified as the cause of the recessive disorder palmoplantar hyperkeratosis. All individuals with this syndrome are phenotypic males (either XY or XX), the first such case of complete XX female-to-male sex reversal (90). RSPO1 is a secreted protein that acts

TABLE 5. Transgenic animals with alterations in sex determination

Transgenic model	Phenotype	Ref.
Rspo1 KO	Development of male-specific coelomic vessel in XX gonad	93, 94
Gata4 KI	No coelomic vessel defects	99
Fog2 KO	No coelomic vessel defects	99
Wnt4 KO	Development of male-specific coelomic vessel in XX gonad	95
Ctnnb1 (β-catenin) cKO (*Nr5a1-Cre*)	Development of male-specific coelomic vessel in XX gonad	97, 99
Ctnnb1 (β-catenin) Tg	XY, male-to-female sex reversal	96
Fst KO	Development of male-specific coelomic vessel in XX gonad	98
Fgfr2 cKO (Heat shock-*Cre*; Sf1-*Cre*)	XY, male-to-female sex reversal	86
Sox9 Tg	XX, female-to-male sex reversal	787
Fgf9 KO	XY, male-to-female sex reversal	83
Sry Tg	XX, female-to-male sex reversal	788
Pdgfra KO	Disrupted testis cord formation and abnormal Leydig cells	789

KO, knockout; cKO, conditional knockout (Cre transgenic); KI, knockin; Tg, transgenic.

extracellularly to increase the signaling of WNT4. The extracellular protein dickkopf homolog 1 (DKK1) binds to a cell surface complex of kringle-containing transmembrane protein 1 (KREMEN1) and low-density lipoprotein receptor-related protein 6 (LRP6) to cause internalization of this ternary complex. However, RSPO1 binds to KREMEN1, dislodging DKK1, and allowing LRP6 access to FRIZZLED (FZD) for binding of WNT4 to the LRP6-FZD coreceptor complex and signaling to increase β-catenin (CTNNB1) levels (91). An alternative model suggests that RSPO1 functions directly to stimulate β-catenin signaling by binding to LRP6 (92), although the activity of RSPO1 alone is much less than in the presence of a WNT ligand (91).

As presented in Table 5, mutations or overexpression of multiple components of the ovary determination cascade [RSPO1, WNT4, β-catenin, or follistatin (FST)] will result in variable degrees of female-to-male sex reversal (93–98). The developing testis contains cords and a coelomic vessel, structures that are absent in the ovary. Mutations in ovary determination cascade genes (*Rspo1*, *Wnt4*, and *Fst*) lead to the presence of a coelomic vessel in the ovary. Besides its role in the regulation of *Sry* expression and testis determination, the GATA4/FOG2 complex also suppresses the expression of *Dkk1*, thereby altering the downstream components of the WNT4 signaling pathway in the ovary (99). Not only WNT4 levels, but also FST levels are suppressed. FST was initially detected as a differentially expressed gene in the developing ovary compared with the testis (100), and absence of FST leads to the development of a coelomic vessel in the developing ovary (98). These findings are milder forms of sex reversal compared with mice lacking RSPO1 or WNT4 where additional alterations are observed, including 17α-hydroxylase/17,20-lyase cytochrome P450 (CYP17A1)-positive, cytochrome P450 aromatase (CYP19A1)-positive adrenal-like cells that produce androgens.

Where do these ovarian and testicular factors converge to influence sex determination? Much data have accumulated to indicate that β-catenin is a central downstream signaling protein in the sex determination pathway. Activation of the WNT4 pathway allows β-catenin to translocate to the nucleus to interact with hepatocyte nuclear factor 1 homeobox A (HNF1A; TCF1) and regulate transcription. The testis differentiation pathway regulates the ovarian differentiation pathway in two ways: 1) human SRY inhibits β-catenin-mediated transcription by direct interaction with β-catenin (101); and 2) SOX9 interacts with β-catenin to cause their mutual degradation. These studies suggest that β-catenin may be the infamous Z factor that is regulated by the testis differentiation pathway. Thus, in the ovarian differentiation pathway, increased levels of β-catenin will result in degradation of SOX9, preventing it from inducing itself or other genes, such as *Fgf9*. Indeed, studies in both humans and mice support a role for β-catenin as a major pro-ovary and anti-testis factor. In humans, a duplication of the region encoding WNT4 and RSPO1 has been shown to cause XY male-to-female sex reversal (102). In mice, overexpression of a stable allele of β-catenin causes XY male-to-female sex reversal (96), whereas conditional knockout (cKO) of β-catenin (97, 99) leads to the presence of a coelomic vessel in XX gonads.

D. Sexually dimorphic changes in the initiation of meiosis

Before entry of PGCs into the urogenital ridge, XX ("female") and XY ("male") PGCs appear to function identically in all aspects. This suggests that there are no sexually dimorphic products for formation, migration, and entry of the PGCs into the genital ridge and that the sex chromosomes are not influencing this process. The first mechanistic difference between an XX and an XY germ cell in the genital ridge is reactivation of the inactive X in the female PGC. Soon after this point, there is a major change in the fate of these PGCs. Whereas XY germ cells arrest in mitosis and do not divide again until postnatally as spermatogonia, the XX germ cells continue to divide and then enter meiosis at approximately E12.5. Subsequently, the female germ cells arrest at the diplotene stage of meiosis I and do not resume meiosis until postnatally during ovarian folliculogenesis. As mentioned in *Section I*, this indicates that the last known dividing oocyte "stem cell" must also disappear at this time. Elegant studies from several laboratories have been able to piece together the molecular details of these important sexual dimorphic processes (Fig. 3).

The first gene necessary to lay the groundwork for these changes is *Dazl* (deleted in azoospermia-like). *Dazl* is expressed in both XX and XY PGCs at the time of arrival at the genital ridge (103). Normally, both XX and XY germ cells begin to express *Sycp3* (synaptonemal complex protein 3) but in the absence of DAZL, SYCP3 is essentially absent, thereby defining a "meiosis-competent" germ cell (104). In the presence of DAZL, this intermediate premeiotic germ cell is responsive to retinoic acid, the meiotic initiation molecule derived from mesonephroi of both sexes (105, 106). In addition, DAZL is an RNA binding protein that specifically binds to the 3′ UTR of the *Sycp3* mRNA to positively regulate its translation (107). Thus, DAZL acts both upstream in the PGC as well as downstream to regulate meiosis.

If both male and female germ cells are "primed" for meiosis and bathed in a similar retinoid acid environment, what causes the sexually dimorphic paths? The gene prod-

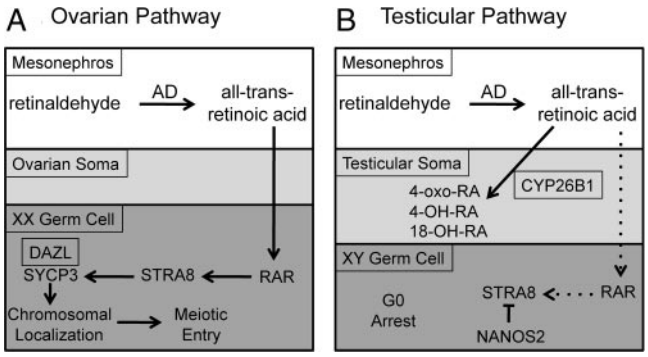

FIG. 3. Sexually dimorphic initiation of meiosis in the embryonic ovary. During embryogenesis, the mesonephroi adjacent to the developing ovary (A) and testes (B) contain several aldehyde dehydrogenases that convert retinaldehyde to all-trans-retinoic acid (RA). The somatic cells of the developing testes contain the enzyme CYP26B1, which degrades RA to pass freely to the germ cell to bind to retinoic acid receptors (RAR). In the developing ovary, RA induces STRA8, which induces SYCP3, which is stably translated in the presence of DAZL and becomes chromosomally localized as the XX germ cell becomes an oocyte and enters meiosis. In male germ cells, the absence of high enough levels of RA early and under the repressive actions of NANOS at later time points, STRA8 is not synthesized and the XY germ cell becomes mitotically arrested.

uct that is responsible for these changes is cytochrome P450 26B1 (CYP26B1), the major protein that degrades retinoic acid. CYP26B1 is down-regulated in somatic cells of the ovary at E12.5 but up-regulated in the testis, thus allowing accessibility of the XX germ cells, but not the XY germ cells, to retinoic acid (105). In the absence of CYP26B1, the XY germ cells of the embryonic testes initiate meiosis, mimicking the XX germ cells. Furthermore, the synthetic retinoid Am580, which is not degraded by CYP26B1, has a similar induction of meiosis in the germ cells of wild-type testes (108), yielding further evidence of the interrelationships of retinoic acid, CYP26B1, and the initiation of meiosis.

How is retinoic acid acting in the germ cell to regulate meiosis? The Page laboratory (109) had previously shown that a known retinoic acid target, *Stra8* (stimulated by retinoic acid gene 8), a bHLH transcription factor, was expressed in the embryo exclusively in XX germ cells before meiotic entry. Using a knockout approach, their laboratory showed that absence of STRA8 blocked entry of the XX germ cells into meiosis, and the meiotic markers *Dmc1* and *Spo11* were undetectable in the E14.5 ovaries (110). Consistent with retinoic acid as the major inducer of *Stra8* in embryonic germ cells, absence of CYP26B1 also leads to increased *Stra8* in the embryonic testes after E12.5 (111) paralleling the *Stra8* rise seen in females. As the levels of CYP26B1 wane after E13.5 in the testes with increased exposure of the XY germ cells to retinoic acid, a "backup" protein, NANOS2, is expressed and represses *Stra8*; absence of NANOS2 in the males leads to induction of *Stra8* (after E13.5), whereas expression of NANOS2 in embryonic female germ cells suppresses *Stra8* (111). Thus, a retinoic acid to *Stra8* pathway results in sexually dimorphic differences in meiotic entry in females *vs.* males. Additional signaling pathways that regulate meiotic arrest and reentry postnatally are discussed in the following section.

III. Ovarian Folliculogenesis

Autocrine, paracrine, juxtacrine, and endocrine factors are essential for ovarian folliculogenesis. Besides the oocyte, the reproductive cargo of the follicle, the somatic cells of the ovarian follicle, namely the granulosa cells that function as the ovarian "nurse" cells and thecal cells that function to supply the granulosa cells with the estrogenic precursor, androstenedione, are recruited to the oocyte and are directly or indirectly necessary for oocyte development, physiology, and survival. As shown in Fig. 4, the major stages of ovarian folliculogenesis are formation of the primordial follicle; recruitment into the growing pool to form a primary, secondary, and tertiary follicle; and lastly ovulation and subsequent formation of a corpus luteum (CL). In this section, we will describe these steps in greater detail. Several of the mutations that cause infertility in humans are also summarized in Table 1.

A. Formation of an ovarian follicle—oocyte survival *vs.* primordial follicle formation

Before formation of an ovarian follicle, oocytes are present within germ cell clusters, also referred to as germ cell cysts or nests. Primordial follicle formation occurs when oocytes that survive the process of germ cell cluster breakdown are individually surrounded with squamous pre-granulosa cells. This represents the first stage of folliculogenesis, and it takes place during the latter half of fetal development in humans and in the days immediately following birth in mice (112, 113). In mammals, the population of primordial follicles serves as a resting and finite pool of oocytes available during the female reproductive life span. Although germ cell cluster breakdown, primordial follicle formation, and subsequent recruitment remain the least understood steps of folliculogenesis, key regulators of these initial stages of follicle development continue to be identified. Furthermore, despite many unanswered questions during this crucial period, the concept of ovarian cross talk between oocytes and somatic cells is apparent from the formation of primordial follicles onward (114, 115).

After differentiation of PGCs, oogonia undergo mitotic proliferation with incomplete cytokinesis, leaving daughter cells connected by intercellular bridges. The ma-

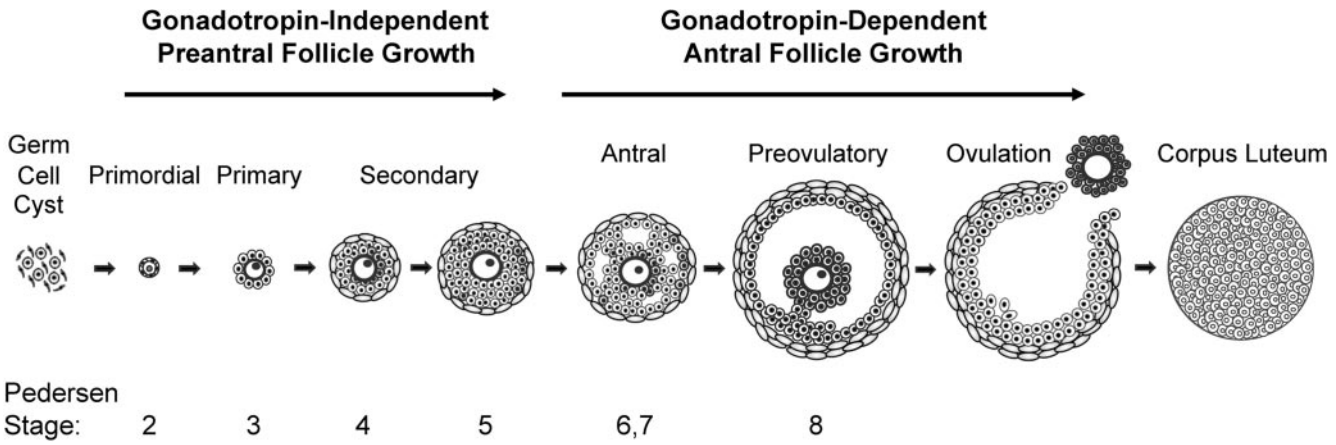

FIG. 4. Classification of the major stages of mammalian folliculogenesis. Primordial follicles form 1–2 d after birth in mice and *in utero* in humans. Preantral follicles begin to develop prenatally in humans, whereas in mice this occurs postnatally. In both mice and humans, preantral follicular development does not require stimulation by the pituitary gonadotropins. By the secondary stage, an additional layer of somatic cells, the theca, forms outside the basement membrane of the follicle. At puberty, FSH secreted by the pituitary promotes further granulosa cell proliferation and survival. Ovulation of the dominant follicle occurs in response to a rise in the other pituitary gonadotropin, LH. After ovulation, the remaining granulosa and theca cells undergo terminal differentiation to form the CL. In most cases, primordial, primary, secondary, preantral, and antral are names commonly used to refer to the different stages of folliculogenesis; however, a classification system described by Pedersen and Peters (768) is also used. Pedersen stages are determined based on the size of the oocyte and the number of granulosa cells in cross-section for any given follicle. Although not shown, certain Pedersen stages are subdivided (*i.e.*, 3a, 3b, 5a, 5b) depending on the number of granulosa cells surrounding the oocyte.

jority of germ cells in a cluster divide synchronously such that a single germ cell cluster contains 2^n germ cells (116). Germ cells subsequently enter meiosis, becoming oocytes. Individual oocytes within these nests lack surrounding somatic cells, and the majority of the oocytes will undergo apoptosis as the germ cell clusters break down to give rise to primordial follicles (117).

Interestingly, although intercellular bridges appear to be evolutionarily conserved structures connecting germ cells from insects to mammals, they do not appear to be essential for follicle formation or female fertility in mice. Testis-expressed gene 14 (TEX14) was discovered as the first essential protein in the postnatal intercellular bridge that interconnects differentiating male germ cells during spermatogenesis (118). Whereas male mice lacking TEX14 are sterile due to postnatal defects in spermatogenesis (118), TEX14 null females have normal fertility over a 6-month breeding period and follicles in all stages of folliculogenesis at 1 yr of age, suggesting that intercellular bridge formation during embryonic germ cell development is not essential for female fertility (119). Nevertheless, intercellular bridges may have a role in determining the initial pool of oocytes because postnatal day (P) 2.5 *Tex14*$^{-/-}$ ovaries have fewer oocytes relative to control ovaries.

Members of the B cell lymphoma/leukemia (BCL) protein family have opposing functions in regulating germ cell apoptosis (120). Although BCL2 and BCLX protect against apoptosis, BAX promotes cell death. Deletion of antiapoptotic BCL2 results in fewer oocytes and primordial follicles at 6 wk of age, with no differences in the number of primary and preantral follicles (121). Neonatal ovaries, however, have not been examined; this would be useful to determine the initial reserve of primordial follicles. As has been observed for BCLX (122), BCL2 may influence survival during PGC development rather than, or in addition to, during germ cell cluster breakdown and primordial follicle endowment. For example, loss of proapoptotic BCL member *Bax* increases the number of germ cells at E13.5, before the start of meiosis and germ cell apoptosis (123), but its role during primordial follicle formation is controversial. Although 6-wk-old *Bax*$^{-/-}$ females have increased nonatretic primordial follicles compared with wild-type controls and, accordingly, a prolonged reproductive life span (124), there are conflicting reports regarding the roles of BAX postnatally during primordial follicle endowment. Postnatal day 4 (P4) *Bax*$^{-/-}$ females were originally reported to have similar numbers of primordial follicles; however, others documented an increase in primordial follicles at P4 (123). This discrepancy may be due to criteria used in classifying primordial follicles because Greenfeld *et al.* (123) counted follicles with a mixture of squamous and cuboidal granulosa cells as primordial, rather than as transitioning into primary follicles.

One regulator of BAX is the aryl hydrocarbon receptor (AHR), a ligand-activated member of the PER-ARNT-SIM family of transcription factors that is activated by polycyclic aromatic hydrocarbons (PAHs). The mouse *Bax* promoter contains two consensus AHR response elements, and exposure of female mice to PAHs induces *Bax*

and subsequently apoptosis in primordial and primary oocytes (125). In support of a role for BAX in establishing primordial follicles, ovaries from *Ahr* null mice have approximately a 2-fold increase in primordial follicles at P2-P4 (126, 127), but no differences in germ cell number before birth on E18 (126). Interestingly, in ovaries from $Ahr^{-/-}$ mice, there are no differences in the number of primordial follicles by P8, and there are actually fewer antral follicles at 8 wk (126), whereas ovaries from $Bax^{-/-}$ females at P7 still contain significantly more primordial follicles (123), suggesting that additional regulators of BAX are active in oocytes during this period. The AHR-BAX pathway is required for PAH-induced oocyte death in mice (125), suggesting that activation of AHR in humans exposed to environmental toxins may contribute to premature ovarian failure (POF), also known as primary ovarian insufficiency.

A second apoptotic pathway that operates in cells involves activation of caspases, which are proteases that upon activation cleave a number of cellular proteins leading to apoptosis. Targeted disruption of the caspase 2 gene (*Casp2*) resulted in significantly more primordial follicles at P4 in $Casp2^{-/-}$ ovaries, and oocytes from $Casp2^{-/-}$ mice were resistant to the chemotherapeutic agent doxorubicin (128). Thus, during fetal development and in the perinatal period, apoptosis is important in establishing the primordial pool, and apoptotic cell death continues throughout folliculogenesis in oocytes and granulosa cells during follicular atresia.

FIGLA (factor in the germline α) is a germ cell-specific bHLH transcription factor that is required for initial follicle formation. The bHLH family of transcription factors functions by forming homo- or heterodimers that bind to gene regulatory regions containing E-box consensus sequences (CANNTG). Female mice lacking *Figla* are sterile secondary to a complete absence of follicles and oocytes (129). Upon closer inspection, although ovaries from E18 *Figla* null and wild-type females have similar numbers of germ cell clusters in the perinatal period, primordial follicles fail to form, and oocytes are depleted by P2 in *Figla* null ovaries. FIGLA was first identified through *in vitro* studies as a regulator of all three zona pellucida genes, *Zp1*, *Zp2*, and *Zp3* (130). The zona pellucida proteins comprise the glycoprotein-rich matrix that surrounds developing oocytes and is essential for fertilization. Although mice deficient in *Zp1*, *Zp2*, or *Zp3* are either infertile or subfertile (131–133), follicles lacking any of the zona pellucida genes can progress through all stages of folliculogenesis. Furthermore, the zona pellucida matrix does not form until oocytes begin to grow, suggesting that FIGLA has additional downstream targets whose misregulation prevents early oocyte-somatic cell interactions. Human *FIGLA* is also expressed only in female germ cells and, like mouse FIGLA, can heterodimerize with the ubiquitous E12 bHLH transcription factor and bind E-box consensus elements in the human *ZP2* promoter (134, 135). Human *FIGLA* transcripts are detectable by 14 wk gestation and dramatically increase at midgestation (19 wk), corresponding to the time of human primordial follicle formation.

Gene expression studies comparing ovaries from $Figla^{-/-}$ and wild-type mice at four different time points showed the greatest number of differentially expressed genes when comparing newborn ovaries, consistent with the onset of primordial follicle formation (136). Of the altered genes, 165 were decreased, and 38 were increased in $Figla^{-/-}$ ovaries, and a large percentage of these genes code for transcription factors or proteins with nucleic acid binding functions. Interestingly, many genes normally expressed in the testis were up-regulated in $Figla^{-/-}$ ovaries, suggesting a role for FIGLA in repressing male germ cell-specific genes in oocytes. *Pou5f1*, which postnatally is germ cell-specific and expressed in growing oocytes, was decreased, and its postnatal up-regulation occurs just after FIGLA is expressed (137), suggesting that FIGLA is one regulator of this important transcription factor during this key time period. Members of the oocyte-specific NLRP (NACHT, leucine-rich repeat and pyrin domain containing) gene family were also decreased or absent in *Figla* null ovaries. Despite shared structural motifs in the NLRP family, individual proteins do not appear to be functionally redundant; inactivation of a single *Nlrp* gene, *Nlrp5*, also known as Mater, prevents embryo development beyond the two-cell stage (138). Thus, in addition to identifying target genes and pathways during primordial follicle formation, the results of these studies suggest that FIGLA might directly or indirectly be important during the later stages of oocyte development.

Although upstream regulators of FIGLA are unknown, studies have identified a number of signaling pathways important in germ cell cluster breakdown and primordial follicle formation that could potentially regulate FIGLA or other mediators of early folliculogenesis. Previous observations supported a role for maternal hormones in the maintenance of germ cell clusters during mouse fetal development. Multi-oocyte follicles containing two or more oocytes within a follicle boundary might arise from oocyte clusters that fail to separate. Multi-oocyte follicles occur more frequently in mice exposed to estrogens or estrogenic compounds prenatally or in the neonatal period (139, 140). In an *in vitro* ovarian culture system, ovaries from newborn mice cultured over the course of 7 d in the presence of estradiol, progesterone, or genistein, a phytoestrogen, exhibit fewer single oocytes and more germ cell clus-

ters, supporting a role for these steroids in blocking cluster breakdown and primordial follicle formation (141). Newborn rat ovaries cultured in the presence of progesterone or newborn rats injected with progesterone show a similar reduction in primordial follicle formation (142). The authors propose that in developing mice, high maternal steroids provide an inhibitory signal that prevents cluster breakdown, whereas at birth, the drop in these steroids allows clusters to break apart (141, 142). However, whereas mice lacking *Cyp19a1*, which are deficient in estradiol, have a reduction in primordial follicles at 10 wk of age (143), ovaries in the neonatal period have not been examined, and the chief defect in folliculogenesis in CYP19A1-deficient females is a block at the antral follicle stage (144). Likewise, mice lacking the progesterone receptor (PR) have normal follicular development, and the primary ovarian defect is inability to ovulate (145). Furthermore, in humans, primordial follicle formation occurs during the last half of fetal development when maternal estradiol and progesterone are high, suggesting a different mechanism controlling primordial follicle formation in humans. Studies in nonhuman primates suggest that estradiol actually promotes primordial follicle formation. Late gestation fetuses from baboons treated with an aromatase inhibitor show a reduction in primordial follicles and an increase in germ cell clusters (146). Thus, the effects and physiological relevance of estradiol and progesterone during follicle formation are unclear, and further studies are warranted. It is possible that the effects of progesterone in rodents are partly due to signaling through a membrane progesterone receptor, rather than the classical nuclear receptor. In rats, the inhibitory effect of progesterone on primordial follicle assembly is not completely reversed when the nuclear PR antagonist, RU-486, is also added (142).

Whereas estradiol and progesterone may partially inhibit primordial follicle formation in rodents, NOTCH signaling appears to promote it. There are four NOTCH receptors in mammals that interact with two families of ligands, DELTA-like and JAGGED, on neighboring cells. Ligand binding of NOTCH leads to proteolytic cleavage by the ADAM-family of metalloproteases, followed by cleavage by γ-secretase to free the NOTCH intracellular domain (NICD). The NICD translocates to the nucleus and interacts with the DNA-binding CSL [CBF1, Su(H), and Lag-1] transcription factor and its coactivator, Mastermind, to promote transcription (147). In neonatal mouse ovaries, the NOTCH2 receptor is expressed in pregranulosa cells, the NOTCH ligand JAGGED1 is expressed in germ cells, and NOTCH target genes *Hes1* and *Hey2* are in both cell types. Using a similar *in vitro* ovarian culture system, blocking NOTCH signaling with γ-secretase inhibitors decreases primordial follicle formation and increases the germ cells remaining in clusters (148). Further support for NOTCH signaling during primordial follicle formation and later stages of folliculogenesis is evident in lunatic fringe (*Lfng*) null mice. Lunatic fringe is a modulator of the NOTCH pathway and is expressed in granulosa and thecal cells of developing follicles. Although most *Lfng* null mice die shortly after birth, females that survive are infertile with follicular defects that include multi-oocyte follicles (149).

B. Maintenance of primordial follicles and initial recruitment

Follicle recruitment is generally subdivided into two broad categories: initial activation of primordial follicles, which occurs throughout life until menopause; and, after puberty, cyclic recruitment of a limited number of small follicles from the growing cohort, from which a subset is selected for dominance and ovulation (150). Although the initial recruitment of follicles from the primordial into the growing pool remains a poorly understood process, in recent years mutant mouse models have led to the identification of several key transcription factors and signaling pathways that regulate this early step in folliculogenesis. The transition from primordial to primary follicle is marked histologically by a morphological change in granulosa cells from squamous to cuboidal.

NOBOX (newborn ovary homeobox), SOHLH1 (spermatogenesis and oogenesis helix-loop-helix 1), and SOHLH2 are critical transcription factors during the transition from primordial to primary follicles. *Nobox* and *Sohlh1* were both identified using an *in silico* subtraction strategy to identify expressed sequence tags that are preferentially expressed in oocytes but not in other mouse cDNA libraries (151). *Sohlh2* was subsequently discovered using the BLAST program of the National Center for Biotechnology Information to search for bHLH domains that share homology with *Sohlh1* (152). All three genes are expressed in germ cell clusters, primordial follicles, and primary follicles in females, whereas *Sohlh1* and *Sohlh2* are also expressed in spermatogonia. Whereas SOHLH1 and SOHLH2 disappear rapidly as oocytes reach the secondary follicle stage, NOBOX continues to be expressed throughout folliculogenesis. Mice lacking any of these three transcription factors are sterile (153–156). Although ovaries from newborn $Nobox^{-/-}$ or $Sohlh1^{-/-}$ mice contain similar numbers of germ cell clusters and primordial follicles relative to controls, progression beyond the primordial follicle stage is disrupted. By P3, control mice have formed primary follicles, but $Nobox^{-/-}$ and $Sohlh1^{-/-}$ mice lack primary follicles and begin to demonstrate an early postnatal loss of oocytes. Ovaries from $Sohlh2^{-/-}$ are remarkably similar to $Sohlh1^{-/-}$ and $Nobox^{-/-}$ ovaries, although occasional follicles escape early postnatal

death and progress to multilayered follicles. Thus, mice deficient in *Nobox*, *Sohlh1*, or *Sohlh2* have defects in the primordial to primary follicle transition, and these genes do not appear to function redundantly during early folliculogenesis.

Molecular analysis of *Nobox*$^{-/-}$ ovaries shows that expression levels of key oocyte-specific genes, including *Gdf9*, *Bmp15*, *Mos*, *Pou5f1*, and several *Nlrp* family members, are dramatically decreased (157). Furthermore, *Gdf9* and *Pou5f1* appear to be directly regulated by NOBOX, and several other down-regulated genes also contain putative NOBOX consensus binding elements in their promoters (158). Although *Figla* expression and some FIGLA targets, such as *Zp1*, *Zp2*, and *Zp3*, are not altered in *Nobox*$^{-/-}$ ovaries, many of the gene changes observed in *Nobox*$^{-/-}$ ovaries were also found in *Figla*$^{-/-}$ ovaries (136), including an up-regulation of several testis-specific genes, which suggests that these transcription factors function in both individual and redundant regulatory pathways during oogenesis.

Ovaries from *Sohlh1*$^{-/-}$ mice have gene changes similar to those observed in *Nobox*$^{-/-}$ ovaries, but also show a significant reduction in *Nobox*, *Figla*, *Zp1*, and *Zp3* (155). In contrast, *Sohlh1* is not significantly affected in *Nobox*$^{-/-}$ ovaries, suggesting that SOHLH proteins function upstream of *Nobox* and *Figla*. The ovarian physiology in *Sohlh1*$^{-/-}$ mice, however, is less severe than in *Figla*$^{-/-}$ mice, which may be due to persistent low-level expression of *Figla* in these mice. An additional transcription factor, *Lhx8*, which encodes a LIM homeodomain protein, is also down-regulated in *Sohlh1*$^{-/-}$ ovaries and the ovarian phenotype in *Lhx8*$^{-/-}$ mice phenocopies *Sohlh1*$^{-/-}$ mice (155, 159). By chromatin immunoprecipitation and reporter assays, SOHLH1 appears to directly regulate *Lhx8*, *Zp1*, and *Zp3* through conserved E-box promoter elements, but not *Nobox* or *Zp2*. Newborn ovaries from *Sohlh2*$^{-/-}$ mice have very similar molecular changes as those from *Sohlh1*$^{-/-}$ mice, consistent with data suggesting that SOHLH1 and SOHLH2 form heterodimers (160). Interestingly, *Sohlh2* is down-regulated in *Sohlh1*$^{-/-}$ ovaries, and likewise, *Sohlh1* is down-regulated in *Sohlh2*$^{-/-}$ ovaries, supporting a further role for transcriptional cross-regulation (153). Unlike the findings in *Nobox*$^{-/-}$ ovaries, in newborn ovaries from *Sohlh1*$^{-/-}$ or *Sohlh2*$^{-/-}$ mice, *Kit* receptor is down-regulated (153). *Kit* is also decreased in newborn *Figla*$^{-/-}$ ovaries (136), and in *Lhx8*$^{-/-}$ newborn ovaries, both *Kit* and its ligand, *Kitl*, are reduced (159).

Interactions between KIT ligand and the KIT tyrosine kinase receptor appear to be critical in early folliculogenesis. During postnatal ovarian development, KIT is expressed in oocytes and KIT ligand is expressed in pregranulosa and granulosa cells throughout folliculogenesis (161–165). The importance of KIT/KIT ligand signaling during folliculogenesis was first identified in mutant mice and later extended by *in vivo* function blocking and *in vitro* culture studies. As mentioned in *Section II*, many different mutations of *Kit* or *Kitl*, encoded by the *W* and *Sl* loci, respectively, result in defects in PGC development. A number of alleles have been identified in both loci that have differential effects on female fertility. In particular, the *Steel Panda* (Sl^{pan}) and *Steel Contrasted* (Sl^{con}) mutations, which result in reduced expression of normal *Kitl* transcript in the gonads (166, 167), provide insight into the roles of KIT/KIT ligand during early folliculogenesis. In addition to a reduction in the number of germ cells, mice homozygous for the Sl^{pan} and Sl^{con} alleles have fewer oocytes in the growing pool, and the majority of those that develop arrest at the primary follicle stage. Female mice with the *Steel Transfer* (Sl^{t}) mutation display a similar arrest of folliculogenesis with many primordial follicles present, but few growing follicles (168). Because Sl^{con} females might have a single litter and have occasional follicles that progress beyond the primary stage, despite increased atresia at the antral follicle stage, there may be a threshold level of KIT ligand necessary for primordial follicle recruitment and later follicular development. The Sl^{pan}, Sl^{con}, and Sl^{t} mutant mice might have sufficient levels of KIT ligand produced for some follicles to reach the primary stage, but insufficient levels of KIT ligand lead to early arrest or increased atresia.

In vivo and *in vitro* studies further support a role for KIT/KIT ligand interactions during initiation of follicular growth from the primordial pool. Newborn mice injected with an antibody to KIT (ACK2) that blocks interaction with KIT ligand have a block at the primordial stage of follicle development. When P2 mice, which have formed primordial follicles, are injected with ACK2 antibodies, primary follicle development is only slightly interrupted, although these mice show a delay in antral follicle development (165). Similar inhibition of primordial follicle development is seen in an *in vitro* neonatal rat ovary organ culture system. In contrast, treatment of neonatal rat ovaries with recombinant KIT ligand accelerates the primordial to primary follicle transition, resulting in an increased number of growing follicles (169). These studies and the *Kitl* mutant mice suggest that intact KIT signaling is not essential during germ cell cluster breakdown, but it is necessary during the transition from primordial to primary follicles and in later stages of follicle development.

The mechanisms by which KIT ligand/KIT signaling contribute to the primordial to primary follicle transition are not entirely known; however, evidence suggests that the KIT ligand/KIT pathway induces the PI3K/AKT path-

way, leading to phosphorylation and inactivation of forkhead box O3 (FOXO3; FKHRL1), an inhibitor of primordial follicle activation. PI3K catalyzes the conversion of the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2) to the second messenger, phosphatidylinositol 3,4,5-trisphosphate (PIP3), which leads to AKT activation. FOXO3 is a member of the FOXO subfamily of forkhead transcription factors, which are downstream targets of the PI3K/AKT pathway; activation of the PI3K/AKT pathway functionally suppresses FOXO transcription factors secondary to phosphorylation and nuclear exclusion (170). When mouse or rat oocytes are treated with KIT ligand, FOXO3 is phosphorylated in a PI3K/AKT-dependent manner because treatment with a PI3K inhibitor prevents AKT activation and subsequent FOXO3 phosphorylation (171).

The functional consequence of FOXO3 inactivation is illustrated in mice lacking *Foxo3*. Although *Foxo3* is expressed in multiple tissues, the chief phenotypic defect in $Foxo3^{-/-}$ mice is a rapid decline in fertility and ultimately sterility by 15 wk of age. Histologically, by 2 wk of age, ovaries from $Foxo3^{-/-}$ mice are enlarged relative to controls, have a marked increase in growing and atretic follicles, and have an absence of primordial follicles (172). By 8.5–9.5 wk of age, ovaries from $Foxo3^{-/-}$ mice have abundant zona pellucida remnants (172, 173), suggesting widespread follicular activation, followed secondarily by atresia. Because forkhead box O1 (FOXO1) mRNA and protein are expressed in mouse oocytes (174, 175), it appears that FOXO1 regulates different functions or is not expressed in sufficient quantities to substitute for FOXO3. Thus, deletion of *Foxo3* removes an oocyte activation brake, leading to premature recruitment of follicles and a complete depletion of the primordial pool before sexual maturity. Further support for the inhibitory role of FOXO3 on follicular activation is seen in transgenic mice expressing constitutively active *Foxo3* under control of the *Zp3* promoter, which is active beginning at the primary stage of folliculogenesis. *Zp3-Foxo3* transgenic females are severely infertile due to impeded follicular development beyond primary and secondary follicles (176).

Additional genetic studies support a critical role for the PI3K/AKT pathway upstream of FOXO3. Oocyte-specific deletion of *Pten* (phosphatase and tensin homolog deleted on chromosome 10), which opposes the actions of PI3K by converting PIP3 to PIP2, causes premature activation of primordial follicles, with an ovarian phenotype nearly identical to *Foxo3* deletion (177, 178). Loss of *Pten* in oocytes results in enhanced PI3K activity, AKT hyperactivation, and functional suppression of Foxo3 secondary to hyperphosphorylation and nuclear export. Concurrent loss of *Pten* and *Foxo3* in oocytes does not have a synergistic effect on follicle activation (178), and the PI3K inhibitor LY294002 suppresses primordial follicle activation in ovaries with *Pten*-deficient oocytes but has no effect in *Foxo3* mutant ovaries (177), suggesting a linear PTEN-PI3K-AKT-FOXO3 pathway. The initial fertility of *Foxo3* and conditional *Pten* mutant females suggests that this pathway is not essential for later steps of folliculogenesis, ovulation, or fertilization. The critical role for this pathway in regulating primordial follicle activation throughout the reproductive life span has been demonstrated using a tamoxifen-inducible germ cell-specific Cre mouse model ($Vasa\text{-}Cre^{ERT2}$). Administration of tamoxifen to adult $Vasa\text{-}Cre^{ERT2}$ $Foxo3^{flox/flox}$ or $Vasa\text{-}Cre^{ERT2}$ $Pten^{flox/flox}$ mice causes the same global activation of primordial follicles that is seen in the ubiquitous *Foxo3* and *Pten* knockout or conditional models (177). It is unknown whether KIT is an upstream modulator of this pathway *in vivo*, or at least one of many receptor tyrosine kinases that might initiate this signaling cascade to promote primordial follicle recruitment. However, female mice with a mutated KIT receptor (Kit^{Y719F}) that prevents binding and activation of PI3K have a retardation of folliculogenesis beyond the primary stages of development (179), similar to mice with constitutively active FOXO3 (176). It would be interesting to determine whether FOXO3 is predominantly nuclear in these mice and whether loss of *Foxo3* would rescue the observed histological findings.

How nuclear FOXO3 prevents primordial follicle activation is unknown. It has been proposed to arrest growth through increased expression of cyclin-dependent kinase inhibitor 1B (CDKN1B; also known as $p27^{Kip1}$) (180), which is retained in the nuclei of 20-d-old mice expressing constitutively active *Foxo3* (176), and FOXO3 regulation of $p27^{Kip1}$ has been documented in other systems (181). Indeed, $p27^{Kip1}$ also functions as a suppressor of primordial follicle activation because $Cdkn1b^{-/-}$ females exhibit premature activation of primordial follicles (182). However, whereas $Cdkn1b^{-/-}$ and $Foxo3^{-/-}$ both have increased recruitment from and ultimate depletion of the primordial follicle pool, mice lacking both genes show a synergistic acceleration of follicle activation, suggesting that they can function independently to suppress primordial follicle activation. Furthermore, by Western blot analysis, $p27^{Kip1}$ levels are normal in oocytes from $Foxo3^{-/-}$ mice, and $p27^{Kip1}$-deficient oocytes have normal total and phospho-FOXO3 (182), although immunolocalization of each protein in the respective mutants may have been more conclusive. It is likely that FOXO3 has multiple targets in primordial oocytes that contribute to the quiescent state until the appropriate signal initiates activation and oocyte growth.

Finally, recent work has identified an additional critical mediator of PI3K activation, 3-phosphoinositide dependent protein kinase-1 (PDPK1; PDK1). Binding of PIP3 leads to activation of PDK1 and subsequent phosphorylation of AKT and other kinases of the AGC family (protein kinases A, G, and C) (183). Oocyte-specific deletion of *Pdk1* caused infertility; despite no difference in ovarian morphology and follicle count through P23, pubertal ovaries were smaller and contained fewer follicles at all stages, secondary to depletion of primordial follicles (184). Whereas KIT ligand stimulated AKT phosphorylation in cultured wild-type oocytes, it did not in *Pdk1* cKO oocytes, thereby preventing FOXO3 phosphorylation (and thus presumably primordial follicle activation). However, phosphorylation of ribosomal protein S6 kinase (RPS6KB1; S6K1), an additional PDK1 target downstream of PI3K activation, was also disrupted. This prevented phosphorylation and activation of the 40S ribosomal protein, RPS6, which is necessary for ribosome biogenesis and protein translation (185). Liu and colleagues (184) predicted that RPS6 may be downstream of PI3K-AKT-S6K1 and important in oocyte growth after primordial follicle activation. Indeed, conditional deletion of *Rps6* resulted in a more profound ovarian defect with *Rsp6* cKO ovaries already smaller by P23 and completely devoid of follicles by 8 wk of age (184). Thus, the suppressed AKT signaling in PDK1-deficient oocytes appears to both prevent primordial follicle activation through retained nuclear FOXO3 and negatively impact oocyte survival through decreased RPS6 activity. This also suggests tight control over the PI3K-PDK1-AKT signaling pathway in maintaining and activating the pool of primordial follicles.

Whereas FOXO3 is the key oocyte factor critical for suppressing primordial follicle activation, another forkhead domain transcription factor, forkhead box L2 (FOXL2), is crucial in the transition from squamous to cuboidal granulosa cells that occurs during the primordial to primary transition. Nonsense mutations in *FOXL2* cause type I blepharophimosis/ptosis/epicanthus inversus syndrome (BPES) and *FOXL2* duplications cause type II BPES (186). Type I BPES is also associated with POF. In the ovary, FOXL2 is expressed in pre-granulosa cells surrounding primordial follicles and in granulosa cells throughout folliculogenesis. *Foxl2*$^{-/-}$ mice form primordial follicles, but differentiation of granulosa cells from the squamous to cuboidal state is blocked, granulosa cell proliferation is interrupted, oocyte growth is retarded, and secondary follicles fail to form (187, 188). At 2 wk of age, the majority of primordial follicles are activated in *Foxl2*$^{-/-}$ ovaries, as demonstrated by expression of TGFβ family member growth differentiation factor 9 (*Gdf9*), a marker of oocyte activation (see *Section III. C*). However, this activation is accompanied by widespread follicular atresia and a near absence of primordial follicles by 8 wk of age because the defective granulosa cells fail to support growing oocytes (187). Another TGFβ superfamily member, AMH (Müllerian-inhibiting substance, MIS), shows reduced expression in *Foxl2*$^{-/-}$ compared with wild-type ovaries (187); however, this is likely secondary to the general perturbation of folliculogenesis in *Foxl2*$^{-/-}$ ovaries.

AMH induces regression of the Müllerian ducts during male fetal sex differentiation (189). In the ovary, AMH produced by granulosa cells of growing follicles also appears to suppress primordial follicle recruitment. In the rodent and human ovary, AMH and its type II receptor, AMHR2, are expressed in granulosa cells of primary and growing preantral follicles (190, 191). Although female mice lacking AMH are fertile, *Amh*$^{-/-}$ juveniles show an increase in growing follicles, and by 4 months this increase is reflected in a reduction of primordial follicles compared with wild-type littermates. By 13 months of age, *Amh*$^{-/-}$ females have few remaining primordial follicles and correspondingly few growing follicles (192). *In vitro* studies support the *in vivo* findings because neonatal ovaries cultured in the presence of recombinant AMH show fewer growing follicles (193). Thus, AMH appears to inhibit the growth of primordial follicles, and in its absence, there is a faster depletion of growing follicles, although it is unknown how AMH functions to repress primordial follicle recruitment (*i.e.*, whether this is a direct or indirect effect of AMH).

Clinically, serum AMH may be a useful biomarker of ovarian reserve (190). In women and mice, serum AMH declines with increasing age. Whereas it is difficult to establish a direct link between serum AMH and the primordial follicle pool in humans, antral follicle number is positively correlated with AMH (194). In mice, there is a strong correlation between serum AMH and the number of primordial follicles (195).

Neurotrophins are soluble growth factors whose functions in development extend beyond the nervous system and include regulation of early folliculogenesis. At least four of the five neurotrophins are expressed in the ovary, including nerve growth factor (*Ngf*), brain-derived neurotrophic factor (*Bdnf*), neurotrophin 3 (*Ntf3*), and neurotrophin 5 (*Ntf5*). In addition, all four neurotrophin receptors are present in the ovary, including neurotrophic tyrosine kinase receptor types 1 through 3 (*Ntrk1*, *Ntrk2*, and *Ntrk3*; also known as *TrkA*, *TrkB*, and *TrkC*, respectively), and *Ngfr* (*p75*), which recognizes all neurotrophins with low affinity (Ref. 196; and reviewed in Ref. 197). *Ngf* expression in the somatic cells and oocytes precedes follicle formation, and ovaries from 7-d-old *Ngf*$^{-/-}$

mice contain mostly primordial follicles with few primary follicles, whereas wild-type ovaries show numerous primary and secondary follicles at this age (198). Similar to observations in *Foxl2*-deficient ovaries (187, 188), *Ngf*-deficient ovaries have reduced somatic cell proliferation, suggesting that NGF signaling is also important in the differentiation of squamous pre-granulosa cells to cuboidal granulosa cells during primordial follicle activation. Although mice lacking the common p75 NGF receptor are fertile with no defects in follicle formation (199), mice lacking the high-affinity NGF receptor, NTRK1, are perinatal lethal (200, 201), and the ovarian effects of *Ntrk1* deficiency remain unknown, although it would be possible to transplant *Ntrk1*-deficient ovaries under the kidney capsule of wild-type mice to assess postnatal follicular development, as described with the *Ntrk2*-deficient ovaries (202).

In addition to the above genetic models and the *in vitro* studies that complement the *in vivo* findings, multiple other *in vitro* studies suggest that several pathways converge to activate or repress primordial follicle recruitment. Ovaries from 4-d-old rats cultured *in vitro* show an increase in growing follicles and fewer primordial follicles when basic FGF2, keratinocyte growth factor (FGF7), BMP4, leukemia inhibitory factor (LIF), platelet-derived growth factor (PDGF), or glial-derived neurotrophic factor (GDNF) are added to the culture medium (Refs. 203 and 204; and reviewed in Ref. 205). Injection of BMP7 into the ovarian bursa of rats also results in fewer primordial follicles and, correspondingly, more growing follicles (206). At least some of these factors, including FGF2, PDGF, and LIF, may promote the primordial to primary transition by up-regulation of *Kitl* in granulosa cells. In primordial follicles, FGF2 and PDGF are primarily expressed in oocytes, whereas LIF is found in pre-granulosa and somatic cells (207). Besides AMH, the only other factor shown to inhibit primordial follicle recruitment is the chemoattractive cytokine, CXCL12. Both CXCL12 and its receptor, CXCR4, are predominantly expressed in primordial and activated oocytes. When neonatal mouse ovaries are treated with CXCL12, there is a reduction in growing follicles and a higher density of primordial follicles (208). CXCL12/CXCR4 interactions are also important during PGC migration when CXCR4 is expressed in migrating germ cells and CXCL12 is expressed in the dorsal body wall. In CXCR4-deficient embryos, fewer PGCs reach the genital ridge (209).

Whether any of the above observations with added growth factors have physiological relevance *in vivo* remains to be determined. For example, *Fgf2*$^{-/-}$ mice are viable and fertile (210); *Fgf7*$^{-/-}$ mice have abnormal hair development, but fertility defects have not been reported (211); *Lif*$^{-/-}$ mice have defects in implantation, but normal folliculogenesis and ovulation (212). Disruption of the genes encoding many of the other putative regulators of primordial follicle activation results in embryonic or perinatal lethality. Furthermore, given the ability of many of these growth factors to influence *Kitl* expression, it is possible that redundant pathways have evolved so that one factor may compensate for deficiency of others. Thus, validation of the *in vitro* findings would require cKO models, as well as generation of mice lacking two or more genes in the appropriate ovarian cell type.

Although the molecular events that control primordial follicle formation and maintenance *in vivo* remain poorly defined, a number of mouse models in recent years have helped to identify master regulators of this critical period in folliculogenesis. The findings in these mutant mice are summarized in Table 6. Moreover, essential mediators of these processes are candidate genes for POF. The clinical criteria for POF are amenorrhea for at least 4 months before 40 yr of age with two serum FSH measurements in the menopausal range (213). In addition to patients with type I BPES that have nonsense mutations in *FOXL2* (186), rare function-disrupting mutations in *NOBOX* and *FIGLA* have been observed in Caucasian and Chinese women, respectively, with nonsyndromic ovarian failure (214, 215). Because these women were heterozygous for *NOBOX* or *FIGLA* mutations, further work is needed to determine whether these mutations are sufficient to cause ovarian failure. *In vitro* studies suggest that at least one of the missense mutations in NOBOX had a dominant negative effect on the ability of wild-type NOBOX to bind DNA.

C. Preantral folliculogenesis

Preantral folliculogenesis is characterized by oocyte growth, granulosa cell proliferation, and acquisition of an additional somatic cell layer, the theca. Preantral follicle growth in mice begins 10–12 d after birth when a cohort of developing follicles reaches the secondary stage of folliculogenesis. Secondary follicles contain oocytes in midgrowth stages surrounded by two or more layers of granulosa cells. Growth of preantral follicles is dependent on autocrine and paracrine regulatory factors but appears to be gonadotropin-independent. Mice deficient in the FSHβ subunit (216) or the FSH receptor (FSHR) (217, 218), as well as hypogonadal mice with naturally occurring mutations in GnRH, which results in a marked reduction in synthesis of FSH and LH from the anterior pituitary (219), have normal preantral follicle growth despite defective antral folliculogenesis.

During preantral folliculogenesis, the complex bidirectional communication between the oocyte and the somatic compartments of the follicle becomes more apparent. Although the oocyte relies on surrounding somatic cells to sup-

TABLE 6. Mouse models with defects in early folliculogenesis

Gene	Reproductive phenotype	Fertility status	Ref.
Folliculogenesis-specific basic helix-loop-helix (*Figla*; FIGa)	No primordial follicles develop at birth and oocytes die	Decreased reproductive lifespan	129
LIM homeobox protein 8 (*Lhx8*)	Primordial to primary follicle block and oocyte loss	Infertile	155
NOBOX oogenesis homeobox (*Nobox*)	Primordial to primary follicle block and oocyte loss	Infertile	156
Spermatogenesis and oogenesis-specific basic helix-loop-helix 1 (*Sohlh1*)	Primordial to primary follicle block and oocyte loss	Infertile	155, 790
Spermatogenesis and oogenesis-specific basic helix-loop-helix 2 (*Sohlh2*)	Primordial to primary follicle block and oocyte loss	Infertile	153, 160
B-cell leukemia/lymphoma 2 (*Bcl2*)	Fewer oocytes/primordial follicles in the postnatal ovary	Subfertile	121
Aryl-hydrocarbon receptor (*Ahr*)	Increased primordial follicles early; decreased numbers of antral follicles in adults	Subfertile	126, 127
Bcl2-associated X protein (*Bax*)	Increased oocytes and primordial follicles	Prolonged reproductive lifespan	123, 124
Kit ligand (*Kitl*; Steel)	*Panda*, *contrasted*, and *transfer* mutants have reduced germ cells and block in folliculogenesis at primary stage	Infertile	166–168
Anti-Müllerian hormone (*Amh*)	Early depletion of primordial follicles	Decreased reproductive lifespan	192, 193
Forkhead box O3 (*Foxo3a*)	Global follicular activation and early follicular depletion	Progressive infertility	172
Forkhead box L2 (*Foxl2*)	Ovarian failure due to absence of germ cell proliferation and differentiation	Infertile	187, 188
Phosphatase and tensin homolog deleted on chromosome 10 *(Pten)* (cKO)	Global follicular activation and early follicular depletion	Progressive infertility	177, 178
3-Phosphoinositide-dependent protein kinase-1 (*Pdpk1*; Pdk1) (cKO)	Ovarian failure due to depletion of primordial follicles, presumably through decreased survival	Infertile	184
Ribosomal protein S6 (*Rps6*) (cKO)	Ovarian failure due to depletion of primordial follicles, presumably through decreased survival	Infertile	184
Growth differentiation factor-9 (*Gdf9*)	Folliculogenesis arrest at the one-layer follicle stage	Infertile	223, 225
Nerve growth factor (*Ngf*)	Fewer primary and secondary follicles; reduced granulosa cell proliferation	Infertile	198

port its growth and development, the rate of follicular development is critically dependent on the oocyte. This dominant role for the oocyte in directing folliculogenesis has been demonstrated in elegant reaggregation experiments performed by Eppig *et al.* (220) where oocytes isolated from secondary follicles of P12 mice were combined with somatic cells from newborn ovaries. The reaggregated ovaries exhibited accelerated folliculogenesis and contained antral follicles 9 d after grafting beneath the renal capsule. Furthermore, the granulosa cells from these antral follicles underwent cumulus expansion when recovered cumulus-oocyte complexes were treated with FSH, and the oocytes from these isolated cumulus-oocyte complexes could resume meiosis and undergo fertilization. In contrast, reaggregated control ovaries in which both the oocytes and somatic cells were from newborn mice contained only secondary follicles 9 d after grafting. Although the precise mechanism by which the oocyte orchestrates follicular development is not known, oocyte-secreted factors appear to have crucial roles.

The first oocyte-derived growth factor demonstrated to be critical for somatic cell function *in vivo* was GDF9. In mice, GDF9 is first expressed in the oocytes of primary follicles with persistent expression until after ovulation (221, 222). Consistent with this expression pattern, *Gdf9* null mice form primordial and primary follicles, but have a block in follicular development at the primary stage of folliculogenesis (223). Histologically, whereas the oocytes in $Gdf9^{-/-}$ ovaries grow more rapidly compared with controls (224), the granulosa cells show reduced proliferation and defects in differentiation with eventual development of an abnormal steroidogenic phenotype (225), and a theca layer fails to develop (223). In addition, very few granulosa cells in $Gdf9^{-/-}$ ovaries undergo apoptosis. Despite similar levels of *Kit* expression in the oocyte, granulosa cell levels of *Kitl* and inhibin α are dramatically increased (225), suggesting that GDF9 from the oocyte negatively regulates granulosa cell production of these growth factors. The increase in inhibin α could prevent

proliferation of granulosa cells at the primary follicle stage because mice lacking both inhibin α and GDF9 develop multilayered follicles (226). The up-regulation of *Kitl* may lead to enhanced signaling through oocyte-expressed KIT, contributing to the increased oocyte size observed in *Gdf9*$^{-/-}$ ovaries (224).

An oocyte-granulosa cell regulatory loop has been postulated where KIT ligand from granulosa cells promotes oocyte growth until a specific size is reached, upon which time GDF9 secretion from enlarged oocytes suppresses *Kitl* expression in cumulus cells to slow or stop further oocyte growth (114). As previously mentioned, in addition to the role of KIT/KIT ligand signaling during initial recruitment of primordial follicles, mutations affecting KIT or KIT ligand often show defects at the primary follicle stage, similar to what is observed in GDF9 null ovaries. For example, the *Sl*^{pan} mutant mice, with a hypomorphic *Kitl* allele, exhibit decreased oocyte recruitment, and the majority of follicles fail to develop beyond the early primary stage. Furthermore, whereas addition of KIT ligand to follicles growing in culture enhances oocyte growth (227), treatment of granulosa cells isolated from preantral and antral follicles with recombinant GDF9 suppresses *Kitl* expression (228). However, *Gdf9* is not misexpressed (*i.e.*, decreased) in the oocytes of mice with a mutated KIT receptor (179), suggesting that KIT signaling does not directly regulate *Gdf9* in such a feedback loop.

BMP15 is another oocyte-secreted TGFβ superfamily member that was identified using a homology-based cloning strategy to identify BMP homologs (229). In addition to 52% amino acid identity, BMP15 and GDF9 share interesting features. *Bmp15* mRNA has an expression pattern identical to *Gdf9* in mouse oocytes, and BMP15 and GDF9 proteins lack a conserved cysteine residue found in other TGFβ superfamily members. This cysteine is required for intermolecular disulfide bond formation that occurs during dimerization of other family members, indicating that BMP15 and GDF9 form noncovalent homo- and/or heterodimers. Despite these characteristics, unlike GDF9, BMP15 is not required during preantral folliculogenesis in mice. *Bmp15*$^{-/-}$ are subfertile; however, this is secondary to decreased ovulation and fertilization rates, rather than disrupted folliculogenesis (230). GDF9 and BMP15 may, however, have redundant roles during folliculogenesis, with GDF9 being the dominant growth factor in mice. Ovaries from *Gdf9*$^{+/-}$ *Bmp15*$^{-/-}$ mice have more defects than *Bmp15*$^{-/-}$ mice, including fewer late-stage follicles and CLs and increased oocyte loss.

There appear to be species-specific differences as to the importance of oocyte-secreted GDF9 and BMP15 during folliculogenesis. To date, five polymorphisms causing nonsense or missense mutations in BMP15 and one causing a missense mutation in GDF9 have been identified in several breeds of sheep. These mutations are associated with increased ovulation in heterozygous carriers, but sterility in homozygous carriers (231, 232). The ovarian phenotype of sheep with homozygous X-linked mutations [Fecundity X (*FecX*)] is similar to that of *Gdf9* null mice with failure of follicles to develop beyond the primary follicle stage and uncoupling of oocyte growth relative to granulosa cell proliferation (233). Linkage analysis, however, mapped the *FecX* allele to the *BMP15* gene locus (234), which is in an orthologous location on mouse and human X chromosomes (229). *In vitro* studies suggest that certain BMP15 missense mutations, such as the V31D substitution found in the Inverdale strain of sheep (234), may impair proteolytic processing and secretion of GDF9 (235). However, despite oocyte and granulosa cell abnormalities, antral follicles are present in ovaries from sheep homozygous for a point mutation in the *GDF9* gene, which causes a nonconservative amino acid substitution in a region of the mature protein that is predicted to interact with its type I receptor (232). Thus, whereas mouse BMP15 does not appear to have a critical function in early folliculogenesis, in sheep it is essential for early follicular development, and GDF9 may be more important in later stages. Although they are rare, mutations in *BMP15* and *GDF9* that affect secretion or function when evaluated in *in vitro* assays have been reported in women with POF (236–240).

The neurotrophins NTF5 and BDNF, which signal through NTRK2 on the oocyte, are expressed in human and mouse primordial follicles (202, 241) and appear to have redundant roles in preantral folliculogenesis. Loss of *Ntf5* alone does not alter follicle number through the secondary stage; however, concomitant loss of *Ntf5* and *Bdnf* causes a significant reduction in the number of secondary follicles, which is also seen in *Ntrk2* null ovaries. Although not significant, the number of primary follicles is also decreased, whereas the population of primordial follicles is unaffected in P7 *Ntrk2*$^{-/-}$ mice (202). When *Ntrk2* null ovaries from P4-P5 mice are transplanted under the renal capsule of adult wild-type females, after a period of 2 wk the ovaries are nearly depleted of oocytes and those that remain are degenerating, in contrast to transplanted control ovaries, which contained follicles that have reached the antral stage (202). Thus, although NTF5, BDNF, and NTRK2 do not appear to be critical for initial follicle recruitment, progression beyond the primary stage is interrupted. Because *Gdf9* and *Kitl* levels are unchanged in *Ntrk2* null ovaries, NTF5 and BDNF appear to affect the transition to secondary follicles independent of these pathways.

In addition to the multiple paracrine factors involved in the intricate dialogue between the somatic cells and oocyte of a developing follicle, direct connections via intercellular membrane gap junction channels are also essential during folliculogenesis. Gap junctions allow the transfer of ions, metabolites, and small molecules between neighboring cells. Connexins are the core proteins that make up gap junctions. Several connexins are expressed in the mammalian ovary (reviewed in Refs. 242 and 243), and at least two, connexin 43 (CX43; GJA1) and connexin 37 (CX37; GJA4), have essential and distinct roles during folliculogenesis. CX43 forms gap junctions between granulosa cells throughout folliculogenesis (244), whereas CX37 localizes to oocyte-granulosa cell gap junctions beginning in the primary follicle stage (245). *Gja1* knockout mice die in the early postnatal period due to severe cardiac malformations (246). Neonatal *Gja1*$^{-/-}$ ovaries are small secondary to germ cell deficiency that occurs as early as E11.5 (244). To determine whether the remaining germ cells could participate in folliculogenesis, ovaries from fetal and newborn *Gja1*$^{-/-}$ mice were cultured *in vitro* or transplanted under the kidney capsule of wild-type mice (244, 247). These CX43-deficient ovaries showed a block at the primary follicle stage with impaired granulosa cell proliferation and retardation of oocyte growth. The oocytes were also morphologically abnormal with defects in meiotic maturation.

Oocytes in mice lacking CX37 also have defects in meiotic competence and do not grow to a normal size (248), but follicular development progresses to the later preantral stage (245). Despite a near-complete absence of large antral (Graafian) follicles, CX37-deficient ovaries have numerous small CL-like structures, suggesting that communication via gap junctions is a major mechanism regulating CL formation; when oocyte-granulosa cell coupling is disrupted, premature luteinization occurs. This is perhaps logical, given that luteinization normally occurs after ovulation, a natural disruptor of oocyte-granulosa cell gap junctions. Thus, whereas CX37 gap junctions are essential for the preantral to antral follicle transition, CX43 gap junctions are required for granulosa cell proliferation earlier in folliculogenesis to form multilayered follicles, and both types of junctions support proper oocyte development.

Although we have highlighted a few growth factors and signaling pathways as well as the importance of intercellular connections during preantral folliculogenesis, numerous mouse models with defects in preantral follicular development have been characterized. These mutant mice are summarized in Table 7.

D. Theca formation and physiology

Once the follicle achieves two layers of granulosa cells, an additional morphologically distinct layer of somatic cells, the theca, differentiates as the outermost layer of the follicle (113). Cells of the theca interna layer, which forms just outside the basement membrane surrounding the granulosa cells, have ultrastructural features, including numerous mitochondria with tubular cristae, smooth endoplasmic reticulum, and abundant lipid vesicles, that cor-

TABLE 7. Mouse models with defects in preantral folliculogenesis

Gene	Reproductive phenotype	Fertility status	Ref.
FSH receptor (*Fshr*)	Preantral block in folliculogenesis	Infertile	218
FSH β (*Fshb*)	Preantral block in folliculogenesis; rescued by exogenous gonadotropins	Infertile	216
Cyclin D2 (*Ccnd2*)	Failure of granulosa cell proliferation	Infertile	292
Discoidin domain receptor family, member 2 (*Ddr2*; *slie*)	Spontaneous mutant; smaller pituitaries and gonadal dysfunction, dwarfism	Infertile	791
IGF-I (*Igf1*)	Hypogonadal; impaired antral follicle formation	Infertile	290
Nitric oxide synthase 1, neuronal (*Nos1*)	Impaired central hormonal regulation of reproductive function; decreased ovary weight, decreased CLs	Infertile	792
Phosphate cytidylyltransferase 1, choline, b isoform (*Pcyt1b*)	Multiple follicular defects; reduced ovarian follicles and CLs	Subfertile	793
SH2B adaptor protein 1 (*Sh2b1*)	Small, anovulatory ovaries with reduced numbers of developing follicles	Subfertile	794
Rous sarcoma oncogene (*Src*)	Defect in antral follicle development; anovulation	Infertile	795
TAF4B RNA polymerase II, TATA box binding protein-associated factor (*Taf4b*; TAFII105)	Defects in follicular development, oocyte maturation and fertilization	Infertile	308–310
Thrombospondin 1 (*Thbs1*)	Increased VEGF and ovarian hypervascularization, increased follicle numbers but decreased size of preantral and antral follicles	Subfertile	796
Ubiquitin protein ligase E3A (*Ube3a*; E6-AP)	Ovarian hypoplasia; defects in ovulation	Subfertile	797

VEGF, Vascular endothelial growth factor.

respond with their principal function as a source of androgens for neighboring granulosa cells to convert to estrogens (249). The theca externa, composed of fibroblasts, smooth muscle-like cells, and macrophages, is important during ovulation. Cells that contribute to the theca differentiate from mesenchymal precursor cells present in the ovarian stroma, adjacent to developing follicles (250). Like preantral folliculogenesis, theca formation is gonadotropin-independent because thecal precursor cells lack LH receptors and the theca layer still forms in the ovaries of FSH-deficient mice (216). Upon formation of a discernible theca interna layer, however, LH principally controls thecal cell androgen production.

Although the factors that regulate thecal cell differentiation are unknown, they appear to be small molecules in the 20- to 25-kDa range secreted by growing follicles. When undifferentiated theca-interstitial cells were cultured in conditioned medium from rat preantral follicles with two to five layers of granulosa cells, markers of theca differentiation, including mRNAs for LH/choriogonadotropin receptor (*Lhcgr*) and the *Cyp17a1* family member, were expressed and androgens were produced (251). Candidate factors that may contribute to thecal cell differentiation include IGF, KIT ligand, and GDF9. In cultured rat thecal cells, IGF-I increased expression of *Lhcgr*, *Cyp11a1*, and 3β-hydroxysteroid dehydrogenase (*Hsd3b1*), but mRNAs for other thecal cell markers, including steroidogenic acute regulatory protein (*Star*) and *Cyp17a1*, were only up-regulated when KIT ligand was also added (252). As mentioned in *Section III. E*, a thecal layer fails to form in *Gdf9*$^{-/-}$ ovaries (223), which show a lack of thecal cell markers *Cyp17a1*, *Lhcgr*, and *Kit*, despite an abundance of presumed thecal cell precursors in the interstitium and increased circulating FSH and LH (225). Whether GDF9 regulates thecal cell recruitment and/or differentiation directly or indirectly through regulation of preantral granulosa cell development is unknown. Recombinant mouse GDF9 has been shown to up-regulate *Igf1* in cultured granulosa cells (253, 254), suggesting that at least some of the effects of GDF9 on theca development are indirect. Furthermore, GDF9 may be more important in thecal differentiation because *Inha Gdf9* double knockout mice form a morphological theca layer, but thecal cell markers are not expressed in this layer (226).

Although long believed to exist, putative thecal stem cells have recently been isolated from neonatal mouse ovaries. Using a procedure that had previously been successful in isolating male germline stem (GS) cells from neonatal testes (255), Honda *et al.* (256) hoped to isolate putative and controversial female GS cells. Instead, the authors isolated somatic cell colonies that were positive for alkaline phosphatase (a stem cell marker) and proliferated and remained in an undifferentiated state in serum-free GS cell media. When the cells were treated with serum, LH, IGF-I, and KIT ligand, or with conditioned medium from granulosa cells, they underwent cytological changes consistent with steroidogenic ability and secreted androstenedione into the culture medium. Transplantation of EGFP-positive, undifferentiated stem cells into host ovaries showed colonization of interstitial areas and inner and outer theca layers around fully grown follicles.

Members of the hedgehog family of secreted morphogens are also candidate regulators of early thecal cell differentiation and may be especially important in regulating differentiation of smooth muscle cells in the theca externa during ovulation. In mammals, there are three secreted hedgehog ligands, including Indian hedgehog, desert hedgehog, and sonic hedgehog (IHH, DHH, and SHH). Hedgehog ligands bind and functionally inactivate transmembrane patched (PTCH1 and PTCH2) receptors on responsive cells, causing derepression of the Smoothened (SMO) seven pass transmembrane receptor, ultimately regulating the activity or levels of Gli family transcription factors (GLI1, GLI2, and GLI3) (reviewed in Ref. 257). Beginning in the primary follicle stage, *Ihh* and *Dhh* mRNAs localize to granulosa cells of growing follicles, decreasing before ovulation, whereas *Ptch1* and *Gli1* are in the mesenchymal stromal cells surrounding primary follicles and increased in the theca layer of larger follicles (258). Although *Dhh* knockout mice do not have an ovarian phenotype, *Ihh* may have redundant or dominant roles during follicular development. To study constitutive activation of hedgehog signaling, a transgenic mouse (*SmoM2*) has been developed that conditionally expresses a dominant SMO protein that is not inhibited by patched receptors (259). Dominant activation of SMO in granulosa and thecal cells using *Amhr2-Cre* (260, 261) caused defective formation of the smooth muscle layer found in the theca externa and severely impaired ovulation (262). The observation that excess hedgehog signaling prevented smooth muscle differentiation is in agreement with other organ systems where mesenchymal cells furthest from an epithelial hedgehog source differentiate into smooth muscle (262). Thus, in wild-type mice, higher levels of PTCH1 in thecal cells may limit hedgehog signaling in the follicle, allowing differentiation of the theca externa. Because there is extensive cross talk between TGFβ and hedgehog pathways in several cell types and tissues (263), it would be interesting to determine whether components of hedgehog signaling are altered in *Gdf9* null mice, contributing to the absence of thecal differentiation as a result of GDF9 deficiency.

Granulosa cells of antral follicles are the chief source of estradiol production, yet they lack the biosynthetic en-

zyme (CYP17A1) necessary to produce the aromatizable androgen and estradiol precursor, androstenedione. Although cells of the theca interna express CYP17A1, they are deficient in CYP19A1, the key enzyme in the conversion of androstenedione to estrogens that is expressed by granulosa cells of later-stage follicles (264). Expression of each of these enzymes is controlled by pituitary gonadotropins, forming the basis of the two-cell, two-gonadotropin concept of estradiol production (reviewed in Ref. 249, and summarized in Fig. 5). In response to LH stimulation, thecal cells express key steroidogenic enzymes, including CYP11A1, HSD3B1, and CYP17A1. LH also promotes up-regulation of STAR, which facilitates delivery of cholesterol to the inner mitochondrial membrane where CYP11A1 is located. Granulosa cells respond to FSH by up-regulating CYP19A1 and 17β-hydroxysteroid dehydrogenase (HSD17B1).

Because estradiol is not essential until later stages of folliculogenesis and ovulation (see *Section III. E*), but thecal cells express LH receptor starting at the secondary follicle stage, it is important to suppress excess androgen biosynthesis in preantral and small antral follicles. To modulate the stimulatory effect of LH on theca androgen production in smaller follicles, granulosa cells secrete factors, such as activins, that inhibit androstenedione production (265, 266). KIT ligand from granulosa cells may also up-regulate factors in thecal cells, including TGFβ, TGFα, FGF7, and hepatocyte growth factor, that have autocrine inhibitory effects on androstenedione production (reviewed in Ref. 267). On the other hand, a threshold level of androgens from thecal cells may be necessary for preantral follicular growth, and production may be controlled by GDF9 (268). In a rat follicle culture system, intra-oocyte injection of *Gdf9* morpholino antisense oligonucleotides suppressed preantral follicle growth, *Cyp17a1* expression, and testosterone production, and these effects were attenuated by exogenous GDF9 (268). The androgen receptor antagonist flutamide also blocked GDF9-induced follicle growth. There are, however, conflicting reports on the effects of recombinant GDF9. Treatment of rat theca-interstitial cells with recombinant GDF9 stimulates androstenedione production (269), whereas in bovine thecal cell culture, GDF9 increased thecal cell proliferation but decreased IGF-I-induced steroidogenesis (270). Many of these observations may be indirect effects of GDF9 and could also be due to differences in culture conditions.

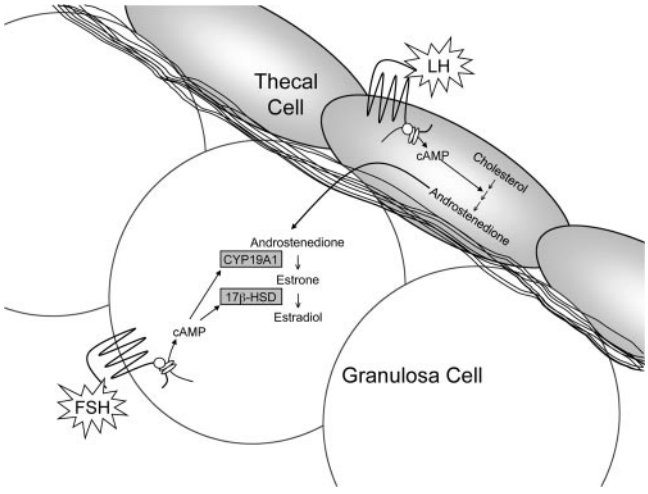

FIG. 5. The two-cell, two-gonadotropin concept of follicular steroid production. The main function of thecal cells during folliculogenesis is the production of steroids. Although thecal cells are capable of *de novo* production of androgens, they lack aromatase (CYP19A1), which is required to convert androgens into estradiol. Thecal cells respond to basal levels of LH by up-regulating biosynthetic enzymes involved in steroid production, including STAR, CYP11A1, CYP17A1, and 3β-hydroxysteroid dehydrogenase (3β-HSD). STAR facilitates the transport of cholesterol to the inner mitochondrial membrane, where it is converted to pregnenolone by CYP11A1. Pregnenolone is converted to dehydroepiandrosterone (DHEA) by CYP17A1. Finally, 3β-HSD converts DHEA into androstenedione, which diffuses across the basement membrane to granulosa cells. In response to stimulation by FSH, granulosa cells up-regulate CYP19A1 and 17β-hydroxysteroid dehydrogenase (17β-HSD), which convert androstenedione into estradiol (249).

Although we have focused our discussion on factors involved in normal theca formation and function, thecal cells, through excess androgen biosynthesis, contribute to polycystic ovarian syndrome. In addition to clinical features of hyperandrogenism due to excess ovarian and adrenal androgen production, this heterogeneous disorder is accompanied by ovarian dysfunction, including ovulatory defects and/or polycystic ovaries (271). For a more extensive review of the pathophysiology and potential genes involved in polycystic ovarian syndrome, the reader is referred to previous work in this journal (272, 273).

E. Antral follicle formation, FSH, and estradiol

A number of important changes take place in the follicle during formation of the antrum. During antral folliculogenesis, multiple small, fluid-filled spaces eventually coalesce to form a single antral cavity that separates two functionally distinct granulosa cell populations. The newly formed mural granulosa cells line the wall of the follicle and are critical for steroidogenesis and ovulation, whereas the cumulus granulosa cells surround the oocyte, promoting its growth and developmental competence. These two cell types appear to be defined by opposing gradients of FSH from outside the follicle and oocyte-secreted factors from within (274). The transition from preantral to antral follicle marks a change from principally intraovarian to extraovarian regulation of folliculogenesis as the hypothalamic-pituitary-gonadal (HPG) axis starts functioning. Although preantral follicles are responsive to FSH, during antral folliculogenesis, FSH becomes essential (216) not

only to prevent granulosa cell apoptosis and follicular atresia (275), but also for granulosa cell proliferation, estradiol production, and LH receptor expression (276).

FSH and LH are the pituitary gonadotropins that coordinate antral follicle development and ovulation. These heterodimeric glycoprotein hormones have a unique β-subunit and a common α-subunit that is also shared with TSH and chorionic gonadotropin. A number of positive and negative feedback loops in the HPG axis coordinate follicle maturation and dominant follicle selection with sexual behavior and preparation for pregnancy. In addition to the negative feedback of estradiol on the HPG axis, the ovary also produces growth factors, including activins, inhibins, and FSTs, that modulate pituitary FSH secretion (277), but also act locally to regulate follicular development.

To circumvent the multiple defects observed in mice lacking the common α-subunit (and thus lacking FSH, LH, and TSH) (278), and the difficulty in studying FSH function independent of LH in hypogonadal mutants (219), mice lacking the FSHβ subunit and therefore circulating FSH were generated (216). Whereas FSH is not essential for male fertility, FSH-deficient females were infertile due to a block in folliculogenesis before antral follicle formation. Despite the absence of antral follicles and no ovulation in FSH-deficient mice, ovaries at 6 wk contained all earlier stages of follicles, including primordial, primary, and multilayered preantral follicles, supporting the notion that early folliculogenesis is gonadotropin-independent. The lack of large antral follicles and CLs probably accounts for the smaller-sized ovaries. Juvenile FSH-deficient mice responded to exogenous administration of pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG), with similar numbers of oocytes recovered from control and knockout females, suggesting that ovulatory competence was not affected by the absence of FSH. Mice lacking FSHR are also infertile with very similar ovarian and uterine findings (217, 218). Although mutations in *FSH* and *FSHR* in humans are rare, the clinical features in humans are similar to the defects observed in mouse models (279–284).

The regulation and functions of FSH in mammals appear to be evolutionarily conserved. When a 10-kb human *FSHβ* transgene that contained gonadotrope-specific, GnRH-responsive, and steroid-responsive elements was introduced into the FSHβ knockout mice to create an interspecies FSH heterodimer hybrid (mouse α:human FSHβ), defects in folliculogenesis were rescued and fertility of transgenic mice was restored to wild-type levels (285). Bitransgenic mice were also engineered that expressed the α-subunit of hCG and the FSHβ subunit under the control of the metallothionein 1 (MT1) promoter, resulting in expression of human FSH from multiple tissues in FSHβ-deficient mice. Although fertility was restored in some females (30%), litters were smaller and two out of three females died in the postpartum period. The different phenotypes in the two transgenic mice may be due to the nature of FSH secretion, which is constitutive (nonpulsatile) when under control of MT1 but more physiological (pulsatile) in the human *FSHβ* transgenic model.

The classical signaling cascade activated by binding of FSH to the G protein-coupled FSHR is a linear adenylyl cyclase (AC)/cAMP/protein kinase A (PKA) pathway that results in phosphorylation and activation of the transcription factor cAMP-response element-binding protein to regulate a number of target genes, including aromatase, the α- and β-subunits of inhibin, LH receptor, and many more. In recent years, however, a number of additional intracellular signaling pathways, some of which are PKA-independent, have also been identified (reviewed in Refs. 286 and 287). Although FSH up-regulates serum and glucocorticoid-induced kinase 1 (SGK1) at the transcriptional level through the classical PKA pathway, phosphorylation and activation of SGK1 and AKT also occurs in a PKA-independent, PI3K-dependent fashion that still requires cAMP (288). Although activation of PI3K in this setting was proposed to be mediated by cAMP-regulated guanine nucleotide exchange factors that activate RAS-like small GTPases upstream of PI3K, more recent studies point to FSHR activation of a SRC tyrosine kinase-dependent pathway (289).

IGF-I also activates the PI3K pathway in granulosa cells. FSH and IGF-I signaling pathways impact proliferation, differentiation, and survival of granulosa cells, in part by distinct regulation of the levels of FOXO1 mRNA and protein (174, 286). *Igf1* null mice are infertile, with an arrest at the preantral follicle stage similar to FSHβ- and FSHR-deficient ovaries (290, 291). Interestingly, although *Igf1* and *Fshr* mRNAs colocalize in healthy gonadotropin-responsive follicles, *Igf1* is not altered in ovaries from FSH-deficient females, but *Fshr* and *Cyp19a1* are reduced in IGF-I knockout mice (291). Thus, IGF-I appears to enhance granulosa cell responsiveness to FSH by augmenting levels of FSHR.

FSH, IGF-I, and estradiol signaling cascades control granulosa cell proliferation through modulation of the cell cycle. The D- and E-type cyclins positively regulate entry into the cell cycle by binding cyclin-dependent kinases (CDK4/6 and CDK2, respectively) and activating a cascade that promotes the G_1/S transition. CDK inhibitors, such as $p27^{Kip1}$, block cell cycle progression by inactivating cyclin-CDK complexes. Mice null for cyclin D2 (*Ccnd2*) have impaired granulosa cell proliferation and demonstrate an arrest in folliculogenesis at the preantral

stage (292), similar to *Fshb*$^{-/-}$ and *Igf1*$^{-/-}$ mice. Treatment of rat granulosa cells with PMSG up-regulates *Ccnd2* mRNA in a cAMP-dependent manner, and *Ccnd2* null ovaries show a minimal response to exogenous FSH, in contrast to rapid granulosa cell proliferation observed in controls. Surprisingly, in FSHβ null mice, *Ccnd2* is only modestly decreased (and cell cycle inhibitor mRNAs are not up-regulated) (293), whereas *Igf1* is unchanged in granulosa cells (291) and serum estradiol is not altered (216). *Ccnd2* also showed little change in FSHR knockout mice (218). Thus, despite the obvious requirement for cyclin D2 during antral follicle formation, these findings suggest a dynamic interdependence of FSH, IGF-I, estradiol, and other pathways in regulation of granulosa cell proliferation. For example, *in vitro* studies in rat granulosa cells suggest that both FSH signaling to remove FOXO1 repression of *Ccnd2* and SMAD2/3 signaling are required to up-regulate cyclin D2 (294).

As shown in Fig. 5, estradiol production in the ovary relies on an interplay between thecal and granulosa cells, and the final biosynthetic step requires aromatase to convert androgens to estrogens. *Cyp19a1* null mice are unable to produce estradiol (144), and therefore provide insight into the role of this sex steroid on folliculogenesis. Ovaries from 12- to 14-wk-old *Cyp19a1* null mice contained follicles of all types; however, the mice were infertile and CLs were absent, suggesting impaired ovulation. In addition, many antral follicles were histologically abnormal with uneven granulosa cell layers and increased apoptosis. Follicular atresia increased with age, and many antral follicles that remained were cystic and hemorrhagic (295). Consistent with a role for estradiol in the negative feedback regulation of gonadotropin production, serum FSH and LH were elevated in *Cyp19a1* null mice, and the high LH likely contributed to hyperplastic ovarian stroma, as well as markedly increased serum testosterone levels. A second *Cyp19a1* knockout mouse model had similar findings and suggested that the increased atresia could be due to up-regulation of proapoptotic genes, including *p53* and *Bax* (296).

The effects of estradiol on folliculogenesis are mediated by two estrogen receptors, ERα (*Esr1*) and ERβ (*Esr2*). These classical ERs are members of the nuclear receptor superfamily of ligand-activated transcription factors. A membrane-bound G protein-coupled receptor, GPR30 (*Gper*), might mediate rapid, nongenomic estradiol signaling (297) and is implicated in maintenance of meiotic arrest in fish oocytes (298). In ovaries from 5-month-old GPR30-deficient mice, however, fertility is unimpaired and folliculogenesis appears normal, with follicles in all stages of development and CLs present (299). In contrast, mice lacking ERα (300), ERβ (301), or both (302, 303) have several ovarian findings consistent with their patterns of expression. ERβ is expressed in granulosa cells of growing follicles and is regulated by gonadotropins, whereas ERα is predominantly expressed in thecal and interstitial cells (304). Absence of ERα causes infertility, and mutant ovaries contain enlarged, cystic, and hemorrhagic follicles and no CLs or evidence of ovulation, similar to *Cyp19a1* null mice and the findings in polycystic ovarian syndrome. The elevated androgen, estradiol, and LH levels in the serum of *Esr1* null mice indicate that loss of ERα significantly affects the negative feedback of estradiol on the HPG axis. Intraovarian feedback of estradiol on thecal cell androgen production also appears to be disrupted in *Esr1* null follicles, which occurs through loss of repression of *Cyp17a1* by ERα, and consequently increased androstenedione production (305). Mice lacking ERβ are subfertile, with the principal defect being reduced ovulation, which may be attributed to reduced expression of LHCGR in granulosa cells (306) because induction of LHCGR in these cells is highest in preovulatory follicles and depends on the synergistic interactions of FSH and estradiol, as shown originally in hormonally primed hypophysectomized immature rats (307). As discussed in *Section III. F*, LH is a critical mediator of events in the periovulatory period. Ovaries from young adult ERβ knockout mice have follicles at all stages of development, but fewer CLs and more atretic follicles. As might be expected based on the individual ER knockouts, mice lacking both ERα and ERβ ($\alpha\beta$ERKO) are anovulatory, but they also exhibit a phenotype distinct from each single ER knockout. Ovaries from prepubertal $\alpha\beta$ERKO mice exhibit precocious maturation with adult-like antral follicles. In adult $\alpha\beta$ERKO ovaries, however, most follicles only reach the small antral stage, CLs are absent, and there are many sex-reversed follicles with degenerating or absent oocytes and the presence of Sertoli-like cells. Similar Sertoli-like and Leydig-like cells are observed in aromatase knockout mice (144). In summary, the ER and CYP19A1 knockout models suggest that unlike FSH, estradiol is not essential for antral follicle formation but is critical for granulosa cell growth and differentiation to maintain antral follicles and promote ovulation.

TAF4B is a gonad-enriched transcriptional coactivator subunit of the TFIID core transcriptional complex that may be an important cofactor in regulation of FSH target genes. The TFIID complex consists of the TATA-binding protein and a number of TATA-binding protein-associated factors (TAFs) that function as coactivators to recruit RNA polymerase II to specific gene promoters. In the ovary, TAF4B primarily localizes to granulosa cells of large preantral follicles (308, 309) in adult mice, but it is also detected in the oocytes of embryonic and prepubertal

mice (310). *Taf4b* null females are infertile with elevated serum FSH and multiple defects throughout folliculogenesis, including a reduction in primordial, preantral, and antral follicles in prepubertal and adult TAF4B-deficient ovaries, as well as less proliferation and increased apoptosis of granulosa cells (309, 310). *Ccnd2* and *Cyp19a1* were also reduced in *Taf4b* null adult ovaries (308), but these findings and the elevated serum FSH could be secondary to a widespread decrease in granulosa cells and ovarian failure that prevents feedback of granulosa cell-produced estradiol and inhibin on pituitary FSH production. To identify TAF4B-dependent promoters, TAF4B was overexpressed in a spontaneously immortalized rat granulosa cell line (311). In this context, TAF4B activated *Ccnd2*, *Inha*, *Inhba*, and *Fst* promoters and also up-regulated the transcription factor c-Jun, which is rapidly induced by FSH (312). These effects were through direct association of TAF4B with target gene promoters. In a human granulosa cell line, increased cAMP leads to PKA-dependent phosphorylation of TAF4B (313), which might affect its function, and in pig granulosa cells FSH increases *Taf4b* expression, which is required for maximal induction of IGF binding protein 3 (*Igfbp3*) by FSH (314). Taken together, these *in vitro* studies and the granulosa cell proliferation and survival defects in TAF4B-deficient ovaries suggest that although TAF4B appears critical during multiple stages of folliculogenesis, it is both a target and a mediator of FSH signaling in later stages.

Androgens may also have a role in priming granulosa cells to respond to gonadotropins because ovaries from 10-d-old androgen receptor (*Ar*) null females have a reduction in *Fshr*, and *Ar* null adults are subfertile, with decreased ovulation rates and defects in cumulus-oocyte complexes (315). In another $Ar^{-/-}$ model (316), *Fshr* trended lower but was not significantly decreased in 8-wk-old ovaries, although by this age a number of hormones and growth factors, including FSH, estradiol, and IGF-I, could have compensated for the absence of AR. *Ar* and *Fshr* mRNAs colocalize to granulosa cells, and testosterone up-regulates granulosa cell FSHR in primate ovaries (317). Further work is needed to determine whether androgens influence follicular response to FSH.

Although FSH signaling is essential for antral follicle formation and survival and we have focused on FSH-regulated pathways in this section, a number of other factors have also been identified that are critically important during this stage of folliculogenesis. Mutant mouse models with alterations in antral follicle development are summarized in Table 8. In addition, mutations in multiple genes have been identified that cause hypogonadism and infertility in women secondary to disruption of gonadotropin-signaling pathways (Ref. 284, and summarized in Table 1).

F. The preovulatory follicle, cumulus expansion, ovulation, and luteinization

Although a majority of follicles in the growing pool will undergo atresia, a select few antral follicles (the number varies by species) in a developing cohort reach the preovulatory stage. Those follicles that survive to this stage were likely most responsive (because of higher relative FSHR expression) to decreasing serum FSH that occurs through negative feedback of estradiol and inhibin on the pituitary. Whereas rising serum estradiol functions to suppress pituitary FSH secretion, increased follicular estradiol production enhances pituitary LH production, resulting in the LH surge. Preovulatory follicles express LHCGR at high concentrations in granulosa cells, enabling them to respond to the LH surge, which initiates a cascade of events leading to oocyte meiotic resumption, cumulus expansion, follicle rupture, and finally terminal differentiation of the remaining granulosa and thecal cells to create the CL.

TABLE 8. Mouse models with defects in antral follicle development

Gene	Reproductive phenotype	Fertility status	Ref.
A disintegrin-like and metalloprotease with thrombospondin type 1 motif, 1 (*Adamts1*)	Defects in preovulatory follicle development	Subfertile	341
Inner mitochondrial membrane peptidase 2-like (*Immp2l*) (transgenic insertion)	Folliculogenesis and ovulation defects	Infertile	798
Insulin receptor substrate 2 (*Irs2*)	Small, anovulatory ovaries with reduced numbers of follicles	Infertile	799
Estrogen receptor α (*Esr1*; ERα)	Enlarged, cystic and hemorrhagic follicles, and no CLs or evidence of ovulation	Infertile	300
Estrogen receptor β (*Esr2*; ERβ)	Block in late antral folliculogenesis and decreased ovulation	Subfertile	301
Superoxide dismutase 1 (*Sod1*)	Folliculogenesis defect; failure to maintain pregnancy	Subfertile	800, 801
SRY-box containing gene 3 (*Sox3*)	Follicular atresia and oogenesis defects	Subfertile	802
TNF type I receptor superfamily, member 1a (*Tnfrsf1a*)	Enhanced prepubertal response to gonadotropins; early ovarian senescence	Subfertile	803

Consistent with the patterns of expression of LHCGR in developing follicles and the cyclic rise in LH that precedes ovulation in mammals, mice deficient in LH (*Lhb* null) (318) or LH receptor (*Lhcgr* null) (319, 320) are infertile with defects in steroidogenesis. As mentioned *in Section III. C*, *Lhcgr* is a marker for thecal cells, and LH induces many enzymes involved in steroid biosynthesis. *Lhb* null females have decreased serum estradiol and progesterone and a reduction in *Cyp11a1*, *Cyp17a1*, and *Cyp19a1*, despite an intact thecal layer. Folliculogenesis is blocked at the early antral follicle stage; healthy large antral and preovulatory follicles and CLs are absent. However, similar to *Fshb* null mice, exogenous gonadotropin administration rescues the follicular defects, and comparable numbers of oocytes are recovered from LH-deficient and control-stimulated females. The histological defects in *Lhcgr* null ovaries mirror those observed in *Lhb* null ovaries, and serum estradiol and progesterone are also decreased in *Lhcgr* null females. Hormone replacement therapy with estradiol and progesterone failed to restore fertility or reverse the follicular defects (319); however, this may partially be attributed to the absence of LH receptor in antral follicles because one of the effects of estradiol, as shown by ERβ-deficient mice, is up-regulation of *Lhcgr* expression (306). Nevertheless, additional ER-independent targets downstream of LH signaling are likely responsible for growth beyond the early antral stage and for triggering ovulation. In recent years, a number of factors that influence the response of follicles to the LH surge have been identified.

Expansion of the cumulus cells on a hyaluronan-rich extracellular matrix surrounding the oocyte is initiated by the LH surge and is required for normal ovulation and fertilization. Regulation of this process is multifactorial and dependent on the activation of MAPK signaling (321, 322), as well as oocyte-secreted paracrine factors (323). After the LH surge, a number of genes involved in formation and stabilization of the extracellular matrix of the cumulus oophorus are up-regulated (324). Although a number of genes are induced in cumulus-oocyte complexes in the periovulatory period (325), we will focus on those that are essential for proper formation of the cumulus matrix; these genes include *Has2*, *Ptgs2*, *Tnfaip6*, and *Ptx3*. As shown in Fig. 6, hyaluronan synthase 2 (HAS2) is required for the production of hyaluronan, which forms the structural backbone of the cumulus matrix. Hyaluronan chains are stabilized through interactions with additional matrix proteins. TNFα-induced protein 6 (TNFAIP6) catalyzes the formation of covalent crosslinks between hyaluronan and the heavy chain of serum-derived inter-α-trypsin inhibitor (IαI) (326). Pentraxin 3 (PTX3) appears to stabilize the cumulus matrix through interactions with IαI (327). Prostaglandin synthase 2 (PTGS2; also known as COX2) is the rate-limiting enzyme in the synthesis of prostaglandins, and genetic studies suggest that prostaglandin signaling through the prostaglandin E receptor 2, subtype EP2 (PTGER2) functions upstream of TNFAIP6 (328). Although it is not entirely understood how these proteins interact to organize the extracellular matrix of the cumulus oophorus, each of them is essential for normal cumulus expansion. Targeted disruption of *Ptgs2* (329, 330), *Ptger2* (331–333), *Tnfaip6* (334), or *Ptx3* (254) results in abnormal or absent cumulus expansion and extreme subfertility (in *Ptgs2*, *Ptger2*, and *Ptx3* null females) or sterility (in *Tnfaip6* null females). *Has2* null mice are embryonic lethal (335), but RNA interference (RNAi)-mediated silencing of *Has2* in cultured cumulus-oocyte complexes reduces cumulus expansion (336). IαI biosynthesis in the liver requires the α-1-microglobulin/bikunin light chain (encoded by *Ambp*) for proper assembly and secretion. *Ambp* null female mice also demonstrate severely impaired cumulus expansion and a marked reduction in fertility, which is rescued by administration of IαI (337, 338). Finally, many additional factors are in the cumulus matrix. Chondroitin sulfate proteoglycan 2 (CSPG2; also known as versican), which can bind hyaluronan, is induced by LH and detected in cumulus-oocyte complexes in the periovulatory follicle (325, 339). *Cspg2* null mice are embryonic lethal (340), so the physiological role of CSPG2 in cumulus expansion has not been determined. However, ADAMTS1 (a disintegrin-like and metallopeptidase with thrombospondin type 1 motif, 1) is a protease known to cleave CSPG2, and *Adamts1* null females are subfertile with defects in preovu-

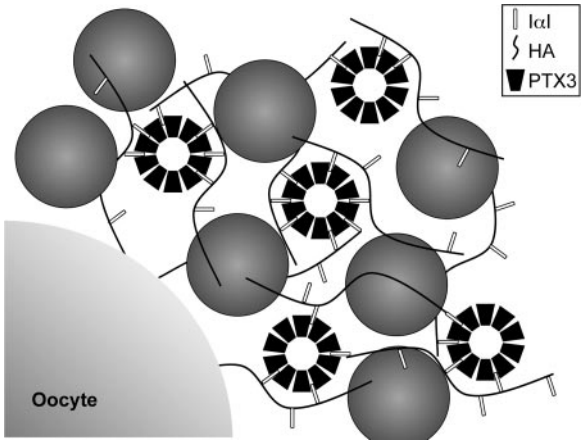

FIG. 6. Hyaluronan-IαI-PTX3 interactions stabilize the cumulus matrix. GDF9 and BMP15 secreted by the oocyte stimulate cumulus cells to produce HAS2, TNFAIP6, and PTX3. HAS2 catalyzes the synthesis of hyaluronan (HA; *curved line*), the structural backbone of the cumulus matrix. Hyaluronan is covalently linked to the heavy chain of IαI (*white box*) by the catalytic activity of TNFAIP6. Multimers of PTX3 (*black trapezoids*) stabilize the hyaluronan matrix by interacting with IαI.

latory follicle development (341), including less extensive cumulus expansion (342).

As previously mentioned, two functionally distinct granulosa cell populations exist in the antral follicle. Interestingly, the mural granulosa cells lining the follicle express high levels of LHCGR, whereas the cumulus cells surrounding the oocyte do not (at least in mice) (274, 343). How then does the LH surge lead to induction of target genes in cumulus cells that are critical for cumulus expansion? Conti and colleagues (344) have shown that the LH surge causes a rapid increase in epidermal growth factor (EGF)-like family members, *Areg*, *Ereg*, and *Btc* (encoding amphiregulin, epiregulin, and betacellulin, respectively), specifically in mural granulosa cells of preovulatory follicles. These ligands are synthesized as integral membrane proteins and are released from the cell surface by proteolytic cleavage of the ectodomain. They then bind and activate EGF tyrosine kinase receptors (EGFRs). All three of these growth factors stimulate cumulus expansion and oocyte maturation *in vitro* in an EGFR-dependent manner. The effect of these EGF-like factors on cumulus expansion occurs through up-regulation of *Ptgs2*, *Has2*, and *Tnfaip6* genes (344), whose products are essential for formation and stabilization of the extracellular matrix of the cumulus oophorus. Within 4 h of hCG stimulation, transcripts for *Areg*, *Ereg*, and *Btc* are also detected in cumulus-oocyte complexes, suggesting that an autocrine regulatory loop is established to maintain EGF-like growth factor expression in cumulus cells (325).

The *in vitro* findings have been verified by *in vivo* analysis in $Areg^{-/-}$ or $Ereg^{-/-}$ mice, which show reduced cumulus expansion in response to exogenous gonadotropins (345). Although $Egfr^{-/-}$ mice are embryonic lethal, mice with a hypomorphic *Egfr* allele ($Egfr^{wa2}$) are viable, and $Areg^{-/-}$ $Egfr^{wa2/wa2}$ double mutant females showed more profound defects in induction of *Ptgs2*, *Has2*, and *Tnfaip6*, cumulus expansion, and ovulation after gonadotropin administration (345). In support of an evolutionarily conserved role for EGF-like growth factors in cumulus expansion, amphiregulin is abundant in human follicular fluid obtained from patients undergoing *in vitro* fertilization (IVF) (346, 347).

As mentioned previously, the LH surge leads to activation of MAPK signaling, which was first implicated in an *in vitro* culture assay to be an important mediator of cumulus expansion. The UO126 inhibitor of MAPK signaling prevented gonadotropin, EGF, and cAMP analog stimulation of cumulus expansion (321). Recently, Richards and colleagues (322) validated these findings *in vivo*. Binding of EGF-like growth factors to EGFR leads to activation of MAPK3 and MAPK1; also known as extracellular signal-regulated kinases 1 and 2 (ERK1/2), respectively. Both *Mapk3* null mice and *Mapk1* cKO mice [using granulosa cell-specific *Cyp19a1-Cre* (264)] are fertile. *Mapk1 Mapk3* double mutant mice (*Mapk1/3* dKO), however, are sterile with defects not only in cumulus expansion but also in ovulation, luteinization, and oocyte meiotic maturation (322). In response to hCG, granulosa cells normally stop dividing and terminally differentiate, resulting in decreased estradiol production and increased progesterone. However, when *Mapk1/3* dKO mice were given hCG, granulosa cells continued to proliferate, and serum estradiol remained elevated because *Cyp19a1* continued to be expressed at a high level, and *Sult1e1*, an estradiol-metabolizing enzyme (348), was not induced, whereas progesterone did not increase due to the lack of induction of *Cyp11a1*, *Star*, and other genes. Thus, MAPK signaling is a critical target and effector of several events triggered by the LH surge in preovulatory follicles. Of further interest, disruption of MAPK1/3 in the pituitary completely blocks the LH surge mode in females but has no effect on basal LH production, thereby rendering the females infertile, whereas the males are fertile (349).

Despite convincing *in vitro* and genetic evidence for EGF-like factors and MAPK signaling as mediators of cumulus expansion in response to the LH surge, the oocyte also has an obligatory role in this process. To circumvent the absence of LH receptors on cumulus cells, *in vitro* expansion of isolated cumulus-oocyte complexes relies on the addition of FSH, cAMP analogs, or EGF. Nearly 20 yr ago, two groups demonstrated that the oocyte is also required to achieve expansion. Microsurgical removal of the oocyte from isolated cumulus-oocyte complexes [referred to as oocytectomized (OOX) complexes] prevented cumulus expansion when FSH or EGF was added to the culture medium (323). Expansion of OOX complexes could be rescued by coculture with denuded oocytes or conditioned medium from denuded oocytes. A similar experiment in which cumulus cells were mechanically dissociated from oocytes and cultured separately showed that these cells failed to synthesize a mucinous matrix in response to FSH, but oocyte coculture or oocyte-conditioned medium restored matrix production (350). These experiments suggested that oocytes secrete a cumulus expansion-enabling factor (CEEF) that allowed oocytes to respond to FSH. Although follicular development in GDF9 knockout mice is arrested at the primary follicle stage, *in vitro* studies have identified critical functions for GDF9 in preovulatory granulosa cells and suggest that it may be the primary CEEF secreted by fully grown oocytes. Recombinant GDF9 up-regulates *Has2*, *Ptgs2*, *Ptger2*, *Tnfaip6*, and *Ptx3* in granulosa cell culture systems (221, 254). Using an RNAi approach, injection of mouse oocytes with *Gdf9* double-stranded RNA, but not *Bmp15*

double-stranded RNA, resulted in lower *Has2* and *Ptgs2* expression as well as limited cumulus expansion when OOX cumulus complexes were cocultured with *Gdf9* knockdown oocytes (351).

GDF9 signals through an unusual heterodimeric complex of a type I TGFβ family receptor, and the type II BMP receptor, BMPRII, to activate SMAD2/3 (352). *In vitro* and *in vivo* evidence suggests that cumulus expansion is dependent on SMAD2/3 signaling. The ALK4/5/7 inhibitor, SB-431542, prevents SMAD2/3 activation and cumulus expansion of OOX complexes cultured in the presence of FSH and GDF9 or oocytes (353), suggesting that one or more of these type I receptors is involved. Conditional deletion of *Smad2* and *Smad3* in granulosa cells using *Amhr2-Cre* (*Smad2/3* cKO) disrupts cumulus expansion *in vivo* (354). Interestingly, although treatment of *Smad2/3* cKO granulosa cells with GDF9 failed to induce *Has2* and *Ptx3*, the other cumulus expansion-related transcripts, *Ptgs2* and *Tnfaip6*, were attenuated but still up-regulated in response to GDF9. These results suggest that GDF9 may function through both SMAD2/3-dependent and SMAD2/3-independent pathways to regulate cumulus expansion. It is plausible that SMAD-independent signaling by GDF9 could involve activation of MAPK pathways because TGFβ family members have been shown to activate MAPK pathways (263), and activation of these pathways is required for cumulus expansion (321, 322).

Although there is convincing evidence that GDF9 is sufficient to function as a CEEF, there is, however, controversy as to whether GDF9 is the sole CEEF (355). BMP15 has also been implicated in the regulation of cumulus expansion and up-regulates expression of EGF-like growth factors in cumulus cells *in vitro* (356). Although the fertility defects in *Bmp15*$^{-/-}$ mice are subtle and *Gdf9*$^{+/-}$ mice are phenotypically normal, *Gdf9*$^{+/-}$ *Bmp15*$^{-/-}$ double mutant mice on a 129SvEV inbred background are infertile with impaired cumulus expansion (230). Furthermore, coculture of *Gdf9*$^{+/-}$ *Bmp15*$^{-/-}$ oocytes with OOX cumulus complexes fails to enable cumulus expansion in the presence of FSH, and the cumulus cells have less activation of MAPK, suggesting that BMP15 acts synergistically with GDF9 as a CEEF (357). BMP15 is believed to signal through the type I activin receptor-like kinase ALK6 (also known as BMPR1B) to activate SMAD1/5/8 pathways (358). *Bmpr1b*$^{-/-}$ female mice are infertile secondary to defects in cumulus expansion, despite a paradoxical increase in *Ptgs2* (359). Finally, BMP15 is found in the follicular fluid of patients undergoing IVF and is associated with increased fertilization and embryo development (360). Although a direct link between BMP15 and cumulus expansion in human cumulus-oocyte complexes was not made, other studies have associated higher levels of *HAS2* and *PTGS2* in human cumulus cells with higher quality embryos (361, 362). Because both *BMP15* and *GDF9* are expressed in human cumulus-oocyte complexes (363), the relative contribution of each factor to human cumulus expansion and developmental potential remains to be determined. Studies in *Bmp15*$^{-/-}$ and *Gdf9*$^{+/-}$ *Bmp15*$^{-/-}$ mice, however, suggest that both growth factors influence developmental competence of oocytes after fertilization (357).

Cumulus expansion and ovulation are not mutually exclusive, as demonstrated by many of the mutant mouse models discussed thus far that have defects in both processes. Ovulation has been compared with an inflammatory-like process, based on the follicular hyperemia, large amount of prostaglandin production, and synthesis of the hyaluronan-rich extracellular matrix that occurs during this stage of follicular development (364). Indeed, many of the matrix-associated genes that are up-regulated in the follicle after the LH surge are also found at sites of inflammation.

A number of transcriptional regulators induced downstream of LH receptor activation are necessary for ovulation. After the LH surge, progesterone receptor (PR) is rapidly induced in the mural granulosa cells of the preovulatory follicle (365, 366). Like ERα and ERβ, PR is a member of the nuclear receptor superfamily of transcription factors. Although progesterone has classically been associated with pregnancy, PR-deficient mice illustrate the importance of progesterone in ovulation as well (145). There are two PR isoforms, PR-A and PR-B, that arise from a single gene (*Pgr*) as a result of transcription from alternative promoters and translation initiation at two alternative start codons in the *Pgr* transcript (367). The original *Pgr* null mouse model eliminated production of either PR isoform, and females were infertile secondary to anovulation, despite cumulus expansion in unruptured follicles (145). CLs were absent; however, subsequent studies showed that granulosa cells in unruptured follicles expressed markers of ovulation and luteinization, including *Ptgs2* and *Cyp11a1*, suggesting that the differentiation response of granulosa cells to the LH surge was intact in *Pgr* null mice (368). Selective ablation of each PR isoform by point mutation of each alternative start codon demonstrated that PR-A, but not PR-B, mediates the ovulatory response to the LH surge (369, 370).

Although it is not entirely understood how PR regulates ovulation, misregulation of a number of gene targets downstream of PR has been identified (371). Genes for the *Adamts1* and cathepsin L (*Ctsl*) proteases are down-regulated, and although only *Adamts1* null mice have impaired ovulation (341), cathepsin L may function redundantly because a number of proteases are expressed in the

periovulatory follicle and many individual knockout models are fertile (372). The vasoactive molecule endothelin 2 (*Edn2*) is induced in preovulatory follicles in a PR-dependent manner (373), and mice treated with endothelin receptor antagonists have a dramatic decline in ovulation secondary to impaired smooth muscle contraction that normally drives follicular rupture (374). *Edn2* and another PR target gene, cGMP-dependent protein kinase (*Prkg2*) (375), may be indirectly regulated by PR through peroxisome proliferator-activated receptor γ (PPARγ, encoded by *Pparg*). *Pparg* is also decreased in *Pgr* null ovaries and conditional deletion of *Pparg* (*Pparg* cKO) in the periovulatory follicle using *PR-Cre* (376) caused subfertility secondary to impaired follicle rupture (377). A subset of PR target genes, including *Edn2*, *Prkg2*, and *Il6*, were decreased in *Pparg* cKO ovaries, and a PPARγ-specific antagonist, GW9662, markedly attenuated induction of these genes in cultured granulosa cells. Another PR target gene is *Snap25*, which regulates vesicle secretion and appears to be important for the release of potent cytokines from granulosa cells as part of the inflammatory and immune-like response of ovulation (364, 378). Highlighting the diverse functions of PR downstream of the LH surge in the periovulatory period, components of many other signaling pathways, including EGF-like growth factors and members of the WNT signaling family, are also altered in PR-deficient mice (371).

Other transcriptional regulators that mediate the ovulatory response to the LH surge include C/EBPβ (CCAAT/enhancer-binding protein β, encoded by *Cebpb*) and several members of the nuclear receptor family (discussed in detail below). C/EBPβ is a member of a family of basic leucine zipper proteins that recognize similar DNA motifs and dimerize with themselves or other basic leucine zipper transcription factors to activate or repress gene transcription. *Cebpb* is strongly up-regulated in periovulatory follicles after hCG administration, and similar to *Pgr* null mice, $Cebpb^{-/-}$ females are sterile and their ovaries contain many unruptured follicles but lack CLs (379). Less is known about how C/EBPβ functions downstream of the LH surge during ovulation; however, both *Cyp19a1* and inhibin α mRNAs fail to be down-regulated after hCG administration to $Cebpb^{-/-}$ females, suggesting that C/EBPβ may be an important transcriptional repressor during the granulosa cell differentiation process initiated by the LH surge (379, 380).

Interestingly, the ovarian phenotype of $Cebpb^{-/-}$ females is similar to *Mapk1/3* dKO females. In response to amphiregulin, C/EBPβ has been shown to induce expression of genes up-regulated in the periovulatory period, including, *Ptgs2*, *Tnfaip6*, *Pgr*, and *Star*, and the up-regulation of these genes is dependent on MAPK activity (322). However, conditional deletion of *Cebpb* in granulosa cells results in subfertility, and CLs are present in some adult ovaries, suggesting that C/EBPβ is not the only transcription factor downstream of MAPK signaling in granulosa cells (322).

Conditional deletion of two related members of the NR5A subfamily, liver receptor homolog 1 (LRH1, encoded by *Nr5a2*) and SF1 (encoded by *Nr5a1*) leads to infertility secondary to anovulation, despite distinct molecular and hormonal changes in each mutant (381, 382). In Lrh1 cKO mice, there is increased CYP19A1 expression and enhanced follicular estradiol production secondary to a decrease in nitric oxide synthase 3 (NOS3), an inhibitor of CYP19A1 expression and activity in mice and humans (383, 384). Chromatin immunoprecipitation analysis identified *Nos3* as a direct target of LRH1 (381). *Ptgs2* and *Tnfaip6* were substantially reduced after hCG treatment, which may partially be due to the enhanced estradiol signaling because this has previously been shown to reduce PTGS2 in mice lacking the estradiol-metabolizing enzyme SULT1E1 (348). Finally, Lrh1 cKO mice also exhibited impaired progesterone synthesis secondary to a decrease in scavenger receptor B1 (*Scarb1*), *Star*, and *Cyp11a1* after hCG administration (381).

Although Sf1 cKO mice are also anovulatory, adult ovaries are substantially smaller compared with wild-type ovaries, which was not readily apparent in Lrh1 cKO mice. In addition, despite similar basal serum estradiol levels in Sf1 cKO *vs.* control mice, the increase in estradiol and progesterone after PMSG was blunted in Sf1 cKO mice. Furthermore, in contrast to Lrh1 cKO mice, CYP19A1 was slightly decreased in Sf1 cKO mice, and there was no difference in *Cyp11a1* in ovaries from untreated or PMSG-stimulated females (382). CYP11A1 did, however, appear to be ectopically expressed in the granulosa cells of large follicles, rather than only the thecal layer or CL. Although mRNA levels for other steroidogenic enzymes critical for progesterone synthesis were not reported, it appears that SF1 and LRH1 have unique roles in the regulation of some ovarian target genes.

Nuclear receptor interacting protein 1 (NRIP1; also known as RIP140), functions as a coregulator of the nuclear receptor superfamily. $Nrip1^{-/-}$ female mice are also infertile secondary to anovulation; however, the unruptured follicles in these mice go on to form CLs with trapped oocytes (385). More detailed histological analysis of *Nrip1* null ovaries demonstrated defects in cumulus expansion, suggesting that NRIP1 is also an important mediator of this process, as well as ovulation. Gene expression profiling showed that after gonadotropin stimulation, *Nrip1* null mice fail to induce many genes already discussed that are important for cumulus expan-

sion, including EGF-like growth factors (*Areg*, *Ereg*, and *Btc*), *Has2*, *Ptgs2*, *Tnfaip6*, and *Cspg2*, whereas many genes involved in cell-cell adhesion are up-regulated and might, therefore, impede expansion. *Nrip1* is most dramatically up-regulated in mural granulosa cells after PMSG administration, whereas *Cebpb* increases after PMSG plus hCG administration (379, 380, 385). Because both FSH and LH activate cAMP/PKA/cAMP response element-binding protein pathways to influence gene expression, the increase in NRIP1 and C/EBPβ in the periovulatory period may function to modulate transcription of the many target genes with cAMP response elements.

In contrast to evidence supporting a role for NRIP1 and C/EBPβ downstream of LH, the testicular orphan nuclear receptor 4 (TR4; also known as nuclear receptor subfamily 2, group C, member 2, *Nr2c2*) may be important in preparing preovulatory follicles for the LH surge. *Nr2c2*$^{-/-}$ mice (386) exhibit subfertility, with smaller ovaries containing fewer preovulatory follicles and CLs, as well as a muted response to superovulation protocols (387). These defects, however, appear to be secondary to a decrease in LH receptor. Furthermore, TR4 appears to regulate *Lhcgr* expression through direct binding to a TR4 response element in the *Lhcgr* promoter, suggesting that TR4 activity is important before the LH surge, perhaps to prepare granulosa cells to respond to the rise in LH that triggers ovulation.

After follicle rupture and release of the cumulus-oocyte complex, the final fate of the remaining granulosa and thecal cells is terminal differentiation to form the CL, a highly differentiated endocrine structure responsible for secreting progesterone to stimulate the uterus and maintain pregnancy. The processes controlling the formation, function, and regression of the CL are complex and have recently been reviewed in this journal (388) and elsewhere (389). Therefore, we will focus on the key regulators of CL formation and maintenance.

The process of CL formation requires the granulosa cells to exit the cell cycle. Although FSH is critical for promoting granulosa cell proliferation through up-regulation of cyclin D2, the LH surge, via obligatory MAPK1/3 activation (322), causes cell cycle arrest by shifting the balance of cell cycle regulators to favor inhibitors of cell cycle progression, such as the CDK inhibitor p27^{Kip1}. In particular, using a rat model system, cyclin D2 is rapidly and robustly down-regulated in the preovulatory follicle after hCG administration (390). Although there is also a transient decrease in p27^{Kip1}, by 24 h after hCG, p27^{Kip1} is markedly increased in terminally differentiated luteal cells. An additional CDK inhibitor, p21^{Cip1} (CDKN1A), is induced within 2–4 h of hCG administration. Loss of function studies suggest that p27^{Kip1} is the principal regulator of luteinization, although p21^{Cip1} likely has a cooperative role. *Cdkn1b*$^{-/-}$ female mice are sterile, and their ovaries exhibit granulosa cell hyperplasia and impaired differentiation to form CLs (391–393). In further support of a luteinization defect, ovulation in response to exogenous gonadotropins is unimpaired, but pregnancy is not maintained (391). Although *Cdkn1a*$^{-/-}$ female mice are fertile (394, 395), in contrast to observations in *Cdkn1b*$^{-/-}$ mice, their ovaries are not enlarged compared with wild-type ovaries. *Cdkn1b*$^{-/-}$ *Cdkn1a*$^{-/-}$ ovaries were even larger than *Cdkn1b*$^{-/-}$ ovaries and had more profound granulosa cell hyperproliferation, suggesting that p27^{Kip1} and p21^{Cip1} function synergistically to promote cell cycle exit in differentiating granulosa cells (396).

An additional cell cycle regulator, CDK4, is indirectly involved in maintaining the CL once it has formed. *Cdk4*$^{-/-}$ female mice (397, 398) are infertile because of insufficient progesterone production by the CL to allow embryo implantation. This is due to a failure of pituitary lactotropes to proliferate and produce prolactin (PRL) (399). Although progesterone is considered the hormone of pregnancy, in rodents PRL has a critical role in CL maintenance and progesterone production. Both isoforms of the PRL receptor (PRLR) are up-regulated during luteinization in an LH-dependent manner (400). *Prlr*$^{-/-}$ mice are sterile secondary to insufficient progesterone production to support implantation and pregnancy (401). A more detailed characterization of *Prlr*$^{-/-}$ ovaries revealed that CLs initially form, but they were disorganized, failed to show signs of neovascularization, and had increased apoptosis (402). Although *Cdkn1b* levels increased as expected in *Prlr*$^{-/-}$ ovaries at 1.5 d post-coitus (dpc), they were not maintained at elevated levels by 2.5 dpc, which may be attributed to a relative decrease in *Lhcgr* expression over the same time period. Transcripts for numerous enzymes involved in steroidogenesis that are known to be induced by LH, including *Star*, *Cyp11a1*, and *Hsd3b1*, were also reduced. Conversely, *Prlr*$^{-/-}$ ovaries showed an increase in the progesterone-metabolizing enzyme 20α-hydroxysteroid dehydrogenase (AKR1C18, aldo-keto reductase family 1, member C18) in regressing CLs. Although their ovaries were not examined, *Prl*$^{-/-}$ mice are also infertile with an impaired pseudopregnancy response and irregular estrous cycles (403). PRL signaling activates a Janus kinase/signal transducer and activation of transcription (STAT) pathway, specifically STAT5A/B. *Stat5a*$^{-/-}$ *Stat5b*$^{-/-}$ mice are also infertile, and similar to *Prlr*$^{-/-}$ mice, their ovaries show reduced *Cdkn1b* and increased AKR1C18 (404). These mouse models suggest that PRL is important in maintaining CLs and progesterone production through positive regulation of LH receptor and negative regulation of AKR1C18. In women, hCG

secretion by the embryonic trophoblast serves a function analogous to PRL in sustaining the CL during early pregnancy (389).

What might regulate PRLR to prevent premature regression of CLs? Components of the WNT/FZD signaling pathways, including *Wnt4*, *Fzd1*, and *Fzd4*, are highly expressed in periovulatory follicles, and *Fzd4* is particularly increased in CLs. The functional significance of this is demonstrated by $Fzd4^{-/-}$ mice, which are infertile with histological and molecular defects in their ovaries that are quite similar to $Prlr^{-/-}$ mice (405). Moreover, *Prlr* was also reduced at 1.5 and 5.5 dpc, suggesting that WNT signaling is necessary for cells of the CL to be able to respond to PRL. It is also possible that PTEN impacts the response of cells to PRL because in $Pten^{flox/flox}$ *Cyp19a1-Cre* mice that have a conditional deletion of *Pten* in granulosa/luteal cells, the life span of CLs is extended (406).

The timing of luteinization is important for normal reproductive function, and the fact that it occurs after ovulation supports a role for oocyte-derived inhibitors that prevent premature luteinization. As mentioned, oocyte-secreted factors oppose FSH signaling to define two distinct granulosa cell populations in the antral follicle. Specifically, oocyte-secreted proteins signal through Smad2/3 to oppose FSH induction of ovulatory (*Lhcgr*) and steroidogenic (*Cyp11a1*) markers in the cumulus cells close to the oocyte (407), suggesting that a TGFβ family member, such as GDF9, may be an important inhibitor of luteinization. Support for this is seen in mice with conditional deletion of the common SMAD, *Smad4*, in granulosa cells of preantral follicles (408). Although *Smad4* cKO ovaries exhibit a number of follicular defects, including impaired cumulus expansion and ovulation, there were a number of small and large follicles with luteinizing cells surrounding oocytes, and these findings were accentuated after administration of PMSG alone in 3-wk-old mice. Molecularly, in contrast to control mice, granulosa cells from PMSG-stimulated *Smad4* cKO mice had significantly higher levels of genes related to steroidogenesis and luteinization, including *Lhcgr*, *Cyp11a1*, *Hsd17b7*, *Star*, secreted frizzled-related protein (*Sfrp4*), and the prostaglandin F receptor (*Ptgfr*). Although it is also possible that the loss of SMAD4 prevents granulosa cells from responding appropriately to BMP/TGFβ/activin signals from the surrounding stroma that might also inhibit luteinization, Eppig and colleagues (407) provide convincing evidence that activation of SMAD signaling by oocyte-secreted factors is key to preventing premature differentiation of granulosa cells.

Although we have tried to highlight critical mediators of ovulation, cumulus expansion, and luteinization, many additional factors are involved in these processes in the periovulatory follicle. Mutant mouse models illustrating the diverse number of factors that regulate these events are summarized in Table 9.

G. Regulation of meiotic arrest and reentry

Oocytes start meiosis in the embryonic gonad and progress through the diplotene stage of the first meiotic prophase. Meiosis is arrested by E14.5 in mouse oocytes and does not resume until the LH surge, when a number of changes take place in the somatic cells and the oocyte to facilitate meiotic maturation. The resumption of meiosis can be recognized morphologically by dissolution of the nuclear envelope, referred to as germinal vesicle breakdown (GVBD).

In the course of follicular development, oocytes acquire the competence to resume meiosis such that about the time of antrum formation they have synthesized a threshold level of maturation-promoting factor (MPF), which is a complex consisting of CDK1 and cyclin B that regulates the G_2/M transition of the cell cycle in mitosis and meiosis (409, 410). However, MPF promotes meiosis, so to maintain arrest at prophase I until the LH surge, MPF must be kept in an inactive state. It has been known for decades that once mammalian oocytes attain meiotic competence, they rely on the follicular milieu to prevent premature resumption of meiosis. Removal of the oocyte from antral follicles causes spontaneous maturation, as evidenced by GVBD (411, 412). Importantly, this inhibitory signal appears to originate from mural granulosa cells because the oocytes were isolated with their surrounding cumulus cells. Likewise, the requirement of high levels of cAMP in the oocyte to maintain meiotic arrest has been dogma for many years, and a decrease in oocyte cAMP levels is associated with the resumption of meiosis (reviewed in Refs. 409 and 410). For years, however, two questions remained unanswered: how does high cAMP maintain meiotic arrest, and how are cAMP levels controlled within the oocyte?

The net effect of elevated cAMP is activation of PKA signaling pathways that keep MPF in its inactive state, and although the targets of PKA were postulated to be regulators of CDK phosphorylation (409), whether this was by direct or indirect means has only recently been established. CDK1 is negatively regulated through phosphorylation on Thr14 and Tyr15. The WEE1/MYT1 protein kinases phosphorylate and inactivate CDK1, whereas CDC25 phosphatases activate CDK1 by dephosphorylating Thr14 and Tyr15 (413). Using a novel strategy to isolate PKA substrates involved in regulating meiotic maturation, Conti and colleagues (414) performed a small pool expression screen of a mouse oocyte cDNA library and identified *Wee2* (WEE1 homolog 2; Wee1b). *In vitro* assays showed that PKA could phosphorylate WEE2, and *in vivo* reduction of WEE2 by the generation of transgenic mice

TABLE 9. Mouse models with defects in the periovulatory period

Gene	Reproductive phenotype	Fertility status	Ref.
Cumulus expansion and ovulation			
LH β (*Lhb*)	Block in folliculogenesis at the early antral stage	Infertile	318
LH/choriogonadotropin receptor (*Lhcgr*)	Block in folliculogenesis at the early antral stage	Infertile	319, 320
Prostaglandin-endoperoxide synthase 2 (*Ptgs2*; Cox2)	Defects in ovulation, cumulus expansion, and implantation	Mostly infertile	329, 330
Prostaglandin E receptor 2, subtype EP2 (*Ptger2*)	Defects in ovulation and cumulus expansion; decreased fertilization and preimplantation defects	Subfertile	331–333
Pentraxin 3 (*Ptx3*)	Defects in cumulus-oocyte complex integrity and ovulation	Subfertile	254, 804
TNFα−induced protein 6 (*Tnfaip6*)	Impaired cumulus matrix formation resulting in failed cumulus-oocyte complex expansion	Infertile	334
CCAAT/enhancer-binding protein β (*Cebpb*)	Reduced ovulation; fail to form CL	Infertile	379
IL-6 signal transducer (*Il6st*; gp130)	Defect in oocyte maturation/ovulation	Subfertile	805
LFNG O-fucosylpeptide 3-β-N-acetylglucosaminyltransferase (*Lfng*; lunatic fringe homolog)	Multiple defects in folliculogenesis; luteinized follicles with trapped oocytes; defect in meiotic maturation of oocytes	Infertile	149
Nitric oxide synthase 3, endothelial cell (*Nos3*; eNos)	Compromised ovulation, delayed meiotic progression from metaphase I	Subfertile	806
Nuclear receptor interacting protein (*Nrip1*; RIP40)	Ovulation defect; ovaries accumulate luteinized and unruptured follicles	Infertile	385
Nuclear receptor subfamily 2, group C, member 2 (*Nr2c2*; TR4)	Small ovaries with few preovulatory follicles and CLs; decreased ovulation	Variable lethality; subfertile	386, 387
2′-5′ Oligoadenylate synthetase 1D (*Oas1d*)	Defects in folliculogenesis and ovulatory efficiency	Subfertile	807
Phosphodiesterase type 4, cAMP specific (*Pde4d*)	Impaired ovulation; luteinized follicles with trapped oocytes	Subfertile	808
Progesterone receptor (*Pgr*; PR)	Anovulation; CLs absent	Infertile	145
Sirtuin 1 (*Sirt1*; SIR2a)	Small ovaries; anovulation; CLs absent	Variable lethality; extremely subfertile	809
Sulfotransferase family 1E, member 1 (*Sult1e1*)	Abnormal ovulation and cumulus expansion	Subfertile	348, 810
Transformation-related protein 73 (*Trp73*)	TAp73 isoform deficiency; defective ovulation, oocyte meiotic defects, and maternal effects	Variable lethality; infertile	791
Y box protein 2 (*Ybx2*; Msy2)	Follicular atresia, oocyte loss, anovulation	Infertile	811
Peroxisome proliferator activated receptor γ (*Pparg*) (cKO)	Impaired ovulation	Subfertile	377
α1 Microglobulin/bikunin (*Ambp*)	Defects in ovulation and cumulus-oocyte complex integrity	Subfertile	337, 338
Amphiregulin (*Areg*)	Suppressed cumulus expansion, delayed meiotic reentry	Subfertile	345
Bone morphogenetic protein 15 (*Bmp15*)	Defects in cumulus expansion and ovulation	Subfertile	230
Bone morphogenetic protein receptor, type IB (*Bmpr1b*)	Defects in estrous cyclicity, cumulus expansion, and endometrial gland development	Subfertile	359
Epiregulin ($Ereg^{wa2/wa2}$; hypomorph)	Suppressed cumulus expansion, defective meiotic resumption, ovulation defect	Subfertile	345
MAPKs 3 and 1 ($Mapk3^{-/-}$ *Mapk1* cKO)	Defects in cumulus expansion and oocyte meiotic maturation; anovulation and absent CLs	Infertile	322
Nuclear receptor subfamily 5, group 2, member 1 (*Nr5a1*; Sf1, steroidogenic factor 1) (cKO)	Small ovaries with few resting and growing follicles; defects in estrous cyclicity, cumulus expansion, and ovulation; CLs absent	Infertile	382
Nuclear receptor subfamily 5, group 2, member 2 (*Nr5a2*; Lrh1, liver receptor homolog 1) (cKO)	Defects in estrous cyclicity, cumulus expansion, and ovulation; CLs absent	Infertile	381
Phospholipase C-β1 (*Plcb1*)	Decreased ovulation with trapped oocytes; abnormal mating behavior	Subfertile	812
Corpus luteum formation			
Cyclin-dependent kinase 4 (*Cdk4*)	Defects in the hypothalamic-pituitary-gonadal axis disrupts maintenance of CL during pregnancy	Infertile	397, 398

(*Continued*)

TABLE 9. (Continued)

Gene	Reproductive phenotype	Fertility status	Ref.
Cyclin-dependent kinase inhibitor 1B (*Cdkn1b*; $p27^{Kip1}$)	Corpus luteum differentiation failure and granulosa cell hyperplasia	Infertile	391–393
Early growth response 1 (*Egr1*; NGFI-A); (targeted *lacZ* insertion)	Down-regulation of LH receptor, not remedied with gonadotropin treatment	Infertile	813
Early growth response 1 (*Egr1*; NGFI-A); (targeted *neo* insertion)	LH insufficiency, loss of estrous cyclicity, no CLs; rescued by treatment with gonadotropins	Infertile	814
Aminopeptidase puromycin-sensitive (*Npepps*; Psa)	Lack of CL formation and PRL production causes early pregnancy loss	Infertile	815
Prostaglandin F receptor (*Ptgfr*)	Do not undergo parturition, failed luteolysis	Infertile	816
Prolactin (*Prl*)	Irregular estrous cycles and impaired pseudopregnancy response	Infertile	403
Prolactin receptor (*Prlr*)	Compromised ovulation, fertilization, and preimplantation development in of *Prlr* null embryos	Infertile	401, 402
MAD homolog 4 (*Smad4*) (cKO)	Premature luteinization; decreased ovulation with trapped oocytes	Subfertile	408

Although many genes are critical for multiple events during this period, mouse models are categorized based on the principal findings.

expressing a short hairpin RNA (shRNA) corresponding to *Wee2* induced GVBD in about 25% of oocytes recovered after PMSG stimulation, despite genetic [*i.e.*, in $Pde3a^{-/-}$ oocytes (415), see below] or chemical maintenance of high cAMP conditions. *In vitro* injection of *Wee2* shRNA showed a similar rate of GVBD in the presence of high cAMP. That not all oocytes in which *Wee2* was knocked down underwent GVBD suggests that either compensatory WEE1/MYT1 family members may function in the oocyte, or the high cAMP conditions prevented CDC25 phosphatases from activating MPF. Although all three CDC25 phosphatase family members (CDC25A, CDC25B, and CDC25C) are expressed in mouse oocytes (416), only CDC25B appears to be essential for activating CDK1. *Cdc25b* null mice are sterile, and their oocytes cannot activate CDK1 to resume meiosis, but the meiotic arrest defect is rescued by microinjection of *Cdc25b* mRNA (417). $Cdc25c^{-/-}$ mice are fertile (418), and although $Cdc25a^{-/-}$ mice are embryonic lethal (419), RNAi experiments suggest that CDC25A may have a supportive role in meiotic resumption (420). CDC25 was first identified as a PKA substrate in *Xenopus* oocytes. Duckworth *et al.* (421) demonstrated that PKA phosphorylates CDC25C, leading to binding and sequestration in the cytoplasm by 14-3-3. The signal to resume meiosis (in this case, progesterone) led to dephosphorylation of CDC25, followed by dephosphorylation and activation of CDK1. Two groups have recently shown that similar regulation of CDC25B by PKA occurs in mouse oocytes (422, 423), thus establishing that PKA directly targets CDC25B for cytoplasmic sequestration in prophase-arrested oocytes. Hence, despite acquiring meiotic competence by the early antral follicle stage, high cAMP concentrations in the oocytes of preovulatory follicles keep PKA active, thereby inhibiting the CDK1 activator, CDC25B, and activating the CDK1 inhibitor, WEE2.

What controls cAMP levels upstream of PKA? Early studies suggested that oocytes have an endogenous means by which they control intracellular cAMP homeostasis. Inhibitors of cAMP phosphodiesterases (PDEs), such as 3-isobutyl-1-methylxanthine (IBMX), prevent metabolism of cAMP and therefore spontaneous maturation of isolated oocytes (424). Stimulation of ACs or heterotrimeric G proteins that activate ACs with forskolin or cholera toxin, respectively, prevents or attenuates meiotic resumption, and this effect is potentiated in the presence of small concentrations of IBMX (425, 426). Diffusion of cAMP from granulosa cells to the oocyte by gap junctions has been proposed as a second means by which meiotic arrest is maintained (409, 410). Work in recent years has solidified the hypothesis that cAMP homeostasis in the oocyte is under the control of endogenous factors; however, there also appears to be a role for gap junctional communication between follicular cells in the regulation of meiotic arrest.

After initially establishing a role for stimulatory G proteins ($G\alpha_S$) (427) within the oocyte in maintaining meiotic arrest, Mehlmann *et al.* (428) searched an expressed sequence tag database from a prophase I-arrested oocyte cDNA library and identified the orphan G protein-coupled receptor, GPR3, as a candidate activator of $G\alpha_S$. GPR3 was particularly interesting because of its constitutive activation of AC activity when overexpressed in HEK293 cells (429). The majority of oocytes within antral follicles of $Gpr3^{-/-}$ females demonstrate premature resumption of meiosis before the LH surge (428, 430). As a consequence, $Gpr3^{-/-}$ females are subfertile and develop POF (430). Taking advantage of the turnover properties of

$G\alpha_S$ and a green fluorescent protein-linked $G\alpha_S$ molecule, GPR3 was shown to directly activate $G\alpha_S$ (431). The putative AC downstream of GPR3/$G\alpha_S$ in the oocyte was determined using a degenerate cloning strategy. AC3 (*Adcy3*) was identified as the predominant oocyte AC in rats, whereas AC1 (*Adcy1*) and AC9 (*Adcy9*) were most frequently amplified in mouse oocytes (432). However, AC3 was also abundant in mouse oocytes, and although most *Adcy3*$^{-/-}$ mice died soon after birth (433), those females that reached maturity exhibited reduced fertility and fecundity. Histologically, *Adcy3*$^{-/-}$ ovaries showed premature meiotic resumption in about 50% of the oocytes (432). The incomplete penetrance of this phenotype could be due to the other ACs present in oocytes. Nevertheless, there is strong evidence that GPR3 signals through $G\alpha_S$ to activate an AC that keeps cAMP elevated before the rise in LH.

If GPR3 is truly a constitutively active G protein-coupled receptor, how is the cAMP signal terminated to allow activation of MPF? In *in vitro* follicle culture, LH does not change the localization of $G\alpha_S$ from the oocyte plasma membrane to the cytoplasm, suggesting that GPR3/$G\alpha_S$ signaling is not inhibited in response to LH. The other ways to regulate the cAMP signal could be increasing cAMP turnover or targeting the cAMP effector PKA. PDEs catalyze the hydrolysis of cAMP, and they appear to have an evolutionarily conserved function in vertebrate oocytes. PDE3-specific inhibitors block meiotic resumption and oocyte maturation in *Xenopus* oocytes and cultured murine and human oocytes (434–437). Although there are two PDE3 isoforms, PDE3A and PDE3B, PDE3A appears to be the major form expressed in mammalian oocytes (435, 436) and an essential function for PDE3A *in vivo* is seen in *Pde3a*$^{-/-}$ female mice, which are sterile because ovulated oocytes remain arrested in the germinal vesicle (GV) stage of meiosis I (415). Inhibition of PKA, however, restored meiotic maturation in *Pde3a*$^{-/-}$ oocytes *in vitro*. These studies indicated that one means by which oocytes resume meiosis is through PDE3A-mediated cAMP hydrolysis, leading to a reduction in PKA activity.

PKA activity can also be mediated by localization away from substrates by A-kinase anchoring proteins (AKAPs). AKAP1 localizes primarily to the mitochondria in wild-type oocytes; whereas the RIIα PKA subtype is primarily cytoplasmic in GV-stage oocytes, in meiosis II (MII) oocytes, AKAP1 and RIIα PKA colocalize to the mitochondria (438). *Akap1*$^{-/-}$ females are severely subfertile with ovulated oocytes that remain arrested in the GV-stage and maintain a cytoplasmic distribution of RIIα PKA. This suggests that upon receiving the signal to resume meiosis, PKA is sequestered to the mitochondria by AKAP1, allowing meiotic maturation to proceed. There also appears to be an additional unidentified AKAP that facilitates the cytoplasmic distribution of PKA in GV-stage oocytes. Injecting an inhibitor (HT31) that disrupts PKA/AKAP interactions into GV-stage oocytes caused GVBD, despite maintaining high levels of cAMP with the PDE inhibitor IBMX. Taken together, the resumption of meiosis after a rise in LH is facilitated by at least two mechanisms that reduce PKA activity: a reduction in cAMP by PDE3A, and redistribution of PKA away from its targets by AKAP1. The mediators of this critical stage in oocyte development are summarized in Table 10.

A second path to meiotic resumption after the LH surge appears to involve the regulation of gap junctions. Although it is clear that oocytes are capable of endogenous cAMP production, it is unclear whether somatic cells also contribute cAMP to the oocyte by way of gap junctions, and that the two sources of cAMP are required for preventing premature MPF activation. The disruption of gap junctions with chemical inhibitors has demonstrated that they are important in preventing meiotic maturation (439). It is known that oocyte maturation depends on cumulus cell MAPK activity (321, 322), and Jaffe and colleagues (440) recently demonstrated that LH causes a rapid MAPK-dependent phosphorylation and closure of the CX43 gap junctions between the follicular somatic cells, whereas the CX37 junctions between oocytes and cumulus cells remain open. Importantly, inhibition of MAPK activation (using low doses of the U0126 MAP Kinase Kinase inhibitor) prevented LH-induced channel closure, but not GVBD in response to LH, suggesting that gap junction

TABLE 10. Mouse models with defects in meiotic arrest or reentry

Gene	Reproductive phenotype	Fertility status	Ref.
G protein-coupled receptor 3 (*Gpr3*)	Abnormal resumption of meiosis in oocytes	Subfertile	428, 430
Adenylate cyclase 3 (*Adcy3*)	Premature meiotic resumption in 50% of oocytes	Subfertile	432
Phosphodiesterase 3A, cGMP inhibited (*Pde3a*)	Presence of trapped oocytes; failure of ovulated oocytes to progress beyond GV stage	Infertile	415
Cell division cycle 25 homolog B (*Cdc25b*)	Oocytes are arrested in meiotic prophase with defects in MPF activity	Infertile	417
Moloney sarcoma oncogene (*Mos*)	Parthenogenetic activation, cysts, and teratomas	Subfertile	453, 454

closure is one of two redundant mechanisms by which LH signaling causes reentry into meiosis.

There are still a number of unanswered questions regarding the reinitiation of meiosis I, namely, how is the LH signal to mural granulosa cells propagated to the oocyte to allow maturation? What are the molecules that pass between gap junctions to maintain arrest? The EGF-like growth factors that are critical for ovulation and cumulus expansion also appear to be involved in promoting oocyte maturation *in vitro* and *in vivo* (344, 345). However this is likely an indirect effect because although they induce oocyte maturation in cumulus-oocyte complexes, they do not have an effect on denuded oocytes (344, 441). Steroids are known to influence meiotic maturation in lower vertebrates and have been implicated as mediators downstream of EGFR activation in cultured mouse cumulus-oocyte complexes. EGFR activation up-regulates steroid production, and with the exception of ERα, the other classical steroid receptors (AR, ERβ, PR) are detected in mouse oocytes, and treatment with their respective ligand agonists induced oocyte maturation (442). However, the physiological significance of steroid receptors in mammalian oocyte maturation remains to be determined, and maturation defects have not been reported in *Ar*, *Esr2*, or *Pgr* null oocytes. Moreover, others have reported inhibitory effects of steroids on oocyte maturation (Refs. 443 and 444; and reviewed in Ref. 410). Follicular fluid-meiosis-activating sterol (FF-MAS), first isolated from human follicular fluid (445), has also been proposed as an inducer of oocyte maturation; however, the timing of the rise in FF-MAS *in vivo* appears later than the onset of GVBD, and FF-MAS may be more important in the meiosis I to metaphase II transition (reviewed in Ref. 410).

In addition to the possibility that cAMP passes through gap junctions into the oocyte, cGMP has been proposed as a competitive inhibitor of PDE3A that enters the oocyte through gap junctions (446). Although inhibiting gap junctional communication did not alter PDE3 activity, the methods for quantifying PDE3 activity may have diluted cGMP, thereby preventing competitive inhibition during *in vitro* assays (439, 440). Intraoocyte cGMP levels decrease during spontaneous maturation of rat oocytes, microinjection of cGMP prevents resumption of meiosis (447), and inhibiting guanylate cyclase activity in rat ovarian follicles induces oocyte maturation (448). More sensitive Förster resonance energy transfer-based cyclic nucleotide assays have recently been used to quantify cAMP and cGMP levels in follicle-enclosed mouse oocytes (449). Exposure of these follicles to LH resulted in a decrease in cGMP in both the oocyte and somatic cells, as well as a decrease in oocyte cAMP. The decrease in cGMP occurred by both gap junction-dependent and -independent mechanisms, resulting in increased PDE3A activity and a corresponding decrease in cAMP, followed by GVBD. Thus, although much work remains to identify the intermediate steps from the LH surge and the elusive *in vivo* inducer of oocyte maturation, the numerous mouse models generated in recent years have established a role for intra-oocyte regulation of cAMP homeostasis as essential for meiotic arrest and reentry. Likewise, there is sufficient evidence that gap junctional communication throughout the somatic compartment and between somatic cells and oocytes is also important.

After activation of MPF and reentry into meiosis, chromosomal condensation and segregation occurs, the nuclear envelope breaks down, and asymmetric division of the cytoplasm results in extrusion of the first polar body. Unlike mitosis, there is no intervening S-phase, and instead, meiosis progresses to a second division to reduce the genome from diploid to haploid. Whereas maintaining MPF in an inactive stage until the LH surge keeps oocytes arrested in prophase I, the activity of a cytostatic factor (CSF) (450) causes metaphase II arrest in the oocyte by stabilization of MPF, and the completion of MII does not occur until fertilization. CSF stabilizes MPF by inhibiting the anaphase-promoting complex/cyclosome (APC/C), an E3 ubiquitin ligase that targets cyclin B for 26S proteosomal degradation to allow the metaphase-anaphase transition (451). Although the existence of CSF was proposed in 1971 (450), it took almost two decades to identify MOS, a serine-threonine kinase, encoded by the Moloney sarcoma oncogene (*Mos*), as a critical component of CSF, first in *Xenopus laevis* oocytes (452), then in the mid-1990s in mouse oocytes. Disruption of the mouse *Mos* gene caused female subfertility, and *Mos* knockout oocytes underwent parthenogenetic activation secondary to a failure of metaphase II arrest (453, 454). MOS may also have a function in the transition to MII because emission of the first polar body is delayed (454) and it is enlarged, which appears to result from failure of the spindle apparatus to translocate to the cortex, resulting in an altered cleavage plane. The enlarged polar bodies frequently went through another round of division, rather than degrading, which is the normal fate in wild-type oocytes (455). Although MOS satisfies the criteria of a CSF component (450), how it functions to inhibit APC/C activity in mammalian oocytes is currently unknown.

More recently, endogenous meiotic inhibitor 2 (EMI2; also known as F-box protein 43, FBXO43) has been identified as an additional protein with CSF activity in *Xenopus* eggs (456) and mouse oocytes (457). FBXO43 functions by inhibiting the APC/C, which in mouse oocytes may be

through direct interaction with the APC/C activator CDC20 (457). RNAi-mediated knockdown of *Fbxo43* in mouse oocytes causes progression through the cell cycle without arresting at metaphase II and results in aberrant cytokinesis. Expressing a stable form of FBXO43 and treating oocytes with a chemical inducer of parthenogenesis ($SrCl_2$) resulted in stable MII arrest. Alternatively, knocking down both *Fbxo43* and its presumed target, *Cdc20*, maintains meiotic arrest. Moreover, knockdown of *Cdc20* alone prevents egg activation after $SrCl_2$ treatment or sperm head injection (457). Thus, FBXO43 also has CSF activity that is manifest through inhibition of CDC20, thereby preventing APC/C activation to allow degradation of cyclin B and progression through the cell cycle. FBXO43 has also been implicated in the entry into MII by stabilizing cyclin B in interkinesis to allow formation of a metaphase II spindle (458).

Because both MOS and FBXO43 have CSF activity and FBXO43 specifically inhibits APC/C activity, could FBXO43 be the missing link between MOS and metaphase II arrest? Although *Xenopus* eggs have been a good model system for studying MOS signaling pathways, there appear to be differences in MOS targets in mammals (459). MOS is an activator of MAPK-signaling pathways, and in *Xenopus*, MOS activation of MAPK leads to activation of the 90-kDa ribosomal protein S6 kinase ($p90^{RSK}$) (460, 461), which was recently shown to phosphorylate *Xenopus* FBXO43 on Ser335/Thr336, causing increased stability and activity, thereby contributing to metaphase II arrest (462, 463). However, $p90^{RSK}$ does not appear to be the downstream mediator of MOS in maintaining metaphase II arrest because oocytes from triple *Rsk* null mice (*Rsk1*, *Rsk2*, *Rsk3*) have a stable CSF arrest, and injection of constitutively active *Rsk1* or *Rsk2* RNA into $Mos^{-/-}$ oocytes does not restore MII arrest (464). Thus, in mammals, a direct linear pathway connecting MOS activation of MAPK signaling to phosphorylation and stabilization of FBXO43 cannot currently be drawn. Generation of $Fbxo43^{-/-}$ mice or overexpression of a stable FBXO43 protein in $Mos^{-/-}$ oocytes may help clarify any connection between FBXO43 and MOS as components of CSF.

The oocyte completes the final stages of meiosis after fertilization. The signal to resume meiosis comes from the sperm, which introduces phospholipase C ζ into the oocyte cytoplasm (465), triggering calcium oscillations that activate APC/C, leading to cyclin B degradation and the completion of meiosis (466). Thus, after being formed in the embryo and nurtured postnatally by granulosa cells as it progressed through multiple stages of folliculogenesis, the oocyte's journey ends with fertilization, and the cycle begins anew.

IV. Ovarian Cancer

Ovarian cancer is the most lethal gynecological malignancy in the United States and the fifth leading cause of cancer death in women, with an estimated 21,650 new cases and 15,520 deaths in 2008 (467). The 5-yr survival rate for women diagnosed with cancer localized to the ovary exceeds 90%; however, nearly 70% of cases are diagnosed after the cancer has metastasized to distant sites, and the 5-yr survival rate for these patients is only 30%. These statistics reflect the lack of an effective screening test for early stage diagnosis and highlight difficulties in the successful treatment of advanced-stage disease. In the following sections, we discuss the three major classes of ovarian cancer: epithelial, sex cord-stromal, and germ cell.

A. Epithelial ovarian cancer

1. Origins and genetics

Approximately 90% of ovarian cancers are epithelial (carcinomas) and are classified by their histological features as serous (50% of ovarian cancers), endometrioid (20%), mucinous (10%), clear cell (5%), transitional, mixed, and undifferentiated (468–470). These cancers are thought to arise from the ovarian surface epithelium (OSE), the single layer of squamous-to-cuboidal epithelial cells that comprises the ovarian surface (reviewed in Ref. 471). Unlike most adult epithelia, the OSE has both epithelial and mesenchymal characteristics that are advantageous for participating in ovarian rupture and repair through repeated ovulatory cycles. These functions may also increase the susceptibility of OSE to genetic mutation and malignant transformation (472–474). Putative precursor lesions are hypothesized to originate in OSE-lined clefts and inclusion cysts in the ovarian cortex, two irregular morphological structures that become more prevalent with age (470, 471, 475). In the progression to malignancy, the OSE differentiates and resembles the lining of different Müllerian duct-derived regions of the female reproductive tract (471). For example, serous, endometrioid, and mucinous ovarian carcinomas are histologically similar to normal epithelia of the fallopian tube, endometrium, and endocervix, respectively. One decade ago, this observation raised an alternative hypothesis that epithelial ovarian cancers do not develop from metaplastic changes in the OSE but instead arise directly from the fallopian tube and secondary Müllerian system, which is composed of microscopic paraovarian and paratubal structures lined by Müllerian epithelium, namely endosalpingiosis, endometriosis, and endocervicosis (476, 477). Although this hypothesis has not gained widespread approval, recent studies suggest that some tumors diagnosed as serous "ovarian" carcinoma more likely originated in the distal

fallopian tube and then implanted on the ovarian surface, particularly in women with *BRCA1* (breast cancer 1) or *BRCA2* (breast cancer 2) mutations (478–480). Additionally, the association between endometriosis and ovarian carcinomas of the endometrioid and clear cell subtypes is well documented (reviewed in Refs. 481–483).

The different histological subtypes and grades of epithelial ovarian cancer are genetically distinct disease entities with unique defects in specific signaling pathways (reviewed in Refs. 470 and 484). Approximately 50–80% of high-grade serous carcinomas demonstrate mutations in the tumor suppressor *p53* (470, 484). On the other hand, low-grade serous carcinomas and serous borderline tumors frequently have mutations in *KRAS*, *BRAF*, or *ERBB2* that constitutively activate MAPK signaling and lead to abnormal regulation of downstream target genes, including overexpression of cyclin D1 (470, 484, 485). The rarity of *KRAS*, *BRAF*, or *ERBB2* mutations in high-grade serous carcinomas has challenged the dogma that these tumors naturally progress from low-grade serous carcinomas. A growing body of molecular evidence now supports the hypothesis that benign serous cystadenomas transform into serous borderline tumors that are the likely precursors of low-grade serous carcinomas, but that high-grade serous carcinomas develop independently and more rapidly from unknown precursors that undergo distinct genetic alterations. Similar to high-grade serous carcinomas, the majority of high-grade endometrioid ovarian cancers have *p53* mutations and arise from unidentified precursor lesions (470, 484). In contrast, low-grade endometrioid carcinomas may come from endometrioid borderline tumors with endometriosis as the likely precursor. Low-grade endometrioid carcinomas are characterized by aberrant activation of the canonical WNT signaling pathway, specifically through activating mutations in β-catenin and less commonly through inactivating mutations in negative regulators of β-catenin such as *APC*, *AXIN1*, and *AXIN2* (470, 484). Deregulation of the PI3K signaling pathway has also been described secondary to activating mutations in *PIK3CA* and inactivating mutations in the tumor suppressor *PTEN* (470, 484). Likewise, *PIK3CA* mutations have been reported in 20–25% of clear cell ovarian carcinomas, with multiple studies showing a strong association with endometriosis (470, 484). Finally, over 75% of mucinous ovarian cancers demonstrate mutations in *KRAS* (470, 484).

Five years ago, Shih and Kurman proposed a new classification scheme for ovarian carcinomas based on their hypothesized patterns of tumor progression (484, 486–488). In this model, tumors are designated as type I or type II. Type I tumors are generally slow growing and localized to the ovary at diagnosis, whereas type II tumors are more aggressive and present as advanced-stage metastatic disease. Type I tumors are composed of genetically distinct low-grade serous and mucinous cancers that develop in an adenoma-borderline-carcinoma sequence, as well as low-grade endometrioid and clear cell cancers that progress from endometriosis. Type II tumors are high-grade serous, endometrioid, and undifferentiated carcinomas that do not have recognizable precursor lesions but commonly harbor *p53* mutations and high levels of chromosomal instability.

2. Modeling epithelial ovarian cancer

Understanding the fundamental mechanisms governing the pathogenesis of epithelial ovarian cancers depends on the ability to develop robust models *in vitro* and *in vivo*. Over the past 30 yr, important advances have been made using rodent and human OSE cultures, human ovarian cancer cell lines, xenograft models, and genetically engineered mouse models (summarized in Table 11). Auersperg and colleagues (471) were pioneers in the isolation, culture, and genetic manipulation of rodent and human OSE, including OSE from women with family histories of ovarian cancer. In 1981, they introduced Kirsten murine sarcoma virus into cultured rat OSE and observed that transformed cells formed endometrioid tumors within 1 wk of sc or ip injection into immunosuppressed female rats (489). These early experiments established a framework for further studying the potential of individual oncogenes to immortalize and transform OSE and promote tumorigenesis. Of note, there are important structural and physiological differences between rodent and human OSE that should be considered when extrapolating results between species. Repeated passaging of rodent OSE cultures results in spontaneous immortalization and malignant transformation based on loss of contact inhibition, substrate-independent growth, and tumor formation in nude mice (473, 474, 490). In contrast, human OSE cultures are more prone to undergo senescence (471), consistent with the observation that human cells generally require more mutations to bypass senescence and growth arrest compared with murine cells (491). This interspecies variation suggests that experiments with cultured human OSE might be more relevant than with cultured rodent OSE. Immortalized ovarian surface epithelial (IOSE) cell lines have been derived from human OSE by transfection with simian virus 40 (SV40) early genes alone or with the catalytic subunit of telomerase (hTERT) (492–496). Subsequent transfection of SV40-immortalized cells with E-cadherin or transfection of SV40/hTERT-immortalized cells with *ERBB2*, activated *HRAS*, or activated *KRAS* causes transformation and tumor development in immunocompromised mice (496–498). Immortalization and malignant transformation of human OSE cells has also been achieved

TABLE 11. Mouse models of epithelial ovarian cancer

Model	Phenotype	Ref.
$p53^{-/-}$ ovarian explants transduced with *Myc*, activated *Kras*, or activated *Akt*, followed by injection of cells into nude mice sc, ip, or intrabursally	Infection with at least two oncogenes causes poorly differentiated carcinomas 8 wk after injection; infection with one oncogene has longer latency of 12 wk to 6 months; intrabursal injection leads to ip and retroperitoneal metastases	503
$p53^{flox/flox}$ $Brca1^{flox/flox}$ ovarian explants transduced with Cre and Myc, followed by ip injection into immunodeficient or immunocompetent mice	Serous carcinomas with hemorrhagic ascites and peritoneal metastases	524
Amhr2-TAg (transgenic; mouse *Amhr2* promoter driving expression of SV40 T antigen)	Bilateral, poorly differentiated ovarian carcinomas with serous components and ip metastases; tumors present in newborn ovaries	504, 509
AdCre-infected $p53^{flox/flox}$ $Rb1^{flox/flox}$ (double cKO)	Serous, poorly differentiated, and undifferentiated metastatic ovarian carcinomas; median survival of 227 d after AdCre injection	510
LSL-$Kras^{G12D/+}$ $Pten^{flox/flox}$ $Amhr2^{cre/+}$ (activation of oncogenic *Kras* allele and conditional deletion of *Pten*)	Serous ovarian carcinomas	513
AdCre-infected *LSL-*$Kras^{G12D/+}$ $Pten^{flox/flox}$ (activation of oncogenic *Kras* allele and conditional deletion of *Pten*)	Endometrioid ovarian carcinomas with peritoneal dissemination	511, 512
AdCre-infected $Apc^{flox/flox}$ $Pten^{flox/flox}$ (double cKO)	Endometrioid ovarian carcinomas with peritoneal dissemination	515

AdCre, ovarian intrabursal injection of recombinant adenovirus expressing Cre recombinase; LSL, LoxP-Stop-LoxP.

by transfection with human papilloma virus type 16 E6/E7 genes (499, 500).

Although OSE cultures are useful for modeling early steps in cancer formation, cell lines established from naturally occurring human ovarian cancers are valuable for exploring advanced-stage disease. Numerous cell lines have been studied *in vitro* and injected sc, ip, and intrabursally (orthotopically) into rodents to compare tumor behavior between various histological subtypes and to analyze the efficacy of novel therapeutics. Advanced noninvasive imaging technologies facilitate *in vivo* visualization of tumor progression and metastasis (501). One caveat to these xenograft models is that the tumors formed *in vivo* may be histologically different from the original cancer that gave rise to the cell line. For example, ES-2 cells were reportedly derived from ovarian clear cell carcinoma but form undifferentiated carcinomas in xenografted mice (502). These incongruities may be secondary to subtle heterogeneity in the parental cancer, variations in tumor microenvironment, or genetic aberrations that accumulate in culture, thus raising the general question of whether culture-based cancer models accurately recapitulate tumorigenesis *in vivo*. Furthermore, xenograft models using either human ovarian cancer cell lines or human OSE cultures require immunodeficient mice and neglect potential roles for the immune system in tumor progression.

Orsulic *et al.* (503) established one of the earliest mouse models for ovarian cancer. They used transgenic mice expressing avian retroviral receptor under control of the keratin 5 promoter, which drives expression in epithelial cells including OSE. Ovarian explants from these mice were cultured transiently and infected with retroviral vectors carrying the oncogenes *Myc*, activated *Kras*, or activated *Akt*. Mutations in all of these genes have been reported in human ovarian cancers. Transduced ovarian cell aggregates were injected sc into nude mice. Eight weeks after injection, recipient mice had poorly differentiated carcinomas when ovarian cells were infected with at least two oncogenes and only on a $p53^{-/-}$ background. Infection with one oncogene on a $p53^{-/-}$ background was also sufficient for tumor formation, but with a longer latency of 12 wk to 6 months, perhaps allowing time for additional genetic mutations to occur. Moreover, when infected cells were implanted under the ovarian bursa of nude mice, widespread ip and retroperitoneal metastases were observed. These features make it a convenient model for testing therapies that may prevent dissemination; however, this system still depends on manipulation of cells in culture and does not model the natural progression of disease from intact ovaries. To overcome these potential confounding factors, stronger genetic approaches were required.

Connolly *et al.* (504) created transgenic mice expressing SV40 T antigen (TAg) under control of the promoter for *Amhr2*. *Amhr2* is expressed in the developing Müllerian ducts, OSE, ovarian granulosa cells, oviducts, and uterus (260, 504–507). In addition, *Amhr2* expression has been reported in human ovarian cancer cell lines and ascites cells isolated from ovarian cancer patients (508). Fifty percent of *Amhr2-TAg* transgenic females developed

bilateral ovarian carcinomas with ip metastases by 6–13 wk of age. The tumors were mostly poorly differentiated, with some regions resembling human serous ovarian cancers. They were also positive for epithelial markers cytokeratins 8 and 19 but negative for the granulosa cell marker inhibin α, suggesting that they were derived from OSE cells and not granulosa cells. On closer examination, ovarian tumors were already present in newborn females, consistent with the embryonic expression of *Amhr2* (509). This makes it difficult to study the earliest steps of cancer initiation using the *Amhr2-TAg* transgenic model and highlights the need for inducible systems in which disease onset can be controlled.

In rodents, encapsulation of the ovary by the bursal membrane creates a protected cavity for the selective delivery of inducing agents to the OSE. Intrabursal injection of recombinant adenovirus-expressing Cre recombinase (AdCre) drives Cre-mediated recombination specifically in the OSE and not in other stromal cells or oocytes. This approach has been effective for conditional deletion of floxed tumor suppressor genes and conditional activation of oncogenes preceded by a floxed stop sequence. Using this technology, Flesken-Nikitin *et al.* (510) deleted the tumor suppressors *p53* and *Rb1* in the OSE. *p53* mutations are common in high-grade human ovarian carcinomas, and involvement of *RB1* has also been described. Targeted inactivation of both genes in murine OSE resulted in serous ovarian cancers as well as poorly differentiated and undifferentiated tumors with metastases. Double knockout mice had a median survival of 227 d after AdCre injection. Deletion of *Rb1* alone did not cause ovarian tumors, whereas deletion of *p53* alone rarely caused ovarian cancer, consistent with the fact that these tumor suppressors regulate interconnected signaling pathways.

The mouse models described above exhibit ovarian carcinomas that are either poorly differentiated or with serous histology, the most common subtype in women. Despite these groundbreaking advances, decoding the morphological and genetic diversity of epithelial ovarian cancers requires models that faithfully represent each major histological subtype. Dinulescu *et al.* (511, 512) used the intrabursal AdCre-loxP system to simultaneously activate an oncogenic *Kras* allele and delete the *Pten* tumor suppressor in murine OSE. Double mutant females developed invasive endometrioid ovarian carcinomas with peritoneal dissemination as early as 7 wk after AdCre injection. The tumors showed activation of the MAPK and AKT pathways, so this model may be instrumental in testing targeted therapies that specifically inhibit these signaling cascades. Interestingly, activation of *Kras* alone was insufficient to provoke tumor formation but instead resulted in peritoneal endometriosis and benign endometriosis-like lesions on the ovary. Deletion of *Pten* alone produced similar benign epithelial ovarian lesions but did not cause endometriosis, although one of 13 *Pten* cKO females demonstrated endometrioid ovarian cancer at 26 wk, raising the possibility that the endometriosis-like lesions on the ovary were potential precursors to ovarian cancer. More recently, Richards and colleagues (513) generated similar *Kras* and *Pten* mutations in OSE cells using mice expressing *Amhr2-Cre* rather than the intrabursal administration of AdCre used by Dinulescu *et al.* (511). Curiously, these mutants developed serous, rather than endometrioid, OSE adenocarcinomas with 100% penetrance. Reasons for the phenotypic differences in these two models expressing the same mutant genes remain to be determined but could be dependent on the stage and/or OSE cell context-specific events initiated by AdCre compared to *Amhr2-Cre*. In women, loss of heterozygosity at the 10q23.3 *PTEN* locus and somatic mutations in *PTEN* have been observed in endometriosis and ovarian carcinomas of the endometrioid and clear cell subtypes (514). In contrast, *KRAS* mutations have not been reported in endometriosis and are rare in endometrioid ovarian cancer, with no evidence to support coexistence or cooperativity with *PTEN* inactivation.

Cho and colleagues (515) examined a large number of primary human endometrioid ovarian cancers and found a significant frequency of coexisting mutations in WNT/β-catenin and PI3K/PTEN signaling pathways. This occurred almost exclusively in tumors that were low-grade and lacking *p53* mutations. To investigate whether these pathways cooperate to drive the malignant transformation of OSE, they used the intrabursal AdCre-loxP system to conditionally inactivate the tumor suppressors *Apc* and *Pten*, which are negative regulators of β-catenin and PI3K, respectively. Whereas no abnormalities were reported for either single mutant, concomitant deregulation of both pathways induced endometrioid ovarian carcinomas with 100% penetrance within 6 wk of AdCre injection. The tumors were accompanied by hemorrhagic ascites, occasional peritoneal dissemination to the liver, and areas of spindle cell morphology that were negative for cytokeratin and E-cadherin immunoreactivity, suggesting epithelial-to-mesenchymal transition. Despite the strong association between endometrioid ovarian cancer and endometriosis in women, there was no evidence of endometriosis-like lesions in the *Apc Pten* cKO. However, alterations in gene expression between the mouse tumors and control ovaries were highly correlated with aberrations in human endometrioid ovarian cancers, in particular to those harboring mutations in WNT/β-catenin and PI3K/PTEN signaling. Hence, this mouse model may be extremely valuable for

identifying therapies specifically tailored to endometrioid ovarian cancer. Furthermore, the phenotype reveals that genetic interactions between canonical WNT signaling and the PI3K/PTEN pathway promote ovarian tumorigenesis. Molecular intersections between these pathways have been described. For example, AKT directly phosphorylates β-catenin, consequently increasing β-catenin transcriptional activity and promoting tumor cell invasion *in vitro* (516). Nevertheless, it remains unknown which mechanisms of cross talk are relevant to ovarian cancer.

Approximately 10% of epithelial ovarian cancers are hereditary with evidence of autosomal dominant genetic susceptibility (reviewed in Ref. 517). Loss of function germline mutations in the tumor suppressor *BRCA1* account for 90% of these cases and are highly penetrant, conferring a 40–60% lifetime risk of developing ovarian cancer, compared with 1–2% in the general population (517, 518). The tumors are typically serous ovarian carcinomas. BRCA1 inactivation also occurs in the majority of sporadic ovarian tumors but usually through mechanisms other than somatic mutation, for instance, epigenetic silencing due to promoter hypermethylation (reviewed in Ref. 519). BRCA1 has critical functions in the DNA damage response and the maintenance of genomic integrity; therefore, defects in BRCA1 have widespread cellular consequences that presumably enhance vulnerability to genomic instability and oncogenic transformation. Indeed, familial ovarian cancers develop at a younger age compared with sporadic ovarian cancers (517). Ovaries removed prophylactically from *BRCA1* carriers and women with a family history of ovarian cancer displayed a higher frequency of morphological alterations in the OSE that may reflect preneoplastic lesions (520–522). Similar trends were observed in murine OSE after targeted deletion of *Brca1* using intrabursal AdCre administration (523). Despite these findings, *Brca1* cKO females did not develop ovarian tumors even 1 yr after injection. BRCA1 inactivation in primary cultures of murine OSE suppressed proliferation, increased apoptosis, and heightened sensitivity to cisplatin (523, 524). On the other hand, concomitant deletion of BRCA1 and p53 enhanced proliferation, whereas inactivation of p53 alone had no effect, suggesting that p53 dysfunction or other oncogenic events are essential mediators of BRCA1-associated tumorigenesis (523). Accordingly, *p53* mutations are more common in hereditary than sporadic ovarian cancers and may be an early initiating event in *BRCA1* carriers (525, 526). Although other players in BRCA1-mediated ovarian cancer remain to be identified, retroviral infection with the *Myc* oncogene (but not *Kras*, *Erbb2*, or *Akt*) was sufficient for transforming OSE cells with combined deficiency of *Brca1* and *p53* (524). BRCA1 binds MYC and represses its transcriptional and transforming activity (229). *MYC* is amplified in BRCA1-associated breast cancer, but its role in BRCA1-associated ovarian cancer is unclear (527, 528).

B. Sex cord-stromal ovarian cancer

Tumors emanating from the gonadal sex cords and stroma comprise about 7% of ovarian cancers and include granulosa-theca cell tumors, thecoma-fibromas, Sertoli-Leydig cell tumors, sex cord tumors with annular tubules, and gynandroblastomas (468, 529). Hormone secretion is a salient feature of these cancers, which often present as a large, unilateral adnexal mass confined to the ovary with signs and symptoms of estrogen or androgen excess. The mainstay of treatment is surgery to remove the tumor, followed by adjuvant chemotherapy in selected patients. Granulosa cell tumors account for approximately 70% of sex cord-stromal ovarian cancers and are classified as adult (95%) or juvenile (5%) (529). The adult variant is more common in postmenopausal women and associated with abnormal vaginal bleeding, endometrial hyperplasia, and endometrial carcinoma, whereas the juvenile type frequently entails gonadotropin-independent isosexual precocious puberty in prepubertal girls (*i.e.*, secondary to estrogen secretion by the tumor). In contrast to epithelial ovarian cancers, the majority of which are diagnosed at advanced stages with poor prognoses, about 80% of adult granulosa cell tumors are diagnosed at stage I and have 5- and 10-yr survival rates exceeding 90% (529). Nevertheless, adult granulosa cell tumors are slow-growing and tend toward late recurrence; although the median time to relapse is around 5 yr after initial diagnosis, recurrences have been reported after 40 yr, and recurrent disease bears a somber prognosis (529, 530).

Sex cord-stromal ovarian cancers are rare, and this is most likely explained by the highly orchestrated removal of apoptotic granulosa cells in follicles undergoing atresia that occurs in most (99%) of all growing follicles. The rarity of these cancers has hindered comprehensive study of the genetic aberrations responsible for tumor formation and progression in women. On the other hand, granulosa cell tumors are the most common spontaneous ovarian tumors in mice (531). Beamer and colleagues have extensively investigated the pathogenesis of spontaneous granulosa cell tumors in SWR and SWXJ mouse strains. Tumors in these mice are grossly visible by 6 wk of age and histologically resemble human juvenile granulosa cell tumors (531, 532). The window of tumor initiation is between 3 and 5 wk of age; beyond this timeframe, unaffected females are no longer at risk. Incomplete penetrance of the phenotype during a restricted period of ovarian maturation indicates a complex interplay between multiple genetic and environmental factors. Detailed linkage studies in these spontaneous mouse models have identified

TABLE 12. Mouse models of sex cord-stromal ovarian cancer

Model	Phenotype	Ref.
SWR/SWXJ strains	Spontaneous granulosa cell tumors by 6 wk of age; incomplete penetrance	531
Inha$^{-/-}$ (inhibin α knockout)[a]	Bilateral, mixed sex cord-stromal ovarian tumors with 100% penetrance as early as 4 wk of age; cancer cachexia syndrome; elevated FSH, estradiol, and activins A and B	542–544
Fshr$^{-/-}$ (FSH receptor knockout)	Unilateral Sertoli-Leydig cell tumors by 1 yr of age; cachexia; elevated FSH and LH; low estradiol	817
bLHβ-CTP (transgenic; chronic hypersecretion of LH)	Strain-dependent formation of granulosa cell tumors with 100% penetrance by 5 months of age; polycystic ovaries	550, 551
Inhα-TAg (transgenic; mouse inhibin α promoter driving expression of SV40 T antigen)	Granulosa cell tumors with 100% penetrance by 6 months of age	552
bLHβ-CTP/Inhα-TAg (double transgenic)	Earlier formation, more rapid progression, and enhanced aggressiveness of granulosa cell tumors compared to either single transgenic model; occasional metastases to lungs and liver	553
MT-hCG (transgenic; mouse metallothionein 1 promoter fused to coding sequences of human chorionic gonadotropin α- and β-subunits)	Hemorrhagic and cystic ovaries; thecomas and stromal cell expansion; enlarged uterine horns; urinary tract abnormalities; elevated estradiol	554
Smad1 Smad5 (double cKO using *Amhr2-Cre*)	Unilateral or bilateral poorly differentiated granulosa cell tumors with 100% penetrance by 3 months of age; metastases to lymph nodes and peritoneum	576
Smad1 Smad5 Smad8 (triple cKO using *Amhr2-Cre*)	Similar to *Smad1 Smad5* double cKO	576
Ctnnb1$^{flox(ex3)/+}$ *Amhr2*$^{cre/+}$ (conditional expression of dominant stable form of β-catenin)	Unilateral or bilateral, hemorrhagic, cystic granulosa cell tumors; detected in mice older than 5 months and prevalence increased with age; precursor lesions; osseous metaplasia	583, 584
Pten$^{flox/flox}$ *Amhr2*$^{cre/+}$ (cKO)	Unilateral or bilateral granulosa cell tumors with low penetrance; ossification and cysts; metastases to lungs	585
Pten$^{flox/flox}$ *Ctnnb1*$^{flox(ex3)/+}$ *Amhr2*$^{cre/+}$ (double conditional mutant)	Bilateral granulosa cell tumors with 100% penetrance; dysplastic cells in newborn ovaries; death prior to 9 wk of age	585
Men1$^{+/-}$ (multiple endocrine neoplasia 1 heterozygote)	Granulosa cell tumors; multiple endocrine tumors; tumors show loss of heterozygosity at *Men1* locus	818–820
OSP1-TAg (transgenic; rat ovarian-specific promoter 1 driving expression of SV40 T antigen)	Unilateral granulosa cell tumors; tumors in multiple other tissues	821

[a] Mouse models related to the inhibin α knockout are described in Table 13.

nine granulosa cell tumor (*Gct*) susceptibility loci (532–536). The *Gct1* locus on distal mouse chromosome 4 surfaced in three independent mapping crosses using divergent mouse strains (532). Loss of this genomic region is a secondary aberration in transgenic mouse models of oligodendroglioma and mammary carcinoma (532, 537, 538). The orthologous region on human chromosome 1p is also deleted in various cancers, strongly suggesting the presence of as yet unidentified tumor suppressor genes (532, 539–541). Additionally, *Gct4* is an X-linked tumor susceptibility locus that modulates responsiveness to androgenic steroids and shows a strong parent-of-origin effect associated with paternal inheritance, raising the possibility that epigenetic effects such as imprinting might also influence tumor formation (534–536).

Further insight into the pathways involved in ovarian sex cord-stromal tumorigenesis stems from a variety of transgenic mouse models (summarized in Table 12). TGFβ superfamily members are critical regulators of ovarian folliculogenesis and granulosa cell function, and include inhibins, activins, BMPs, growth and differentiation factors, and anti-Müllerian hormone. Granulosa cells and Sertoli cells secrete inhibins (α:βA, α:βB) and activins (βA:βA, βB:βB, βA:βB) that suppress (*i.e.*, inhibins) or stimulate (*i.e.*, activins) pituitary production and secretion of FSH. Mice null for the inhibin α-subunit gene (*Inha*$^{-/-}$), and therefore completely inhibin-deficient, develop bilateral, multifocal, mixed, invasive sex cord-stromal tumors with 100% penetrance in both sexes as early as 4 wk of age (542, 543). The mice eventually die secondary to a cancer cachexia syndrome marked by severe weight loss and multiple extragonadal defects (544). Hence, inhibins are secreted tumor suppressors with gonadal specificity. For more than 15 yr, inhibin-deficient mice have been a valuable model system to investigate modifiers of gonadal tumorigenesis, including gonadotropins, sex steroid hormones and receptors, cell cycle regulators, and activin signaling (summarized in Table 13).

FSH and LH are pituitary gonadotropins composed of a common α-subunit and a hormone-specific β-subunit, the

TABLE 13. Mouse models related to the inhibin α knockout

Model	Phenotype	Endocrine abnormalities[a]	Survival relative to *Inha*$^{-/-}$	Ref.
Inha$^{-/-}$ (inhibin α knockout)	Bilateral, mixed sex cord-stromal ovarian tumors with 100% penetrance as early as 4 wk of age; cancer cachexia syndrome	Elevated FSH, estradiol, and activins A and B	n/a	542–544
Gonadotropins				
Inha$^{-/-}$ *Gnrh1*$^{hpg/hpg}$ (gonadotropin releasing hormone)	No tumors, only premalignant ovarian lesions (*e.g.*, seminiferous tubule-like structures without germ cells)	Suppressed FSH and LH	↑ ↑ ↑	545
Inha$^{-/-}$ *Fshb*$^{-/-}$ (FSH β subunit)	Slower growing and less invasive tumors; delayed onset of cancer cachexia syndrome	FSH absent; decreased estradiol and activin A compared to *Inha*$^{-/-}$	↑ ↑	549
Inha$^{-/-}$ *Lhb*$^{-/-}$ (LH β subunit)	Delayed tumor progression	Lower FSH compared to *Inha*$^{-/-}$ at 6 wk of age	↑	555
Sex steroid hormones and receptors				
Inha$^{-/-}$ females treated with flutamide, an androgen antagonist	Delayed tumor development and less hemorrhagic tumors	Decreased FSH compared to untreated *Inha*$^{-/-}$	↑	558
Inha$^{-/-}$ *Esr1*$^{-/-}$ (estrogen receptor α)	More rapid tumor development and earlier onset of cancer cachexia syndrome	Elevated estradiol; suppressed FSH and LH compared to *Inha*$^{-/-}$	↓ ↓	558
Inha$^{-/-}$ *Esr2*$^{-/-}$ (estrogen receptor β)	Similar to *Inha*$^{-/-}$	Estradiol and gonadotropin levels similar to *Inha*$^{-/-}$	↓	558
Inha$^{-/-}$ *Esr1*$^{-/-}$ *Esr2*$^{-/-}$ (estrogen receptors α and β)	Accelerated tumor development and earlier onset of wasting syndrome	Elevated FSH	↓ ↓ ↓	558
Cell cycle regulators				
Inha$^{-/-}$ *Ccnd2*$^{-/-}$ (cyclin D2)	Delayed onset and slower progression of tumors and cancer cachexia syndrome	Elevated FSH	↑ ↑	562
Inha$^{-/-}$ *Cdkn1b*$^{-/-}$ (cyclin-dependent kinase inhibitor 1B, also known as p27^{Kip1})	Accelerated tumor development and earlier onset of wasting syndrome	Elevated FSH and activin A	↓ ↓ ↓	557, 562
Inha$^{-/-}$ *Rb1*$^{-/-}$ (retinoblastoma 1)	Accelerated tumor progression	Higher LH and progesterone compared to *Inha*$^{-/-}$	↓	563
Activin signaling				
Inha$^{-/-}$ *Acvr2a*$^{-/-}$ (activin receptor IIA)	Similar to *Inha*$^{-/-}$ with notable absence of cancer cachexia syndrome	Elevated activins A and B	↑ ↑ ↑	567
Inha$^{-/-}$ *MT-Fst* (mouse metallothionein 1 promoter fused to coding sequence of mouse follistatin)	Less hemorrhagic tumors and less severe wasting syndrome	Elevated FSH compared to *MT-Fst* controls; decreased activin A compared to *Inha*$^{-/-}$	↑	571
Inha$^{-/-}$ treated with ACVR2A-mFc, a chimeric activin antagonist	Slower tumor progression and absence of wasting syndrome	Trend toward lower FSH compared to PBS-treated *Inha*$^{-/-}$ females	↑ ↑ ↑	572
Inha$^{-/-}$ *Smad3*$^{-/-}$ (MAD homolog 3, also known as *Madh3*)	Slower tumor progression and delayed onset of wasting syndrome	Decreased activin A and trend toward lower estradiol compared to *Inha*$^{-/-}$ at 15 wk of age	↑ ↑ ↑	573, 574
Miscellaneous				
Inha$^{-/-}$ *Gdf9*$^{-/-}$ (growth differentiation factor 9)	Similar to *Inha*$^{-/-}$	Not determined	Unchanged	226

n/a, Not applicable.

[a] Serum levels as compared to wild-type mice, unless otherwise specified.

synthesis of which is regulated in part by hypothalamic GnRH. When inhibin α mutants were crossed to mice deficient in GnRH [*i.e.*, the hypogonadal (*Gnrh1*^{hpg}) model], in which FSH and LH levels are suppressed, the resulting double homozygous mutants were strikingly free of tumors and the cancer cachexia syndrome (545). Although the ovaries of *Inha*$^{-/-}$ *Gnrh1*$^{hpg/hpg}$ mice contain seminiferous tubule-like structures similar to lesions in *Inha*$^{-/-}$ females, these structures do not evolve beyond the premalignant stage. In an analogous experiment, Beamer and colleagues (546, 547) showed that an intact hypothalamic-pituitary axis is essential for granulosa cell tumor development in genetically susceptible SWR/SWXJ female mice. These data suggest that gonadotropins are key regulators of ovarian tumorigenesis, consistent with the dramatic rise in the incidence of ovarian cancer around menopause, when gonadotropin levels are elevated (reviewed in Ref. 548).

Further experiments were necessary to delineate the individual contributions of FSH and LH. Inhibin-deficient mice have increased serum FSH levels compared with wild-type mice, consistent with the known function of inhibins to suppress FSH (542). As mentioned earlier, binding of FSH to its receptor on granulosa cells activates cAMP-dependent PKA signaling and other pathways, ultimately promoting the expression of genes that orchestrate granulosa cell proliferation and differentiation. Female mice deficient in inhibins and FSH (*Inha*$^{-/-}$ *Fshb*$^{-/-}$) have increased survival because of slower growing, less invasive tumors along with delayed onset of the wasting syndrome (549). In a complementary gain of function experiment, human FSH α- and β-subunits were simultaneously overexpressed from the mouse MT1 promoter on a wild-type background (549). Transgenic female mice with elevated human FSH do not develop cancer but instead have hemorrhagic and cystic ovaries in addition to urinary tract dysfunction, features reminiscent of human ovarian hyperstimulation and polycystic ovarian syndromes. Together, these models demonstrate that FSH signaling promotes sex cord-stromal tumor progression but is not necessary for tumor formation.

Corroborating evidence from several different mouse models emphasizes the contribution of LH to granulosa cell tumorigenesis. Chronic hypersecretion of LH in transgenic female mice (*bLHβ-CTP*) causes polycystic ovaries and strain-dependent formation of granulosa cell tumors with 100% penetrance by 5 months of age (550, 551). In another transgenic model, granulosa cell tumors invariably develop by 6 months of age when the inhibin α promoter drives SV40 TAg expression (*Inhα-TAg*) (552). Notably, double transgenic *bLHβ-CTP/Inhα-TAg* females display earlier formation and more rapid progression of tumors (553). Related to the *bLHβ-CTP* model, the mouse MT1 promoter was used to simultaneously overexpress α- and β-subunits of hCG, a placental glycoprotein hormone and LH analog that acts through the LH receptor (554). Like *MT-hFSH* mice, *MT-hCG* transgenic females develop hemorrhagic and cystic ovaries coincident with urinary tract defects; thecomas and marked stromal cell expansion are also observed in the ovaries.

In light of these observations and to further understand the distinct phenotypes of *Inha*$^{-/-}$ *Gnrh1*$^{hpg/hpg}$ mice and *Inha*$^{-/-}$ *Fshb*$^{-/-}$ mice, we recently characterized mice deficient in both inhibins and LH (555). *Inha*$^{-/-}$ *Lhb*$^{-/-}$ females develop ovarian cancer but have increased survival because of delayed tumor progression, an effect correlated with elevated tumor expression of p15^{INK4b} (*Cdkn2b*) and a trend toward higher levels of p27^{Kip1}, both genes that repress cell proliferation. Loss of expression of CDK inhibitors p15^{INK4b} and p16^{INK4a} (*Cdkn2a*) have been reported in adult granulosa cell tumors in women, and the loss of p27^{Kip1} exacerbates tumorigenesis in inhibin-deficient mice (556, 557). *Inha*$^{-/-}$ *Lhb*$^{-/-}$ ovarian tumors also have decreased expression of *Cyp17a1*, the LH-induced thecal cell enzyme that converts pregnenolone and progesterone to dehydroepiandrosterone and androstenedione, respectively (555). Lower *Cyp17a1* expression might reduce the levels of these androgens, consequently slowing tumor growth in *Inha*$^{-/-}$ *Lhb*$^{-/-}$ females. This explanation is based on the observation that treatment of inhibin-deficient females with the androgen antagonist flutamide prolongs life span by diminishing tumor progression (558). Conversely, exogenously supplied androgens increase the frequency of granulosa cell tumors in genetically susceptible SWR/SWXJ female mice (534, 546, 559). Importantly, treatment of SWR/SWXJ females with hCG, but not FSH, also induces tumors (547). These experiments collectively suggest that LH signaling is sufficient for ovarian sex cord-stromal tumor formation, in part by triggering androgen production.

Consistent with the fact that granulosa cells are the principal site of estradiol synthesis, granulosa cell tumors in women and mice secrete estradiol. To investigate the role of estrogen signaling in sex cord-stromal ovarian cancers, our group generated mice lacking inhibin α, ERα (*Esr1*), and ERβ (*Esr2*) (558). *Inha*$^{-/-}$ *Esr1*$^{-/-}$ females exhibit a substantial decrease in survival attributed to more rapid tumor progression and earlier onset of the cancer cachexia syndrome. Although *Inha*$^{-/-}$ *Esr2*$^{-/-}$ females are similar to *Inha*$^{-/-}$ single mutants, triple knockout *Inha*$^{-/-}$ *Esr1*$^{-/-}$ *Esr2*$^{-/-}$ females demonstrate the most severe phenotype with 100% death by 9 wk of age, a timepoint at which the majority of *Inha*$^{-/-}$ mice are still

alive. These data indicate that in the absence of inhibins, ER signaling is protective in females. Accordingly, estradiol treatment suppresses granulosa cell tumor incidence in genetically susceptible SWR/SWXJ mice (559). Because *ESR1* and *ESR2* are expressed in human granulosa cell cancers, the protective effect of estradiol may occur through direct action on the tumor by unknown mechanisms (560). Estradiol also imparts negative feedback on the hypothalamic-pituitary axis that is predominantly mediated by ERα (561). Therefore, an alternative explanation is that estradiol reduces LH synthesis and release from the pituitary in an ER-intact mouse, which in turn mitigates tumorigenesis as described earlier.

Signaling cascades activated by gonadotropins and sex steroid hormones ultimately converge on a final common pathway: the cell cycle. The G_1/S transition is regulated by D- and E-type cyclins that complex with CDKs (CDK4/6 and CDK2, respectively) to promote cell proliferation, whereas CDK inhibitors such as $p27^{Kip1}$ block cell cycle progression. The absence of CCND2 causes female sterility because of impaired granulosa cell proliferation in response to FSH (292). On the other hand, sterility in $p27^{Kip1}$ null females is secondary to defects in cell cycle withdrawal and luteinization in response to LH (391–393). *Ccnd2* expression is higher in human granulosa cell tumors compared with other ovarian cancers and normal ovaries (292). Moreover, sex cord-stromal cancers in inhibin-deficient females have elevated *Ccnd2* and *Cdk4* mRNA and lower $p27^{Kip1}$ protein compared with wild-type ovaries, leading to the hypothesis that these alterations might foster tumor growth (557). Indeed, the loss of CCND2 prolonged survival, and the loss of $p27^{Kip1}$ decreased survival in inhibin-deficient mice, correlating with changes in the rate of tumor development and onset of the associated wasting syndrome (557, 562). Granulosa cell-specific deletion of *Rb1* (retinoblastoma 1) on the inhibin α null background also accelerates ovarian tumorigenesis (563). When unphosphorylated and active, RB1 binds to E2F transcription factors and prevents the expression of genes required for cell cycle progression (reviewed in Ref. 564). RB1 also blocks cell proliferation by inhibiting the ubiquitination and degradation of $p27^{Kip1}$ (565, 566). Accordingly, ovarian tumors lacking inhibins and RB1 have lower $p27^{Kip1}$ levels and increased expression of mitotic markers such as CCND2, PCNA (proliferating cell nuclear antigen), and CCNB1 (cyclin B1) compared with tumors from $Inha^{-/-}$ females. Despite these aberrations, double mutants showed only a modest rise in mortality, likely because there was also increased apoptosis that may have limited tumor growth and higher expression of related family members *Rbl1* (retinoblastoma-like 1; p107) and *Rbl2* (retinoblastoma-like 2; p130) that might have partially compensated for loss of *Rb1*.

Along with their endocrine actions as regulators of FSH levels, inhibins and activins secreted from granulosa cells function in autocrine and paracrine fashions to balance growth and differentiation within the maturing follicle. TGFβ superfamily ligands signal through transmembrane serine/threonine kinase receptor complexes that phosphorylate and activate intracellular SMAD proteins, which then translocate to the nucleus to control gene expression. Activins bind ACVR2A and ACVR2B (type II receptors) that recruit activin receptor-like kinases (type I receptors), which signal through SMAD2 and SMAD3. Gonadal tumors in inhibin-deficient mice secrete large amounts of activins into the circulation (544). Because inhibins antagonize activins at the receptor level, inhibin-deficient mice are a useful model for studying the consequences of unchecked activin signaling on tumorigenesis. Deletion of *Acvr2a* on the inhibin α null background does not abolish tumor development but instead prolongs life span by preventing the cancer cachexia syndrome (567). This wasting syndrome is characterized by severe weight loss, lethargy, hepatocellular inflammation and necrosis, and atrophy of the glandular stomach (544). *Acvr2a* is expressed in mouse liver and glandular stomach, and infusion of recombinant activin A in rodents induces apoptosis of hepatocytes around the central vein, a pattern similar to the liver pathology in cachectic $Inha^{-/-}$ mice (567–569). These data indicate that increased activin signaling specifically through ACVR2A in hepatocytes and the glandular stomach causes the wasting syndrome in inhibin-deficient mice.

Although absence of ACVR2A does not affect tumor growth, activin signaling through ACVR2B or other unidentified receptors may promote tumor progression. Activin A stimulates the growth of gonadal tumor cell lines derived from inhibin α- and p53-deficient mice, whereas FST inhibits tumor cell proliferation (570). FST binds to activin β-subunits and interferes with the binding of activins to ACVR2A and ACVR2B. Transgenic overexpression of FST in inhibin-deficient mice expectedly decreases the severity of the wasting syndrome but also produces less hemorrhagic ovarian tumors (571). Similar outcomes were observed when $Inha^{-/-}$ mice were injected with a chimeric activin antagonist consisting of the ACVR2A extracellular domain fused to the Fc region of murine IgG2a (ACVR2A-mFc) (572). Treatment with ACVR2A-mFc completely prevented the wasting syndrome and delayed tumor progression.

The models described thus far modulate activin signaling at the ligand or receptor level. To investigate downstream components of the pathway, two independent

groups generated *Inha*$^{-/-}$ *Smad3*$^{-/-}$ double knockout mice that have increased survival because of diminished ovarian tumor progression (573, 574). SMAD3 regulates *Ccnd2* expression through transcriptional activation at the *Ccnd2* promoter and through potentiation of FSH signaling (294, 574, 575). Double mutant ovarian tumors have lower CCND2 and partial insensitivity to FSH signaling, specifically through PI3K/AKT, leading to a phenotype reminiscent of both *Inha*$^{-/-}$ *Ccnd2*$^{-/-}$ mutants and *Inha*$^{-/-}$ *Fshb*$^{-/-}$ mutants (574). Notably, loss of SMAD3 attenuates tumor development to a greater degree in inhibin-deficient males than females, supporting functional redundancy with SMAD2 in ovarian granulosa cells (354, 573).

Recent work has shown that the oncogenic effects of activin signaling through SMAD2 and SMAD3 are balanced by the tumor suppressive roles of BMP signaling through SMAD1, SMAD5, and SMAD8. Conditional deletion of *Smad1/5* or *Smad1/5/8* in ovarian granulosa cells causes progressive infertility and granulosa cell tumors that metastasize to the lymph nodes and peritoneum (576). The tumors and metastases express inhibin α, and therefore activin signaling is either normal or suppressed because the mutants do not develop elevated activin levels or cachexia. However, the nuclear localization of phospho-SMAD2/3 immunoreactivity indicates activation of SMAD2/3 by the physiologically normal levels of activins or by another ligand such as TGFβ. Multiple TGFβ-induced genes (*e.g.*, *Hmga2*, *Mmp2*, *Tgfbi*) are up-regulated in *Smad1/5* knockout tumors compared with wild-type granulosa cells, and overexpression of TGFβ promotes tumor invasion and metastasis in a variety of human cancers. Overall, this model emphasizes that growth and differentiation of granulosa cells are determined by complex interactions between BMP and TGFβ/activin pathways.

Similar to granulosa cell tumors in women, the ovarian cancers in *Smad1/5* cKO mice and in genetically susceptible SWR/SWXJ mice are inhibin-positive (576, 577). Throughout the literature, this has raised questions about the relevance of the inhibin-deficient mouse model to human ovarian cancers. If inhibins are markers for human granulosa cell tumors, how can they be tumor suppressors? The recently described phenotypes of ACVR2A-mFc-treated inhibin-deficient mice, *Inha*$^{-/-}$ *Smad3*$^{-/-}$ mutants, and in particular the *Smad1/5* cKO have shed light on this apparent paradox. Activation of SMAD2/3 promotes tumorigenesis upon stimulation by TGFβ or activin signaling; hence, inhibins are clearly tumor suppressors in part because they function as activin antagonists. The strong evidence implicating TGFβ superfamily signaling in granulosa cell tumorigenesis and the fact that granulosa cell tumors in women are inhibin-positive (578) suggest that these tumors have mutations that circumnavigate the inhibins, effectively making them inhibin-resistant and ultimately leading to SMAD2/3 activation, SMAD1/5 inactivation, or aberrations in the expression or function of their respective target genes. Validation of this hypothesis awaits investigation of TGFβ superfamily signaling in human granulosa cell cancers. Furthermore, although inhibin α mutations may not be a common initiating event in human granulosa cell tumors, the TGFβ model predicts that the loss of inhibins would exacerbate tumorigenesis by unleashing activin (and FSH) signaling. Indeed, Ala-Fossi *et al.* (579) reported that in contrast to early-stage granulosa cell tumors in women, advanced-stage tumors were negative for inhibin α immunostaining and correlated with shorter survival.

Aside from TGFβ superfamily signaling, WNT pathway components are also expressed in ovarian granulosa cells (580–582), and alterations in WNT signaling have been implicated in granulosa cell tumorigenesis. Richards and colleagues (583) observed nuclear localization of β-catenin in human and equine granulosa cell tumors, indicating aberrant activation of the canonical WNT pathway. To model this scenario, they generated *Ctnnb1*$^{flox(ex3)/+}$ *Amhr2*$^{cre/+}$ mice expressing a dominant stable form of β-catenin in granulosa cells. Mutant females developed unilateral or bilateral tumors composed of sheets of granulosa cells and hemorrhagic cysts lined with granulosa cells. Tumors were only detected in mice older than 5 months, and the prevalence increased with age because eight of 14 females were affected by 7.5 months and two others developed tumors after 1 yr. The tumors apparently evolve from precursor lesions that are present at 6 wk of age and characterized by vascular follicle-like nests of disorganized, pleiomorphic granulosa cells in addition to cavitary and cystic structures. Gene expression analyses revealed that some of these pretumoral lesions progress through an intermediate stage resembling osseous metaplasia, consistent with known roles for WNT signaling in cell fate determination and differentiation (583, 584). These unique observations suggest that metaplastic transformation might be an important premalignant step for human granulosa cell tumors, although this has not yet been described.

Regions of ossification and cystic structures were also reported in granulosa cell tumors of *Pten*$^{flox/flox}$ *Amhr2*$^{cre/+}$ cKO females (585). Only 7% of these mutants developed tumors between 7 wk and 7 months, but the tumors were particularly aggressive and metastasized to the lungs. In contrast, the *Pten*$^{flox/flox}$ *Cyp19a1-Cre* mice do not develop tumors but do exhibit enhanced ovulation rates related to reduced follicular apoptosis (406). Thus, there appears to be a stage-dependent effect of disrupting

Pten in granulosa cells. The tumor suppressor PTEN represses the PI3K/AKT pathway, a major effector of FSH signaling in granulosa cells (586). FSH activation of PI3K/AKT triggers mammalian target of rapamycin (mTOR) signaling and attenuates FOXO1 repression at the *Ccnd2* promoter (294, 586) by rapidly turning off expression of the *Foxo1* gene in granulosa cells (174). Accordingly, tumors resulting from deletion of PTEN in granulosa cells demonstrated loss of FOXO1 expression and elevated mTOR and phospho-mTOR levels compared with Cre-negative granulosa cells (585). Unexpectedly, the tumors also showed loss of phospho-AKT expression, an incongruent finding that may be the end result of negative feedback loops induced by early constitutive activation of the PI3K/AKT pathway. Human and equine granulosa cell tumors do not have alterations in *PTEN* expression but instead show perinuclear and nuclear localization of phospho-AKT, the significance of which remains unclear.

As mentioned earlier, there are several avenues of cross talk between PI3K/AKT and canonical WNT signaling, and these pathways cooperatively induce endometrioid tumors in a mouse model of epithelial ovarian cancer (515). Hence, simultaneous activation of both pathways in granulosa cells might also have synergistic effects on sex cord-stromal tumorigenesis. Boerboom and colleagues (585) tested this hypothesis by generating $Pten^{flox/flox}$ $Ctnnb1^{flox(ex3)/+}$ $Amhr2^{cre/+}$ mice, which developed bilateral granulosa cell tumors with enhanced severity compared with either single conditional mutant. The phenotype was fully penetrant with dysplastic cells present in newborn ovaries and death occurring before 9 wk of age, a rapid course that likely precluded metastasis. Indeed, surgical removal of the tumors from 6-wk-old females prolonged survival and allowed for the emergence of pulmonary metastases, indicating that the tumors were capable of malignant progression. Future dissection of the precise mechanisms regulating tumorigenesis in these models may shed new light on the pathogenesis of human granulosa cell tumors.

Finally, in an exciting and provocative study, Huntsman and colleagues (587) used next generation sequencing technology to comprehensively profile the transcriptomes of four human adult granulosa cell tumors. They identified a single recurrent somatic missense mutation (402C→G, C134W) in the *FOXL2* gene. This variant was confirmed to be present in 86 of 89 independent adult granulosa cell tumors, three of 14 thecomas, and one of 10 juvenile granulosa cell tumors, but absent in multiple sex cord-stromal tumors of other subtypes and numerous unrelated ovarian and breast cancers. As described earlier in *Section III*, FOXL2 is a forkhead transcription factor that is expressed in granulosa cells and essential for their differentiation from squamous to cuboidal morphology during the primordial to primary follicle transition. Previously characterized germline mutations in *FOXL2* cause type I BPES with POF (186), a phenotype recapitulated in *Foxl2* null female mice, which are sterile (187, 188). Although the somatic mutation in the current study occurs on the surface of the forkhead DNA-binding domain, the impact on FOXL2 function remains unclear. Nonetheless, the specificity of the mutation for adult granulosa cell tumors holds promise for future investigation.

C. Germ cell ovarian cancer

Germ cell tumors represent 20–25% of ovarian neoplasms but only 1–2% of ovarian cancers (588). They account for the majority of ovarian tumors in women younger than 20 yr old. Unlike epithelial ovarian cancers, germ cell tumors are frequently diagnosed at stage I disease, perhaps because they grow rapidly and provoke symptoms early, secondary to capsular distension, hemorrhage, or necrosis (589). Also, women with advanced-stage germ cell tumors often respond well to treatment. From 1973 to 2002, the age-adjusted incidence of germ cell ovarian cancer declined, and survival rates improved significantly, with 5-yr relative survival exceeding 80% (589).

Ovarian germ cell tumors are classified as teratomas, dysgerminomas (the female counterpart to the male seminoma), endodermal sinus (yolk sac) tumors, choriocarcinomas, embryonal carcinomas, and mixed (468, 588). Teratomas (from the Greek word "teras," or monster) are the most common type and are further divided into mature (benign), immature (malignant), and monodermal or highly specialized (struma ovarii and carcinoid tumors). Mature cystic teratomas are pathologically intriguing because they contain adult tissues from all three germ layers (ectoderm, mesoderm, endoderm), for example, skin, hair, sebaceous glands, teeth, cartilage, bone, thyroid, and neural tissue. Immature teratomas similarly contain derivatives of all three germ layers, but with less differentiation and closer resemblance to embryonic than adult tissues (468). The majority of malignant ovarian germ cell tumors are immature teratomas, dysgerminomas, or endodermal sinus tumors (589).

Most basic research on germ cell tumorigenesis has centered around mature and immature teratomas. In women and mice, ovarian teratomas arise from parthenogenetic cleavage of oocytes; in other words, oocytes that begin to form embryos in the absence of fertilization by sperm. Cytogenetic analyses have revealed five mechanisms of origin for human ovarian teratomas: meiosis I error; MII error; duplication of the genome of a mature ovum; mitotic division of a premeiotic germ cell; and fusion of two

TABLE 14. Mouse models of germ cell ovarian cancer

Model	Phenotype	Ref.
LT/Sv and LT-related strains (*e.g.*, LTXBO, LTXBJ)	Abnormal metaphase I arrest; spontaneous ovarian teratomas as early as 30 d of age; 50–80% penetrance by 90 d of age	592, 593
Mos$^{-/-}$ (Moloney sarcoma oncogene knockout)	Destabilization of metaphase II arrest; 30% have teratomas between 4 and 8 months of age	453, 454, 603, 604
Inhα-Bcl2 (transgenic; mouse inhibin α promoter driving expression of *Bcl2*)	Mature cystic teratomas in 4 of 20 females	607
TG.KD (transgenic; imprinted transgene integrated on chromosome 8 of FVB/N strain)	Unilateral or bilateral mature and immature teratomas with occasional metastases to lymph nodes and lungs; 15–20% of hemizygous females affected; dominant effect secondary to alterations of endogenous gene(s) at insertion locus	822
Ubiquitin C-hCGαβ (double transgenic; human ubiquitin C promoter driving expression of human chorionic gonadotropin α- and β-subunits)	Teratomas; precocious puberty; enhanced steroidogenesis; infertility	823

ova (590, 591). Scattered reports of spontaneous ovarian teratomas in mice began as early as 1920 (592). Although the scarcity of these tumors has precluded detailed study in mouse models, there are a few exceptions (summarized in Table 14). Stevens and Varnum (592) observed that strain LT/Sv females develop spontaneous ovarian teratomas as early as 30 d of age, with 50% of females affected by the time they are 90 d old. The penetrance jumps to more than 80% in recombinant inbred lines LTXBO and LTXBJ that were generated from intercrossing strains LT/Sv and C57BL/6J (593, 594). This is curious because C57BL/6J females are not predisposed to teratoma formation. The phenotype is likely multigenic and includes a semidominant LT/Sv allele at ovarian teratoma susceptibility locus (*Ots1*) on chromosome 6 and modifier alleles from C57BL/6J (593, 595).

The teratomas in LT/Sv and LT-related strains (hereafter referred to as LT) are mostly benign and stem from parthenotes in the ovary that resemble normal embryos until the blastocyst stage when they become disorganized and form tumors (592). Eppig and colleagues (593, 596, 597) outlined the sequence of events leading from oocyte to tumor in LT females, namely metaphase I arrest, parthenogenetic activation, completion of the first meiotic division, development to the blastocyst stage, and teratoma formation. The mammalian oocyte normally advances without interruption from prophase I to metaphase II by the time of ovulation, remaining arrested in metaphase II until fertilization activates the egg and triggers completion of the second meiotic division. LT oocytes frequently deviate from this paradigm and arrest at metaphase I instead of metaphase II; when this occurs in the ovary (*i.e.*, before ovulation), these oocytes are vulnerable to tumorigenesis (593). Metaphase I arrest in LT oocytes is initiated by sustained activation of the spindle assembly checkpoint, possibly due to meiotic spindle abnormalities or intrinsic defects in the checkpoint machinery (598). Although the checkpoint is eventually inactivated, the delay allows for premature buildup of CSF to levels that maintain metaphase I arrest (599–602). In normal oocytes that are not delayed, CSF does not reach adequate levels to induce arrest until metaphase II. The mechanisms responsible for parthenogenetic activation and teratoma development subsequent to metaphase I arrest in LT oocytes remain unknown.

As mentioned, in normal mammalian oocytes CSF is essential for metaphase II arrest by the time of ovulation. An integral component of CSF is MOS, a serine/threonine kinase with MAPK-stimulatory activity. Whereas metaphase I arrest predisposes LT oocytes to form teratomas, destabilization of metaphase II arrest leads to parthenogenetic activation of oocytes and teratoma development in *Mos* knockout females (453, 454, 603, 604). The teratomas are usually benign and histologically similar to those in LT mice but appear with lower frequency and later onset; specifically, 30% of *Mos*$^{-/-}$ females have tumors between 4 and 8 months of age (604). Hirao and Eppig (594) noted that this incidence is peculiarly low given the large proportion of mutant oocytes that become parthenogenetically activated. However, in contrast to LT parthenotes, *Mos*$^{-/-}$ parthenotes rarely progress to the blastocyst stage, and this might explain their decreased propensity to form tumors (594).

In light of the substantial cross talk between oocytes and somatic cells, it is not surprising that several pieces of evidence have revealed connections between granulosa cells and the development of teratomas. In LT strains, the primary lesions responsible for tumor formation are intrinsic to the oocyte; nonetheless, cumulus cells contribute to the maintenance of metaphase I arrest, induction of parthenogenesis, and eventual progression to metaphase II (605). LT ovaries also contain numerous abnormal fol-

TABLE 15. Characteristics of mammalian small RNAs

Class	Length (nucleotides)	Synthesis	Function
miRNA	21–23	DICER-dependent	Regulation of translation and stability of target mRNAs
siRNA	21–23	DICER-dependent	Transposon suppression and pseudogene regulation of founding source mRNAs
piRNA	24–31	DICER-independent feed-forward amplification loop	Transposon regulation and unknown functions

licles comprised of a single layer of granulosa cells surrounding a disproportionately large oocyte (606). Parthenogenetic embryos that form teratomas are usually found in these granulosa cell-deficient follicles, and the presence of these follicles is necessary but insufficient for germ cell tumorigenesis in LT females. In another mouse model, overexpression of the antiapoptotic protein BCL2 in ovarian somatic cells resulted in mature cystic teratomas in four of 20 transgenic females (607). The predominant phenotype of these transgenic mice is prolonged somatic cell survival and enhanced folliculogenesis, and it was hypothesized that paracrine signals from mutant somatic cells influence oocyte differentiation and in some instances steer oocytes toward tumorigenesis. On the other hand, overexpression of BCL2 in mouse oocytes delays spontaneous apoptosis of oocytes in culture, but does not cause germ cell tumors (608).

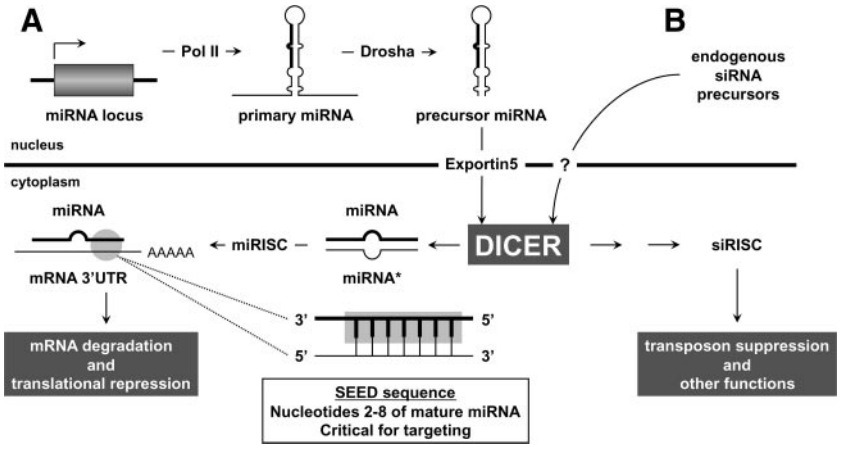

FIG. 7. Dicer-dependent synthesis of miRNAs and endogenous siRNAs. A, miRNA genes are transcribed by RNA polymerase II to generate primary transcripts. These transcripts are cropped by the DROSHA/DGCR8 Microprocessor complex to yield approximately 70-nt precursor miRNAs, which are exported from the nucleus to the cytoplasm by Exportin 5. There, the DICER complex processes the precursor into a 21- to 23-nt duplex consisting of the mature miRNA and its antisense. The mature miRNA is preferentially loaded into the miRNA-induced silencing complex (miRISC), which mediates pairing with complementary sequences in the 3′ UTR of target mRNA transcripts, leading to mRNA degradation and translational repression. The specificity of targeting is especially dependent on nucleotides 2–8 of the mature miRNA, known as the seed sequence. B, Endogenous siRNAs are synthesized from long, double-stranded RNA precursors derived from repetitive sequences, sense-antisense pairs, or inverted repeats that form hairpins. It is unknown whether endogenous siRNA precursors use Exportin 5 to translocate to the nucleus; however, once in the cytoplasm they are processed by the DICER complex into 21- to 23-nt duplexes. Mature siRNAs are incorporated into the siRNA-induced silencing complex (siRISC), which is similar to the miRISC but may also have unique components. Endogenous siRNAs function in transposon suppression and other functions such as pseudogene regulation of founding source mRNAs.

D. Small RNAs in ovarian physiology and cancer

The discovery of small noncoding RNAs that direct gene silencing has unveiled new dimensions in reproductive physiology and disease. There are three major classes of silencing RNAs encoded in the mammalian genome: microRNAs (miRNAs), Piwi-interacting RNAs (piRNAs), and siRNAs. Each of these small RNA categories has unique structural and functional characteristics (summarized in Table 15).

1. Small RNAs in ovarian physiology

As illustrated in Fig. 7, miRNAs are transcribed by RNA polymerase II as long primary transcripts that are processed into approximately 70-nt stem-loop precursors by a complex that includes the ribonuclease (RNase) III DROSHA and the RNA-binding protein DGCR8. Precursor miRNAs are exported from the nucleus to the cytoplasm where they are cleaved by a second RNase III, DICER (*Dicer1*), to produce a 21- to 23-nt duplex structure, one strand of which is preferentially incorporated into the RNA-induced silencing complex (RISC). The RISC guides pairing of the mature miRNA with complementary sequences in the 3′ UTR of target mRNA transcripts. Perfect complementarity between a miRNA and its mRNA target causes mRNA degradation through endonucleolytic cleavage (reviewed in Ref. 609). Although this is often the scenario in plants, target pairing in animal cells is frequently imperfect and also downregulates gene expression by repressing translation, possibly at the initiation or elongation steps, and by accelerating mRNA decay through removal of the poly(A) tail (609). In a complex twist to the miRNA dogma, recent evidence indicates that miRNAs may also activate translation under rare conditions (610). To date, there are approximately 700 human and 500 mouse miRNAs, and many are evolutionarily conserved.

Collectively, miRNAs are predicted to directly regulate over 60% of human protein-coding genes (611). For several years, they were the only small RNAs that had been identified in mammals.

In 2006, five laboratories independently reported the discovery of mammalian piRNAs in mouse and rat testes (612–616). PiRNAs (24–31 nt) are longer than miRNAs and siRNAs (21–23 nt), consistent with the observation that piRNAs are generated without DICER through a feed-forward amplification loop that is still under investigation (reviewed in Refs. 617–619). There are estimated to be over 100,000 PiRNAs in the mammalian genome; they are encoded in relatively few genomic clusters and in a strand-specific manner within each cluster. Although the genomic locations of piRNA clusters are conserved between species, specific piRNA sequences are poorly conserved. Unlike miRNAs and siRNAs, which interact with Argonaute family proteins in the RISC, piRNAs interact with Piwi family proteins (MILI, MIWI, and MIWI2; also known as PIWIL2, PIWIL1, and PIWIL4, respectively) (reviewed in Ref. 620). In mice, homozygous deletion of individual Piwi proteins causes spermatogenesis arrest and male sterility (621–623). Furthermore, *Mili* and *Miwi2* knockout males demonstrate activation of transposable elements, supporting the hypothesis that piRNAs suppress transposon mobilization in the male germline (623, 624). Interestingly, the majority of mammalian piRNAs do not map to annotated repeats such as transposons and retrotransposons, suggesting other potential functions of the piRNA pathway including epigenetic regulation and translational control (617).

If piRNAs guard the male germline against transposon activation, what mechanisms similarly protect the female germline? Initially, only a handful of piRNAs had been cloned from whole mouse ovary, and it was unknown which were expressed in oocytes (625). Moreover, MILI is the only Piwi family protein detected in oocytes (626), but *Mili* knockout females are fertile (622), implying that piRNAs are not the only factors that maintain stability of the oocyte genome. Watanabe *et al.* (616) identified a novel class of oocyte-expressed small RNAs known as endogenous siRNAs. Similar to miRNAs, siRNAs are processed by DICER into 21- to 23-nt species that function in the RISC. However, unlike miRNAs, which have short stem-loop precursors, siRNAs are synthesized from longer double-stranded RNA precursors that are derived from retrotransposons and other sources.

The initial data provided a glimpse into the siRNA population in the female germline and motivated two independent laboratories to comprehensively profile the gamut of small RNAs in mouse oocytes using deep sequencing technology (626, 627). In addition to detecting annotated miRNAs, they uncovered a broad MILI-bound piRNA population resembling piRNAs expressed in early-stage spermatocytes. Even more compelling was the identification of new endogenous siRNAs that are abundantly expressed. Most of these siRNAs are predicted to target specific retrotransposons that are also suppressed by piRNAs, a functional redundancy that could explain the absence of a phenotype in *Mili* knockout females, although retrotransposons preferentially targeted by either piRNAs or siRNAs were observed as well. Apart from retrotransposons, double-stranded RNA precursors of siRNAs are also generated from inverted repeat structures that form hairpins, the pairing of overlapping transcripts that are oppositely oriented, and the pairing of a protein-coding mRNA and an antisense transcript from its corresponding pseudogene. This latter example is most intriguing and suggests that mammalian pseudogenes, previously relegated to the domain of nonfunctional "junk DNA," instead regulate the expression of their founding source mRNAs through the siRNA pathway.

Because DICER is required for the synthesis of both siRNAs and miRNAs, conditional deletion of *Dicer1* in mouse oocytes offered additional insight into the functional roles of these small RNA pathways in the female germline (628, 629). *Dicer1 Zp3-Cre* cKO females are sterile despite the observation that their ovaries are histologically normal and responsive to gonadotropins. Further investigation of mutant oocytes highlighted defects in spindle organization and chromosome cohesion that block completion of meiosis I. Microarray profiling of DICER cKO oocytes revealed up-regulation of genes involved in microtubule dynamics and increased expression of maternal transcripts that are normally degraded during meiotic maturation, suggesting that DICER-dependent small RNA pathways foster oocyte maturation by accelerating mRNA turnover and regulating genes essential for spindle integrity. MiRNAs predicted to target genes involved in microtubule-based processes include mir-103, let-7d, mir-16, mir-30b, and mir-30c. Also, the complete set of up-regulated genes was significantly enriched in putative binding sites for mir-495, mir-126, and mir-302c*.

Transposon levels and oocyte transcripts with transposon sequences embedded in their 3′ UTRs are increased in DICER cKO oocytes; in particular, repetitive sequences such as short interspersed nuclear elements (SINEs) and mouse transcript subfamilies B and C (MTB/MTC) are elevated (626–628). MTB and MTC are almost exclusively regulated by endogenous siRNAs and not piRNAs, indicating that transposon activation in mutant oocytes is secondary to loss of DICER-dependent siRNA biogenesis (627). Putative targets of pseudogene-derived siRNAs are also increased in the absence of DICER, for example, oogenesin 4 (*Oog4*), histone deacetylase 1 (*Hdac1*), and Ran

GTPase activating protein 1 (*Rangap1*). The regulatory target of RANGAP1 is the RAS oncogene family member RAN, which is important for microtubule organization in mouse oocytes (630). Interestingly, mir-30b and mir-30c are predicted to target *Ran*, and accordingly, *Ran* expression is increased in the DICER knockout (628). The putative silencing of *Ran* by miRNAs and *Rangap1* by siRNAs leads to speculation that miRNA and siRNA pathways may coordinately regulate related functional networks that are essential for oocyte maturation.

Whereas current evidence suggests that mammalian piRNAs are critical primarily in the male germline, and roles for endogenous siRNAs may be restricted to oocytes and ES cells, miRNA expression is ubiquitous and essential for the proper function of germ cells and somatic cells (summarized in Table 16). Conditional deletion of *Dicer1* in various somatic cells and tissues of mice results in dramatic phenotypes that are predominantly attributed to loss of specific miRNAs, albeit dysfunction caused by disruption of siRNA biogenesis cannot be definitively excluded. To investigate global roles for miRNA and siRNA pathways in somatic cells of the female reproductive tract, our laboratory and others generated *Dicer1* cKO mice using *Amhr2-Cre* (631–634). Although *Amhr2-Cre* is commonly used for studying gene function in ovarian granulosa cells, it is also expressed postnatally in the smooth muscle and stromal cells of the oviducts and uterus, and embryonically in the mesenchyme of the developing Müllerian ducts that give rise to these structures (260, 505–507). *Dicer1 Amhr2-Cre* females are sterile and have multiple reproductive defects, including prominent bilateral oviductal cysts that act as a reservoir for spermatozoa and oocytes and prevent embryos from transiting the oviduct to enter the uterus for implantation. The uteri of mutant females demonstrate normal decidualization (631); however, they are shorter and have fewer endometrial glands, an observation that correlates with implantation defects after embryo transfer experiments (633). Mutant ovaries are histologically normal except for a subtle but significant increase in granulosa cell apoptosis. In response to gonadotropin stimulation, immature cKO females ovulate significantly fewer oocytes, have oocytes that are trapped in luteinized follicles, and show a marked decreased in the proportion of oocytes that progress to the two-cell stage after overnight culture. Taken together, these observations suggest that DICER is essential for proper development of the oviducts and uterus, and that DICER expression in granulosa cells regulates ovulation and indirectly contributes to oocyte quality because of communication between somatic and germ cell compartments of the ovary.

One possible explanation for the ovulatory defects we observed may be aberrant LH signaling in granulosa cells upon loss of DICER. Fiedler *et al.* (635) compared miRNA expression profiles in granulosa cells isolated from wild-type mice pharmacologically stimulated with PMSG alone or PMSG followed by hCG. Of the 212 miRNAs detected, 13 miRNAs were differentially expressed as early as 4 h after hCG treatment. The expression levels of two up-regulated miRNAs, mir-132 and mir-212, were also increased in granulosa cell cultures treated with cAMP, a principal second messenger downstream of LH. Notably, these two miRNAs share identical seed sequences and hence may target some of the same transcripts. Although specific interactions between granulosa cell miRNAs and mRNAs remain to be identified and validated, these data demonstrate that LH signaling alters granulosa cell miRNA expression, which in turn might influence the regulation of genes that govern ovulation.

It appears that granulosa cell miRNA expression may be dispensable for luteinization because we did not observe luteinization defects after deleting DICER in granulosa cells (631). However, miRNAs may indirectly affect CL function through roles in angiogenesis. Otsuka *et al.* (636) created a viable mouse model with hypomorphic DICER expression and reduced miRNA production. They reported female sterility secondary to luteal insufficiency, characterized by lower serum progesterone levels and decreased ovarian expression of *Lhcgr*, *Cyp11a1*, and *Prlr* after copulation. Mu-

TABLE 16. Dicer mutations with female reproductive phenotypes

Mouse model	Phenotype	Ref.
Dicer1 Zp3-Cre (Dicer cKO in oocytes)	Sterile; defects in spindle organization and chromosome cohesion block completion of meiosis I; increased expression of genes involved in microtubule dynamics, maternal transcripts normally degraded during meiotic maturation, transposons, and putative targets of pseudogene-derived siRNAs	626–629
Dicer1 Amhr2-Cre (Dicer cKO in ovary, oviduct, uterus)	Sterile; bilateral oviductal cysts sequester embryos and prevent transit to the uterus; decreased ovulation; uteri decidualize normally but are shorter with fewer endometrial glands and demonstrate implantation defects after embryo transfer experiments	631–634
Dicer1 hypomorphic mutation	Sterile; luteal insufficiency due to diminished vascularity in CLs	636

tant females showed diminished vascularity in their CLs, along with up-regulation of the antiangiogenic factors tissue inhibitor of metalloproteinase 1 (TIMP1) and platelet factor 4 (PF4). The *Timp1* 3′ UTR has binding sites for two miRNAs, mir-17–5p and let-7b, which decreased expression of a luciferase reporter fused to the *Timp1* 3′ UTR and also reduced TIMP1 activity in cultured endothelial cells. Intrabursal injection of wild-type mice with inhibitors against these two miRNAs increased TIMP1 levels and impaired CL angiogenesis, whereas combined overexpression of mir-17–5p and let-7b in hypomorphic DICER females suppressed TIMP1 levels and improved CL vascularity. Nonetheless, restoration of these two miRNAs failed to maintain pregnancy in DICER mutants, thus highlighting potential roles for other miRNAs.

2. Misregulation of miRNAs in ovarian cancer

The precise functions of ovarian miRNAs remain to be delineated; however, studies in a wide range of tissues and cell types indicate that miRNAs guide fundamental cellular processes such as proliferation, differentiation, and apoptosis. Hence, it is not surprising that aberrations in miRNA expression have been observed in a variety of human cancers. MiRNA profiles reflect the developmental lineages and differentiation states of tumors, sometimes with greater accuracy than mRNA profiles (637). During mammalian development, a global rise in miRNA levels correlates with increasing cellular differentiation. In contrast, cancers generally demonstrate an overall reduction in miRNA expression that corresponds to a less differentiated state. Searching for a causal relationship between these observations, Kumar *et al.* (638) globally repressed miRNA levels in mouse and human cancer cell lines using shRNA-mediated knockdown of three factors required for miRNA processing: DROSHA, DGCR8, and DICER. With knockdown of these gene products, the cells showed increased proliferation, improved colony formation, and better migration, and also formed tumors more rapidly and with greater invasive properties in nude mice. Notably, impaired miRNA processing enhanced tumorigenesis only in cells that were already transformed but was insufficient to cause *de novo* transformation, suggesting that decreases in miRNA expression modify rather than initiate carcinogenesis. Although these experiments broadly implicate miRNAs as tumor suppressors, either through direct targeting or indirect regulation of oncogenes, other studies highlight roles for specific miRNAs as oncogenes such that their overexpression accelerates tumorigenesis, clearly indicating that these small RNAs have dual functions in cancer.

Potential mechanisms of miRNA deregulation in cancer are diverse and include defects in miRNA processing (638), DNA copy number abnormalities (639–641), epigenetic alterations (641, 642), mutations in the miRNA or its mRNA binding site (643), and aberrant transcription (644). Using microarray profiling, a handful of studies have identified genome-wide alterations in miRNA expression in primary epithelial ovarian tumors and established ovarian cancer cell lines. This work has focused predominantly on the most common subtype, serous ovarian carcinoma, presumably because these tumors are more readily procured, although a few endometrioid and clear cell ovarian cancers have also been profiled. Both Iorio *et al.* (645) and Nam *et al.* (646) found up-regulation of mir-141, mir-200a, mir-200b, mir-200c, and mir-21 in ovarian cancers compared with normal ovary, down-regulation of mir-99a, mir-100, mir-125a, mir-125b, mir-143, mir-145, mir-214, and let-7 family members, and aberrations unique to their respective studies. Similarly, Yang *et al.* (647) observed up-regulation of mir-200a and down-regulation of mir-100, mir-125b, and let-7 family members in their analysis of serous ovarian cancers compared with HIOSE118, a human OSE cell line immortalized with SV40 large TAg (648). On the other hand, Dahiya *et al.* (649) reported changes in the opposite direction for mir-99a, mir-100, mir-141, mir-200a, and mir-21 when comparing ovarian cancers to HOSE-B, a human OSE cell line immortalized with human papillomavirus genes E6 and E7. These discrepancies underscore a major challenge in dissecting the molecular signature of ovarian cancer: the choice of "normal" control dramatically influences the outcome of genome-wide expression studies.

Zorn *et al.* (650) systematically illustrated this problem by comparing the gene expression profiles between a common set of serous ovarian carcinomas and five different control groups: OSE brushings; whole ovary; short-term cultures of normal OSE (NOSE); SV40 large TAg-immortalized OSE cell lines (IOSE); and telomerase-immortalized OSE cell lines (TIOSE). For each cancer and normal pairing, the majority of differentially expressed genes were unique to that particular comparison, with no gene present in all five comparisons. Additionally, hierarchical clustering of the controls revealed distinct profiles for each group, with the first major branch point separating the cultured samples (NOSE, IOSE, TIOSE) from those that were not cultured (OSE brushings and whole ovary), pointing to the substantial impact of *in vitro* manipulation on OSE-derived cells. Taken together, these data emphasize that candidate genes and miRNAs that emerge from profiling experiments should be meticulously examined in independent functional assays to evaluate their actual relevance to cancer formation and progression. To date, specific roles for only a few miRNAs have been described in epithelial ovarian cancer (summarized in Table 17).

TABLE 17. MiRNAs with putative functions in epithelial ovarian cancer

MiRNA	Select validated targets	Potential role of miRNA	Ref.
mir-34a; mir-34b; mir-34c	*BCL2; CDK4; CDK6; CCNE2; MET*	Activated by p53; loss of function mutations in *p53* may decrease mir-34 expression	651
mir-200a; mir-200b; mir-200c; mir-141; mir-429; mir-205	*ZEB1; ZEB2*	Repress epithelial-to-mesenchymal transition	659
mir-214	*PTEN*	Overexpression promotes chemoresistance	647
mir-199a-5p	*IKKβ (IKBKB)*	Loss of function activates NF-κB pathway, which may foster a protumor microenvironment	667
let-7 family	*KRAS; HRAS; MYC; HMGA2*	Loss of function may promote tumorigenesis; let-7i knockdown increases chemoresistance	666, 677–680

Given the frequent mutation of *p53* in high-grade human ovarian carcinomas, Corney *et al.* (651) compared miRNA expression profiles between primary cultures of wild-type and *p53*-deficient mouse OSE cells to screen for miRNAs that are regulated in a p53-dependent manner. Mir-34b and mir-34c were significantly down-regulated upon p53 inactivation and are transcribed from a single locus containing an upstream p53 binding site that is conserved between mouse and human. Both miRNAs are markedly decreased in a *p53* null human ovarian cancer cell line (SKOV-3) compared with short-term cultures of human OSE cells, and two profiling studies reported lower levels of mir-34c in primary serous ovarian carcinomas compared with normal ovary (645) or an immortalized OSE cell line (649). Overexpression of mir-34b and mir-34c in *p53* null mouse OSE cell lines suppressed their proliferation and anchorage-independent growth (651). These data are consistent with five independent studies demonstrating activation of mir-34 family members by p53, with mir-34 overexpression inducing cell cycle arrest or apoptosis depending on the cellular context (652–656). Accordingly, predicted mir-34 targets are involved in cell cycle control, apoptosis, and DNA repair, and validated targets include *BCL2*, *CDK4/6*, cyclin E2 (*CCNE2*), and met proto-oncogene (*MET*) (652, 654, 657). Aside from ovarian cancer, reduced mir-34 expression has been reported in neuroblastoma (658), pancreatic cancer (653), and non-small cell lung cancer (652). Thus, the mir-34 family is an integral effector of the p53 tumor suppressor network, and the loss of mir-34 expression, either secondary to *p53* mutation or independently in the case of *p53* wild-type tumors, may be a driving force in ovarian and other cancers.

One of the hallmarks of tumor progression is epithelial-to-mesenchymal transition (EMT), a complex process characterized by loss of E-cadherin-mediated cell-cell adhesion and polarity, and by acquisition of vimentin expression and the ability to migrate and invade, ultimately facilitating metastasis. The zinc finger E-box binding homeobox transcription factors ZEB1 and ZEB2 promote EMT by repressing expression of E-cadherin and other master regulators of epithelial polarity (659, 660). The ratio of ZEB2/E-cadherin expression is higher in stage IV compared with stage III ovarian carcinomas and may predict poor overall survival (661). Several independent studies demonstrated that mir-200 family members (mir-200a, mir-200b, mir-200c, mir-141, mir-429) and mir-205 directly target ZEB1 and ZEB2 and prevent EMT induction (659, 662, 663). In the National Cancer Institute panel of human cancer cell lines (NCI60), which includes several ovarian cancer lines, mir-200 members are selectively expressed in E-cadherin-positive and vimentin-negative cells, further indicating that these miRNAs are strong determinants of the epithelial phenotype (659). Moreover, Park *et al.* (659) found a significant correlation between mir-200c and E-cadherin expression in primary serous ovarian carcinomas. These data raise the possibility that loss of mir-200 family members may contribute to ovarian cancer metastasis, but counterintuitive to this hypothesis is the observation that higher levels of mir-200 family miRNAs were significantly correlated with shorter overall survival in women with serous ovarian carcinoma (646). A formal comparison of mir-200 expression and function between primary and metastatic ovarian lesions has not been done.

Loss of function mutations in *PTEN* are characteristic of endometrioid ovarian carcinomas (664); however, reduced or absent PTEN expression has been described in other ovarian cancer subtypes with intact *PTEN* alleles (665), hinting at alternate mechanisms of PTEN inactivation. Yang *et al.* (647) showed that the *PTEN* 3′ UTR is targeted by mir-214, which is up-regulated in ovarian cancers [although two other studies (645, 646) reported decreased mir-214 expression] and inversely correlated with PTEN protein levels. Overexpression of mir-214 in human ovarian cancer cell lines reduced PTEN expression and conferred resistance to cisplatin-induced apoptosis by activating the AKT pathway, whereas knockdown of mir-214 sensitized cells to cisplatin (647). Although many women with ovarian cancer are initially responsive to ad-

juvant chemotherapy with cisplatin, the majority of patients develop recurrent disease that is drug-resistant. Out of 11 patients with recurrent ovarian cancer, eight had low or undetectable mir-214 levels in their primary tumors but elevated mir-214 expression in their recurrent lesions, suggesting that mir-214 may promote chemoresistance in ovarian cancer. Notably, an independent analysis detected higher mir-214 expression in primary ovarian carcinomas from women with poor responses to chemotherapy compared to those with greater chemosensitivity (666).

Chen *et al.* (667) identified another miRNA-mediated pathway that may be a critical regulator of chemoresponsiveness in ovarian cancer. Nuclear factor-κB (NF-κB) activation directs the constitutive secretion of proinflammatory cytokines that promote tumor progression by stimulating cell proliferation, inducing antiapoptotic proteins, and enhancing chemoresistance. Using ovarian cancer cell lines and primary cells isolated from malignant ovarian cancer ascites and solid tumors, they demonstrated that NF-κB pathway activity is directly dependent on the expression of inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta (IKKβ), part of a protein complex that phosphorylates and degrades an inhibitor of NF-κB. Whereas NF-κB activity varied with IKKβ protein levels, *IKKβ* mRNA expression was relatively constant, indicating posttranscriptional regulation of *IKKβ*. Microarray profiling revealed that the expression of mir-199a was inversely correlated with NF-κB activity, and further experiments showed direct targeting of *IKKβ* by mir-199a. Therefore, low mir-199a expression leads to up-regulation of IKKβ and contributes to NF-κB pathway activation, potentially fostering a protumor microenvironment. Interestingly, mir-199a levels were lower in ovarian cancers compared with normal ovary (645), but higher in ovarian cancers compared with an IOSE cell line (649).

To pinpoint additional miRNAs that modulate chemoresponsiveness, Sorrentino *et al.* (668) analyzed differences in miRNA expression between various human ovarian cancer cell lines that are sensitive or resistant to cisplatin and paclitaxel. Three miRNAs (mir-30c, mir-130a, mir-335) were consistently down-regulated in the drug-resistant cell lines, and each miRNA has a unique set of predicted target genes. Although the levels of these miRNAs were not manipulated to show a causative role in drug response, reporter assays demonstrated that mir-130a targets colony stimulating factor 1 (*CSF1*). The overexpression of CSF1 and its receptor denotes poor prognosis in ovarian and breast cancers (669–671). CSF1 mediates chemoresistance of breast cancer cells and promotes invasion and metastasis of ovarian cancer cells in culture and in a xenograft model (672–674). Although it was previously demonstrated that the *CSF1* 3′ UTR contains an AU-rich element that binds glyceraldehyde-3-phosphate dehydrogenase and controls mRNA stability (675, 676), this is the first report indicating posttranscriptional regulation of *CSF1* by the miRNA pathway.

Extending beyond the profiles of drug-resistant cell lines, Zhang and colleagues (666) identified miRNAs that are differentially expressed between primary ovarian cancers from women with chemosensitive and chemoresistant disease. Among the top miRNAs comprising a distinct signature, let-7i was dramatically reduced in chemoresistant tumors, and lower let-7i levels were significantly correlated with shorter progression-free survival. Knockdown of let-7i in human ovarian cancer cell lines increased resistance to cisplatin, whereas let-7i overexpression enhanced chemosensitivity. These data are corroborated by a wealth of evidence indicating that let-7 family members are potent tumor suppressors in a variety of human cancers. Let-7 is a chief regulator of cell proliferation pathways and directly represses known oncogenes such as KRAS (677), HRAS (677), MYC (678), and HMGA2 (HMG AT-hook 2) (679, 680). HMGA2 is an architectural transcription factor that is undetectable in normal OSE but is expressed in ovarian cancer (681–683). Silencing of HMGA2 in ovarian cancer cell lines suppressed growth and increased apoptosis in culture and significantly reduced tumor burden in nude mice (683). HMGA2 expression is inversely correlated with let-7 expression in the NCI60 cancer cell lines and in primary ovarian carcinomas (681, 682). Patients with high HMGA2 and low let-7 in their tumors have worse progression-free survival compared to women with a low HMGA2/let-7 expression ratio, suggesting that this ratio may be an important predictor of outcome (681, 682).

Let-7 is among a group of miRNAs that are absent in the early embryo but then induced in later stages of development and in adult tissues (682). It has been hypothesized that these miRNAs in part control the onset and maintenance of cell differentiation by suppressing the expression of embryonic proteins, including HMGA2. On the other hand, the loss of let-7 might allow reexpression of the embryonic program and transformation to a dedifferentiated state that drives tumorigenesis. Indeed, reduced let-7 levels determine key properties of breast cancer stem cells, namely self-renewal (through the regulation of *HRAS*) and multipotent differentiation (through the regulation of *HMGA2*), and these findings may have broad implications for other human cancers (684). As mentioned in *Section II*, the posttranscriptional maturation of let-7 primary transcripts is suppressed by the pluripotent factor LIN28 and its homolog, LIN28B (31–35). In agreement with the discussion above, high *LIN28B* expression is significantly associated with increased risk of

disease progression and death in women with epithelial ovarian cancer (685). Furthermore, consistent with roles for *LIN28* in PGC development and oncogenesis, *LIN28/LIN28B* expression is a reliable marker for testicular germ cell tumors (30), and these proteins may likewise be involved in the pathogenesis of germ cell ovarian cancers, although this has not yet been explored.

3. MiRNAs as prognostic and diagnostic biomarkers

The aforementioned studies highlight emerging roles for miRNAs in the pathogenesis of ovarian cancer, including regulating the response to chemotherapy and the potential to metastasize. If aberrations in miRNA function significantly influence tumor behavior, it follows that the expression patterns of specific miRNAs may be powerful prognostic indicators for cancer patients. For women with ovarian cancer, the identification of miRNA-related biomarkers is still in its infancy, but the early data have revealed several candidates whose expression in ovarian tumors portends prolonged (+) or diminished (−) survival, for example, let-7(+), mir-200(−), mir-93(−), and the eight miRNAs(+) clustered in the *Dlk1-Gtl2* domain, a putative tumor suppressor locus on human chromosome 14 (641, 646, 666). These eight miRNAs are mir-337, mir-368, mir-376a, mir-376b, mir-377, mir-410, mir-432, and mir-495 (641).

As described earlier, there is a global reduction in miRNA expression in a variety of human cancers, suggesting deregulation of the miRNA processing machinery. Lower mRNA and protein levels of DROSHA and DICER in advanced-stage, poorly differentiated epithelial ovarian carcinomas compared with benign OSE specimens have been reported (686). Low *DROSHA* expression significantly correlated with suboptimal surgical cytoreduction, whereas low *DICER1* expression significantly associated with advanced tumor stage and was an independent predictor of shorter survival. These data conflict with other evidence demonstrating similar mRNA and protein levels of DROSHA and DICER between early- and late-stage epithelial ovarian cancers, without any correlation to patient survival (641). A third study found that there were no significant differences in the expression of miRNA processing enzymes in multiple types of human cancer (637). Hence, the utility of these factors as prognostic markers remains inconclusive. Finally, a fourth study identified lower expression of eukaryotic translation initiation factor 6 as an independent predictor of reduced disease-free survival, but not overall survival, in women with serous ovarian carcinoma (687). Eukaryotic translation initiation factor 6 associates with the RISC complex and is a key mediator of miRNA-dependent gene silencing (688), suggesting that global impairment of miRNA function may contribute to tumor recurrence.

The stark contrast in 5-yr survival rates for women diagnosed with advanced stage ovarian cancer (30%) compared with localized disease (90%) emphasizes the crucial importance of early detection (467). Recently, several laboratories demonstrated that tumor-derived miRNAs in serum or plasma are stable markers for cancer detection (689, 690), an exciting discovery considering that miRNA expression profiles classify human cancers with high accuracy (637). The mechanisms by which circulating miRNAs are protected from endogenous RNase activity remain unknown, but may involve the packaging of miRNAs into exosomes, vesicles of endocytic origin that are released into the extracellular environment (691, 692). A pilot comparison of serum miRNA expression between healthy women and patients newly diagnosed with epithelial ovarian cancer before treatment revealed up-regulation (mir-21, mir-92, mir-93, mir-126, mir-29a) and down-regulation (mir-155, mir-127, mir-99b) of specific miRNAs in cancer patients (693). These preliminary observations are encouraging and warrant further investigation on a larger scale, especially to determine whether differentially expressed miRNAs are clinically informative in early stage disease. Indeed, the development of minimally invasive miRNA screens to detect and monitor ovarian cancer would be revolutionary.

V. The Assisted Reproductive Technology Laboratory

Infertility is a worldwide problem for individuals wishing to reproduce, affecting about 15% of couples and causing significant economic, social, and psychological distress for the childless couple (694, 695). Approximately 9% of women in the 20- to 44-yr-old age group experience a 12-month period where they are unable to become pregnant (696). Luckily for these women, assisted reproductive technologies (ART) have been developed to help them achieve their dreams of becoming mothers. In this section, we will briefly describe the advances that have been made in the ART laboratory and clinic that have revolutionized reproduction. For a historical perspective on gonadotropin use in the clinic, the reader is referred to a 2004 article by Lunenfeld (697). For a more extensive review of the development of ovarian stimulation agents and their use in the clinic, the reader is referred to a 2006 review in this journal by Macklon *et al.* (698). In the current review, we will give sufficient background to bring the reader up to speed and give an update on key advances in the last few years that we believe will have an important impact on the advancement of female fertility.

A. Hormonal preparations

The first "test tube baby," Louise Brown, celebrated her 31st birthday this year (699). During the past three decades, there have been many procedural changes that have come about to enhance the success rate of ART. Whereas Louise Brown was conceived through a natural ovulatory cycle (699), within a few years, clomiphene citrate and urinary gonadotropins were being used to hyperstimulate the ovaries (700). Clomiphene citrate, a selective ER modulator, inhibits the feedback of estrogen on the pituitary leading to increased release of FSH (a noncovalent α:β heterodimer; the α-subunit is common to the four glycoprotein hormones, whereas the β-subunit is unique to FSH, LH, hCG, and TSH) and stimulation of follicle recruitment. Since the early days of ART, there have been multiple advances in the hormones, their concentrations, and the preparations that are given to women to stimulate their ovaries to produce mature oocytes that can be fertilized *in vitro*. Although clomiphene citrate continues to be used in the setting of controlled ovarian stimulation, multiple preparations of human postmenopausal gonadotropins and recombinant preparations of human FSH, LH, and hCG (viewed as "safer" because they are not prone to any possible transfer of infectious agents such as viruses or prions) have been effectively developed and are being used successfully in the ART clinic. Furthermore, these recombinant preparations are not only used in the ART clinic; in 2008, Pergoveris, a mixture of recombinant FSH and LH, was approved in Europe for the treatment of severe combined FSH and LH deficiencies.

To more closely mimic the longer continuous levels of FSH seen in normal cycles, several recombinant long-acting FSH analogs have been created including FSH-CTP (addition of one or more O-linked C-terminal extensions of hCGβ to the FSHβ C terminus), tandem FSH-CTP (fusion of FSHβ-CTP and the common α-subunit), an N-linked tandem FSH variant (FSHβ-α subunit fusion linked by an N-linked peptide), single-chain FSH-Fc (a human FSHβ-α subunit-linker sequence-IgG_1 Fc domain fusion), and heterodimeric FSH-Fc fusion (human FSHβ-linker sequence-IgG_1 Fc domain-6 His and human α-subunit-linker sequence-IgG_1 Fc domain covalent dimer) (701–703). FSH-CTP (corifollitropin alfa), the first of this new generation of long-acting gonadotropin analogs (also referred to as sustained follicular stimulants) to be tested in feasibility studies in the clinic, has an approximately 2- to 3-fold longer half-life ($t_{½} \approx 65$ h) and essentially identical biopotency compared with FSH, producing similar numbers of oocytes (704). Most importantly, corifollitropin alfa yielded equal numbers of good quality embryos to transfer, the incidence of ovarian hyperstimulation syndrome was low, and only a single initial injection of corifollitropin alfa was required, compared to multi-daily injections of recombinant FSH injections. Results from a randomized phase II trial (NCT00598208) showed that corifollitropin alfa was highly effective in a 1-wk regimen (705). Patients were given 60, 120, or 180 mg of corifollitropin alfa on d 1, a GnRH antagonist on d 5 through the end of the cycle, and daily injections of recombinant FSH (Puregon) beginning 1 wk later until it was time to induce oocyte maturation with hCG. Compared with daily injections of Puregon for the first 7 d of the cycle, there was a statistically significant increase in the number of cumulus-oocyte complexes retrieved when patients were treated with 120 mg and 180 mg of corifollitropin alfa. Likewise, there was an increase in the mean number of good quality embryos obtained, and cumulative pregnancy rate was also higher in these same groups. In July 2008, Schering announced the results of its first phase III trial (NCT00696800, called ENGAGE), the largest double-blind fertility trial ever. In this trial, 1509 women received either a single 150 mg dose of corifollitropin alfa or daily injections of 200 IU of Puregon for 7 d. Other aspects of the study (the GnRH antagonist and follow-up injections of FSH and hCG) were the same as the phase II trial. The findings indicated that the ongoing pregnancy rate with corifollitropin alfa (38.9%) *vs.* Puregon (38.1%) was similar. If results of the additional phase III trials are as promising as the above studies, then corifollitropin alfa protocols could replace many of the older protocols in the clinic with added benefits to the patient. In January 2009, the European Medicine Agency decided to review Schering-Plough's Marketing Authorization Application for corifollitropin alfa. For an extensive review on corifollitropin alfa, the reader should see the recent paper by Fauser *et al.* (706).

One goal of the pharmaceutical companies who are working in the ART area has been to develop orally bioavailable small molecule (low molecular weight) FSH and LH agonists that could replace the injectable glycoproteins. The early days of development of these small molecules have been reviewed by Lunenfeld (697), and the more recent advances have been reviewed by Arey (707). An amazing aspect regarding the development of these small molecular analogs is that they can "substitute" for the large bulky glycosylated LH or FSH protein dimers. It is believed that these small molecules bind to allosteric pockets in the FSH or LH receptors that are distinct from the orthostatic binding sites of the endogenous ligands. Thus, these small molecules are not mimicking the FSH or LH ligand binding, but instead activate the receptor through an independent site to induce a conformational change in the receptor that mimics endogenous ligand binding to the receptor. This feature of the small molecules

has made it easier to find and develop both more potent and specific FSH and LH agonists.

There are three classes of small molecule glycoprotein hormone receptor agonists: the thiazolidine (FSHR agonists), the pyrazole (LH receptor agonists), and the thienopyrimidine (TSH receptor/LH receptor agonist) classes. Members of the thiazolidine class were the first reported small molecule glycoprotein hormone agonists that were shown to act allosterically (708). Although the pyrazole class was shown to be selective for LH receptor agonist activity with an EC_{50} in the low micromolar range (709), discovery and optimization studies resulted in a specific thienopyrimidine analog (Org 43553) with an EC_{50} of 3.7 nM for LH receptor activation (710). Org 43553 is the first orally active LH receptor agonist, is able to induce ovulation in mice and rats when given orally (710), and has been developed as a good radioligand for the identification of more potent analogs (711). It will be interesting to see whether similar orally efficacious FSHR analogs can also be developed, possibly following the leads of Org 43553.

B. *In vitro* fertilization and intracytoplasmic sperm injection

It is estimated that 3.5 million babies have been born through the use of ART. Although children have been born through the injection of sperm into a woman's reproductive tract (intrauterine or intracervical insemination), the most common procedures used in the ART clinic are IVF and intracytoplasmic sperm injection (ICSI). IVF, as its name implies, allows oocytes to be fertilized by spermatozoa *in vitro* and requires the donor's spermatozoa to have all of the necessary characteristics for fertilization (*i.e.*, the spermatozoa must be motile, have the capability to bind to the oocyte zona pellucida, and be able to fuse with the oocyte membrane). ICSI was first reported in 1992, and involves the injection of a single sperm into the cytoplasm of an MII oocyte. Because ICSI bypasses a number of steps in the fertilization process, normal spermatozoa and abnormal (*e.g.*, immotile) spermatozoa or earlier stage elongation and elongated spermatids can be used for the procedure, allowing some men with infertility to pass their genetic material to offspring. In 2008, the International Committee for Monitoring Assisted Reproductive Technologies reported that about 200,000 babies are born annually from ART and the total number of procedures are about 1.3 million. In addition, approximately 50% of ART procedures performed in 2008 were in seven countries (Japan, the United States, France, Germany, Spain, the United Kingdom, and Australia). Based on 2004 data, Denmark has the highest incidence of ART babies born (4.2% of all births) (705). Furthermore, most ART laboratories now use ICSI (63% of cycles) as the routine procedure.

Despite the growing use of ICSI worldwide, there are reports of increased malformations in children born from ICSI including imprinting errors such as Beckwith-Wiedemann syndrome and Angelman syndrome (reviewed in Refs. 712 and 713). However, these malformations are less likely to be associated with the ICSI procedure *per se* than the transfer of sperm containing defective genetic material or, alternatively, the use of oocytes from women who are transmitting genetic abnormalities to their offspring. The Practice Committee of the American Society for Reproductive Medicine and the Practice Committee of the Society for Assisted Reproductive Technology have formulated a number of recommendations on ICSI (714).

In addition to the above studies on ICSI, there are also reports that ART (IVF or ICSI) is associated with birth defects. Using data from the National Birth Defects Prevention Study for babies born in the United States between October 1997 and December 2003, a number of structural birth defects were found to be increased in children whose mothers used ART to become pregnant (715). Among the defects observed in singleton births are septal heart defects, cleft lip with or without cleft palate, esophageal atresia, and anorectal atresia. Similar to ICSI itself, the etiologies of the increase in these birth defects are not clear, although an increase in transmission of defective genetic material and/or epigenetic alterations associated with culturing of embryos under nonphysiological conditions could be causal.

C. Advances in cryopreservation

Freezing of spermatozoa for IVF or ICSI is relatively straightforward. Likewise, excellent strategies have been developed for cryopreserving human and nonhuman embryos. Although challenges remain for the preservation of ovarian tissue (see below), a major breakthrough has been made for cryopreserving oocytes.

Why is the cryopreservation of oocytes (or ovarian tissue) important? Oocytes are highly sensitive to toxins, including polycyclic aromatic hydrocarbons (120, 716), as well as drugs used for cancer chemotherapy and radiation treatment. Furthermore, in some countries, there are strict guidelines for human embryo freezing. For example, the German embryo protection law that was passed in 1991 states that no more than three embryos can be produced in each IVF cycle and all embryos, irrespective of their quality, must be transferred, cannot be frozen, and cannot be discarded. However, this has led to an increased incidence of fetal reductions. Italy subsequently enacted nearly identical legislation restricting embryo freezing and limiting the number of oocytes fertilized to three. Thus, there are practical and ethical reasons why oocyte cryopreservation should be used.

In 1986, the first baby was born from a frozen human oocyte (717). Despite the millions of children that have been born through ART procedures, only a few hundred have been born through fertilization of a frozen oocyte. This low incidence is secondary to the poor survival of oocytes cryopreserved with the typical slow-freezing cryoprotectant solutions. Both the slow speed of the freezing methods and the long-term exposure of oocytes to the solutions used for cryoprotection are associated with significant trauma to the human oocyte, making this methodology impractical in the ART laboratory (reviewed in Ref. 718).

Fortunately, scientists and clinicians have been able to take an alternative approach for oocyte cryopreservation which involves vitrification, the rapid freezing of specimens (*e.g.*, oocytes or embryos) in ultrasmall volumes (719–726). The original study by Kuwayama *et al.* (719) demonstrated higher oocyte survival, fertilization, and pregnancy outcome when a slightly lower concentration of ethylene glycol (5.0 mol/liter) was used and when cumulus cells were not removed from the oocytes, suggesting that this method could also be used in the future for freezing GV oocytes. In the 4 yr since this initial report, the vitrification approach for freezing human oocytes has become remarkably efficient in multiple ART laboratories (725). One possible reason for the success of the vitrification method over slow freezing is the more rapid recovery of the meiotic spindle with vitrification (727, 728), allowing fertilization after thawing to proceed in a more timely manner. In addition, various groups have also begun to use straws or CryoLoops that are closed to the liquid nitrogen (729, 730), thereby avoiding any possible contamination (infectious or otherwise) of the vitrified human oocytes. Likewise, vitrification has also been shown to be highly efficient for the cryopreservation of d 3 human embryos (731); 94.8% of vitrified embryos survived the procedure compared with slow freezing (88.7%), and significantly more developed to the blastocyst stage with vitrification (60.3%) *vs.* slow freezing (49.5%). These vitrification strategies are also beginning to be used for freezing human ovarian cortex (732) for the preservation of fertility in women who are subjected to chemotherapy or radiation therapy for cancer treatment. However, with this cohort of women, a strategy for the *in vitro* culture of human primordial follicles to fertilizable MII oocytes is a necessary first step to ensuring future fertility.

D. Choosing the best oocyte—morphological and molecular analysis

In vivo or *in vitro*, the best embryo will obviously develop from the best oocyte. Abnormalities in oocytes, whether genetic or metabolic, are not beneficial to a healthy embryo or a healthy offspring. A major problem for the ART laboratory is how to decide which oocyte is "best." Unlike chorionic villus biopsies or amniocentesis, where there are an abundance of cells available for molecular and chromosomal analysis, the genetic status of the oocyte DNA that joins with the sperm cannot be evaluated. Over the last few years, there have been several advances made for noninvasive analysis of the oocyte, and these strategies are discussed below.

In theory, all oocytes that come from a chromosomally normal female should be capable of progressing through meiosis to become a normal haploid gamete that is capable of fusing with a haploid sperm to become a normal diploid embryo. Meiosis in females has several stages as follows: oocytes enter meiosis during embryogenesis and arrest in diplotene of meiosis I; after follicle recruitment, oocytes in antral follicles will reenter meiosis after the LH surge, release the first polar body but subsequently arrest at metaphase of MII; meiosis is completed upon fertilization with a sperm and release of the second polar body (Fig. 1). This abrupt stop-and-go pattern in female meiosis would not be considered advantageous to producing a normal haploid germ cell. With age, the oocytes in women are more likely to demonstrate chromosomal defects (733), increasing the risk of miscarriage, newborn death, or the birth of a child with chromosomal abnormalities. Although it is impossible to evaluate these possibilities at the time of oocyte-sperm fusion *in vivo* in the fallopian tube, it has become possible to perform some analysis of the oocytes *in vitro* in the ART clinic. Because the majority of aneuploidies arise in oocytes, the polscope has been used in some ART laboratories for noninvasive analysis of human oocytes over the last decade (reviewed in Ref. 734). The polscope is a polarized light microscope that can visualize birefringent structures in the oocyte. The two major structures that can be analyzed are the meiotic spindle, an important component necessary for producing a normal chromosomal complement, and the zona pellucida, the large, abundant, glycoprotein coat that surrounds the oocyte. By visualizing these two structures, the polscope allows a clinician to make judgments about the "normality" of an oocyte, and several groups have been able to make generalities about the best oocyte. In the original publication by Keefe and colleagues (735, 736), 61.8% of oocytes were fertilized when obvious meiotic spindles were observed with the polscope *vs.* 44.2% fertilized when the spindles were not observed with the polscope. Unlike fluorescent labeling techniques, the short time of exposure (10–20 sec) of the oocytes to the polarized light does not have any detrimental effects on the oocyte and its ability to produce viable embryos. Other studies have demonstrated that oocytes with longer spindle lengths (>12 nm) and wider inner zona pellucida layers (10–12 nm) resulted in more ICSI

fertilized oocytes that progressed to the blastocyst stage (737). Meta-analysis from multiple studies also confirmed the above findings that visualization of the meiotic spindle resulted in higher fertilization rates and better embryos based on multiple parameters (738). Surprisingly, there was no correlation with clinical pregnancy or implantation rate, although the polscope may be useful when there is a governmental limit to the number of oocytes that can be fertilized in one cycle.

A second method that has been used to make predictions about oocyte chromosomal quality is polar body biopsy (reviewed in Ref. 739). Polar body biopsy can be viewed as noninvasive because the removal of the first polar body does not alter the oocyte. Removal of the polar body can subsequently permit the analysis of the chromosomal remains after the first meiotic division by methods such as fluorescent *in situ* hybridization or comparative genomic hybridization. Thus, in situations where prenatal genetic diagnosis of a blastomere is either not permitted or is unwanted, this could be a viable noninvasive option for the couple. Similar to other noninvasive methodologies, this method cannot detect any meiotic defects that result at the second meiotic division, although biopsies of the second polar body are also possible, nor can it evaluate chromosomal defects that may arise from the paternal side at fertilization. However, first polar body biopsy has been used in combination with oocyte vitrification, and in this scenario may allow clinicians to make additional judgments about which oocyte is best (740).

Based on studies of cumulus cell-oocyte interactions in the mouse, our group and others have evaluated gene expression patterns of human cumulus cells as another noninvasive strategy to predict oocyte quality. Our studies were directed at three of the major targets of the oocyte-secreted growth factors GDF9 and BMP15, namely *HAS2*, *PTGS2*, and the BMP antagonist gremlin 1 (*GREM1*) (361). Because cumulus cells are stripped off the oocyte before ICSI and their gene expression is regulated by these oocyte-secreted growth factors, we hypothesized that poor quality or immature oocytes would not regulate expression of these cumulus cell genes to the same extent as high quality or mature oocytes. Our findings showed that expression of *PTGS2*, *HAS2*, and *GREM1* in cumulus cells derived from oocytes that were fertilized and developed into morphologically high-grade embryos (grades 3, 4, and 5) were 6-fold, 6-fold, and 15-fold higher, respectively, compared with cumulus cells derived from oocytes that gave rise to low-grade embryos (grades 1 and 2). A follow-up study by Cillo *et al.* (362) confirmed the *HAS2* and *GREM1* findings. Several groups have taken these studies one step further. Sirard's group (741) used cDNA microarrays and Affymetrix oligonucleotide microarrays to correlate gene expression in mural granulosa cells with oocyte quality and pregnancy. These studies identified 115 genes associated with competent follicles. Hamamah and colleagues (742) used Affymetrix microarrays to correlate cumulus cell gene expression with pregnancy outcome. These authors identified two genes, *BCL2L11* (BCL2-like 11) and *PCK1* (phosphoenolpyruvate carboxykinase 1), as being up-regulated and *NFIB* (nuclear factor I/B) as being down-regulated in cumulus cells of oocytes that led to a positive pregnancy outcome.

In summary, we believe that all of the above methods could be used to make the best predictions of oocyte quality, especially in environments where there are restrictions on embryo freezing or where all fertilized eggs, regardless of quality, must be transferred to the mother.

E. Stem cells and nuclear cloning

As mentioned in *Section I. D*, during embryogenesis there are sexually dimorphic differences in the pathways that male and female germ cells take; whereas male PGCs in the current somatic cell environment will arrest in mitosis, female PGCs will go through one last mitotic division and then enter and arrest in meiosis. Male germ cells will eventually become spermatogonial stem cells that can be isolated and shown to propagate in culture (although not yet successful for humans). However, because female germ cells enter meiosis, it makes it difficult to imagine a scenario where these differentiated cells can be dedifferentiated to become oocyte stem cells capable of replenishing a new ovarian environment. This may be one reason for the rapid depletion of oocytes over time and the eventual entry into menopause, which is in contrast to spermatogonial stem cells that remain throughout the lifetime of most male mammals.

Alternative strategies to reproduce "oocytes" *in vitro* have now been described (743). These studies demonstrate that follicle-like structures can form *in vitro* during mouse ES cell cultures and that cells that mimic oocytes (*i.e.*, express several oocyte markers such as GDF9, FIGLA, ZP1, ZP2, and ZP3 and can enter meiosis) are observed to form. Although fertilization and key events to recapitulate normal oocyte physiology have not been observed, these observations hold promise for future endeavors into this area including nuclear cloning (see below). At this point, studies have not been reported where human "oocytes" can be derived from human ES cells.

In parallel with the above situation, at least in mice, it has been possible for many years to convert PGCs into pluripotent embryonic germ cells (744–746). The growth factors LIF, KIT ligand, and FGF2 appear to be critical for this dedifferentiation event (747). Key reprogramming events during the formation of embryonic germ cells are the suppression of PRDM1 and the subsequent derepression of *Myc* and *Klf4* (Krüppel-like factor 4), the move-

ment of PRMT5 out of the nucleus and into the cytoplasm, and the early activation of the LIF/STAT3 pathway, with translocation of STAT3 into the nucleus. These studies not only make it possible to understand how PGCs form from embryonic germ cells by studying the process in reverse, but now also make it possible to go all the way back to totipotency by starting out with PGCs. Interestingly, it is now possible to produce pluripotent stem cells from mouse testis (748, 749) and adult human testis (750). Although not described so far, it is also possible that these XY stem cells could be used to make "oocytes" for additional manipulation *in vitro*.

Major advances in nuclear cloning have occurred recently, and it has even become possible to produce induced pluripotent stem (iPS) cells from multiple lineages (751, 752). One shortfall for mammalian nuclear cloning studies and in particular the production of designer ES cells for each individual has been the scarcity of oocytes for these studies. The new advances in the development of iPS cells may help to overcome this deficit. However, if iPS cells are not viewed as "replacement" for ES cells, the advent of the above nuclear cloning involves the injection of a diploid nucleus into an oocyte in which the nucleus was removed. This strategy was first used to create the sheep Dolly, the first mammal created via cloning (753). However, described techniques for oocyte cryopreservation may now make it possible to address the availability of oocytes as a limiting factor for nuclear cloning (754). Thus, continued advances in the ART laboratory may again help advance broad areas of medicine and healthcare.

VI. Future Perspectives

This review has focused on genetic models of ovarian development and folliculogenesis, and along the way, we have touched on numerous growth factors and signaling pathways implicated in these processes. Mouse ES cell technology has made it possible to study hundreds of genes in the ovary. By designing conditional alleles and taking advantage of the numerous transgenic Cre recombinase lines now available for studying gene function during formation of the germline and the gonad, and in oocytes and somatic cells of the ovary at different stages of postnatal development, we will continue to expand our knowledge of normal and pathological ovarian biology. Using *in silico* strategies and online databases to identify candidate genes with similar expression patterns to known ovarian genes is one way to find novel genes that may influence follicular development (755). The Ovarian Kaleidoscope Database is a useful online resource with up-to-date information on the expression, function, and regulation of genes in the ovary (756).

The prevalence of POF increases with age such that one in 100 women are affected by the time they are 40 yr old (213, 757). Frustratingly, the causes of POF in the vast majority of affected women remain unknown. Although women who are *FMR1* (fragile X mental retardation 1) premutation carriers make up the largest identified group with POF, they account for only 2% of nonfamilial cases (758), and the pathogenesis of ovarian failure in this cohort is still unknown. It is not even known whether the abnormalities in the *FMR1* premutation carriers leading to POF are primarily due to hormonal, somatic cell, or germ cell defects, or a combination of the three.

As reproductive biologists continue to identify genes with critical functions in folliculogenesis, we may also gain insight into additional candidate genes that contribute to POF, which may help physicians diagnose and treat women with this condition, as well as idiopathic infertility. Furthermore, the sequencing of the human genome, the follow-up International HapMap Project that identified polymorphisms within distinct ethnic populations (759), and the availability of multiple other tools for genome-wide analysis of patient DNA will help researchers and clinicians hone in on genomic regions associated with fertility disorders. This information, in conjunction with already available or easily generated mouse models, should contribute to a better understanding of the etiology of human infertility and potentially identify therapeutic targets for treating this condition. For example, the HapMap consortium identified several single nucleotide polymorphisms (SNPs) in *ACVR1*, which encodes a type I receptor (ALK2) for some TGFβ family members, in over 5% of Caucasians. Although there was no difference in the frequency of *ACVR1* SNPs between normovulatory women and women with polycystic ovarian syndrome, the SNPs were associated with elevated levels of AMH and follicle number in the women with polycystic ovarian syndrome (195). Thus, although the current approach to determining the etiology of nonsyndromic POF includes karyotype analysis, testing for premutations in *FMR1*, and testing for autoimmune disorders (including adrenal and thyroid) (213), additional genetic tests may become available in this postgenomics era.

Although there are many agents that reduce reproductive life span and induce reproductive senescence (*e.g.*, cigarette smoking), one of the major hurdles for reproductive biologists and clinicians is how to prolong and enhance the reproductive life span of a woman. Although dietary restriction and low body fat shut down reproductive cycling, as observed in long-distance runners, it is possible that these effects may not only prolong somatic life span but also reproductive (ovarian) life span as recently suggested (760, 761). Thus, these transient halts may ac-

tually be advantageous to the body and ovary alike. An intriguing article by Schmidt *et al.* (762) showed that male mice housed with females had a 20% longer reproductive life span than bachelor males. The reverse experiment has not been reported, so the question remains: Does the presence of a man extend a woman's reproductive life span?

In addition to employing genomic tools when evaluating fertility disorders, the advent of high-throughput genomic technologies, particularly next generation sequencing, will also broaden our knowledge of genetic events that lead to ovarian cancer. In a search for novel mutations, The Cancer Genome Atlas has sequenced 6000 candidate oncogenes and tumor suppressors, including miRNA-encoding loci, in an extensive panel of human serous epithelial ovarian tumors and matched normal controls (http://cancergenome.nih.gov). Large-scale collaborations are also under way to identify chromosomal aberrations, gene fusions, and cancer-initiating "stem" cells in serous and other ovarian carcinoma histotypes. These data will inspire the creation of novel mouse models to investigate tumorigenic mechanisms, especially for the less common mucinous and clear cell subtypes for which there are no genetic models. In parallel to mining the cancer genome, the merits of sequencing the cancer transcriptome are exemplified by the recent report that *FOXL2* mutations are pathognomonic for human adult granulosa cell tumors (587). Relatively few tumors need to be sequenced in the discovery phase of these studies, suggesting that a similar strategy may lead to the identification of specific mutations in human juvenile granulosa cell tumors, conceivably in TGFβ pathway genes or regions orthologous to the *Gct* susceptibility loci, based on insight gleaned from mouse models.

The discovery of specific functions for miRNAs in ovarian cancer has made them enticing therapeutic targets, especially as sensitizing agents for existing treatments considering their ability to modulate chemosensitivity. Although overexpression of miRNAs *in vivo* might be accomplished using shRNA constructs along with conventional gene delivery methods (*e.g.*, viral and liposomal), the inhibition of specific cancer-associated miRNAs may be achieved using modified oligonucleotides with high affinity for complementary RNA (antimiRs). Several groups have demonstrated efficient silencing of liver-specific mir-122 in mice using independently designed antimiRs (763–765). Importantly, Kauppinen and colleagues (766) reported similar results in nonhuman primates without any associated toxicities. By antagonizing mir-122, hepatitis C replication can be inhibited, thereby uncovering a novel approach to prevent chronic hepatitis C infection, a major cause of hepatocellular carcinoma (767). These groundbreaking studies suggest that miRNAs are promising and tangible molecular targets for the treatment of human cancers, including ovarian cancer.

Advances in the ART laboratory continue to occur. The rapidity with which recombinant FSH, LH, and hCG have become available for clinical use is remarkable, especially because the first reports demonstrating that these dimers can be effectively secreted were published in the mid-1980s. These recombinant proteins have played a major role in the fertility clinic, and the next generation of long-acting FSH analogs are making their way onto the scene, with corifollitropin alfa leading the way. The recent successes with the identification and synthesis of orally active small molecule LH receptor agonists and the findings of specific small molecule FSHR agonists suggest that a new arsenal of allosteric drugs may be available in the next decade to complement and expand existing treatment options for infertile couples. Future goals of ART should be to increase births of singletons and reduce multigestation pregnancies. New noninvasive methodologies could include identification of cumulus cell gene expression signatures that differentiate good and bad oocytes and the development of advanced metabolic and proteomic assays for oocyte- and embryo-secreted substances. All of this progress will undoubtedly increase the chances of a couple bringing a healthy newborn into their lives.

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