Deconstructing mammalian reproduction: using knockouts to define fertility pathways

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Abstract

Reproduction is the *sine qua non* for the propagation of species and continuation of life. It is a complex biological process that is regulated by multiple factors during the reproductive life of an organism. Over the past decade, the molecular mechanisms regulating reproduction in mammals have been rapidly unraveled by the study of a vast number of mouse gene knockouts with impaired fertility. The use of reverse genetics to generate null mutants in mice through targeted disruption of specific genes has enabled researchers to identify essential regulators of spermatogenesis and oogenesis *in vivo* and model human disorders affecting reproduction. This review focuses on the merits, utility, and the variations of the knockout technology in studies of reproduction in mammals.

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Introduction

Living forms are defined by their ability to reproduce. Although different organisms produce progeny using diverse methods (asexual and sexual reproduction, parthenogenesis, etc.), the basic principles of reproduction, both at the molecular and the organismal levels, have remained the same since the dawn of life: formation of gametes to facilitate the transfer of genetic material and epigenetic information to the next generation. Indeed, in several model organisms, orthologous genes have been identified that perform similar roles in reproductive physiology, thereby highlighting a conservation of this complex biological process.

Historically, two major obstacles have impeded the study of reproductive biology in mammals (reviewed by Staub 2001). First, germ cells in mammals develop in a microenvironment of supporting stromal cells of somatic origin that interacts with the former through autocrine/paracrine mechanisms as well as direct cell-to-cell interactions (Matzuk *et al.* 2002). Devoid of this support, isolated germ cells in culture fail to survive and maintain their characteristics, making it highly difficult to study molecular interactions and pathways that are unique to these cells. The second, but related, hindrance has been the inability to develop a proper cellular context *in vitro* that would allow undifferentiated germ cells to differentiate into specialized gametes that retain their ability to

fertilize and produce healthy offspring. Recently, interest has exploded in the field of *in vitro* germ cell differentiation (Lacham-Kaplan 2004, West & Daley 2004) due to exciting findings by several investigators who have been able to generate meiosis-competent germ cells from undifferentiated embryonic stem cells (Hubner *et al.* 2003, Toyooka *et al.* 2003, Geijsen *et al.* 2004). Although these gametes have been shown to fertilize oocytes and produce blastocysts, no data are available yet to suggest that these can progress to produce live offspring. In addition, Feng and coworkers (2002) have generated an undifferentiated spermatogonial cell line immortalized by the ectopic expression of the catalytic component of telomerase. The cell line was also shown to differentiate *in vitro* to morphologically similar haploid round spermatids but the authors did not investigate whether these haploid germ cells were competent to initiate fertilization and zygotic development when injected into oocytes. So, although the future looks promising, more careful analysis of the physiological state of these *in vitro*-formed gametes, including the identification of parent-of-origin-specific imprints, would be critical for enhancing the utility of these techniques.

Our understanding of the molecular mechanisms of mammalian reproduction therefore has been largely dependent on loss-of-function mutagenesis in mice. Ever since researchers developed techniques to culture mouse embryonic cells *in vitro* (Evans & Kaufman 1981) and

methods were developed to inject cultured embryonic pluripotent cells into blastocysts to produce germ-line chimeras (Bradley *et al.* 1984), the field of mouse transgenic technology has undergone a veritable explosion. Around the same time, researchers were successfully targeting exogenous DNA into endogenous loci based on limited amounts of homology between the two DNA sequences in cultured mammalian cells (Smithies *et al.* 1985). Simultaneously, Thomas and colleagues (Thomas *et al.* 1986, Thomas & Capecchi 1987) had developed a highly efficient method of targeted mutagenesis of cultured embryonic stem cells and other mammalian cells by using homologous recombination between endogenous genes and exogenously introduced DNA (targeting vector), a technique that was shown to be highly specific by Doetschman and coworkers (Doetschman *et al.* 1987, 1988). Our present-day knockout technology, a cornerstone of functional genomic studies, got its final form when these two technologies were harnessed together to generate the first targeted knockout mouse model through homologous recombination in embryonic stem cells (Koller *et al.* 1989).

A knockout mouse model is typically designed to produce a null mutation in the target gene and thus uncover an essential function. In dissecting gene functions, a hypomorphic mutation or the generation of an allelic series can at times be beneficial to researchers testing for genetic interactions between candidate genes. However, a null mutation is preferred in identifying non-redundant and essential functions of genes, as exemplified by the generation of approximately 300 mouse knockouts that manifest defects in reproduction (see below and online table that accompanies Matzuk & Lamb 2002).

Among the earliest mouse knockouts generated that were found to affect different aspects of reproduction were *Inha* (inhibin α), *Lif* (leukemia inhibitory factor), and estrogen receptor α (Matzuk *et al.* 1992, Stewart *et al.* 1992, Lubahn *et al.* 1993). While LIF-deficient females had defects in implantation, knockout males and females of inhibin α were infertile and had gonadal tumors. Female mice null for estrogen receptor α were infertile, and males had reduced fertility. Over the last decade, several hundred mouse knockouts have been created that affect different stages of reproduction (reviewed in Matzuk & Lamb 2002), including primordial germ cell (PGC) formation and migration into the gonads, gonad development and sex determination, postnatal gametogenesis, sperm-egg binding and fertilization, and early embryonic development. For more comprehensive background on these topics and the mouse models that have shed light on these stages of reproductive development, readers are referred to these excellent reviews: Bendel-Stenzel *et al.* (1998), Matzuk & Lamb (2002), Matzuk *et al.* (2002), Primakoff & Myles (2002), Zhao & Garbers (2002), de Rooij & de Boer (2003), Brennan & Capel (2004) and Park & Jameson (2005).

In this review, we focus, in a stage-specific manner, on specific knockouts that have proved pivotal in our

understanding of the biology of reproduction, with special emphasis on a few *in vivo* models that have clarified or refuted conflicting findings from *in vitro* experiments. We then briefly describe the principle of conditional mutagenesis and highlight its use in reproductive studies. Finally we discuss some of the current deficits of these technologies that have tempered the enthusiasm of researchers embracing them as their primary tools in functional genomics.

Germ-line specification: what seals the fate?

In most model organisms studied so far, the germ line is specified in embryos by a maternally derived source of egg cytoplasm referred to as the germ plasm. In mammals, however, germ-cell determination appears to be regulated within the classical developmental paradigm of cell signaling and cell adhesion.

In mice, PGCs are the founder cells of the germ line that are allocated from a pool of proximal epiblast cells at around embryonic day 7.2 (E 7.2; reviewed in Zhao & Garbers 2002). By using mice that were deficient for bone morphogenetic protein (BMP), member 4 (*Bmp4*^{-/-} mice), Lawson and colleagues (1999) provided clear evidence that germ-cell competence is induced in a subset of proximal epiblast cells in response to BMP4 secreted from the extra-embryonic ectoderm. In *Bmp4*^{-/-} mice PGCs were completely absent, and in *Bmp4* heterozygotes the PGC number was either greatly reduced or completely absent, suggesting that the effect of BMP4 is dose-dependent (Lawson *et al.* 1999). Deficiency of the related transforming growth factor β (TGF β) superfamily members, BMP8b and BMP2, led to a very similar phenotype of reduced or absent PGCs on a mixed genetic background (Ying *et al.* 2000; Ying & Zhao 2001). Interestingly, mice null for SMAD5 or SMAD1 (Chang & Matzuk 2001, Tremblay *et al.* 2001), the intracellular effectors of BMP signaling, have pronounced defects in PGC formation as well. More recently, knockout mice carrying a targeted mutation in the type I BMP receptor, activin receptor-like kinase 2 (ALK2), were found to have a complete absence of PGCs, further highlighting the importance of this signaling pathway in germ-cell specification (de Sousa Lopes *et al.* 2004).

While these studies suggest that an intact BMP signaling system in the embryonic stages is necessary for PGC fate specification, what is not clear is whether such a signaling mechanism is actually sufficient to induce PGC fate. What are the primary targets of BMP4 and BMP8b signaling in the epiblast? Do they signal directly to the PGC precursors? Is there a master regulator of PGC fate? These are some of the questions that remain unsolved. However, a recent study, utilizing a knockout in the mouse zinc-finger-containing transcriptional repressor PRDM1 (PR domain containing 1, with zinc-finger domain), has uncovered an essential role for this protein in PGC specification. Embryos lacking the PRDM1 protein have a complete absence of

PGCs while heterozygotes have a drastic reduction in PGC number (Vincent *et al.* 2005). Since *Prdm1* transcripts are expressed in PGC precursors as early as E 7.0, this is the first transcriptional factor to be identified that plays an essential role in PGC specification. So, is PRDM1 the master regulator that seals the fate of PGC precursors into entering a germ-cell lineage? The answer to that question awaits further studies, which would address key questions regarding the sufficiency of PRDM1 in specifying a germ-cell fate upon non-committed epiblast cells and whether its action is cell-autonomous in these precursors.

Studying gene function through knockouts has proved highly significant in the field of reproduction not only because of the uncovering of new genes and pathways but also because these *in vivo* studies on many occasions served to prove or refute several models that were based on either cell-culture-based assays or on evidence from forward-genetics approaches. One instance is noteworthy: by analyzing the expression pattern of genes from solitary nascent PGCs, Saitou and colleagues (2002) proposed a mechanism for acquisition of germ-cell competence and specification in PGC precursors in the epiblast which was based on essential roles for two highly expressed and unique cDNAs (named *fragilis* and *stella*) in PGC fate determination. However, when the *stella* gene was knocked out in mice, there appeared to be no defects in the specification, maintenance or proliferation of PGCs (Payer *et al.* 2003, Bortvin *et al.* 2004); instead, knockout males were fertile although null females failed to sire normal numbers of offspring as the gene fulfilled an essential role in the pre-implantation development of embryos as a maternally derived factor. In this context it would be interesting to know the phenotype of mice with a null mutation in *fragilis*.

PGC proliferation, migration and survival

Subsequent to their specification from precursor cells, PGCs undergo several rounds of proliferation till E 13.5, around when they enter cell-cycle arrest in G₀ within the

male gonad or meiotic prophase I in the female gonad. In between, they migrate to the gonads by E 10.5 (Molyneaux & Wylie 2004) after traveling through the hindgut and the dorsal mesentery in response to secreted cues from the environment.

Through knockout approaches, researchers have identified several new genes that play essential roles in some or all of these processes. Targeted disruption of a peptidyl-prolyl isomerase-encoding gene, *Pin1*, uncovered an unexpected role for this gene in the regulation of the cell cycle and PGC proliferation. On an isogenic C57BL/6J background, mice null for *Pin1* were found to have reduced PGC numbers during the mid-to-late migratory phases (E 9.5–12.5) compared with wild-type mice (Atchison *et al.* 2003); by analyzing BrdU labeling, this reduction seemed to be due to a delay in progression through the cell cycle rather than due to a cell-cycle arrest or enhanced apoptosis in these cells.

Specific genes regulate the survival of the migratory germ cells: the POU domain transcription factor, *Oct4* (*Pou5f1*; Mouse Genome Informatics), is a critical determinant of pluripotency in the inner cell mass that causes embryonic death when mutated (Nichols *et al.* 1998, Niwa *et al.* 2000). To circumvent the early embryonic death and to examine the effect of deletion of *Oct4* from PGCs, investigators have used *TNAP^{Cre}* (tissue non-specific alkaline phosphatase – Cre), which encodes the Cre recombinase only in the PGC population prior to E 10.5 (Kehler *et al.* 2004; Table 1). Surprisingly, loss of OCT4 protein from PGCs leads not to differentiation but to enhanced apoptosis in these cells prior to their colonization of the gonads.

Nanos3, a mouse homolog of the *Drosophila nanos* gene, represents another interesting knockout model in which the mouse homolog was found to perform an evolutionarily conserved role in flies and mammals (Forbes & Lehmann 1998). In *Nanos3^{-/-}* mice, PGC proliferation and/or survival during migration through the hindgut was severely disrupted, leading to sterility in both sexes. In further examples of evolutionary conservation of these

Table 1 Conditional mutants with defects in reproduction.

Gene	Cre driver	Tissue affected	Null phenotype	References
<i>Oct4</i> (<i>Pou5f1</i>)	<i>TNAP</i>	PGC	Increased PGC apoptosis	Kehler <i>et al.</i> (2004)
<i>Dax1</i> (<i>Nr0b1</i>)	<i>CMV</i>	Preimplantation embryo	Aberrant spermatogenesis, male infertility	Yu <i>et al.</i> (1998)
<i>Sox9</i>	<i>Sf1</i>	Fetal gonad	Male–female sex reversal	Chaboissier <i>et al.</i> (2004)
<i>Bmpr1a</i>	<i>Amhr2</i>	Müllerian duct	Persistent Müllerian-duct structures in males	Jamin <i>et al.</i> (2002)
<i>Sf1</i> (<i>Nr5a1</i>)	<i>Amhr2</i>	Leydig cells and granulosa cells	Semi-descended testis, impaired spermatogenesis; hemorrhagic cystic ovary	Jeyasuria <i>et al.</i> (2004)
<i>Ar</i>	<i>Amh</i>	Sertoli cells	Increased germ-cell apoptosis, spermatogenic arrest, testicular atrophy	Chang <i>et al.</i> (2004), De Gendt <i>et al.</i> (2004)
<i>Fst</i>	<i>Amhr2</i>	Granulosa cells	Decreased ovarian follicles, ovarian failure	Jorgez <i>et al.</i> (2004)
<i>GP130</i> (<i>Il6st</i>)	<i>TNAP</i>	Oocytes	Defective oocyte maturation/ovulation	Molyneaux <i>et al.</i> (2003a)
<i>Pig-a</i> (<i>Piga</i>)	<i>Zp3</i>	Oocytes	Defective sperm–egg binding, infertility	Alfieri <i>et al.</i> (2003)
<i>Pten</i>	<i>TNAP</i>	PGC	Testicular teratoma, increased embryonic germ cell proliferation	Kimura <i>et al.</i> (2003)
<i>Dnmt3a</i>	<i>TNAP</i>	Spermatogonia, oocytes	Absence of <i>de novo</i> parental imprints, sterility	Kaneda <i>et al.</i> (2004)

mechanisms knockout mice deficient in the chemokine stromal cell-derived factor-1 (Sdf1) and its cognate receptor CXCR4 (chemokine C-X-C motif receptor 4) show defective colonization of the gonads by PGCs (Ara *et al.* 2003, Molyneaux *et al.* 2003b).

PGCs that successfully navigate to the genital ridges enter cell-cycle arrest, although at different stages in the two sexes, as mentioned above. Heterozygosity for the transcription factor zinc-finger protein 148 (*Zfp148*) has been shown to increase apoptosis and cause complete loss of germ cells from the male gonad at the E 13.5 stage (Takeuchi *et al.* 2003), an effect that is probably due to improper regulation of the p53 protein in these cells. Thus *Zfp148* is one more addition to the growing list of genes that seem to affect PGC specification and maintenance in a dosage-dependent manner in mice.

Sex determination

Acquisition of reproductive competence in mammals involves the development of both germ cells and the supporting somatic cells that together constitute the gonad. The development of either the testis or the ovary from a bipotential gonad is a complex developmental process that is influenced by several transcriptional pathways. The first gene that was identified to play an essential role in determining the sex of the gonad was the Y-chromosome-encoded putative transcription factor *Sry*. In mice the *Sry* gene product has been shown to be both necessary and sufficient to impose a testis fate on the bipotential gonad (Lovell-Badge & Robertson 1990, Koopman *et al.* 1991). Although a recent report described a role for SRY in pre-mRNA splicing (Ohe *et al.* 2002), downstream targets of this master sex-determining gene remain elusive. Our understanding of sex determination and gonadal organogenesis therefore have been dependent on targeted mutagenesis of factors that have been implicated to regulate *Sry* or to affect gonad formation in other model organisms or through spontaneous mutations leading to sex-reversal phenotypes in humans and mice (reviewed in Brennan & Capel 2004). Among these, the X-linked gene encoding the orphan nuclear receptor DAX1 (NR0B1, nuclear receptor subfamily 0, group B, member 1; Mouse Genome Informatics) was initially believed to cause XY sex reversal

in mice when over-expressed in a *Sry* hypomorphic mutant background (Swain *et al.* 1998). Since over-expression of DAX1 led to the development of female reproductive structures from a bipotential gonad, albeit on a sensitized background, it was assumed that this gene was critical for ovarian development. Intriguingly, targeted deletion of *Dax1*, employing a conditional allele, does not lead to failure of ovarian development; on the contrary, postnatal spermatogenesis is impaired while null females are fully fertile (Yu *et al.* 1998). Furthermore, when the *Dax1*^{+/-} females were mated to males with the hypomorphic *Sry* allele, all XY offspring were sex-reversed and their external and internal genitalia were completely indistinguishable from their wild-type XX siblings (Meeks *et al.* 2003), suggesting that this gene interacts with *Sry* to determine gonadal sex in mice and is required not for ovarian development but for spermatogenesis, probably through its action in Sertoli cells.

It is critical to note, however, that the role of DAX1 is highly dependent on the genetic background of the mice under investigation. A recent study by Eicher and colleagues demonstrated that although DAX1 function is essential on the C57BL/6 (B6) background for testis development in XY mice, it is completely dispensable on the DBA/2J background (Bouma *et al.* 2005). In B6 *Dax1*⁻/Y mice, there was a failure of up-regulation of *Sox9*, which is another important gene in the sex-determination pathway (see below), even though *Sry* expression initiated normally.

The homeobox gene *Sox9* is the earliest marker of pre-Sertoli cells following *Sry* expression. Through the use of a conditional knockout model SOX9 has been shown to be essential for normal testis differentiation and sex-cord formation in the testis (Chaboissier *et al.* 2004). SOX9 regulates the differentiation of Sertoli cells and the production of male-specific markers *Amh* (anti-Müllerian hormone) and P450scc (cytochrome P450 side-chain cleavage), both of which are decreased in homozygous knockout mice. Indeed, this finding is a corroboration of earlier studies in which targeted disruption of the SOX9-binding site in the mouse *Amh* promoter caused a failure of transcription of *Amh* (see Table 2) that resulted in non-regression of the Müllerian duct structures in XY mice and the formation of pseudo-hermaphrodites, confirming that SOX9-mediated up-regulation of AMH is a critical and specific step that is

Table 2 Point mutagenesis, knockins and tissue-specific promoter ablations reveal promoter regions/specific isoforms essential for reproductive organ development and/or physiology.

Gene/regulatory element	Mutation	Phenotype	References
<i>Amh</i> promoter	SOX9-binding site	Pseudo-hermaphrodites	Arango <i>et al.</i> (1999)
<i>Pgr</i> (A isoform)	Mutation in AUG	Female infertility	Mulac-Jericevic <i>et al.</i> (2000)
<i>Wt1</i> (+KTS isoform)	Exon 9 splice site	XY sex reversal	Hammes <i>et al.</i> (2001)
<i>Wt1</i> (-KTS isoform)	Exon 9 splice site	Gonadal agencies	Hammes <i>et al.</i> (2001)
<i>Inhba</i>	<i>Inhbb</i> knockin	Delayed fertility in males, females subfertile	Brown <i>et al.</i> (2000)
<i>Dnmt1o</i>	Deletion of oocyte-specific promoter and exon 1	Loss of allele-specific imprints, embryonic lethality	Howell <i>et al.</i> (2001)

essential in the male sex-determination pathway (Arango *et al.* 1999). Precisely how AMH mediates regression of Müllerian duct structures *in vivo* has been characterized by the generation of a conditional knockout for one of the type I BMP receptors, *Bmpr1a* (Jamin *et al.* 2002); male mice lacking these receptors in mesenchymal cells of the Müllerian duct fail to respond to AMH and have persistent uteri and oviducts.

Two isoforms of the WT1 (Wilms' Tumor 1) protein, produced by alternative splicing, that differ in the presence or absence of 3 amino acids (KTS; the +KTS and -KTS forms) play unique roles in gonad development and sex determination. By introducing point mutations in the gene to create mutants expressing either one of the two isoforms, deficiency of the -KTS form was found to cause gonadal agenesis in both sexes whereas a loss of the +KTS form uncovered a critical role for this isoform in male sex determination as both XX and XY homozygotes developed along the female pathway (Hammes *et al.* 2001; Table 2). Since *Sry* expression was reduced in the KTS-ablated WT1 homozygotes, it can be inferred that WT1 + KTS is important for *Sry*-mediated XY gene program establishment in the bipotential gonad; whether this down-regulation is a direct result of WT1 + KTS loss or as a result of decreased numbers of *Sry*-expressing pre-Sertoli cells awaits further experiments.

Another gene involved in ovary development is *Wnt4*, which is required for Müllerian duct formation in both sexes prior to sex determination (Vainio *et al.* 1999); female mice lacking *Wnt4* exhibit partial female-male sex reversal. Since *Fst*-null ovaries have a similar defect and since *Fst* expression is absent in *Wnt4*-null XX gonads, *Fst*, operating downstream of *Wnt4*, has been implicated in the suppression of BMP and/or activin signaling to suppress the male-specific coelomic vessel (Yao *et al.* 2004).

Molecular control of pre-antral folliculogenesis

Folliculogenesis in the mammalian ovary is one of the most intricately regulated developmental processes in biology. Not only does normal folliculogenesis entail the complex interplay of both long-range endocrine cues and short-range paracrine and autocrine factors, the development of viable offspring from fertilization depends, to a large extent, on the structural and functional integrity of the mature oocyte (Matzuk & Lamb 2002, Matzuk *et al.* 2002). Physiologically, it is useful to visualize ovarian follicles as divided into two broad groups, each being characterized by the presence or absence of a morphological antrum (cavity). Follicles prior to the formation of an antrum are independent of the pituitary gonadotropins for growth and survival; these pre-antral follicles undergo an extensive growth phase once they have been recruited into the active pool although they remain arrested in a prolonged diplotene stage of meiosis throughout this phase. In contrast, around the period of antrum formation,

oocytes regain meiotic competence and oocytes in early antral follicles depend on pituitary follicle stimulating hormone (FSH) for survival and growth.

The formation of primordial follicles in the newborn mouse ovary is preceded by the development of germ-cell clusters in the embryonic ovary in which the dividing germ cells undergo incomplete cytokinesis to remain connected to each other through intercellular bridges and their subsequent breakdown perinatally (Pepling & Spradling 2001). The search for active players that regulate the formation of primordial follicles in mammals has resulted in the discovery of a bHLH (basic helix-loop-helix) transcription factor called FIG α (factor in the germ line α ; *Figla*) present specifically in the female germ cells. Mice carrying a targeted disruption of the *Figla* gene (*Figla*^{-/-}) had comparable number of germ cells in their ovaries with respect to wild-type ovaries at the E 18–19 stage (Soyal *et al.* 2000). However, by postnatal day 1 (PD 1) the oocytes in the *Figla*^{-/-} ovaries failed to grow in size and began to disappear; by PD 2 the null ovaries were completely devoid of primordial follicles. The *Figla*^{-/-} oocytes do progress through meiosis I, but fewer cells survive beyond the diplotene stage, suggesting that FIG α regulates the expression of survival factors/growth factors at this stage.

While *Figla* represents an oocyte-derived factor critical for the formation of primordial follicles, data from another mutant provide evidence that primordial follicle formation is regulated by the somatic components as well. Mutants lacking the *Wnt4* gene product die shortly after birth due to defects in kidney development; however, at day 1 the mutant ovaries have a dramatic reduction in the number of meiotic-stage oocytes and most oocytes degenerate shortly thereafter (Vainio *et al.* 1999). A similar finding has emerged from mice with follistatin-deficient ovaries (Yao *et al.* 2004), highlighting the functional interactions between these two proteins (see above).

Shortly after birth a subset of primordial follicles is selected from a resting pool of quiescent follicles to undergo the follicular activation and growth. Follicular activation is characterized by oocyte growth and the transition of squamous to cuboidal granulosa cells, which signals the formation of a primary follicle followed by granulosa cell proliferation. Work from our group and others has recently identified several proteins believed to be critical at this step. A homeobox transcription factor, NOBOX, which is specific to the oocyte in the newborn ovary (Rajkovic *et al.* 2004), is essential in this process. In *Nobox*^{-/-} ovaries, primordial follicles do not develop into primary follicles and beyond; instead, by PD 14, the majority of the primordial follicles are lost leading to ovarian failure and infertility. Future studies will illustrate the target genes for this transcription factor and uncover other oocyte-specific genes that play critical roles in this process.

Primordial follicles are recruited in cohorts for growth, differentiation, and maturation through a series of highly regulated processes. However, primordial follicles in

Foxo3a^{-/-} ovaries undergo global activation so that by PD 14 the null ovaries do not have any quiescent primordial follicles but have an almost 2-fold increase in the number of primary and secondary follicles as compared with wild-type ovaries. Significantly, the null ovaries also have an almost 4-fold increase in atretic follicles and an approx. 14-fold increase in the amount of zona pellucida remnants, suggesting that the majority of the growing follicles undergo atresia following the formation of the zona pellucida by the oocyte. Not surprisingly, therefore, this sequence of global activation and atresia leads to a premature depletion of the pool of primordial follicles and secondary infertility by 15 weeks of age (Castrillon *et al.* 2003, Hosaka *et al.* 2004).

Mutations in the human forkhead transcription factor *FOXL2* cause an autosomal dominant condition referred to as Blepharophimosis/ptosis/epicanthus inversus syndrome (BPES), associated with premature ovarian failure (Crisponi *et al.* 2001, De Baere *et al.* 2001). Mice with a targeted disruption in the murine ortholog (*Foxl2*^{-/-} mice) of the human gene are infertile and provide a mechanism to explain the etiology of BPES. In *Foxl2*^{-/-} mice granulosa cells in the mutant ovaries do not make the squamous-cuboidal transition and consequently the mutants lack any secondary follicles and are infertile (Schmidt *et al.* 2004). Another granulosa-cell derived factor that regulates the rate of follicle recruitment, in a subtler manner, is the anti-Müllerian hormone (AMH), which, when secreted from the granulosa cells of secondary follicles, acts in a paracrine fashion within the follicle to inhibit the recruitment of primordial follicles. In *Amh*^{-/-} females there is a derepression of the follicular activation process causing mutant ovaries to be depleted of primordial follicles earlier than in wild-type ovaries (Durlinger *et al.* 1999).

Growth differentiation factor 9 (*Gdf9*) is an oocyte-derived mitogenic paracrine factor that plays a critical role in folliculogenesis by regulating the somatic components of the follicle. *Gdf9* encodes for a member of the TGF β superfamily of secreted proteins that play important roles in cell proliferation and differentiation in mammals (Chang *et al.* 2002). Members of this family, such as GDF9, BMP15, AMH, and activins, exquisitely regulate early stages of folliculogenesis. In mice, a null mutation in *Gdf9* causes complete infertility in female mice (Dong *et al.* 1996). Histopathological examination of null ovaries demonstrate a total lack of normal appearing secondary follicles indicating that GDF9 secreted from oocytes acts upon the granulosa cell population to augment the proliferation/differentiation of these follicular supporting cells. Thus, GDF9 is essential for the primary-to-secondary follicle transition in mice. The oocyte that has already entered the growth phase following activation continues to grow in these follicles (Carabatsos *et al.* 1998); however, devoid of the close support from the granulosa cell mass there is atresia of both the oocyte and eventually the somatic cells.

Another member of the TGF β superfamily of proteins that appears to play an important role in pre-antral stages of folliculogenesis is BMP15. BMP15 is secreted from oocytes and regulates the proliferation and differentiation of the granulosa cell population. Analysis of a null mutation in *Bmp15* generated in our laboratory revealed that *Bmp15*^{-/-} mice are subfertile and display defects in ovulation and fertilization (Yan *et al.* 2001). However, studies in sheep have shown that BMP15 may also play important roles at the primary to secondary follicle transition (Galloway *et al.* 2000) or, alternatively, the mutant BMP15 may antagonize the functions of GDF9 (Liao *et al.* 2004).

Spermatogenesis

In adult mammals, spermatogenesis is one of the most highly productive biological processes; one study (Johnson *et al.* 1992) done on human testis specimens obtained at autopsy estimated that the daily sperm production ranges between 3.5 and 7.5 million sperm/g of testis parenchyma. This tremendous capacity to produce spermatozoa throughout the life of an individual rests solely on the self-renewing capacity of spermatogonial stem cells, which constitute a small group of pluripotent cells present in the mature testis. Concomitant with the identification of molecular markers that identify the spermatogonial stem-cell pool (Kubota *et al.* 2004) has come new knowledge on the regulation, survival and proliferation of these rare populations of adult stem cells *in vivo* through knockout studies. Here we briefly review some of these models that have illustrated the utility of the targeted knockout approach.

Among the first mutations that were identified to have a defect in spermatogonia were the *W* and *Sl* mutations (Russell *et al.* 1979) that affect the Kit receptor, which is present on differentiating spermatogonia and its ligand, the Stem cell factor, which is produced in the Sertoli cells. However, mutations in the Kit signaling system affect only the differentiated spermatogonia as the receptor Kit is not expressed on earlier spermatogonial populations (Donovan & de Miguel 2001). Recently, glial cell-derived neurotrophic factor (GDNF), another Sertoli cell-derived factor distantly related to the TGF β family of growth factors, was shown, in a dose-dependent manner, to control the cell-fate decision of undifferentiated spermatogonia; reduced levels of GDNF caused spermatogonial differentiation and depletion of the stem-cell pool while high levels *in vivo* caused a differentiation block and increase in the numbers of spermatogonia (Meng *et al.* 2000). Germ-cell factors that play equally important roles in the proliferation of undifferentiated spermatogonia have also been identified through mouse knockouts; prominent has been the identification of a zinc-finger-containing transcriptional repressor, *Plzf*, that is essential for stem-cell self-renewal in the adult testis (Buas *et al.* 2004, Costoya *et al.* 2004). Although the precise genes that are regulated

by this transcription factor are not yet known, future studies utilizing transcriptome analysis of spermatogonia similar to the approach used by Costoya and colleagues (2004) should lead to further insights into the molecular mechanisms of germ-line stem-cell biology.

Knockouts have been created that identify essential genes at almost all steps of spermatogenesis (reviewed in Matzuk & Lamb 2002, de Rooij & de Boer 2003); literally hundreds of mutant mice have been characterized with arrest at various stages such as a spermatogonial differentiation block, pachytene stage meiotic arrest, and defects in spermiogenesis and sperm function.

Several genes that are expressed in post-meiotic cells have been found to be essential for spermiogenesis in a dose-sensitive manner. Notable among these are mutations in the genes encoding protamines 1 and 2 (*Prm1* and *Prm2*), Kelch-like 10 (*Klhl10*) and sperm antigen 16 (*Spag16*), haploinsufficiency of which are incompatible with male fertility (Cho *et al.* 2001, Yan *et al.* 2004, Zhang *et al.* 2004). Male mice that are high-percentage chimeras of these genes are unable to transmit the mutant alleles through the germ line due to either severe morphological defects (as in the *Prm1* or *Prm2* mice) or severe reduction of sperm count and defects in sperm morphology and function (*Klhl10* or *Spag16*). During spermiogenesis, chromatin packaging in the sperm head requires the replacement of histones by the transition proteins and protamines. Similar to protamines, deficiency of both transition proteins 1 and 2 also leads to severe defects in sperm morphology and infertility (Zhao *et al.* 2004), thereby highlighting the importance of proper chromatin remodeling during spermatogenesis.

Defects in sperm motility also lead to infertility; analysis of knockouts of *CatSper1* (Ren *et al.* 2001) and *CatSper2* (Quill *et al.* 2003), which are cationic channels present on the sperm membrane, a sperm-specific isoform of a glycolytic enzyme, *Gapdhs* (Miki *et al.* 2004), and of structural components of the axoneme (*Spag6*, *Tekt2*) have revealed interesting models of asthenozoospermia (Sapiro *et al.* 2002, Tanaka *et al.* 2004) that lead to infertility in male mice. Finally, knockouts have uncovered genes that play essential functions in the final step of sperm function, sperm-egg binding (reviewed in Evans 2001, Primakoff & Myles 2002). The plethora of single-knockout models that impair spermatogenesis at so many discrete steps is in addition to numerous models that cause a hitherto undefined or non-specific block in spermatogenesis and as a whole serve to illustrate both the complexity of this process and the huge number of potential targets for development of contraceptives in humans.

Endocrine, paracrine and autocrine regulation of gametogenesis

Germ cells are dependent on the somatic cells of the gonad (Sertoli and Leydig cells in the testis; granulosa and

theca cells in the ovary) for development, which are in turn regulated by gonadotropins secreted by the pituitary, namely FSH and luteinizing hormone (LH). Deficiency of the pituitary gonadotropins leads to hypogonadism in both sexes, as in the *hpg* (hypogonadal) mouse, which carries a naturally occurring deletion in the gonadotropin-releasing hormone (GnRH) gene and consequently are infertile with reduction in the size of the gonads and accessory sex organs as well as a block in gametogenesis (Mason *et al.* 1986). Since *hpg* mice lack both LH and FSH, the relative contributions of each of these gonadotropin hormones to the phenotypic spectrum was not clear until our group and others created knockouts of four different genes in mice: *Fshb* (FSH β subunit), *Fshr* (FSH receptor), *Lhb* (LH β subunit) and *Lhr* (LH receptor). Analyzing the effects of these single knockouts revealed that FSH and LH had non-redundant roles in both sexes. In males, loss of FSH signaling resulted in smaller testis size, reduced spermatogenesis, and impaired sperm motility although the males were fertile (Kumar *et al.* 1997, Dierich *et al.* 1998). In contrast, females deficient for a functional FSH signaling pathway are sterile with thin uteri and hypoplastic ovaries due to a block in folliculogenesis prior to antrum formation (Kumar *et al.* 1997). An intact LH signaling pathway, however, is essential in both sexes as both males and females deficient for either LH β subunit or its cognate receptor develop hypogonadism and infertility (Lei *et al.* 2001, Zhang *et al.* 2001, Ma *et al.* 2004). Furthermore, folliculogenesis is blocked in LH-deficient ovaries at an early antral stage, suggesting that the progression from secondary to early antral follicle stage is dependent on FSH while LH is required for further development. Similarly in males, spermatogenesis is blocked at the round spermatid stage, revealing an absolute requirement for only LH in further maturation of the haploid gametic cells.

Sex hormones (estrogen and progesterone in females, testosterone in males) have been long believed to play essential roles in the development, differentiation and proper function of female and male reproductive systems. Targeted knockouts of their respective receptors have revealed precisely how these steroid hormones function and have uncovered hitherto unsuspected roles for estrogen signaling in males and testosterone in females. Estrogen acts through two nuclear hormone receptors, ER α and ER β ; whereas deletion of ER β leads to subfertility (Krege *et al.* 1998) due to defects in granulosa cell development (Dupont *et al.* 2000), disruption of ER α results in female infertility due to a complete lack of ovulation with the null ovaries displaying hemorrhagic cysts (Lubahn *et al.* 1993, Dupont *et al.* 2000). Interestingly, ER α -knockout males are infertile as well due to a defect in fluid reabsorption in the epididymis that leads to degeneration of seminiferous tubules and disruption of spermatogenesis (Eddy *et al.* 1996, Hess *et al.* 1997), thereby identifying a male-specific role for estrogen. Progesterone, which also acts through two receptor isoforms (PR-A and PR-B), was believed to be important for the establishment and

maintenance of the pregnant state; however, deletion of the progesterone receptor in females led to infertility due to defects in ovulation with associated uterine inflammation and disruption of mammary gland morphogenesis (Lydon *et al.* 1995). Identifying which of these effects were mediated by the two receptor isoforms required some subtle manipulations because both isoforms were generated from the same gene from alternative promoters and translation initiation sites. In elegant experiments, Mulac-Jericevic and colleagues (Mulac-Jericevic *et al.* 2000, 2003) mutated the two AUG initiation codons in embryonic stem cells and generated PR-A and PR-B knockouts (Table 2). Analysis of these mutants revealed essential roles for PR-A in ovulation and uterine physiology while PR-B was important for mammary gland development.

Studies from the spontaneous *tfm* (testicular feminized) mutant and from androgen receptor-knockout mice (Yeh *et al.* 2002) have shown androgen signaling to be essential for the descent of testes, development of male accessory sex organs, and spermatogenesis beyond the pachytene spermatocyte stage. Recently, generation of Sertoli cell-specific knockout of the androgen receptor have clarified the roles of testosterone signaling by demarcating androgen signaling in these cells as essential for spermatogenesis but not for non-gonadal functions of testosterone (Chang *et al.* 2004, De Gendt *et al.* 2004). Interestingly, signaling through the androgen receptor has also been found to have important roles in female fertility

as null females show longer estrous cycles and reduced fertility (Hu *et al.* 2004).

Studies from our group and several others have also discovered the essential roles played by multiple TGF β family members in reproduction (reviewed by Pangas & Matzuk 2004, Jorgez *et al.* 2005; see Fig. 1). Among these, the *Inha* mouse model revealed unexpectedly important roles for the protein in gonadal cell-cycle regulation as null animals developed gonadal tumors of granulosa cell/Sertoli cell origin (Matzuk *et al.* 1992). As identified by our group and others, also important in reproduction are the activin signaling components; activin receptor type II mutants develop testicular hypoplasia and activin β B subunit mutants have defective parturition (Vassalli *et al.* 1994, Matzuk *et al.* 1995). In order to analyze the functional interaction between activin β A and activin β B, our group also created a knockin allele of β B into the β A locus (Table 2). The knockin rescued the neonatal lethality of the β A-knockout mice, thus allowing dissection of the reproductive phenotype (Brown *et al.* 2000). While male knockin mice had delayed fertility, female mice had severely reduced fertility, thus highlighting the important roles played by activin β A in mammalian reproduction. Also significant is the follistatin conditional knockout model in which the activin/BMP/GDF antagonist follistatin is deleted only in the granulosa cells of the ovary. Female mice with a granulosa cell-specific deletion of follistatin have severely reduced fertility due to reduced numbers of ovarian follicles and defects in ovulation and fertilization

Figure 1 TGF β superfamily signaling in germ-cell formation, sex determination, and ovarian folliculogenesis. Null mutations in several members of this family result in an arrest in the development of the reproductive system. Whereas *Smad5*^{-/-} embryos lack PGCs at E 8.0 (Chang & Matzuk 2001), *Fst*^{-/-} mice have a loss of germ cells at E 18.5 (Yao *et al.* 2004). Oocytes from *Bmp15*^{-/-}/*Gdf9*^{+/-} double mutants display defective cumulus-cell adherence to the oocytes (Yan *et al.* 2001). WT, wild type; ME, medulla; CO, cortex; ACVR2, activin receptor type 2.

(Jorgez *et al.* 2004). These and other models (reviewed by Chang *et al.* 2002) have identified an enormous number of proteins belonging to the TGF β superfamily with essential roles in reproduction.

Early embryo: the maternal effect

In the early embryonic period prior to zygotic genome activation, maternally supplied proteins play critical roles in pre-implantation development. The first such maternally derived factor identified was Mater (*Nalp5*). Embryos lacking maternal NALP5 protein arrested at the two-cell stage and degenerated, thereby suggesting an essential role of maternal NALP5 protein in embryonic development (Tong *et al.* 2000). Maternally supplied oocyte-specific isoform of the DNA methyltransferase 1 (*Dnmt1o*) was also found to be essential for maintenance of methylation at imprinted loci in embryos; heterozygous embryos of null females died *in utero* due to alteration of allele-specific expression from imprinted loci (Howell *et al.* 2001; Table 2). Methyltransferases that are implicated in initiation of methylation at imprinted loci (*Dnmt3l* and *Dnmt3a*) have also been demonstrated to be essential maternally supplied proteins for embryonic viability (Bourc'his *et al.* 2001, Kaneda *et al.* 2004). Recently our group and others have added to the list of maternal effect genes in mice. First, female mice null for an oocyte-specific gene *Zar1* (zygote arrest 1) were found to be infertile due to a failure of progression of fertilized embryos from the one- to two-cell stage (Wu *et al.* 2003); in the arrested embryos the parental genomes remain separated and there is a marked reduction in the synthesis of the transcription-requiring complex. Thus *Zar1* seems to act at an earlier time-point than *Mater* in embryonic development. Next, a mouse homolog of *Xenopus* nucleoplasmin (nucleoplasmin 2, *Npm2*) was identified to be critical in the process as well. Embryos from *Npm2*-null females had defects in nuclei and nucleolar organization specifically evident in the loss of heterochromatin from these organelles (Burns *et al.* 2003). Although, some embryos are able to proceed through the two-cell stage and diminished numbers of offspring are born from null females, most embryos show reduced cleavage to the two-cell stage. Another recent maternal-effect factor to be identified is Stella or *Dppa3* (developmental pluripotency-associated 3). Embryos from *Dppa3*^{-/-} females progress to the four-cell stage but are defective in further cleavage and in pre-implantation development (Payer *et al.* 2003, Bortvin *et al.* 2004). Future experiments utilizing *in silico* approaches to identifying early-embryo-enriched genes would provide more candidate genes that might play important roles during this phase of embryonic development.

Conditional mutagenesis

Although a knockout mouse is a valuable resource to analyze the effects of a null mutation in a tissue of

interest, in a large number of cases the study of the phenotype is precluded by a more widespread effect caused by the mutation (for example, embryonic or perinatal lethality of homozygotes). Also, distinguishing between the cell-autonomous and cell-non-autonomous nature of action of a gene of interest is not possible in a ubiquitous knockout. Researchers have long recognized this drawback of the traditional knockout approach and have sought to develop technologies that would allow ablation of a gene function in a specific tissue and at a specific time: this is the rationale behind the conditional mutagenesis approach. Over the years refinement and innovation have led to a whole set of new tools with which conditional knockouts are achieved *in vivo* at present (see Lewandoski 2001 for a detailed review); here we discuss the advantages and pitfalls of the use of the binary Cre-*loxP* technology in the generation of conditional mutants within the context of reproduction. For a list of available Cre lines, go to www.mshri.on.ca/nagy/Cre-pub.html and see Table 1; for a list of available floxed alleles, go to www.mshri.on.ca/nagy/floxed.html.

The binary Cre-*loxP* method is so designed that the bacteriophage recombinase Cre is driven by a tissue- or stage-specific promoter in a restricted spatio-temporal manner; the deletion of gene function in that tissue is then dependent on the Cre recombinase to bind to its cognate recognition sequences (*loxP*) sites and effect a DNA recombination event between any two of such *loxP* sites, which can lead to deletion, inversion and other genomic rearrangements based on the position and orientation of such sites. The gene (or more commonly, the gene region) to be deleted is flanked by *loxP* ('floxed') sites in a targeting event similar to a conventional knockout; floxed mice are then bred with mice expressing Cre under the regulation of a promoter of choice to obtain tissue-specific knockouts in the offspring. Using this method several conditional knockouts have been generated that display reproductive defects (see Table 1); indeed, some of them were created after conventional knockouts resulted in embryonic or post-natal lethality (*Oct4* and *Fst*).

One apparent deficit of this technology in reproduction is the paucity of available Cre- driver lines that are specifically expressed in reproductive tissues of interest. Another problem relates to the fact that our restricted knowledge of gene-regulatory elements results in use of promoter regions that prove to be 'leaky', meaning that they drive Cre expression and consequently gene deletion outside of their specific spatio-temporal zone (for example the *Sf1*-Cre line), thus defeating the very purpose of creating a conditional knockout. The last problem, and not a trivial one, is the issue of the efficiency of Cre-mediated recombination. Cre lines often express the recombinase at levels that are not sufficient to achieve complete recombination between *loxP* sites, thus resulting in the generation of a hypomorph. Attention has been focused on producing

more efficient deleter Cre lines (Shimshek *et al.* 2002) but we are still some distance from achieving mastery over this elegant but at times difficult technology. Nevertheless, with the intensive efforts of various groups and the advent of newer technologies, for example inducible Cre lines, the *Flp-Frt* system, it is conceivable that in the future, instead of one cure for all ills, the field will witness the use of a multitude of technologies each best tailored in a particular situation to deliver the proper result: that of generation of a null mutation in the tissue of interest.

In the future

Worldwide, 15% of couples are unable to conceive within 1 year of starting unprotected intercourse (Matzuk & Lamb 2002). Although fertility defects may be the secondary manifestation of systemic illnesses (endocrinopathies, neurological disorders, anatomic malformations), which may or may not be treatable, often the defect is primary to the reproductive system and requires specialized evaluation of urologists and/or gynecologists and fertility specialists for possible treatment. However, even with advanced clinical tools for diagnosis of reproductive defects, about a quarter of clinical infertility cases are diagnosed as idiopathic (Matzuk & Lamb 2002). Since the identification of the genetic bases of a complex disorder such as infertility cannot be done through traditional genetic approaches of pedigree analysis and linkage studies, our understanding of the genetics of reproduction in humans has remained minimal and is limited to a few documented examples such as the discovery of Y-chromosome microdeletions in azoospermic men or the findings of cystic fibrosis transmembrane conductance regulator (CFTR) mutations in patients with congenital bilateral absence of the vas deferens. This raises the possibility that some of these 'idiopathic' cases are likely due to as-yet unidentified genetic anomalies.

In this review we have illustrated some examples of how knockouts in mice have greatly enhanced our understanding of this complex biological process. This is not meant to be a comprehensive survey of all knockouts with reproductive defects but rather an illustrative essay of the utility of the knockout approach towards understanding a process. Challenges remain plenty in both our understanding of the process and in the use of this technology itself, but the emergence of exciting technologies and projects such as RNA interference in mammals (reviewed in Mittal 2004), the MICER project for genome-wide large-scale insertional mutagenesis from indexed targeting vectors (Adams *et al.* 2004), and the recent conceptualization of a whole-genome knockout mouse project, or KOMP, by an international collaboration (Austin *et al.* 2004) will broaden the scope and increase the enthusiasm of researchers in the power of knockouts and knockdowns in evaluating the roles of additional proteins in the reproductive axis *in vivo*.

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