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Minireview

Oocyte-Specific Knockout: A Novel In Vivo Approach for Studying Gene Functions During Folliculogenesis, Oocyte Maturation, Fertilization, and Embryogenesis[1]

Qing-Yuan Sun,[3,4] Kui Liu,[5] and Kazuhiro Kikuchi[2,4]

State Key Laboratory of Reproductive Biology,[3] Institute of Zoology, Chinese Academy of Sciences, Beijing 100080, China
Division of Animal Sciences,[4] National Institute of Agrobiological Sciences, Tsukuba, Ibaraki 305-8602, Japan
Department of Medical Biochemistry and Biophysics,[5] Umeå University, SE-901 87 Umeå, Sweden

ABSTRACT

Knockout mice have been highly useful tools in helping to understand the functional roles of specific genes in development and diseases. However, in many cases, knockout mice are embryonic lethal, which prevents investigation into a number of important questions, or they display developmental abnormalities, including fertility defects. In contrast, conditional knockout, which is achieved by the *Cre-LoxP* system, can be used to delete a gene in a specific organ or tissue, or at a specific developmental stage. This technique has advantages over conventional knockout, especially when conventional knockout causes embryonic lethality or when the function of maternal transcripts in early development needs to be defined. Recently, a widely used practice has been used to specifically delete genes of interest in oocytes: *Zp3-Cre* or *Gdf9-Cre* transgenic mouse lines, in which Cre-recombinase expression is driven by oocyte-specific zona pellucida 3 (*Zp3*) promoter or growth differentiation factor 9 (*Gdf9*) promoter, are crossed with mice bearing floxed target genes. This novel in vivo approach has helped to increase the understanding of the functions of specific genes in folliculogenesis/oogenesis, oocyte maturation, fertilization, and embryogenesis. In this minireview we discuss recent advances in understanding the molecular mechanisms regulating major reproductive and developmental events as revealed by oocyte-specific conditional knockout and perspectives on this technology and related studies.

conditional knockout, Cre-recombinase, embryo, embryogenesis, fertilization, follicular development, folliculogenesis, gamete biology, meiosis, oocyte

INTRODUCTION

Knockout technology allows scientists to replace or remove a specific gene of interest in order to define its function. This process includes the construction of targeting vectors, introduction of the vectors into embryonic stem (ES) cells, and injection of selected ES cells bearing mutated genes into blastocysts, followed by the production and propagation of germ-line transmission knockout mice. This manipulation knocks out both alleles and causes complete absence of the gene (null) from the cells. Knockout mice have been widely used to determine the role of a specific gene in development and its contribution to a particular disease. Conventional knockout technology is particularly useful for determining the in vivo function of tissue- or cell type-specific genes. For example, knockout mice lacking the oocyte-specific proteins GDF9 or ZP3 are infertile due to a block in folliculogenesis at the primary follicle stage and an absence of zona pellucida, respectively [1, 2]; knockout of *Figlα*, a transcription factor implicated in postnatal oocyte-specific gene expression, causes absence of ZP1, ZP2, or ZP3 expression, massive depletion of oocytes, and sterility [3]; additionally, spermatozoa from beta 1,4-galactosyltransferase-null mice are refractory to ZP3-induced acrosome reaction and have a low ability to penetrate the zona pellucida [4]. Although conventional knockout technology is useful, it has limitations, as many knockout mice die at different stages of embryo development before investigators are able to use them for experimentation or they have developmental abnormalities, including fertility defects.

Conditional knockout, which is based on tissue- and cell type-specific deletion of the gene of interest, has advantages over conventional knockout, especially when the conventional knockout causes embryonic lethality. The most widely used approach is the *Cre-LoxP* system, which involves a "floxed" mouse line bearing alleles of the gene to be deleted with recombinase-specific sites (i.e., two *LoxP* repeats flanking critical exons) and a transgenic mouse line expressing the Cre-recombinase guided by a promoter with a desired temporal and/or spatial pattern. The gene of interest to be deleted is flanked by *LoxP* sites in a targeting event similar to a conventional knockout, and the gene function remains normal in targeted animals. Cre is a 38-kDa recombinase that recognizes the *LoxP* (locus of crossover of P1) site, which is a 34-kb sequence consisting of two 13-bp inverted repeats separated by an 8-bp directional spacer [5]. Cre-recombinase is a type1 topoisomerase, and it mediates the DNA recombination between two *LoxP* sites with the same orientation into a single *LoxP*,

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[2]Correspondence: Kazuhiro Kikuchi, Division of Animal Sciences, National Institute of Agrobiological Sciences, Kannondai 2-1-2, Tsukuba, Ibaraki 305-8602, Japan. FAX: 81 298 38 7447;
e-mail: kiku@affrc.go.jp

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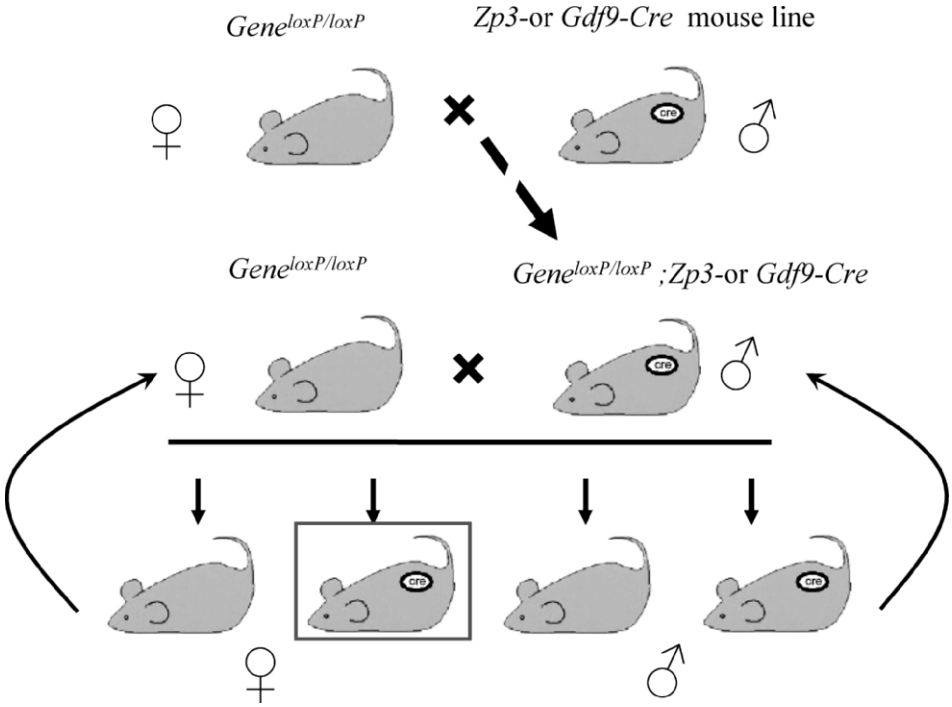

FIG. 1. An example of breeding schemes to generate oocyte-specific gene knockout mouse lines. Males carrying *Zp3-Cre* or *Gdf9-Cre* transgene are crossed for two generations (shown by the broken arrow) with females in which the target gene is flanked by *LoxP* sequences to obtain homozygous *Gene$^{loxP/loxP}$;Cre* mouse lines. Females with this genotype (boxed) are used for experimentation to study the function of the target gene in oocytes or early embryos, and males can be used for further breeding. In each generation, mouse genotypes are determined by genotyping. Specific for oocyte-specific knockout experiments, only males (but not females) carrying the *Cre* transgene can be used for breeding, because in females the CRE activity in oocytes can cause DNA recombination in the zygotes, and thus universal knockout of target genes in the whole body, which is not different from the traditional knockout. Both *Zp3-Cre* and *Gdf9-Cre* are not expressed in male germ cells.

allowing the excision of the target gene. Cre-recombinase expression guided by a tissue- or cell type-specific promoter exerts its functions, which allows examination of gene function in a specified tissue or cell type. Both the Cre-recombinase expression level in the transgenic mice and the selection of the targeted construct are critical for the successful deletion of the gene. After the germ line transmission occurs and the mice are bred to homozygosity, the floxed mice are bred with Cre mice to null the gene of interest. These mutant mice usually have no evident developmental abnormalities and thus can be used for studies on a gene's function in a specified tissue or cell type. Figure 1 shows an example of breeding schemes to generate oocyte-specific gene knockout mouse lines. There are several ways to detect whether oocyte-specific depletion was successful. One can collect wild-type and mutant oocytes and perform RT-PCR or Western blot analysis to detect the expression levels of mRNA or protein products of target genes. In some cases, one can also perform PCR to detect the deletion in genomic DNAs.

In this minireview, we have summarized the advantages of the conditional knockout technology and the application of oocyte-specific deletion of targeted genes as a novel in vivo approach for understanding molecular mechanisms regulating folliculogenesis, oocyte maturation, fertilization, and embryogenesis as well as related recent progress.

ADVANTAGES OF *Cre/LoxP* TECHNOLOGY OVER TRADITIONAL KNOCKOUT TECHNOLOGY

Examining the Function of Genes Whose Knockout Causes Lethality or Defect

In many cases, deletion of genes by conventional knockout causes embryonic and perinatal lethality or the surviving offspring exhibit developmental defects. The early lethal phenotypes prevent us from elucidating functions of genes in later development and in adulthood. This problem can be overcome by selective deletion of a gene in a chosen tissue or cell type [6]. Conditional knockout, achieved by crossing a line of floxed mice with normal expression of a targeted gene and a line with Cre-recombinase expression driven by a tissue- or cell type-specific gene promoter, allows us to analyze the pathophysiological functions of a gene of interest without affecting survival of the animal.

Examining the Role of Maternal Transcripts

A mature mammalian oocyte is transcriptionally quiescent, and early embryo development depends on translation of maternal mRNAs that have been synthesized during oocyte growth. Depending on the species, embryonic genome activation occurs at different stages of early embryo development, for example, at the 2-cell stage in mice and the 8–16 cell stage in cows. Maternal mRNAs are required for the final phase of oocyte maturation, fertilization, and early embryo development [7]. Deletion of many maternal transcripts will cause infertility and/or subfertility of homozygous females, which limits the study of the function of maternal transcripts [8]. By using *Zp3* or *Gdf9* promoter, it is possible to direct *Cre* expression and inactivate target genes in the female germ cells, thus allowing analysis of the function of transcripts in embryogenesis.

Other Advantages

The *Cre-LoxP* system can also be used to analyze cell lineage in mammals. Using an enhanced green fluorescent protein cDNA as a noninvasive reporter and *lacZ* gene as a histochemical marker, Sato et al. [9] found that this approach is of practical use in tracing certain embryonic cells that have been genetically marked by transiently expressed Cre protein at least during preimplantation development in the mouse. In addition, the *Cre-LoxP* system could be used as a tool for conferring increased levels of tissue-specific gene expression from a weak promoter. Most tissue-specific promoters are weak compared with stronger but constitutively expressing viral promoters. Recently, Nakamura et al. [10] established a system by combining a weak tissue-specific promoter with the

Cre-LoxP system to enhance the strength of tissue-specific promoters in vitro and in vivo, which may have a potential application in studying gene functions in ovarian cells.

OOCYTE-SPECIFIC DELETION OF TARGET GENES THROUGH *Zp3-Cre* AND *Gdf9-Cre*

There are numerous genes expressed in oocytes that are not oocyte-specific, and their deletion by conventional knockout causes embryonic lethality. For example, many genes function in both mitosis and meiosis, and knockout of these genes will cause failure of embryo development. Even if germ line deletion of these oocyte-expressing genes could bypass embryo lethality, the female reproductive phenotypes (if there are any) could be affected by the loss of genes in other tissues related to reproduction, such as the hypothalamus and pituitary [11]. Oocyte specific inactivation of ovarian genes using *Cre/LoxP* technology provides an excellent approach for understanding their physiological roles during folliculogenesis/oogenesis, fertilization, and embryogenesis [11].

Zp3-Cre, *Gdf9-Cre*, and *Msx2-Cre* transgenic mouse lines, in which *Cre* gene is driven by oocyte-specific *Zp3* promoter, *Gdf9* promoter, and homeobox gene *Msx2* promoter, respectively, have been generated [11, 12]. *Zp3-Cre* and *Gdf9-Cre* transgenic mice have been widely used to delete genes in oocytes (Fig. 1). Zona pellucida protein synthesis starts in primary follicles, reaches the maximum in growing follicles, and decreases in fully grown oocytes [13]. The gene is specifically expressed in oocytes. In *Zp3-Cre* transgenic mice, Cre-recombinase activity is not observed in oocytes of primordial follicles, but its activity is first observed in oocytes of primary follicles and is highest in growing follicles but less in fully grown follicles [14]. *Msx2-Cre* is expressed from secondary follicle stages on, and both *Msx2-Cre* and *Zp3-Cre* mice are not suitable for deletion of gene expression in oocytes at the primordial follicle stage and early primary follicle stage [11, 14, 15]. *Zp3-Cre* functions only after the completion of homologous recombination. A recent study using *Zp3-Cre* to delete separase showed that follicles/oocytes developed normally in $Espl1^{flox/flox}$;*Zp3-Cre* mice. Therefore, the *Zp3-Cre* system should be applicable to genes that have a function during later stages of oocyte development [16].

Growth differentiation factor 9 (GDF9), an important oocyte-specific protein, is critical for both early and late follicle growth and cumulus functions [17–19]. In *Gdf9-Cre* mice, Cre-recombinase expression occurs earlier than in *Zp3-Cre* and *Msx2-Cre* mice [11]. In *Gdf9-Cre* mice, Cre-recombinase expression is exclusively expressed in oocytes of primordial follicles and in later developmental stages [11]. Crossing of *Gdf9-Cre* mice with floxed mice proved that a gene of interest can be completely deleted from the oocytes without affecting survival of the $Cre^{+}/target^{+}$ animal. The sequential oocyte-specific Cre-recombinase expression driven by different promoters will allow us to use them for different purposes. For example, when investigating a gene's function in follicle activation or recruitment from a primordial follicle pool, *Gdf9-Cre* founder mice instead of *Zp3-Cre* mice should be used.

In addition, tissue-nonspecific alkaline phosphatase (*TNAP*)–*Cre* knockin mice, which express the Cre recombinase in primordial germ cells, can be used for investigating gene functions in reproduction [20]. Another transgenic mouse line, *Vasa-Cre*, where Cre is efficiently and specifically expressed in both male and female germ cells, can also be useful for genetic analysis of diverse aspects of gametogenesis [21].

NEW UNDERSTANDING TOWARD MAJOR REPRODUCTIVE AND DEVELOPMENTAL EVENTS AS REVEALED BY OOCYTE-SPECIFIC KNOCKOUT

Folliculogenesis/Oogenesis

Perinatally, individual oocytes are surrounded by granulosa cells within the ovary to form primordial follicles. Although a few follicles resume growth during the fetal and neonatal period, regular growth occurs only after puberty. Each day a few dormant primordial follicles resume growth to constitute a follicle pool. However, only a limited number of primordial follicles are recruited, whereas the majority will undergo atresia. It is well known that primordial follicles start growth independent of any extraovarian factors [22]. The role of the oocyte in the initial recruitment of follicles has been established, but the exact mechanisms propelling the primordial follicles to leave the resting pool remain elusive [23]. Recently, Reddy et al. [24], by using *Gdf9-Cre/LoxP* technology, found that oocyte-specific deletion of *Pten* (phosphatase and tension homolog deleted on chromosome 10), an important repressor of the phosphoinositide 3 (PI3) kinase pathway, causes activation of the entire primordial follicle pool prematurely. In the $Pten^{loxP/loxP}$;*Gdf9-Cre* mice, all primordial follicles are depleted in early adulthood, suggesting that PTEN functions as a suppressor of primordial follicle recruitment. Further evidence showed that intraoocyte PTEN-PI3 kinase signaling appears to play an important role in the initiation of follicle growth [24]. By using a similar approach, glycoproteins are also proved to be suppressors of female fertility because oocyte-specific depletion of core 1-derived O-glycans leads to a sustained increase in fertility. The conditional knockout mice ovulated 30%–50% more eggs and had an increased litter size when compared to control mice. The increase in ovulated egg numbers is not caused by more follicles entering the growing follicle pool or the occurrence of multi-oocyte follicles, but is caused by modified follicle development and the maturation and ovulation of more follicles [25]. Although the factors driving primordial follicle recruitment are still unknown, investigations are underway to reveal the downstream signaling pathway(s) initiating follicle activation by this in vivo approach.

Several oocyte-specific molecules, including bone morphogenetic protein 15 (BMP15) and GDF9, have been proved to regulate follicle development and oocyte-cumulus complex function [26]. Oocyte-specific germ cell nuclear factor (GCNF), exclusively expressed in oocytes in the follicle at various stages except for primordial follicle stages [27, 28], may regulate oocyte function. However, *Nr6a1*-null mice showed embryonic lethality [29, 30], which prevents gaining knowledge of GCNF's functions by conventional knockout. Oocyte-specific knockout of GCNF was achieved by using *Zp3-Cre* mice. These mice showed hypofertility, and oocytes from these mice displayed increased expression of BMP15 and GDF9 at the diestrous phase. The steroidogenesis in follicles was also disrupted. Abnormal double-oocyte follicles, indicative of aberrant *Bmp15* and *Gdf9* expression, were observed in conditional knockout females. These in vivo results suggest that GCNF affects female fertility by regulating oocyte *Bmp15* and *Gdf9* expression [15].

In growing follicles, bidirectional communications between the oocyte and granulosa/cumulus cells are important for follicle growth and oocyte maturation. It is generally believed that GJA4, also known as connexin 37 (Cx37), constitutes gap junctions between the oocyte and cumulus cells, while GJA1, also known as connexin 43 (Cx43), constitutes gap junctions

between cumulus cells. However, this notion is still controversial. It has recently been found that *Gja1*$^{loxP/loxP}$*;Zp3-Cre* mice did not express GJA1 in oocytes, though they showed normal oogenesis/folliculogenesis, ovulation, and early embryo development. However, crossing *Gja1*$^{loxP/loxP}$*;Zp3-Cre* females with wild-type males resulted in impaired embryo implantation and decreased litter size [31]. It has been shown that mice lacking *Gja4* are sterile, but a recent report showed that oocyte-specific replacement of *Gja4* with *Gja1* by *Zp3* promoter-guided transgene in *Gja4*-null mutant mice restored oocyte growth and maturation as well as fertility [32]. These results suggest that GJA4 and GJA1 may have redundant roles in regulating follicle growth.

Follicle Growth and Ovulation

As mentioned above, communication between oocytes and follicle cells is bidirectional, and the interplay is essential not only for oocyte development but also for follicular development and ovulation [33]. Both germ cell-specific and granulosa-specific deletions were applied to investigate gene regulation of follicular growth and ovulation. For example, IL6ST, also known as GP130, is a shared receptor for members of the IL6 family of cytokines, and deletion of *Il6st*, specifically in germ cells, by the *Cre-LoxP* system resulted in a fertility defect in females. These animals had a major defect in ovulation [34]. On the other hand, an important approach is also to specifically delete target genes in granulosa and cumulus cells to study follicle growth and ovulation by crossing mice carrying a floxed target gene with mice expressing Cre-recombinase driven by *Amhr2* promoter (*Amhr2-Cre*) or *Cyp19a1* promoter (*Cyp19-Cre*) [35–37]. The *Cre-loxP* system was used to null the *Fst* (encoding follistatin) gene specifically in the granulosa cells of the postnatal ovary using *Amhr2-Cre* transgenic mice. The *Fst* conditional knockout females showed reduced numbers of ovarian follicles, decreased ovulation, and fertilization defects [37]. Small G-protein RAS is critical for FSH-induced signaling events and the regulation of target genes in cultured granulosa cells, but the in vivo function of RAS in granulosa cells has not yet been defined. In conditional knockin mouse models in which granulosa cells express a constitutively active *Kras*^{G12D} after Cre-mediated DNA recombination (using either *Amhr2-Cre* or *Cyp19a1-Cre*), the mutant females were subfertile and showed indications of premature ovarian failure. The ovaries contained numerous abnormal follicle-like structures devoid of mitotic and apoptotic cells as well as cells expressing granulosa cell marker genes. Most follicles that proceeded to the antral stage failed to ovulate and exhibited impaired responses to eCG and hCG [35]. Thus, transient but not sustained activation of RAS in granulosa cells is critical for directing normal follicle development and specific events associated with the ovulation process. Complementary to the functions of the PI3K/PTEN pathway in oocytes mentioned above, the PI3K/AKT pathway is also activated by FSH and thereby enhances granulosa cell differentiation in culture. To identify the physiological role of the PI3K pathway in vivo, Fan et al. [36] disrupted the PI3K suppressor, *Pten,* in granulosa cells of developing follicles. *Pten*$^{loxP/loxP}$ mice were mated with transgenic mice expressing CRE recombinase driven by *Cyp19a1* promoter (*Cyp19a1-Cre*). The resultant *Pten* mutant mice were fertile, ovulated more oocytes, and produced moderately more pups compared to control mice. These physiological differences in the *Pten* mutant mice were associated with hyperactivation of the PI3K/AKT pathway, decreased susceptibility to apoptosis, and increased proliferation of mutant granulosa cells [36]. These findings provide novel evidence showing that *Pten* affects the survival of granulosa cells and that its loss results in facilitated ovulation.

Oocyte Meiotic Maturation

Correct segregation of homologous chromosomes during the first meiosis and of sister chromatids during the second meiosis is prerequisite for avoiding aneuploidy. Segregation of chromosomes during first meiosis is triggered by separase (encoded by *Espl1*) cleavage of the cohesin's REC8 subunit along chromosome arms, and cleavage of centromeric cohesion by separase and other factors is required for faithful sister chromatid separation during the second meiosis [38]. Since separase is essential for chromosome separation in mitosis, separase deficiency causes embryonic lethality. Homozygous mutants could not be obtained by crossing of heterozygotes, and homozygous mutants could not develop beyond Embryonic Day (E) 8.5 in the uterus, although heterozygous mutant mice generated from ES cells were intact and fertile [39]. Another report showed that when heterozygous *Espl1*$^{-/+}$ mice were intercrossed, progenies included *Espl1*$^{+/+}$ and *Espl1*$^{-/+}$, but not *Espl1*$^{-/-}$, suggesting that a single copy of the *Espl1* gene is both necessary and sufficient for embryonic development. A further experiment showed that *Espl1*-null mice died before 6 days after copulation, which implies that separase is required for early embryogenesis [40]. To investigate the physiological function of separase in oocyte meiosis in vivo, Kudo et al. [16] deleted a floxed allele of *Espl1* specifically in mouse oocytes by using *Zp3-Cre*/*LoxP* technology. The specific deletion of separase did not affect follicular growth, and fully grown oocytes from *Espl1*$^{loxP/loxP}$*;Zp3-Cre* mice underwent germinal vesicle breakdown and reached the metaphase I stage; however, separase depletion inhibited removal of Rec8 from homologous chromosome arms, prevented resolution of chiasmata, and thus caused infertility.

Mitosis and meiosis share numerous molecules for regulating cell cycle progression, and conventional knockout of these mitotic genes may cause embryonic lethality, which therefore presents difficulties for investigating functions of these mitotic genes in meiotic cell cycle regulation in vivo. For example, many spindle checkpoint proteins are common for both mitosis and meiosis, but their mutation causes abnormal cell cycle progression and cell proliferation and thus embryo lethality. With the *Cre-LoxP* technology, we can specifically delete these genes in oocytes and investigate their functions in meiosis in vivo when floxed mice are used.

Fertilization

Sperm-oocyte fusion is one of the most important events in mammalian fertilization and it is believed to be mediated via specific molecular interactions. Due to different approaches employed to study the molecules involved in gamete fusion, conflicting results have been reported, and the molecules that mediate this fusion event have been the topic of numerous debates. Using conventional gene deletions, a few cell surface proteins in both eggs and spermatozoa have been identified as essential for gamete fusion [41]. Oocyte-specific knockout has also been used for related studies. For example, glycosylphosphatidylinositol (GPI)-anchored proteins on the oocyte membrane have been proposed as candidate molecules involved in sperm-oocyte fusion on the basis of in vitro experiments [42]. However, in vivo evidence is required. Oocyte-specific knockout mice were created using the *Cre-loxP* system to investigate the role of GPI-anchored protein in gamete fusion

TABLE 1. Ovarian-specific mutants with defects in reproduction.

Cre driver	Gene	Phenotype	References
Oocyte-specific mutation			
Zp3	*Nr6a1*	Upregulated *Bmp15* and *Gdf9* expression in oocyte, abnormal steroidogenesis, and hypofertility	[15]
	Espl1	Prevention of chiasmata resolution in oocyte meiosis	[16]
	Cigalt1	Maturation and ovulation of more follicles	[25]
	Cja1	Normal oogenesis and ovulation, hypofertility	[31]
	Piga	Failure of egg-sperm fusion, infertility	[43]
	Smarca4	Two-cell arrest of embryo development	[44]
	Rps6	Retarded follicle growth, peri-gastrulation death of heterozygous embryos	[45]
	Rbpj	Embryo death around midgestation	[46]
Gdf9	*Pten*	Premature activation of primordial follicle pool	[24]
Granulosa cell-specific mutation			
Cyp19a1	$Kras^{G12D}$	Failure of ovulation	[35]
	Pten	Facilitated (increased) ovulation	[36]
Amhr2	*Fst*	Reduced ovarian follicles, ovulation, and fertilization defects	[37]
Primordial germ cell-specific mutation			
TNAP	*Dnmt3a*	Death in utero of offspring from mutant females	[20]
	116st	Defective oocyte maturation and ovulation	[24]

in vivo. A portion of the *Piga* gene, which encodes an enzyme involved in GPI anchor biosynthesis, was deleted, and the conditional *Piga*-knockout females were infertile. Eggs from knockout mice lost their ability to fuse with sperm, suggesting that GPI-anchored proteins are required for gamete fusion [43].

Embryo Development

Maternal gene products drive early development when the newly formed embryo is transcriptionally inactive. During the maternal-zygotic transition, embryonic transcription is initiated and many maternal RNAs are degraded. Conventional knockout of many maternal genes does not affect early embryo development but causes embryo lethality in later developmental stages. In such cases, *Cre-LoxP* technology provides a powerful tool for studying the functions of these genes during embryogenesis.

Although little is known about the molecular basis of zygotic genome activation (ZGA), oocyte-derived mRNAs and proteins that alter chromatin structure are likely to be crucial. To test this hypothesis, a maternal-effect mutation of *Smarca4*, also known as *Brg1*, that encodes a catalytic subunit of SWI/SNF-related complexes was generated by utilizing *Cre-loxP* gene targeting. In conditional-mutant females, *Smarca4*-depleted oocytes completed meiosis and were fertilized. However, embryos derived from those oocytes exhibited 2-cell arrest. Therefore, it is proved that *Smarca4* is a gene required for ZGA in mammals [44].

There is a large amount of nascent ribosome synthesis that can maintain early embryo cleavage in mice. Nascent ribosome biogenesis is re-activated in the 6–8 cell stage, and it is speculated that it may play an important role during development. To prove this hypothesis, one *Rps6* allele was specifically deleted in growing oocytes by activation of *Zp3-Cre* transgene. $Rps6^{loxP/loxP}$;*Zp3-Cre* mice showed retarded follicle growth; however, S6-heterozygous embryo development until E5.5 was not affected, but the embyros showed inhibition of M phase entry and apoptosis after E5.5 and died at the perigastrulation stage, suggesting that active ribosome biogenesis is important for embryo survival after E5.5 [45].

The Notch pathway is implicated in regulating embryo cell specification. In order to deplete both maternal and zygotic expression of *Rbpj*, a key element in the Notch pathway, oocyte-specific knockout of *Rbpj* was achieved by crossing *Zp3-Cre*-transgenic mice with floxed *Rbpj* mice. $Rbpj^{-/-}$ zygotes deprived of maternal transcript resulted in blastocysts that implant and develop normally. However, after gastrulation these embryos died around midgestation, providing in vivo evidence that the Notch pathway is indispensable for three-germ-layer differentiation [46].

Imprinted genes are epigenetically marked during gametogenesis. Although de novo DNA methyltransferases of the *Dnmt3* family are implicated in maternal imprinting, the lethality of *Dnmt3a* and *Dnmt3b* knockout mice has precluded further studies. By disrupting *Dnmt3a* and *Dnmt3b* specifically in germ cells through conditional knockout using tissue nonspecific alkaline phosphatase *TNAP-Cre* knock-in mice, which express the Cre recombinase in primordial germ cells, it was found that offspring from *Dnmt3a* conditional mutant females die in utero, suggesting an essential role for de novo DNA methyltransferase Dnmt3a in parental imprinting and offspring survival [20].

CONCLUSION AND PERSPECTIVE

Major reproductive developmental events are regulated by numerous mitotic genes, in addition to germ cell-specific genes, and conventional knockout of these mitotic genes can cause embryonic or perinatal lethality of homozygotes. In addition, it may cause alterations in the physiology of many organs, which complicates the studies especially for reproduction. Deletion of some maternal-effect genes also causes failure in embryonic development. In such cases, investigators are not able to study the functions of these genes in reproduction in vivo by conventional knockout approaches. Oocyte-specific knockout achieved by crossing a line of floxed mice with normal expression of targeted gene and a line which has Cre-recombinase expression driven by *Zp3* or *Gdf9* promoter allows the in vivo analysis of patho-physiological functions of these mitotic or maternal-effect genes without affecting the general survival of the animal. This will be a particularly useful and reliable in vivo tool for defining the roles of mitotic genes and maternal transcripts in folliculogenesis, oocyte development, maturation, and fertilization as well as embryogenesis. This approach highly depends on the efficiency of target gene

deletion and the availability of floxed mouse lines. Although oocyte-specific knockout technology has been used to reveal the functions of numerous genes in folliculogenesis/oogenesis, oocyte maturation, fertilization, and embryonic development (Table 1), this technology will have more potential in clarifying the functions in vivo of many more genes, especially mitotic and maternal-effect genes, in major reproductive and developmental events in the future. A list of available Cre lines and floxed lines is available at http://jaxmice.jax.org/literature/models/crelox.pdf; http://www.emmanet.org/strains/list.utf8.php?sublist=Cre; http://www.emmanet.org/strains/list.utf8.php?sublist=TMCM; www.mshri.on.ca/nagy/Cre-pub.html; www.mshri.on.ca/nagy/floxed.html.

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