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REVIEW

New Understandings on Folliculogenesis/Oogenesis Regulation in Mouse as Revealed by Conditional Knockout

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ABSTRACT

In comparison to conventional knockout technology and *in vitro* research methods, conditional gene knockout has remarkable advantages. In the past decade, especially during the past five years, conditional knockout approaches have been used to study the regulation of folliculogenesis, follicle growth, oocyte maturation and other major reproductive events. In this review, we summarize the recent findings about folliculogenesis/oogenesis regulation, including the functions of four signaling cascades or glycoprotein domains that have been extensively studied by conditional gene deletion. Several other still fragmented areas of related work are introduced which are awaiting clarification. We have also discussed the future potential of this technology in clarifying gene functions in reproductive biology.

KEYWORDS: Conditional knockout; Folliculogenesis; Oogenesis; Mouse

1. INTRODUCTION

When an egg becomes exposed to spermatozoa, fertilization takes place. The resulting zygote will then develop into an embryo, and eventually result in live birth of an offspring. As an indispensable prerequisite for fertilization, a functional egg must be generated through processes that are highly complex. From the activation of the primordial follicle, to the primary follicle, secondary follicle, tertiary follicle and mature follicle, as well as oocyte maturation and ovulation, every step is critical and involves cascades of molecular interactions. Errors that take place during these processes may result in diseases, such as premature ovarian failure (POF), or infertility. Therefore, it is very important to study how a follicle/oocyte develops and which factors affect the specific processes.

Knockout technology has been employed in research on folliculogenesis/oogenesis for decades, which allows replacing or removing the gene of interest in order to determine its

Abbreviations: GC, granulosa cell; PN, postnatal; PI3K, phosphatidylinositol 3-kinase; PIP2, phosphatidylinositol (4,5)-bisphosphate; PIP3, phosphatidylinositol (3,4,5)-trisphosphate; PTEN, phosphatase and tensin homolog deleted on chromosome 10; PDK1, 3-phosphoinositide-dependent protein kinase-1; AKT, acute transforming retrovirus thymoma protein kinase; mTORC, mammalian target of rapamycin complex; FOXO, forkhead winged helix box O; TSC, tuberous sclerosis complex; rpS6, ribosomal protein S6; S6K1, p70 S6 kinase1; LH, luteinizing hormone; AREG, amphiregulin; EREG, epiregulin; EGFR, epidermal growth factor receptor; ERK1/2, extracellular signal-regulated kinases 1 and 2; COC, cumulus−oocyte complex; C/EBP, CCAAT/enhancer-binding protein; MAPK14, p38MAPKα; PGE, prostaglandin E; CTNNB1, β-catenin; SAC, spindle assembly checkpoint; SCC, sister chromatid cohesion; APC/C, anaphase-promoting complex; Rec8, meiotic recombination protein REC8 homolog; Mps1, monopolar spindle 1; Mad, mitotic arrest deficient; Bub, budding uninhibited by benomyl; BubR1, Mad3/Bub1b/Bub1-related kinase; GlcNAc, *N*-acetylglucosamine; GlcNAcT-I, *N*-acetylglucosaminyltransferase I; GalNAc, *N*-acetylgalactosamine; T-synthase, core 1 β1,3-galactosyltransferase; T-antigen, Galβ1−3GalNAcα1-Ser/Thr; Core 1-derived, core-1 and core-2; MOF, multiple-oocyte follicles.

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function. Although conventional knockout technology is useful, it has limitations, as it deletes the gene in embryonic stem (ES) cells and often causes embryonic lethality. This lethality can be avoided if the gene is deleted later in life or if it is deleted in a particular tissue-specific manner. Therefore, in recent years, conditional knockout has been applied and has become a popular approach. In the study of folliculogenesis/oogenesis, especially during the last five years, the conditional knockout approach has been applied frequently and has helped to discover the mechanisms that play a role in follicle activation, oocyte meiotic maturation, ovulation, and luteinization. For example, in the process of follicle activation, the role of granulosa cells (GCs) had not been appreciated for a very long time. However, by deleting genes specifically in GCs, GCs proved to be no less important than the oocyte during folliculogenesis (Fan et al., 2008b, 2009).

The most widely used conditional knockout approach is the *Cre-LoxP* system. Cre is a 38 kDa recombinase that mediates site-specific recombination between *loxP* sites. The 34 bp *loxP* consensus sequence consists of two 13 bp inverted repeats separated by an 8 bp asymmetric spacer region (Xu and Fu, 2005). If two *loxP* sequences are in the same orientation, Cre will mediate excision of the intervening DNA between the repeats, and only one *loxP* site will remain. The *Cre-LoxP* system involves a floxed mouse line and a transgenic mouse line. The floxed mouse line is designed to have two *loxP* sites flanking one or more exons of the gene of interest and positioned in the surrounding introns. Floxed mice are then crossed to the transgenic mouse line expressing Cre recombinase based on the pattern of Cre expression that can be controlled by cell type specific promoters (Gama Sosa et al., 2010). The resulting mutant mice usually have no evident developmental defects and thus can be used to study a gene's function in a specific tissue or cell type. Although the *Cre-LoxP* system is widely used, it also might have some drawbacks: Cre may be expressed non-specifically in other tissues; the efficiency of Cre recombinase varies in different kinds; the mammalian genome contains many pseudo *Loxp* sites that potentially could be recombined by Cre with totally unknown effects; and the *Cre-LoxP* system is not reversible (Matthaei, 2007).

In the study of folliculogenesis/oogenesis, Cre mice are often utilized and include *Zp3-Cre*, *Gdf9-Cre*, *Amhr2-Cre* and *Cyp19-Cre* transgenic mouse lines. Both ZP3 (zona pellucida 3) and GDF9 (growth differentiation factor 9) are specifically expressed in oocytes. The synthesis of ZP3 starts in primary follicles, reaches the maximum in growing follicles, and decreases in fully-grown oocytes, which makes *Zp3-Cre* only suitable for deletion of gene expression in oocytes from primary follicle stages on. *Gdf9-Cre* is expressed in oocytes of primordial follicles and in later developmental stages, as GDF9 is critical for both early and late follicle growth and cumulus functions (Sun et al., 2008). Unlike ZP3 and GDF9, AMHR2 (anti-Mullerian hormone receptor, type II) is highly expressed in the GCs of preantral and small antral follicles in the adult ovary (Arango et al., 2008); thus *Amhr2-Cre* is often used to generate conditional knockout in GCs of the ovary. Another kind of Cre mice often used for GC-specific gene targeting is the *Cyp19-Cre* mouse line. CYP19 (cytochrome P450, family 19) is an important factor for sexual development and mainly expressed in the fetal placental layer and ovarian GCs but also in other organs such as brain and testis. Since *Amhr2* is also known to be expressed in other reproductive tissues, including ovarian surface epithelial cells and the uterus, the *Cyp19-Cre* mouse line is generated more highly specific for GCs than *Amhr2-Cre* (Fan et al., 2008b). In brief, *Gdf9-Cre* and *Zp3-Cre* are used to delete genes in the oocyte after postnatal day 3 (PN3) and PN5, respectively (Hammond and Matin, 2009); *Amhr2-Cre* and *Cyp19-Cre* are used to delete genes in GCs after PN6 and PN10, respectively. All of these four Cre mice are proved to have high efficiency for inducing DNA excision (Lan et al., 2004; Fan et al., 2008b) and have been widely and frequently applied in conditional knockout in the mammalian ovary.

In this review, we have summarized recent progress in molecular mechanisms regulating folliculogenesis and oocyte maturation as revealed by conditional knockout technology in the mouse ovary.

2. NEW ADVANCES IN FOLLICULOGENESIS/ OOGENESIS STUDIES

2.1. Conditional knockout technology unveils the pivotal roles of PI3K pathway in primordial follicle activation

Signaling through PI3K (phosphatidylinositol 3-kinase) is fundamental for the regulation of cell proliferation, survival, growth, and migration, and also for pathological processes such as cancer and diabetes. PI3K phosphorylates PIP2 (phosphatidylinositol (4,5)-bisphosphate) to produce PIP3 (phosphatidylinositol (3,4,5)-trisphosphate) at the intracellular membrane. PTEN (phosphatase and tensin homolog deleted on chromosome 10) is a lipid phosphatase that reverses this process; thus it functions as a major negative regulator of the PI3K pathway. PDK1 (3-phosphoinositide-dependent protein kinase-1) activates AKT (acute transforming retrovirus thymoma protein kinase) through co-binding to PIP3. The PIP3-bound AKT can also be phosphorylated by mTORC2 (mammalian target of rapamycin complex 2). Activated AKT promotes cell survival, proliferation, and growth by phosphorylating and inactivating downstream "negative" targets, such as FOXO3 (forkhead winged helix box O 3) and TSC2 (tuberous sclerosis complex 2). FOXO molecules promote the transcription of pro-apoptotic genes. TSC2 negatively regulates the activity of mTORC1 that positively regulates cell growth and proliferation (Zheng et al., 2011) (Fig. 1).

For a long time, it was not well known how primordial follicles are preserved and activated; however, recent studies using conditional gene knockout have shown that the PTEN/PI3K signaling pathway plays an essential role in manipulating the dormancy and activation of mammalian primordial follicles.

Reddy et al. (2008) found that the entire primordial follicle pool became activated and premature ovarian failure was induced in mice lacking PTEN in their oocytes ($Pten^{fl/fl}$;*Gdf9-Cre* mice). Further evidence showed that the loss of PTEN in

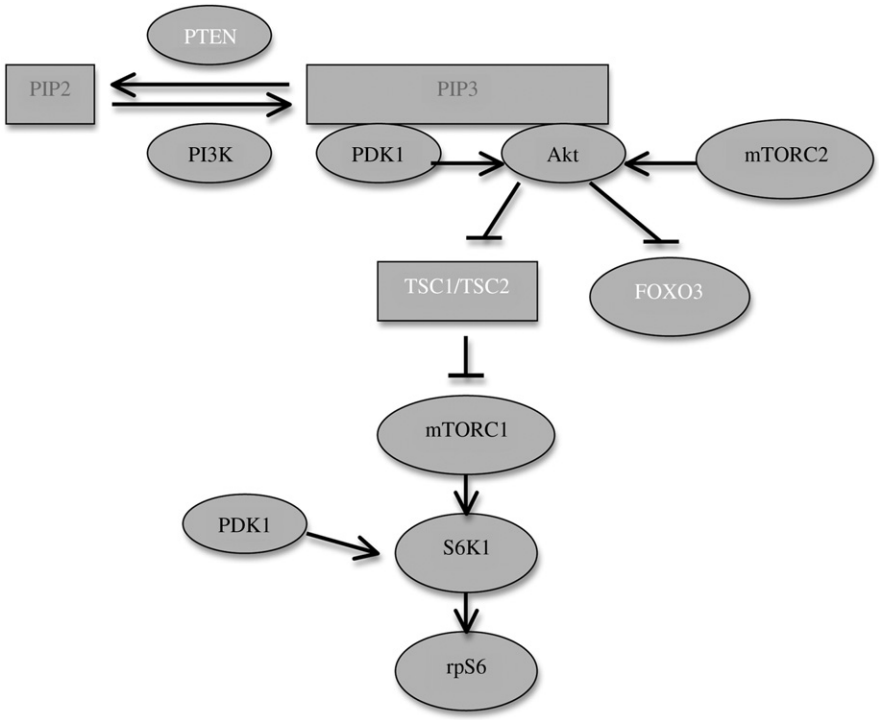

Fig. 1. PTEN/PI3K signaling pathway.
Molecules including PI3K, PDK1, mTORC1, S6K1, and rpS6 (black) regulate the survival of primordial follicles. However, molecules including PTEN, FOXO3, and the TSC1/TSC2 complex (white) suppress the activation of primordial follicles and maintain their dormancy.

oocytes led to elevated levels of phospho-AKT and enhanced expression and phosphorylation of rpS6 (ribosomal protein S6), suggesting that the PTEN-PI3K-AKT-rpS6 signaling cascade appears to play a critical role in the initiation of oocyte growth. In addition, the activation of rpS6 in mutant oocytes was dependent on the activities of mTOR, as phosphorylation of rpS6 was sensitive to the mTOR-specific inhibitor rapamycin.

The same group further conditionally deleted the *Pten* gene from oocytes of primary and further developed follicles by using *Zp3-Cre* mice, and revealed that there exists a stage-specific function of PTEN/PI3K signaling based on the facts that properly controlled PTEN/PI3K-AKT signaling is necessary for primordial follicle development, but overactivation of PI3K signaling does not affect the development of growing follicles (Jagarlamudi et al., 2009).

Depletion of *Pdk1* gene and *Rps6* gene in mouse oocytes (*Pdk1*$^{fl/fl}$*;Gdf9-Cre* mice, *Rps6*$^{fl/fl}$*;Gdf9-Cre* mice) showed that the PDK1-AKT-S6K1(p70 S6 kinase 1)-rpS6 signaling pathway was critical for primordial follicle survival (Reddy et al., 2009). Together, the PI3K/PTEN-PDK1 molecular network in oocytes controls the physiological course of primordial follicles and determines the reproductive life span in females.

On the other hand, Adhikari et al. (2009) have provided evidence that TSC2 activity in oocytes helps to maintain dormancy of primordial follicles, but mTORC1, which is negatively regulated by TSC2, promotes follicular activation. Deregulation of TSC/mTOR signaling in oocytes may cause infertility and POF, as in mutant mice lacking the *Tsc2* gene in oocytes (*Tsc2*$^{fl/fl}$*;Gdf9-Cre* mice), and the pool of primordial follicles is activated prematurely due to elevated mTORC1 activity in oocytes.

More recently, it was showed that dormancy and activation of primordial follicles require synergistic, cooperative functioning of both TSC/mTORC1 signaling and PTEN/PI3K signaling, and their deregulation in oocytes led to POF and infertility (Adhikari et al., 2010).

John et al. (2008) discovered that FOXO3 functioned specifically within oocytes to suppress primordial follicle activation, which was regulated by PI3K-AKT signaling. They confirmed that primordial follicle activation was regulated *via* the linear pathway PTEN-PI3K-AKT-FOXO3 by using *Vasa-Cre;Foxo3*$^{-/L}$ female mice.

In addition, Fan et al. (2008a) used *Pten*$^{fl/fl}$*;Cyp19-Cre* mice to induce targeted disruption of *Pten* in ovarian GCs, and provided novel evidence that *Pten* is required for the life span of granulosa/luteal cells and its loss results in persistence of nonsteroidogenic luteal structures in the adult mouse ovary.

2.2. Application of conditional knockout in GCs enriches the functions of the LH pathway

In mammals, a surge of LH (luteinizing hormone) triggers initiation of oocyte maturation, ovulation, and luteinization for successful reproduction. The LH surge can induce the expression of the EGF−like factors (AREG (amphiregulin) and EREG (epiregulin)) and then activate the signaling molecules: EGFR (epidermal growth factor receptor), RAS and ERK1/2 (extracellular signal-regulated kinases 1 and 2).

In GCs of preovulatory follicles, this pathway may mediate COC (cumulus−oocyte complex) expansion and oocyte maturation. Activation of small G-proteins within the RAS superfamily impacts several downstream signaling cascades, including the ERK1/2 pathway and PI3K pathway in many tissues. Transient activation of RAS can promote cell proliferation and cell differentiation (Fan and Richards, 2010) (Fig. 2).

LH is known to induce ovulation and luteinization, but how and through which signaling pathway(s) it exerts its effects are major questions that are currently being addressed by several investigators. Recent studies using conditional gene targeting have provided new information on this LH-regulated signaling pathway and its roles in ovulation- and luteinization-related events.

Fan et al. (2008b) generated conditional knock-in mouse models (*LSL-Kras^{G12D};Amhr2/Cyp19-Cre* mice) in which the GCs expressed a constitutively active Kras^{G12D}. The mutant mice were subfertile and showed POF symptoms. The mutant cells showed reduced ERK1/2 phosphorylation, elevated levels of phosphor-AKT and progressive decline of FOXO1. Thus, improper activation of KRAS blocks granulosa cell differentiation and causes ovulation failure.

Subsequently, they made an important discovery that the ERK1/2 pathway was essential for female fertility (Fan et al., 2009). They disrupted *Erk1/2* in mouse GCs by conditional knockout technology (*Erk1$^{-/-}$;Erk2$^{fl/fl}$;Cyp19-Cre* mice). *In vivo* evidence showed that ERK1/2 is pivotal for oocyte meiosis resumption, ovulation and luteinization induced by LH. In addition, they conditionally disrupted the *Cebpb* gene in GCs (*Cebpb$^{fl/fl}$;Cyp19-Cre* mice), and showed that C/EBPβ (CCAAT/Enhancer-binding protein β) played important roles downstream of ERK1/2 activation.

The same group presented further function of C/EBPα and C/EBPβ in GCs. *Cebpb$^{gc-/-}$* mutant mice (*Cebpb$^{fl/fl}$;Cyp19-Cre* mice) were subfertile whereas *Cebpba/b$^{gc-/-}$* double-mutant females were sterile (Fan et al., 2011). Follicles failed to ovulate and ovaries were devoid of corpora lutea.

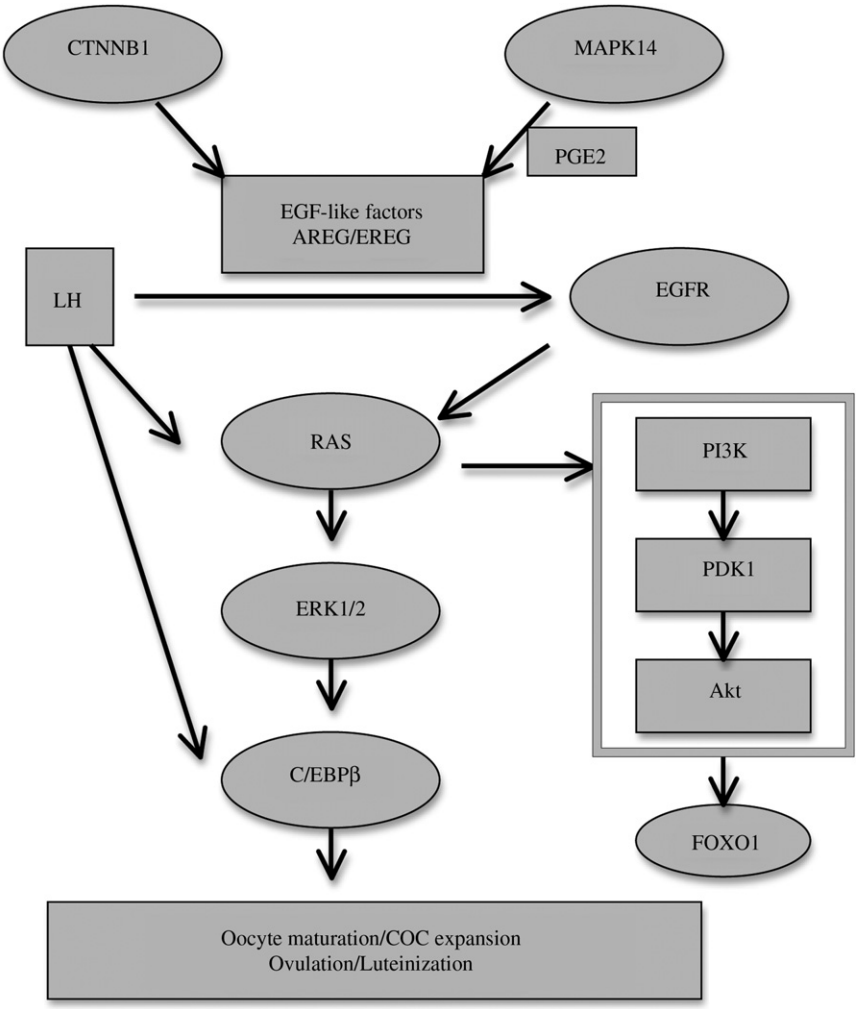

Fig. 2. LH-induced pathway.

LH surge can induce the expression of the EGF-like factors and then activate the signaling molecules—EGF receptor, RAS and ERK1/2—in GCs of preovulatory follicles, which may mediate COC expansion, oocyte maturation and other events. Activation of RAS impacts several downstream signaling cascades, including the ERK1/2 pathway and PI3K pathway.

They investigated several potentially related genes and reached the conclusion: C/EBPα/β regulate the terminal differentiation of GCs during luteinization.

They also expanded their work and enriched the LH-induced EGFR/RAS/ERK1/2 pathway (Fan et al., 2010). GCs in *Ctnnb1 (Ex3) gc$^{fl/fl}$;Cyp19-Cre* mice showed reduced responses to the LH surge and decreased *Areg* and *Ereg* expression. CTNNB1 (β-catenin) can enhance FSH and LH actions in antral follicles, but CTNNB1 overactivation inhibits LH-induced ovulation and luteinization. Disruption of *Mapk14* (encoding MAPK14 (p38MAPKα), a kinase which is critical for FSH and PGE2 (prostaglandin E 2) signaling cascades in GCs and COCs) selectively altered the expression of *Areg* and *Ereg* (Liu et al., 2010).

Recently, Hsieh et al. (2011) investigated the LH responses in GCs with targeted ablation of EGFR (*Egfr$^{delta/f}$;Cyp19-Cre* mice). Oocyte maturation and ovulation were disrupted, and LH-induced phosphorylation of ERK1/2, p38MAPK, and connexin-43 was impaired in GCs in this mouse model.

2.3. New methods for old issues: cohesin/SAC (spindle assembly checkpoint) in oocyte maturation

In mitosis or meiosis, sister chromatids are connected by SCC (sister chromatid cohesion). At the metaphase-to-anaphase transition, SCC has to be destructed to ensure sister chromatid separation (Nasmyth and Haering, 2009). In mammalian somatic cells, SCC depends on the essential cohesin complex that consists of four proteins, SMC1a, SMC3, RAD21, and SA1 (or SA2), whereas in germ cells, the meiotic cohesin complex is composed of Rec8, STAG3, and SMC1b. Dissolution of SCC is dependent on the removal of Rec8 (meiotic recombination protein REC8 homolog) by separase, activation of which is regulated by SAC proteins and APC/C. During each cell division, before anaphase, SAC verifies whether all kinetochores are properly attached to the opposite poles of the bipolar spindle and whether they are under proper tension, thus ensuring accurate metaphase-to-anaphase transition. Core SAC proteins include Mps1 (monopolar spindle 1), Mad1/2 (mitotic arrest deficient 1/2), Bub1/3 (budding uninhibited by benomyl 1/3) and BubR1 (Mad3/Bub1b/Bub1-related kinase).

Most of our knowledge about cohesin/SAC was gained through *in vitro* studies, but recent studies employing conditional knockout have provided new insights on how cohesin/SAC function *in vivo*.

Kudo et al. (2006) deleted a floxed allele of separase specifically in mouse oocytes through expressing Cre recombinase by *Zp3* promoter. Results showed that separase deficiency prevented Rec8 removal from chromosome arms and chiasmata resolution, and also inhibited extrusion of the first polar body (PBE), leading to female infertility.

Recently, Revenkova et al. (2010) specifically and highly efficiently inactivated the mouse *Smc1β* gene at the primordial follicle stage shortly after birth (*Smc1β$^{fl/fl}$;Gdf9-iCre* mice), to test the hypothesis that a steep increase in chromosome missegregation and aneuploidy in aging oocytes may be caused by loss of SCC through slow deterioration of cohesin. As a result, *Smc1β* inactivation in mouse oocytes at the primordial follicle stage did not affect chiasma positions, SCC, litter size, or number of offspring at any age tested. Thus, SMC1β cohesin only needs to be expressed during prophase I prior to the primordial follicle stage to ensure SCC establishment up to the aging stages.

Concerning SAC, McGuinness et al. (2009) depleted Bub1 protein specifically in oocytes (*Bub1$^{F/F}$;Zp3-Cre* mice). Bub1 depletion largely accelerated chiasmata resolution and extrusion of polar bodies. It also resulted in chromosome missegregation at meiosis I, and loss of cohesion precociously between sister centromeres. Furthermore, deletion of APC/C (anaphase-promoting complex) specifically in mouse oocytes (*APC2$^{F/F}$;Zp3-Cre* mice) showed that the APC/C is essential for the first meiotic division, and SAC controls the timing of APC/C and separase activation in oocytes.

Most recently, the role of Mps1 in meiotic progression and checkpoint control in meiosis I was addressed, using a conditional loss-of-function approach (*Mps1$^{fl/fl}$;Zp3-Cre* mice) (Hached et al., 2011) addressed. It is demonstrated that Mps1 is required for the timing of prometaphase and SAC function in meiosis I.

2.4. New research topic: glycans in folliculogenesis

By attaching to glycoproteins, specific glycans contribute to glycoprotein functions such as ensuring appropriate folding, secretion and receptor−ligand interactions. Complex and hybrid *N*-glycans contain sugar residues that have been implicated in fertilization, embryo compaction and implantation. Complex and hybrid *N*-glycans are generated from oligomannosyl *N*-glycans by the addition of a GlcNAc (*N*-acetylglucosamine) catalyzed by GlcNAcT-I (*N*-acetylglucosaminyltransferase I). GlcNAcT-I is encoded by the *Mgat1* gene; thus deletion of *Mgat1* prevents the synthesis of complex and hybrid *N*-glycans. Core 1-derived *O*-glycans are required for embryonic development after E12.5. The core 1 *O*-glycan is initiated by the transfer of GalNAc (*N*-acetylgalactosamine) to Ser or Thr residues in glycoproteins, which is extended with Gal by T-synthase (core 1 β1, 3-galactosyltransferase) to generate T-antigen (Galβ1−3GalNAcα1-Ser/Thr). Core 2 *O*-glycans are generated by adding a GlcNAc residue to core 1 *O*-glycans. Deletion of the *C1galt1* gene that encodes T-synthase removed T-synthase and prevented the generation of core 1-derived *O*-glycans (Williams and Stanley, 2011b).

So far, glycans have not been of interest to scientists studying oogenesis; however, recent research employing the conditional knockout approach provided evidence to determine roles of complex and hybrid *N*-glycans and core 1-derived (core-1 and core-2) *O*-glycans in oogenesis, which is a fresh and new approach to research on oocytes.

On one hand, Shi et al. (2004) deleted the oocyte-specific gene *Mgat1* by using a *Zp3-Cre* recombinase transgene to prevent the generation of complex and hybrid *N*-glycans in mouse oocytes, and found that mutant eggs showed thinner zona pellucida and mutant females yielded fewer eggs than controls.

Recently, Williams and Stanley (2009) further studied how oocyte-specific deletion of complex and hybrid *N*-glycans leads to reduction in the ovulation rate. Their data indicated that the decreasing ovulation rate was due to abnormal development of preovulatory follicles. In addition, cumulus mass development was also unusual.

On the contrary, oocyte-specific loss of core 1-derived *O*-glycans in mice by using a similar approach led to a sustained increase in fertility (Williams and Stanley, 2008). The increase was caused by prolonged follicle development, maturation and ovulation of more follicles and MOF (multiple-oocyte follicles) formation at late stages of folliculogenesis.

Most recently, Williams and Stanley (2011a) used females carrying floxed alleles of both the *C1galt1* (T-syn) and *Mgat1* glycosyltransferase genes and a *Zp3-Cre* transgene, and generated oocytes lacking complex *O*- and *N*-glycans following oocyte-specific deletion at the primary follicle stage. Double-mutant females showed an absence of developing follicles, ovary dysfunction, reduced testosterone and inhibin A, and elevated FSH, indicating that the loss of complex *N*- and *O*-glycans from oocyte glycoproteins affects hypothalamic−pituitary−gonadal feedback loops. The double-mutant females also represent a new mouse model for the study of follicular POF.

Table 1
Oocyte-specific mutant mice with defects in reproduction

Cre driver	Gene	Phenotype	References
Amhr2-Cre	*Gata4*	Impaired fertility and cystic ovarian changes: smaller ovaries released fewer oocytes and expressed less CYP19	Kyrönlahti et al., 2011
Zp3-Cre	*Yin-Yang1*	Severely reduced expression of both GDF9 and BMP15; infertility, failure of estrus cycling and altered reproductive hormone levels; altered levels of several oocyte-specific factors	Griffith et al., 2011
Zp3-Cre	*Hsp90b1*	Thinner zona pellucida but no obvious anomalies in follicular growth, meiotic maturation or fertilization; zygotes obtained from mutant oocytes were unable to reach the 2-cell stage	Audouard et al., 2011
	Nobox	Disrupting early folliculogenesis and the expression of oocyte-specific genes in mice; *Pad6* was down-regulated in *Nobox*-null ovaries	Choi et al., 2010
Amhr2-Cre	*Bmpr1a/ Bmpr1a Bmpr1b*	Subfertile with reduced spontaneous ovulation/granulosa cell tumors developed	Edson et al., 2010
Amhr2-Cre	*Dicer1*	An increased primordial follicle pool endowment, accelerated early follicle recruitment and more degenerate follicles; different expression of some follicle development-related genes	Lei et al., 2010
Zp3-Cre	*Dnmt3a*	Paternal and maternal imprinting disrupted, hypomethylation of imprinted genes	Kaneda et al., 2010
Gdf9/Zp3-Cre	*Mll2*	Anovulation and oocyte death, with increased transcription of *p53*, apoptotic factors, and *Iap* elements	Andreu-Vieyra et al., 2010
MisRII/Gdf9-Cre	Androogen receptor (AR)	GC-specific ARKO mice had premature ovarian failure and were subfertile, with longer estrous cycles and fewer ovulated oocytes. However, fertility, estrous cycles, and ovarian morphology of oocyte-specific ARKO mice were normal	Sen and Hammes, 2010
Amhr2-Cre	*Wnt4*	Reduced ovary weights and smaller litters, less number of healthy antral follicles	Boyer et al., 2010
Cyp17-iCre	*Esr1*	Erratic pattern of estrous cycles and infertile before reaching the age of 6 months; fewer corpora lutea but more antral follicles; elevated levels of serum testosterone and FSH, lower or undetectable LH level; fewer oocytes and multiple hemorrhagic cysts when superstimulated.	Lee et al., 2009
Amhr2-Cre	*β-catenin*	Infertile, oviducts were grossly distended, with fewer but healthy oocytes	Hernandez Gifford et al., 2009
Amhr2-Cre	*SmoM2*	Infertile and ovarian and reproductive tract defects; oocytes were rarely recovered from the oviducts of superovulated mice and remained trapped in preovulatory follicles; cumulus expansion appeared disorganized	Ren et al., 2009
	Insr/Igf1r	Female reproductive functions were not affected	Pitetti et al., 2009
	Sohlh2	Infertile due to lack of ovarian follicles; limited oocyte growth, no differentiated GCs structures; oocytes rapidly lost; misexpression of numerous germ cell- and oocyte-specific genes in primordial oocytes	Choi et al., 2008
Zp3-Cre	*Gja1*	No histological abnormalities were detected in the ovaries; mating with wild-type males resulted in a reduced rate of parturition and a substantial decrease in litter size	Gershon et al., 2008
Amhr2-Cre	*Smad4*	Subfertile with decreasing fertility over time and multiple defects in folliculogenesis; disrupted regulation of steroidogenesis; increased levels of serum progesterone; severe cumulus cell defects	Pangas et al., 2006
TNAP-Cre	*Gp130*	A slight reduction in the number of primary follicles and a major defect in ovulation	Molyneaux et al., 2003
Zp3-Cre	*GCNF*	Hypofertility due to prolonged diestrus phase of the estrous cycle and aberrant steroidogenesis; abnormal double-oocyte follicles.	Lan et al., 2003
Zp3-Cre	*Pig-a*	Infertile, and eggs recovered from the females after mating are unfertilized	Alfieri et al., 2003

2.5. Others

Except for the work mentioned above, there are still other studies on folliculogenesis/oogenesis using conditional gene targeting. However, these are relatively independent and uncorrelated and might need further research for clarifications. We list them in Table 1.

3. CONCLUSIONS AND PERSPECTIVES

Gene knockout offers remarkable advantages when compared to conventional *in vitro* research methods. Knockout mice serve as perfect model animals for the *in vivo* study of gene function during development, organogenesis and aging, as well as the evaluation of therapeutic strategies for human disease and the investigation of disease progression in a manner not possible in human subjects.

In the oocyte research field, conventional *in vitro* techniques, such as oocyte or follicle culture *in vitro*, inhibitor treatment, specific siRNA or morpholino injection, as well as others, have been developed and widely used. These approaches are fast and convenient for gene function research. Furthermore, the *in vitro* environment is simplified compared to *in vivo* studies and can be controlled by the investigator. For these reasons, *in vitro* studies can easily be repeated to confirm the results. However, these conventional *in vitro* techniques might not fully resemble the body's environment and completely simulate *in vivo* conditions; thus the results might be less trustworthy or less valid. Therefore, *in vivo* techniques are necessary and gene knockout meets the demand. Unfortunately, some gene knockouts from ES cells cause embryo lethality; therefore conditional knockout in a specific tissue or organ provides a better choice. In comparison to conventional knockout, conditional knockout technology has the advantages of temporal and spatial specific control of gene functions, which allows the study of a gene's function at different stages or in different tissues, especially concerning the genes whose knockout causes lethality or defects. In addition, the conditional knockout approach can also help to examine the role of maternal transcripts and analyze cell lineages in mammals (Sun et al., 2008). However, no approach is perfect and conditional knockout also has its disadvantages. Its power depends on the efficiency of the cell type-specific Cre recombinase excitation and the availability of floxed mouse lines. Besides, under some circumstances, for example, if the expression of Cre starts at the earlier stages of folliculogenesis/oogenesis, the phenotypes observed by conditional knockout may be very difficult to interpret due to the complexity of the body environment and the phenotypes reflecting a long-term effect of losing the target gene. In conclusion, if conditional knockout technology is combined with *in vitro* approaches, more reliable and convincing results may be obtained.

In this review, we have summarized the new findings on the role of four signal cascades or glycoprotein domains in folliculogenesis/oogenesis using conditional knockout technology. There is still plenty of fresh ground waiting to be explored. For example, considering that the functions of many genes cannot be studied by conventional knockout as their loss will cause embryo death, such genes would present excellent targets for conditional knockout experiments. Besides, conditional gene inactivation may also be used to verify the findings obtained in *in vitro* studies or clarify controversies based on *in vitro* studies. In summary, this technology will have increasing potential and play increasingly important roles in clarifying gene functions in reproductive biology.

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