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Orphan nuclear receptor function in the ovary

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1. ABSTRACT

Orphan nuclear receptors such as germ cell nuclear factor (GCNF), steroidogenic factor 1 (SF-1) and liver receptor homolog-1 (LRH-1), are emerging as important ovarian factors in regulating female reproduction. Within the ovary, GCNF (NR6A1) expression is restricted to the oocyte, while SF-1 (NR5A1) is expressed only in the somatic cells, such as granulosa, thecal and luteal cells, and interstitial cells. LRH-1 (NR5A2), an orphan receptor closely related to SF-1, is expressed only in the granulosa cells of the follicles and luteal cells within the ovary. Recent studies using conditional knockout strategies to bypass the embryonic lethality of *GCNF* and *SF-1* null mice have uncovered important roles of GCNF and SF-1 in the oocyte and granulosa cells, respectively. In this review, we will summarize the major findings of GCNF and SF-1 in the ovary from the studies of conditional *GCNF* and *SF-1* knockout mice. The potential role of LRH-1 in the ovary is also briefly discussed. Understanding the ovarian functions of these orphan nuclear receptors may lead to the development of new agents for regulation of female fertility and new medicines for the treatment of female idiopathic infertility, premature ovarian failure, polycystic ovarian syndrome and ovarian cancer.

2. INTRODUCTION

In the mammalian ovary, follicles are the principal functional units which provide the support system necessary for production of female germ cells (mature oocytes) during postnatal life (1). The process of follicular development after birth is termed folliculogenesis and the production of fertilizable eggs is referred to as oogenesis. During reproductive life, folliculogenesis and oogenesis are highly coordinated to ensure the production of fertilizable eggs. These processes require intercellular communication between many cell types such as oocytes, granulosa and thecal cells within the ovary (2, 3). Many intraovarian growth factors and endocrine factors such as steroids, insulin-like growth factor-1, epidermal growth factor, members of the transforming growth factor beta (TGFβ) family, the Wnt/Frizzled family and pituitary gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH), are involved in these processes, affecting female reproduction (2-9). Understanding steroid or other nuclear receptor signaling in the ovary has been, and remains to be, one of major research goals in the field. In the past two decades, mouse genetic studies have uncovered the essential roles of classical steroid nuclear receptors [*e.g.* estrogen receptor (ER)-α, ERβ, progesterone receptor (PR)-A and PR-B, androgen receptor

(AR)] in the ovary. For instance, both ERα and ERβ are required for the maintenance of germ and somatic cells and the formation and release of mature (Graafian) oocytes in the postnatal ovary (10-14). PRs, more specifically the PR-A, are essential for ovulation of mature oocytes, but not folliculogenesis, as female mice lacking both PR-A and PR-B (PRKO) fail to ovulate but have different stages of follicles in the ovary, and only PR-A knockout mice, but not the PR-B knockouts, exhibit the severe impairment of ovulation (15-18). Recently AR knockout mice have been generated and display female subfertility, defective folliculogenesis and premature ovarian failure, suggesting that AR function is needed for normal folliculogenesis and female fertility (19, 20). Besides these classical steroid nuclear receptors, several orphan nuclear receptors, whose ligands (if present) were unknown at that time of cloning, are emerging as important molecules that regulate ovarian function, affecting female reproduction.

Nuclear receptors are one of the largest families of transcription factors, with diverse physiological functions such as homeostasis, reproduction, development and metabolism (21-23). There are 48 and 49 identified nuclear receptor members in human and mouse genomes, respectively (23, 24). The common structural feature of this superfamily is a tripartite domain structure consisting of a hypervariable amino-terminus that contributes, in some instances, to transactivation function; a highly conserved DNA-binding domain (DBD), which is responsible for DNA response element recognition and dimerization; and the conserved carboxyl-terminus ligand binding domain (LBD), which is involved in many overlapping functions including ligand binding, nuclear localization, dimerization, silencing and transactivation (21-23). The high degree of conservation within the DBD or LBD of steroid receptors has led to the discovery of many more structurally related receptors, which are termed orphan nuclear receptors, since both a ligand and a cellular function were unknown at the time of cloning (25). The nuclear receptor superfamily has been systematically divided into seven subgroups, which includes the classical *bona fide* receptors such as steroid receptors, vitamin D receptor, thyroid hormone receptor and retinoid receptors, and a large *ad hoc* group of orphan receptors, which spans every subgroup of the superfamily (23, 24, 26). In the past two decades, identification of putative ligands and characterization of physiological roles of orphan nuclear receptors have been intensively investigated (27, 28). A number of previously denoted orphan nuclear receptors such as SF-1, LRH-1, peroxisome proliferator-activated receptors (PPAR)-α/γ/δ, liver X receptor (LXR), farnesoid X receptor (FXR), constitutive androstane receptor (CAR) and steroid xenobiotic receptor (SXR) are no longer regarded as orphan nuclear receptors, as their ligands have been identified (28-31). However, a number of nuclear receptors such as GCNF remain to be orphan nuclear receptors (28). To keep consistency with literature, we still regard those nuclear receptors with unknown ligands at the time of cloning (*e.g.* SF-1 and LRH-1) as orphan nuclear receptors in this review, even though they are no longer "orphan" anymore. Recent studies of these orphan nuclear receptors using molecular and mouse genetic techniques have provided new insight about the roles of these orphan nuclear receptors in the ovary. This mini-review will briefly summarize a few important studies of orphan nuclear receptors GCNF, SF-1 and LRH-1 in the ovary.

3. GERM CELL NUCLEAR RECEPTOR (GCNF:NR6A1)

GCNF (Retinoid receptor related Testis specific Receptor/Neuronal Cell Nuclear Factor, NR6A1) is a novel orphan member of the nuclear receptor superfamily as it is more distantly related to other members and forms a sixth and separate sub branch of the family (23, 32). It functions as a transcription factor, that binds to a direct repeat of the sequence AGGTCA with zero base pair spacing between the half sites (DR0) element to repress gene transcription *in vitro* and *in vivo* (32-35). GCNF, more specifically the DBD of GCNF, is essential for normal embryonic development and repression of *Oct4* expression in somatic cells of early mouse embryos (35-37). In addition, GCNF is important for the differentiation of mouse embryonic stem cells (38), neuronal precursor cells (39) and human teratocarcinoma NT2 cells (40). The action of GCNF in repressing gene transcription is likely via its interaction with Nuclear Receptor Corepressor (NcoR), Silencing Mediator for Retinoid and Thyroid receptor (SMRT) (35), RAP80, methyl DNA binding proteins, MBD3 and MBD2, and/or DNA methyltransferases (41-43).

In adult vertebrates, *GCNF* is predominantly expressed in the gonads of several species, including mouse, rat, and human (32, 44-46). In the testis, GCNF is predominantly expressed in round spermatids in rodents (32, 44, 45, 47) and pachytene spermatocytes in human (46). GCNF can bind to DR0 elements in the promoters of postmeiotic spermatogenic genes, *protamine 1* and *2*, and repress their promoter activity *in vitro* (48, 49), indicating that GCNF will have a role in spermatogenesis. In the ovary, *GCNF* is exclusively expressed in oocytes in mouse, xenopus and zebrafish (32, 44, 50, 51). In the mouse ovary, *GCNF* is expressed in the oocytes of primary, secondary and preovulatory follicles, but not primordial follicles at both the mRNA and protein levels (32, 44, 47). GCNF is also present in ovulated oocytes and preimplantation embryos (47). The oocyte-specific expression pattern indicates that GCNF will have a role in regulating some aspect of oocyte functions, affecting female fertility. This notion has been supported by our recent studies which are summarized in the next paragraph (52).

To characterize the role of GCNF in the ovary, we adopted the *Cre/loxP* conditional knockout strategy (53) to specifically delete GCNF in growing oocytes (52). In brief, we generated floxed *GCNF* mice which can bypass embryonic lethality and display normal expression of GCNF in growing oocytes (54). Then, an oocyte-specific *Cre* transgenic mouse line, *zona pellucida protein 3 (Zp3)-Cre* (55), was crossed with floxed *GCNF* mice to obtain oocyte-specific *GCNF* knockout mice (52). Using this strategy, we have successfully generated oocyte-specific *GCNF* knockout mice, as demonstrated by the absence of GCNF mRNA in growing oocytes. These oocyte-specific

GCNF knockout mice are developmentally normal but display reduced fertility with reduced litter sizes and reduced numbers of litters per month after being bred with fertile males for one year. An abnormal estrous cycle with a significant increase of the length of the estrous cycle, more specifically the diestrus of the cycle, is observed in oocyte-specific *GCNF* knockout mice. The prolonged diestrus in these *GCNF* mutant mice likely accounts for the reduced number of litters per month. The prolonged estrous cycle in oocyte-specific *GCNF* knockout mice appears to result from defects in ovarian somatic cells such as granulosa, thecal and luteal cells. In fact, aberrant steroidogenesis is observed in oocyte-specific *GCNF* knockout mice. This is concluded from the reduced levels of circulating steroid hormones including estradiol, progesterone and testosterone, reduced expression of steroidogenic enzyme genes, *steroidogenic acute regulatory protein* (*StAR*) and *3β-hydroxysteroid dehydrogenase I* (*3βHSD I*), and elevated expression of *17-α hydroxylase* (*17αOH*), a metabolic enzyme for progesterone, at the diestrus stage of the estrous cycle in the oocyte-specific *GCNF* knockout mice. Since GCNF is only expressed in the oocyte, mis-expression of *StAR*, *3βHSD I* and *17αOH* in oocyte-specific *GCNF* knockout ovaries is not the direct effects of *loss of GCNF* in the oocyte. The direct target genes of GCNF in the ovary are two oocyte-secreted TGF-β family members, *bone morphogenetic protein 15* (*BMP-15*) and *growth differentiation factor 9* (*GDF-9*). GDF-9 and BMP-15 are restrictedly expressed in the oocyte within the ovary and are involved in the regulation of adjacent somatic cell function in the ovary (3, 5, 56, 57). *GDF-9*$^{-/-}$ mice are infertile with defects in follicular development beyond the primary stage and thecal cell development (58-60). Elevated progesterone levels and mis-expression of ovarian steroidogenic genes in somatic cells such as *17αOH* in thecal cells have been reported in *GDF-9*$^{-/-}$ mice (58, 60). Regulation of steroidogenesis by recombinant GDF-9 has also been documented not only in granulosa cells (61-63) but also in thecal cells (64, 65). BMP-15, another oocyte-secreted TGFβ family member, also modulates follicular development and steroidogenesis in ovarian somatic cells (56, 66-68). BMP-15 stimulates granulosa cell proliferation, and inhibits FSH-induced progesterone production by suppressing FSH-induced expression of steroidogenic enzymatic genes such as *StAR* and *3□HSD in vitro* (66, 67). *BMP-15* null mutant mice are subfertile with reduced ovulation and fertilization. Introduction of a *GDF-9* null allele into *BMP-15* null mice causes more severe fertility and ovarian defects than *BMP-15* null mutant mice, indicating that GDF-9 and BMP-15 act synergistically in the ovary (68). In the oocyte-specific *GCNF* knockout mice, expression of *GDF-9* and *BMP-15* are significantly elevated at the diestrus of the estrous cycle. Abnormal double oocyte follicles, indicative of aberrant GDF-9/BMP-15 expression, are also observed in oocyte-specific *GCNF* knockout mice. More importantly, GCNF can bind to multiple DR0 elements in the promoters of *GDF-9* and *BMP-15 in vitro*, and repress both *GDF-9* and *BMP-15* promoter activities in a dose-dependent manner in cultured cells. These results demonstrate that *GDF-9* and *BMP-15* are *bona fide* GCNF target genes in the oocyte.

Besides *GDF-9* and *BMP-15*, the proto-oncogene *c-mos* is another potential GCNF target gene in the oocyte. Like *GDF-9* and *BMP-15*, *c-mos* is also specifically expressed in the growing and mature oocytes within the ovary (69, 70). Female *c-mos* knockout mice are infertile with defective oogenesis (71-73). Recently, it has been shown that recombined GCNF can bind to the GCNF response element in the *c-mos* promoter and regulate *c-mos* transcription *in vitro*, indicating that *c-mos* may be a direct target gene of GCNF in the oocyte (74). However, we did not observe a significant change in the expression levels of *c-mos* at the diestrus of the estrous cycle in oocyte-specific *GCNF* knockout mice by RT-PCR analysis (52). Whether the expression of *c-mos* is affected in the oocytes at other stages of the estrous cycle and ovulated oocytes remains to be characterized in these oocyte-specific *GCNF* knockout mice.

In summary, an oocyte-specific *GCNF* knockout mouse model displays hypofertility, abnormal estrous cycle, aberrant steroidogenesis, and double oocyte follicles in the ovaries (52). This mouse model has uncovered a new regulatory pathway in ovarian function. A paracrine regulatory pathway exists among oocytes and adjacent somatic cells to modulate the estrous cycle during female reproduction, and this function is mediated by BMP-15 and GDF-9, whose expression are regulated by GCNF in oocytes. In addition, maternal GCNF (expressed in ovulated oocytes) may play a role in regulating gene transcription during early embryonic development.

4. STEROIDOGENIC FACTOR-1 (SF-1:NR5A1)

SF-1 was initially identified as a key regulator of the expression of the cytochrome P450 steroid hydroxylases in steroidogenic cells lines [review in (27, 75)]. It is an orphan nuclear receptor and designated NR5A1 (23). Recently, phosphatidyl inositols have been identified as ligands for SF-1 (30, 31). SF-1 can bind the consensus DNA sequences T/CCAAGGTCA and activate gene transcription (27, 76). Its DNA binding and transactivation properties are regulated by protein kinases-induced phosphorylation (27). It can interact with coactivators such as steroid receptor coactivators (SRCs) or corepressors such as the dosage-sensitive, sex-reversal adrenal hypoplasia congenital critical region on the X chromosome gene 1 (Dax-1, NR0B1) and small heterodimer partner (SHP, NR0B2) to regulate gene expression (27, 76). Many SF-1 target genes have been identified. Some of these genes are steroidogenic enzymes, *mullerian inhibiting substance (MIS)* and *its receptor, the pituitary glycoprotein a-subunit*, *LH β-subunit*, *ACTH receptor*, *StAR*, *oxytocin*, *Dax-1* and *inhibin alpha* (27, 76, 77). SF-1 acts at multiple levels of the hypothalamic-pituitary-gonadal/adrenal axes to regulate expression of genes that are important for steroid metabolism and sex differentiation [reviews in (27, 75, 78)]. *SF-1* null mutant mice are born without gonads, adrenal glands and pituitary gonadotropes, and then die at 8 days after birth due to adrenal insufficiency (27, 75, 78), which confirms the essential role of SF-1 in mammalian gonad development and differentiation of steroidogenic tissues such as adrenals.

SF-1 is expressed in ovarian cells of various species including mouse (79-81), rat (80, 82, 83), bovine

(82), equine (84) and human (77, 85). In immature and adult rodents, SF-1 mRNA and proteins have been detected in all types of ovarian cells including granulosa, thecal, luteal and interstitial cells (79-83). Expression of SF-1 in granulosa cells of rodent ovaries is regulated by hormones such as gonadotropins and estrogen (80, 81). Similarly, bovine SF-1 protein has been detected in graffian follicles and corpora lutea (82), and equine SF-1 in granulosa cells and theca interna (84). Although the cell-specific expression of SF-1 in the human ovary has not been determined, SF-1 has been shown to be present in human granulosa cells (77, 85). The expression of SF-1 in various types of ovarian cells indicates the potential ovarian role of SF-1 during steroidogenesis and female reproduction. Recent molecular studies have shown that *StAR* and *aromatase*, that are required for synthesis of estrogens in granulosa cells within the ovary, and *inhibin alpha*, which is important for maintaining follicular architecture in the ovary, are likely SF-1 direct target genes in granulosa cells (77, 80, 81, 85). A recent study (86) from the Parker laboratory highlights the importance of granulosa cell-expressing SF-1 in female fertility. They generated gonad-specific *SF-1* knockout mice by crossing floxed *SF-1* mice with *anti-Mullerian hormone type 2 receptor (Amhr2)-Cre* mice which express Cre in granulosa cells of adult ovary and embryonic gonads. Female gonad-specific *SF-1* knockout mice bypass the perinatal lethality observed in *SF-1* null mice with no distinguishable ovarian phenotype in embryos or at birth. However, adult gonad-specific *SF-1* knockout females are sterile, and their ovaries have limited numbers of follicles and an absence of corpora lutea. Many of these follicles contain hemorrhagic cysts, similar to the phenotypes observed in *ERα* and *aromatase* null mutant mice, indicating that this mouse model may have the defect in the synthesis of estrogen in the ovary. Since *Amhr2-Cre* mice express Cre recombinase only in granulosa cells, not in thecal or interstitial cells within adult ovary, the ovarian defects observed in gonad-specific *SF-1* knockout mice are most likely due to the deletion of SF-1 in granulosa cells of the adult mouse ovary; however further studies are required to confirm the absence of SF-1 in granulosa cells in this mouse model. This study suggests that granulosa cell-expressing SF-1 is important for normal follicular development and ovulation, and is essential for female fertility. Further histological and morphological studies are needed to determine whether these mice have any defects in folliculogenesis and oogenesis. In addition, analyses of steroid hormone and molecular marker gene expression will confirm whether there is a defect in steroidogenesis in the ovary and whether *StAR, aromatase* and *inhibin alpha* are *bona fide* SF-1 downstream target genes in granulosa cells within the ovary. Whether SF-1 has a role in other types of ovarian cells (*e.g.* thecal and luteal cells) remains to be characterized using similar conditional knockout strategies.

5. LIVER RECEPTOR HOMOLOG-1 (LRH-1:NR5A2)

LRH-1 (NR5A2) is an orphan nuclear receptor that is evolutionarily closely related to SF-1 (23, 87). Mouse LRH-1 was initially identified because of its homology to the *Drosophila Fushi tarazu factor 1* (NR5A3) (88). Homolog have been identified in many species such as rat, horse, and human [see review in (76)]. Recently, phosphatidyl inositols have been identified as ligands for LRH-1 (29-31). Similar to SF-1, LRH-1 can bind to the consensus DNA sequence T/CCAAGGT/CCA/G and transactivate target gene transcription by recruiting coactivators such as SRC-1, SRC-3 and P300. Corepressors such as SMRT, Dax-1 and SHP can inhibit the transcriptional activity of LRH-1 by directly binding to its LBD through canonical LXXLL motifs. LRH-1 is expressed in tissues derived from endoderm and play important role for embryonic development, reverse cholesterol transport, bile-acid homeostasis and steroidogenesis (76). Embryonic lethality of *LRH-1* null mutant mice supports the essential role of LRH-1 during embryonic development (87, 89, 90). In addition, LRH-1 is required to maintain expression of *Oct4* in pluripotent embryonic stem cells (87).

Similar to SF-1, LRH-1 is also expressed in the ovaries of mouse (80, 81, 91), rat (80, 92), equine (84) and human (93). Unlike SF-1, which is expressed throughout the ovary with more abundant expression in thecal and interstitial cells, LRH-1 is more restrictedly expressed in granulosa cells of primary to preovulatory follicles and luteal cells of the newly formed corpus luteum within the rodent ovaries (80, 81, 92). Expression of LRH-1 in the ovary is regulated during the estrous cycle, by estradiol and FSH during follicular growth, LH during ovulation, and LH and prolactin during luteinization and maturation of the corpus luteum (80, 92). During pregnancy, high levels of LRH-1 expression, but not SF-1, are observed in the corpus luteum within rodent ovaries (80, 81, 92). The expression of LRH-1 in granulosa and luteal cells suggest that LRH-1 may have specific roles in regulating gene expression in those cells during folliculogenesis and ovulation (76). The potential direct target genes of LRH-1 in those cells within the ovary are *aromatase (Cyp19), 3β-hydroxysteroid dehydrogenase type II (HSD3B2)*, *StAR*, and *inhibin alpha*. Recent studies have shown that LRH-1 can bind to its response elements in the promoters of these ovarian genes to activate their transcription in cultured cells (77, 81, 94, 95). These results suggest that LRH-1 will have a critical role in granulosa and luteal cells within the ovary, affecting female fertility. This conclusion is in part supported by the female sterility and severe ovarian defects in folliculogenesis and ovulation observed in the *TATA-box-binding protein (TBP)-associated factor II 105 (TAFII105)*-null mice, in which *LRH-1* expression is down-regulated (3.5-fold) in the ovary (96). Generation and characterization of granulosa or luteal cell-specific *LRH-1* knockout mice are required to define the physiological roles of LRH-1 in the ovary and female reproduction.

6. PERSPECTIVE

The orphan nuclear receptors, GCNF, SF-1 and LRH-1, are emerging as important factors in controlling gene expression in the oocyte and somatic cells within the ovary, directly affecting female reproduction. Oocyte-expression of GCNF is important for maintaining normal paracrine signaling from the oocyte to somatic cells during

folliculogenesis and oogenesis, while SF-1 and LRH-1 are involved in steroidogenesis in somatic steroidogenic cells (*e.g.* granulosa, thecal and luteal cells) within the ovary. More-detailed characterization of oocyte-specific *GCNF* and granulosa cell-specific *SF-1* knockout models, together with the use of differential gene array technologies to identify tissue-specific downstream target genes, should facilitate dissection of the molecular signaling pathways of these nuclear factors in the ovary. Generation of thecal or luteal cell-specific *SF-1* and granulosa or luteal cell-specific *LRH-1* knockout or knockdown mice using the *Cre/loxP* and RNA interference strategies (53, 97) will help to determine the exact functions of SF-1 and LRH-1 in the specific cells within the ovary. Identification and characterization of the factors in controlling the expression of these orphan nuclear receptors, and the coregulators of GCNF, SF-1 or LRH-1 and the cross-talk between SF-1 and LRH-1 in regulating gene expression in the ovary in the presence or absence of their putative ligands such as phosphatidyl inositols will provide insight into understanding the mechanism of actions of these orphan nuclear receptors. Knowledge gained by these approaches will eventually facilitate a better understanding of ovarian functions and female fertility. This understanding will help to uncover the pathogenesis of certain types of female idiopathic infertility, premature ovarian failure, polycystic ovarian syndrome and ovarian cancer, and may lead to the development of new agents for regulation of female fertility with reduced side-effects and new medicines for the treatment of these diseases.

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