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Application of Growth Differentiation Factor 9 for the Control of Ovarian Follicular Development in Mammals

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Summary

Intraovarian factors derived from the theca layer, granulosa cells, and oocytes play important roles in regulating ovarian follicular development. Of the intraovarian factors, growth differentiation factor 9 (GDF-9) is one of the main regulators of follicular development in mammals. GDF-9 was discovered from mouse genomic DNA by PCR using degenerate oligonucleotides corresponding to conserved regions of known TGF-β family members. Animals deficient in GDF-9 showed an arrest of follicle development beyond the primary stage with one layer of granulosa cells. In this review, we focus on the role of GDF-9 during follicular development in mammalian ovaries, and the manipulation of follicle development by using GDF-9 gene and protein.

Key words: GDF-9, Ovary, Follicle Development

Follicles are the functional units of the ovary; each follicle consists of an oocyte surrounded by one or more layers of somatic cells, and develops through the primordial, primary, preantral, and antral stages (McGee and Hsueh, 2000). The proliferation, cytodifferentiation, and follicular atresia are regulated by complex interactions between a host of intrafollicular factors and pituitary gonadotropins. Transition through the initial stages of follicle development (from primordial follicles to primary follicles) can be observed in hypophysectomized rats (Hirshfield, 1985), which suggests that this process does not require pituitary gonadotropins. Initiation of follicle growth from the primordial to primary stage is characterized both by changes in the shape of granulosa cells and an increase in

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oocyte size (Anderson and Hirshfield, 1992). This process is characterized by differentiation and proliferation of granulosa cells and by enlargement of the oocyte (Braw-Tel and Yossefi, 1997) and is regulated by several growth factors (McGee and Hsueh, 2000).

Growth differentiation factor-9 (GDF-9) belongs to the transforming growth factor-β (TGF-β) superfamily and is secreted by the oocyte (Erickson and Shimasaki, 2000). GDF-9-deficient mice, similar to steel panda mutants of the kit ligand gene (Huang *et al.*, 1993), show follicular growth arrest at the primary follicle stage (Dong *et al.*, 1996). The ovaries of GDF-9 null animals lack several theca cell markers, including 17-alpha-hydroxylase (CYP17) and c-kit (Elvin *et al.*, 1999). Thus, the oocyte-derived GDF-9 plays an important role in follicular development of mammalian ovaries.

GDF-9, like other ligands in the TGF-β family, activates type II and type I serine/threonine kinase receptors that leads to the phosphorylation of Smad proteins (Massague, 1998). There are 5 type II serine/threonine kinase receptors : bone morphogenetic protein receptor type II (BMPRII), anti-mullerian hormone receptor type II (AMHRII), TGF-β receptor type II (TGFRII), and activin receptor type II (ActRIIA and ActRIIB). In addition, there are 7 type I receptors designated activin receptor-like kinases (ALKs) (ten Dijke *et al.*, 1993). Type II and type I receptors are organized into an amino terminal extracellular ligand-binding domain with 10 or more cysteine residues, a transmembrane region, and a carboxy-terminal serine/threonine kinase domain. Type II receptors have autophosphorylation activity (Attisano *et al.*, 1996). GDF-9 transmits signals to ovarian cells via BMPRII and ALK-5 (Mazerbourg *et al.*, 2004). This review will discuss the current state of knowledge regarding the role of GDF-9 in ovarian physiology in mammalians.

Role of GDF-9 in Follicular Development and Function

The mRNA and protein of GDF-9 is expressed in the oocytes of primary and large follicles in rats (Jaatinen *et al.*, 1999), mice (Dong *et al.*, 1996), and humans (Aaltonen *et al.*, 1999). These findings suggest that since follicles can progress to the primary stage in GDF-9 null mice (Dong *et al.*, 1996), once GDF-9 is produced by the oocyte of a given primordial follicle, this follicle can begin to grow. GDF-9 has been shown to promote the expression of kit ligand (Nilsson and Skinner, 2002) that stimulates theca cell recruitment from the surrounding stromal cells (Parrott and Skinner, 2000) and is implicated in early oocyte growth (Manova *et al.*, 1993).

In vitro treatment with GDF-9 promotes the survival as well as the progression of human follicles to the secondary stage in organ culture (Hreinsson *et al.*, 2002). In experiments using cultured granulosa cells, GDF-9 promotes granulosa

cell proliferation and stimulates basal estradiol synthesis in differentiated and undifferentiated granulosa cells and stimulates basal progesterone synthesis in differentiated granulosa cells (Vitt *et al.*, 2000a). In contrast, GDF-9 inhibits follicle-stimulating hormone (FSH)-induced steroidogenesis and luteinizing hormone (LH) receptor expression (Vitt *et al.*, 2000b). The inhibition of FSH-induced differentiation by GDF-9 could explain the inhibitory role of oocytes in FSH-induced steroidogenesis and LH receptor expression (Vanderhyden and Tonary, 1995).

Follicles in GDF-9 null mice lack selective theca cell markers such as CYP17, LH receptors, and c-kit receptors (Huang *et al.*, 1993). In contrast, *in vivo* treatment with GDF-9 increases ovarian CYP17 content (Vitt *et al.*, 2000a). Studies using small-follicle theca cells have indicated that GDF-9 decreases the abundance of the LH receptor and cytochrome P450 side-chain cleavage enzyme (CYP11A1) mRNA, but has no effect on CYP17 mRNA (Spicer *et al.*, 2008). GDF-9 also increases the number of theca cells and DNA synthesis in theca cells of small follicles but does not alter the percentage of cells that express CYP17 protein or the levels of CYP17 mRNA (Spicer *et al.*, 2008). These data suggest that GDF-9 increases the number of steroidogenic theca cells.

Studies using recombinant GDF-9 have showed that GDF-9 increases prostaglandin E_2 production in granulosa cells and hyaluronic acid synthetase and cyclooxygenase 2 (COX2) productions in cumulus cells (Elvin *et al.*, 1999). Moreover, knockdown of GDF-9 using RNAi inhibits cumulus expansion and decreases COX2 and hyaluronic acid synthetase levels (Gui and Joyce, 2005). These findings suggest that GDF-9 is associated with cumulus expansion.

Expression and Localization of GDF-9 Receptors

GDF-9 transmits its signals through specific receptors in the ovarian cell membrane. The phosphorylated type-I receptor, in turn, transphosphorylates a set of intracellular substrate signaling proteins called Smads (Attisano and Wrana, 2002). The specificity of Smad signaling is determined by the type I receptors, rather than the type II receptors (Attisano and Wrana, 2002). GDF-9 transmits the signals to ovarian cells via BMPRII and ALK-5 (Mazerbourg *et al.*, 2004). Expression of these receptor types has been confirmed in granulosa cells of the primordial and primary follicles (Juengel and McNatty, 2005). Studies using bovine follicles (Jayawardana *et al.*, 2006) indicated that the levels of BMPRII and ALK-5 mRNAs in the granulosa cells were significantly higher in the post-selection follicles (POF) than in the pre-selection dominant follicles (PRF). The mRNA expression of BMPRII and ALK-5 in the theca tissues showed no significant differences between PRF and POF. Thus, these studies suggest that BMPRII and ALK-5 may be physiologically relevant factors for transducing

signals critical for follicular development in mammals and such signaling could well be the result of the action of GDF-9.

Hormonal Regulation on the Expression of BMPRII and ALK-5

Studies using cultured bovine granulosa cells (Jayawardana *et al.*, 2006) indicated that a high concentration of E_2 (100 ng/ml) significantly increased the expression of BMPRII mRNA in granulosa cells, while a lower concentration of E_2 (10 ng/ml) significantly increased the level of ALK-5 mRNA. In contrast, treatment with FSH alone down-regulated the expression of the BMPRII and ALK-5 genes in cultured bovine granulosa cells, but FSH (5 ng/ml) in combination with a significantly lower E_2 concentration (1 ng/ml) up-regulated BMPRII and ALK-5 mRNA levels. These results suggest that E_2 is required for expression of BMPRII and ALK-5 during follicular development and provide strong evidence that FSH and E_2 cooperatively play physiological roles in regulating GDF-9 type I and type II receptor expression in the granulosa cells during bovine follicular development.

Manipulation of Early Folliculogenesis by GDF-9 Gene Injection

GDF-9 mRNA and GDF-9 protein are expressed not only at the primary follicle stage but are also present in oocytes throughout growth to the ovulatory phase (Elvin *et al.*, 2000). In GDF-9 knockout mice (Dong *et al.*, 1996), the absence of GDF-9 resulted in a block of folliculogenesis at the type 3b stage (late, one-layer primary follicle stage), which indicates that GDF-9 is associated with early folliculogenesis. *In vivo* treatment with recombinant GDF-9 has been shown to induce primordial follicle growth and enhance the transition from the primordial and primary to the small preantral follicular stage (Vitt *et al.*, 2000a). To investigate the function of GDF-9 in early follicular development, we directly injected porcine GDF-9 gene fragments into the ovary (Shimizu *et al.*, 2004). After injection of GDF-9 gene fragments, the percentage of primordial follicles in the ovaries decreased significantly compared with that in the control. Furthermore, the percentages of primary, secondary and tertiary follicles in the ovaries injected with GDF-9 gene fragments were significantly higher than those in the control (Fig. 1). Quantitative real-time PCR analysis showed that the expression of GDF-9 mRNA was greatly increased in ovaries that were treated with GDF-9 gene fragments, as compared to the controls. These results indicate that the injection of GDF-9 gene fragments increases the number of primary and secondary follicles, concomitant with a decrease in the number of primordial follicles. These findings are consistent with the previously reported findings that the *in vivo* application of GDF-9 led to an increase in the number of primary and preantral

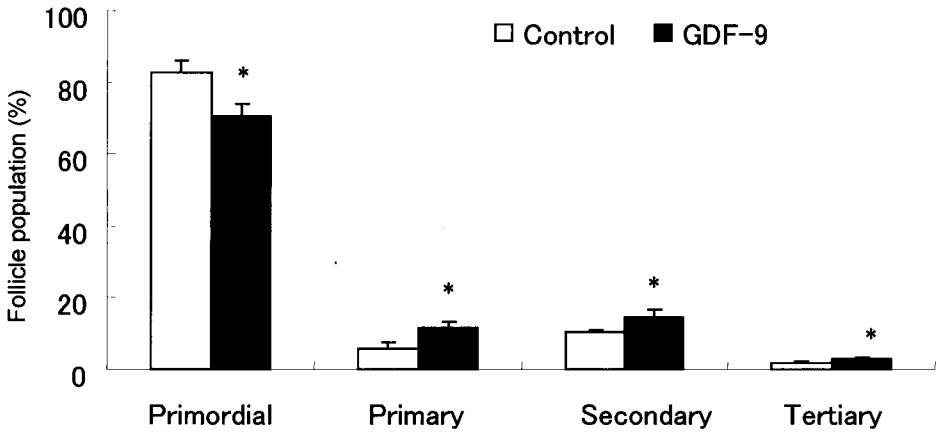

FIG. 1. Changes of folliculogenesis in the ovaries injected with or without porcine GDF-9 gene fragments. The percentages of primary, secondary and tertiary follicles in the ovaries injected with GDF-9 gene fragments were significantly higher ($p<0.05$, chi-square test) than those in the control, whereas the percentage of primordial follicles in the ovary decreased significantly compared with that in the control. Adapted from Shimizu *et al.* (2004).

follicles (Vitt *et al.*, 2000a).

Conclusion

Current ovarian stimulation protocols for infertility treatment influence antral follicle growth mainly by using gonadotropins (Diedrich and Felberbaum, 1998). However, in poor responders to gonadotropin stimulation (Scott, 1996), it is difficult to enhance the ovulation rate using the currently available infertility therapies. We are proposing the induction of follicular development by using a combination treatment of GDF-9 with other factors. Indeed, we have reported that the combination treatment of GDF-9 and vascular endothelial growth factor (VEGF) stimulates the development of antral follicles and a large number of oocytes could be collected from an individual animal (Fig. 2; Shimizu *et al.*, 2008). Thus, exogenous gene fragment injection may be an effective protocol to maximize the potential of ovarian function.

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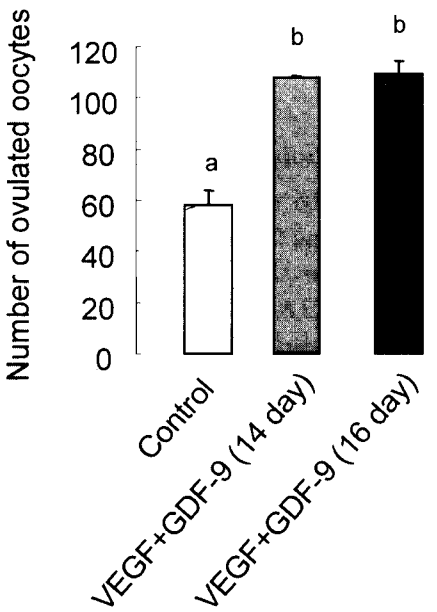

FIG. 2. The number of ovulated oocytes from immature rats with combined VEGF and GDF-9 gene fragments injection. Immature rats (14 or 16 days old) were injected with GDF-9 gene fragments into the ovary and were then dorsally injected with the VEGF gene at 21 days old. Different letters represent a significant difference ($p<0.05$). Adapted from Shimizu *et al.* (2008).

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