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Gonadotropin and intra-ovarian signals regulating follicle development and atresia: the delicate balance between life and death

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1. ABSTRACT

Regulation of mammalian follicular development is tightly regulated by both cell death and survival signals, including endocrine (e.g. gonadotropin) and intra-ovarian regulators (e.g. Nodal and GDF9). The destiny of the individual follicle (growth/ovulation or atresia) is dependent on a delicate balance in the expression and action of factors promoting follicular cell proliferation, growth and differentiation, and of those promoting programmed cell death (apoptosis). Development of the follicle from the primordial to preantral stage is regulated by oocyte-derived factors including GDF9 and BMP15, and is not dependent on gonadotropin support (gonadotropin-independent stage). As the follicle transits into the early antral stage it becomes responsive to gonadotropin (gonadotropin–responsive stages) and further development renders the follicle completely dependent on the presence of gonadotropin while modulated by intra-ovarian regulators (gonadotropin-dependent). Follicle fate is also regulated by pro-apoptotic factors such as the intraovarian regulator Nodal, which is secreted by the theca and promotes apoptosis of differentiated granulosa cells through a mechanism involving Smad2 signaling and suppression of the PI3K/Akt pathway. The intracellular protein prohibitin (PHB) appears to have a dual role during folliculogenesis; acting as a cell survival factor in undifferentiated cells, and as a pro-apoptotic factor following differentiation. Further investigations of the interplay between these endocrine and ovarian regulators will lead to a better understanding into the regulation of follicular development and atresia, allowing development of new techniques for assisted reproduction.

2. INTRODUCTION: ATRESIA AND FOLLICUALR CELL APOPTOSIS

Mammalian ovarian follicular development is tightly regulated by crosstalk between cell death and survival signals, which include both endocrine and intra-ovarian regulators (e.g. growth factors, cytokines and gonadal steroids). Whether the follicle ultimately ovulates or undergoes atresia is dependent on the expression and actions of factors promoting follicular cell proliferation, growth, differentiation or apoptosis. Growth of the follicle can be classified into distinct stages, each of which is influenced by a different subset of factors (Figure 1). For example, primordial to secondary follicle development does not require gonadotropin support and is hence termed gonadotropin-independent. Transition of the follicle from the preantral to early antral stage is primarily controlled by intraovarian regulators [e.g. growth differentiation factor 9 (GDF9)]. While gonadotropin promotes follicle growth during this stage it is not required (termed gonadotropin-responsive). Continual growth past antrum formation to the preovulatory stage is FSH-dependent, as the follicles require FSH for continual growth and suppression of apoptosis (1). While these stages have been well classified in terms of FSH responsiveness, the contributions of other factors during follicle growth are less well understood. Since ovarian dysfunction, such as premature ovarian failure, polycystic ovarian syndrome and gonadotropin poor-responsiveness, are consequences of stage-specific dysregulated follicle growth, understanding the molecular and cellular mechanisms in the control of follicular development may provide important insight into the pathophysiology of these conditions. This review will focus

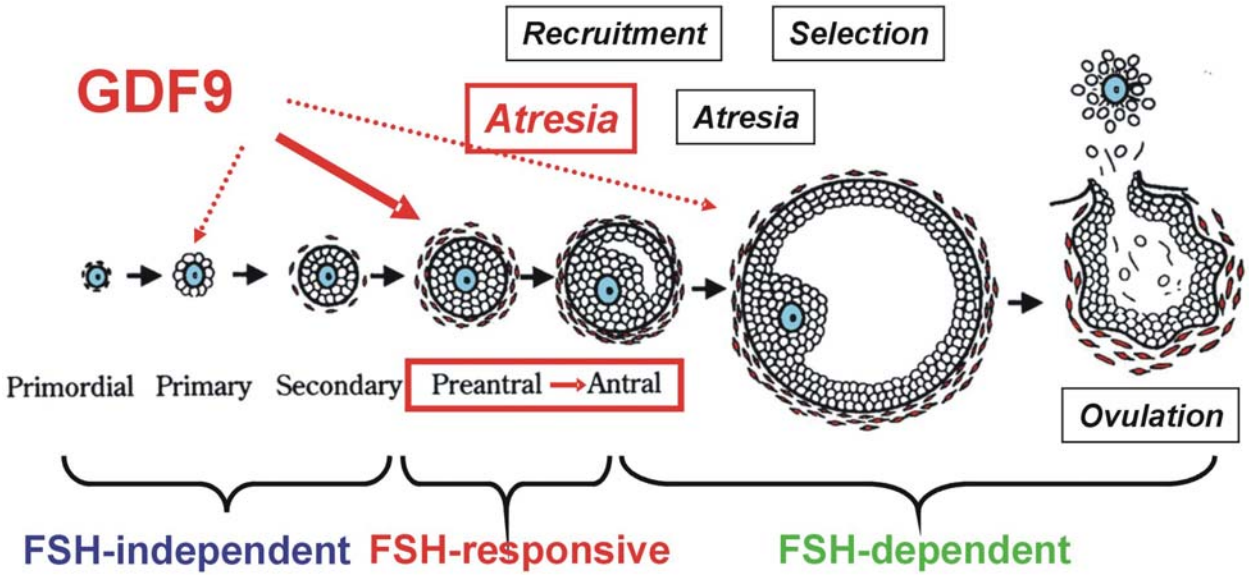

Figure 1. Model of follicular development and atresia highlighting the relative contributions of FSH and GDF9 to different stages of development.

on recent progress that has been made in understanding the roles of gonadotropin, Nodal, and GDF9 in control of follicle growth and atresia, as well as some of the intracellular signaling pathways which mediate the effects of these endocrine and intra-ovarian factors, as well as suggesting directions for future research, particularly in the context of ovarian pathology.

Only a small proportion of the primordial follicles present in the female mammalian ovary at birth will reach the ovulatory stage, while the rest will succumb to follicular atresia. Follicular atresia is mediated by apoptosis, which is initially observed in the granulosa cell layer (2,3), followed by apoptosis of the theca cells (4). It is well established that follicular atresia during antral stage of development is a consequence of follicular cell apoptosis (5). Apoptosis can be mediated via either the extrinsic (death receptor) or intrinsic (mitochondrial) pathway (6, 7), both of which are inducible in ovarian granulosa cells (5). The extrinsic death receptor pathway is activated by ligand-bound death receptors such as members of the TNF family and Fas-FasL (5), while the intrinsic pathway is activated from within the cell, and is characterized by permeabilization of the outer mitochondrial membrane resulting in the release of pro-apoptotic factors (e.g. cytochrome C, Smac, OMI) to the cytosol and loss of mitochondrial functions necessary for cell survival (7). Mitochondrial membrane permeabilization is primarily controlled by members of the Bcl-2 family (6-8), and has been described in the ovary (5). Characteristics of apoptosis include cytoplasmic and chromatin condensation, membrane blebbing and phagocytosis by neighboring cells (2). Late stage apoptosis is characterized by the cleavage of genomic DNA into oligonucleosomal length fragments by a Ca^{++}/Mg^{++}-dependent endonuclease which gives the distinctive appearance of nuclear fragmentation. It is well established that apoptosis is triggered by activation of a series of cysteine aspartate-specific proteases (caspases; (6, 8). The caspase family includes initiator caspases (caspase-8 and -9), which activate effector caspases (*i.e.* caspase-3, -6, and -7). Cleavage of the inactive pro-caspase to its active form is induced by either pro-apoptotic or insufficient anti-apoptotic signaling. Activated caspases are then able to cleave multiple cellular targets including poly (ADP-ribose) polymerase, DNA-dependent kinases, as well as cytoskeletal cytoplasmic and nuclear proteins. Caspase-3 is the most characterized effector caspase, and its activation leads to the final stages of cellular death. Granulosa cell caspase-3 content increases in preovulatory follicles undergoing atresia (2), and activated caspase-3 is detectable in granulosa cells of atretic follicles (9). Caspase-3 is required for granulosa cell apoptosis, as follicles from caspase-3 null ovaries do not show granulosa cell apoptosis in response to serum starvation (9).

3. FSH

FSH is a well characterized endocrine hormone which is required for follicle survival past antrum formation (1). *In vivo*, equine chorionic gonadotropin (eCG) suppresses granulosa cell apoptosis, promotes mitotic activity and follicular growth (10), while gonadotropin withdrawal via antibody neutralization induces granulosa cell apoptosis and follicular atresia (2). Indeed, we have shown that gonadotropin withdrawal leads to up-regulation of both Fas and FasL and is associated with follicular atresia (11). Granulosa cells from eCG-treated rats show reduced Fas and FasL content (11), while increased Fas content is evident in granulosa cells from atretic mouse follicles (12). *In vitro*, activation of Fas induces both cumulus (13) and granulosa cell apoptosis (14). In the goat, FSH promotes development of medium follicles, an event accompanied by reduced apoptosis *in vivo* (15), while heat stress inhibits rat granulosa cell

expression of FSH receptor (FSHR), and increases Bax expression and apoptosis (16). Interestingly, unlike the well established anti-apoptotic role of gonadotropin in developing early antral follicles, LH and FSH have been shown to increase caspase -3 and -7 activities in cultured large rat preovulatory follicles, which is accompanied by increased apoptosis in theca, but not granulosa cells (17). This latter finding confirms the earlier notions that theca apoptosis is the physiologic process underlying gonadotropin-induced ovulation (18).

3.1. Signaling Mechanisms

Akt (PKB) is a well known survival factor activated by growth factors in a phosphatidylinositol 3-OH-kinase (PI3K)-dependent manner (19). Akt promotes cell survival and suppresses apoptosis in a number of cell types. We have previously shown that gonadotropic stimulation increases granulosa cell phospho-Akt content both *in vivo* and *in vitro* (20, 21), and it has since been suggested that an adaptor protein, APPL1, mediates crosstalk between FSHR and Akt. APPL1 interacts with both the FSHR (22), the p110α catalytic subunit of PI3K and with inactive Akt (23), suggesting that APPL1 may function as an adaptor or docking protein to coordinate inputs from multiple signaling pathways (22).

The inhibitor of apoptosis proteins (IAPs) are a family of cell survival proteins, first identified in baculovirus, which are a key determinant of cell fate by modulating post-mitochondrial death signaling. This family includes X-linked IAP (XIAP), human IAP-1 (HIAP-1), human IAP-2 (HIAP-2), neuronal apoptosis inhibitory protein (NAIP), Survivin, Livin, TS-IAP and Apollon/Bruce (24). We have shown that over-expression of XIAP in small antral follicles by adenoviral sense expression results in enhanced follicular growth and reduced apoptosis, while its down-regulation elicited opposite responses (1). This effect was also observed in cultured primary granulosa cells, further supporting the important role of XIAP in the regulation of ovarian follicular development (1). We have shown in ovarian cancer cells that XIAP inhibits caspase-3 activity, and modulates the Bax/cytochrome *c* pathway by inhibiting caspase-9 (25-27).

Small antral follicles cultured in the presence of FSH exhibit increased XIAP expression, suppressed apoptosis, and increased follicular growth (1). A causal relationship between up-regulation of XIAP content by FSH and reduced apoptosis is supported by the observation that down-regulation of XIAP induced apoptosis, and prevented follicular development despite FSH stimulation. These findings suggest that XIAP plays an important role in FSH-stimulated follicular development and serves as an anti-apoptotic factor in rat ovarian follicles. It is also known that eCG administration up-regulates granulosa cell HIAP-2 expression in immature rats, suppresses granulosa cell apoptosis, and induces follicular growth, whereas gonadotropin withdrawal suppresses HIAP-2 expression and induces apoptosis and follicular atresia (28). NAIP expression may also contribute to granulosa cell survival, as its down-regulation in granulosa cells by antisense expression results in decreased number of morphologically normal ovulated oocytes, implying an indirect involvement of NAIP in germ cell development via enhancement of granulosa cell survival (29).

One of the main intracellular signals produced by FSH binding to its receptor is the up-regulation of cAMP/PKA, leading to increased progesterone synthesis, and aromatase and LH receptor expression (30). Although the details of these pathways are still being elucidated, it is clear that FSH stimulates the PKA-dependent phosphorylation of many intracellular targets, such as CREB and histones H3 and H1, resulting in changes in granulosa cell gene expression (30). In addition, FSH activates ERK in a PKA-dependent manner, via the dissociation of an inhibitory tyrosine phosphatase from ERK upon its phosphorylation by PKA (30). Activation of ERK leads to the phosphorylation of immediate early genes (e.g. c-jun, c-fos and JunB), transcription factors (e.g. SF-1), and other kinases (e.g. RSK and MSK) (30).

4. INTRAOVARIAN REGULATORS

4.1. GDF9

In addition to gonadotropin, factors synthesized and secreted within the follicle have a direct action on granulosa cell proliferation, differentiation and apoptosis, or modulate gonadotropic control of these processes. These factors include insulin-like growth factor-1, epidermal growth factor, transforming growth factor α and β, and tumour necrosis factor α, and are secreted by granulosa and theca cells (5, 31, 32). Oocyte-derived GDF9 and bone morphogenic protein 15 are essential for ovarian follicular development and female fertility (33). GDF9 deletion results in decreased granulosa cell proliferation, abnormal oocyte growth, and failure of follicles to develop past the primary stage (34). GDF9 stimulates rat granulosa cell proliferation, cumulus expansion, and preantral follicle growth *in vitro* (35), while suppresses FSH-induced cAMP production and steroidogenesis. Although $GDF9^{-/-}$ mouse ovaries do not show increased apoptosis (36), this may be due to follicle arrest at the primary stage, prior to when apoptosis is most common. We have recently demonstrated that down-regulation of GDF9 by intra-oocyte injection of a GDF9 antisense morpholino attenuates both basal and FSH-induced follicle growth, while the addition of recombinant GDF9 enhances basal and FSH-induced follicular growth (Figure 2; (37)). In addition, down-regulation of GDF9 content increases caspase-3 activation and granulosa cell apoptosis (Figure 3; (37)). Interestingly, GDF9 was sufficient to suppress ceramide-induced apoptosis in primary granulosa cells from early antral, but not large/preovulatory follicles (37), suggesting that GDF-9 is an important granulosa cell survival factor during the preantral to early antral transition, but may play a lesser role in follicle survival past antrum formation. Interestingly, there may be considerable crosstalk between GDF9 and FSH during the FSH-responsive stage, as a GDF9 is required to maintain FSH receptor expression (37), and GDF9 receptors (BMPRII and ALK-5) are up-regulated by co-treatment of estrogen and FSH (38).

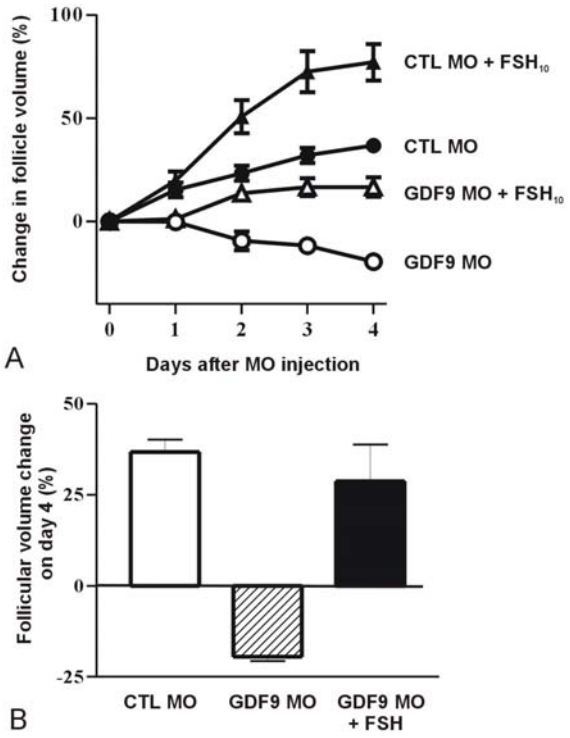

Figure 2. Down-regulation of GDF9 suppresses basal and FSH-induced preantral follicle growth *in vitro*. GDF-9 antisense Morpholino (GDF9 MO) or its control (CTL MO) were injected into the oocyte of isolated preantral follicles at Day 0. Follicles were treated with or without FSH (10 ng/ml) and GDF-9 (100 ng/ml) on Day 1 and cultured for another 3 days. Follicular diameter was measured daily and results were expressed as change in follicular volume. The percentage change of follicular volume on Day "n" of culture is defined as the volume difference between Day "n" and Day expressed as a percentage of the volume at Day 0. Results represent the means ± SEM of a total of 16 follicles from four or five independent experiments. Different superscripts indicate statistical significance ($P<0.01$). Reproduced with modifications from (37). Copyright 2006, The Endocrine Society.

4.2. Nodal

Nodal, a member of the TGFβ superfamily, has been shown to be an essential factor during embryo development; its knock-out is embryo lethal due to defects in primitive streak formation (39). During induction of dorsal mesoderm Nodal plays an important role in anterior patterning and formation of left-right asymmetry (39-41). In extra-embryonic tissues, Nodal inhibits differentiation of trophoblast stem cells (41) and regulates human placental development (42). A pro-apoptotic and growth inhibitory function of Nodal has been reported in human trophoblast cells (43) and ovarian epithelial cancer cells (44). Over-expression of Nodal or a constitutively active form of its type I receptor ALK7 (ALK7-ca) in both ovarian cancer cells and trophoblast cells resulted in a significant decrease in the number of metabolically active cells (43, 44). Both Nodal and ALK7 are expressed in the rat ovary, however interestingly they are only co-localized in granulosa cells of atretic follicles (45). In healthy follicles, Nodal protein is detectable primarily in the theca cell layer while ALK7 is detected in granulosa cells. However, the induction of follicular atresia via gonadotropin withdrawal results in the co-localization of Nodal and ALK7 to the granulosa cell layer (45). Treatment of primary granulosa cells from large antral follicles with recombinant Nodal, or forced over-expression of Nodal or ALK7-ca results in the induction of apoptosis (Figure 4), yet this effect is attenuated by a dominant negative form of ALK7 (45). Over-expression of either Nodal or ALK7-ca activated both caspase-3 and caspase-9, inhibited cell proliferation and increased apoptosis (43-45). Nodal-induced apoptosis is mediated, at least in part, through the ALK7 receptor and the Smad signaling pathways, as it can be blocked by dominant negative mutants of ALK7, Smad2, or Smad3 (43-45). Consistent with Nodal and ALK7 signalling in ovarian cancer cells (44, 46), ovarian epithelial cells (46) and trophoblast cells (43), either addition of recombinant Nodal or forced expression of Nodal or ALK7-ca in primary granulosa cells induces phosphorylation and nuclear accumulation of Smad2, as well as down-regulation of phospho-Akt (Figure 5) and XIAP content (45), supporting the hypothesis that Nodal/ALK7 signaling pathway is involved in promoting follicular atresia.

4.3. Signaling Mechanisms of Nodal and GDF9

As members of the TGFβ family, Nodal and GDF9 signal through herteromeric receptor complexes of type I and type II seriene/threonine kinase receptors. Binding of these ligands to their type II receptor recruits and induces phosphorylation of type I receptors and receptor-regulated Smads (R-Smads; Smad2/3). Activated R-Smads form a complex with the common mediator Smad (Smad4; (47)), which undergoes cytoplasmic to nuclear translocation and modulates transcription by binding with the *cis*-acting elements in the promoter region of the target gene (48).

While GDF9 activates the complex of ALK5 (49) and BMPIIR (50), and Nodal activates a complex of ALK4 or ALK7 and ActRIIA or ActRIIB, both receptor complexes are involved in the activation of Smads 2 and 3 (45, 49, 51, 52). Ovaries of Smad 3 knockout mice show increased atretic follicles, degenerated oocytes, and low expression of Bcl-2 (53), supporting the role of GDF9 in granulosa cell survival and the importance of Smad3 signaling in follicular development and atresia. In preantral rat follicle cultures, down-regulation of GDF9 attenuates both basal and FSH-induced follicle growth and increases granulosa cell apoptosis, while exogenous GDF9 also prevents ceramide-induced apoptosis in primary granulosa cells (37). This survival effect is mediated, at least in part, via the PI3K/Akt pathway, as recombinant GDF9 increases phospho-Akt content, and GDF9-mediated survival was prevented by the PI3K inhibitor LY294002 (37). In addition, forced expression of a dominant negative form of Akt prevented the pro-survival actions of GDF9.

Although the Smad pathway is activated by both GDF9 and Nodal, they have opposite effects on cell fate. This may be in part due to crosstalk with the PI3K/Akt

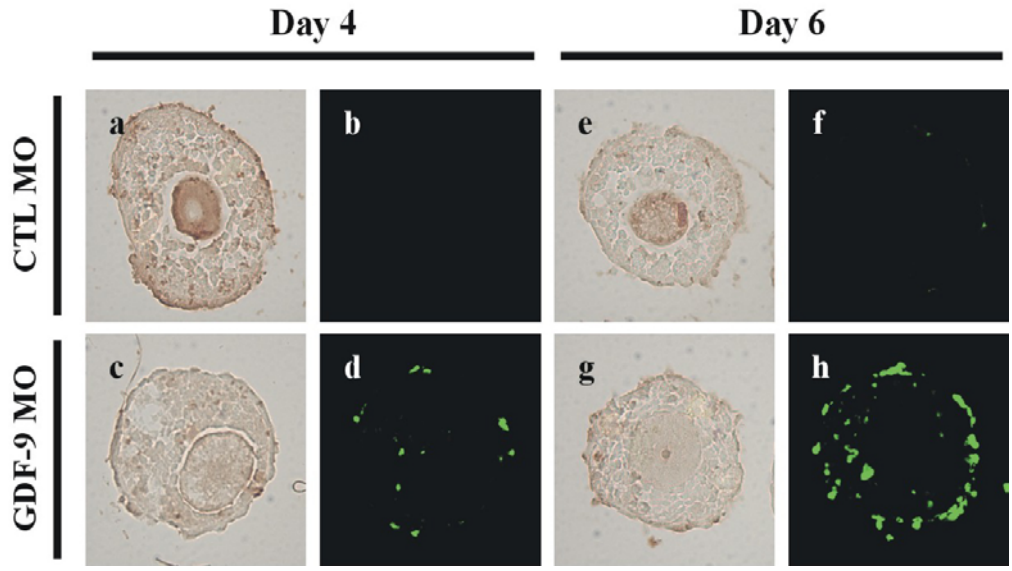

Figure 3. GDF9 suppresses granulosa cell apoptosis. GDF-9 antisense Morpholino or its control were injected into the oocyte of cultured preantral follicles at Day 0. GDF-9 expression (IHC) and apoptosis (TUNEL) were monitored on Day 4 (a-d) and Day 6 (e-h). An image from 10 representative follicles is shown for each treatment group. Reproduced with modifications from (37). Copyright 2006, The Endocrine Society.

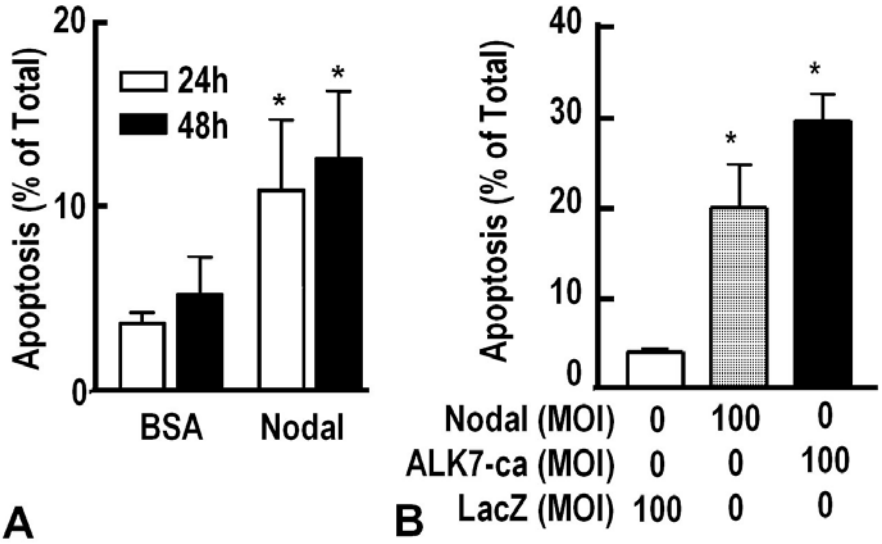

Figure 4. Nodal and ALK7 activation induce granulosa cell apoptosis *in vitro*. Granulosa cells from large antral/preovulatory follicles were cultured with (A) recombinant mouse Nodal 24 or 48h, or (B) infected with adenoviral Nodal or ALK7-ca for 48h. Apoptosis was determined by Hoechst staining analysis of nuclear morphology. (n=3, *p<0.05). Reproduced with modifications from (45). Copyright 2006, The Endocrine Society.

pathway. It has recently been shown that Akt has the ability to interacts directly with unphosphorylated Smad3, sequestering it outside the nucleus, and preventing its phosphorylation and nuclear translocation (54, 55). This Akt-Smad3 interaction results in the inhibition of both TGFβ-induced Smad3-mediated gene transcription and apoptosis (54, 55). While this mechanism appears to be Akt kinase activity-independent, it requires the phosphorylation of Akt (55). We have observed that phospho-Akt content in primary granulosa cells is decreased by Nodal (Figure 5; (45)), but is increased by GDF9 stimulation (37), suggesting a potential involvement of GDF9 and Akt in modulating the Nodal pathway. While this may provide a potential explanation for how Nodal and GDF9 can have both pro- and anti-apoptotic actions respectively, this hypothesis requires further investigation.

4.4. Prohibitin

The intracellular protein prohibitin (PHB; PHB1) is ubiquitously and abundantly expressed, suggesting an important role in cellular physiology. PHB is involved in diverse physiological functions including cell-cycle control

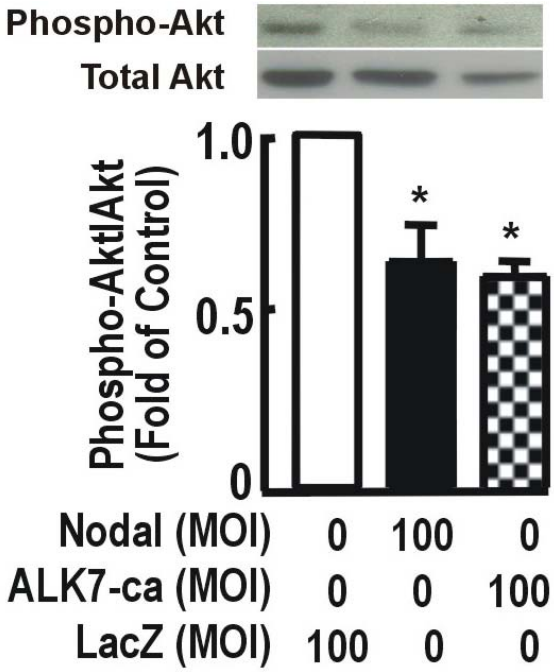

Figure 5. Nodal and ALK7 down-regulate granulosa cell phospho-Akt content. Granulosa cells from large antral/preovulatory follicles were infected with adenoviral Nodal or ALK7-ca for 48h. Phospho-Akt content was assessed by Western and expressed as a ratio of total Akt (n=4, * P<0.05). Reproduced with modifications from (45). Copyright 2006, The Endocrine Society.

(56-61), senescence (62-65), anti-proliferative activity (66-68), and cytodifferentiation (69-73). Although PHB is expressed in all cells of the ovary, including granulosa cells (72), its role in follicular development and atresia is poorly understood. Granulosa cell PHB content increases during follicle development, and during the initial events of granulosa cell apoptosis (72). Interestingly, eCG treatment *in vivo* increases both total PHB content as well as the acidic form of the protein (believed to be phosphorylated) in granulosa cells (69, 72), however the kinase responsible for this putative phosphorylation, and its role in the ovary is unknown.

Although PHB is primarily localized in the mitochondria (70, 71, 74), it is also present in the nucleus (75-77). In ovarian granulosa cells PHB is detectable in both the mitochondria (70) and nucleus (our unpublished data). Mitochondrial PHB is believe to play an important role in mitochondrial structure, function and inheritance (64, 71, 72, 76-82), while nuclear PHB has been reported to regulate cell proliferation through interaction with E2F family members, Rb (76) and p53 (75). PHB physically interacts with p53 in breast cancer cells, and enhances p53-mediated gene transcription (75), suggesting these two molecules may work together in the regulation of apoptosis. In addition, apoptotic stimuli induces co-translocation of PHB and p53 to the mitochondria (75), raising the possibility that PHB may modulate the mitochondrial function of p53 during apoptosis. Interestingly, a putative coiled-coil domain of PHB is sufficient to repress E2F1-mediated transcription and induce apoptosis in breast cancer cells (78).

It appears that the action of PHB in granulosa cells is dependent on the stage of differentiation. For instance, infection of undifferentiated granulosa cells from preantral follicles with a PHB adenoviral construct resulted in over-expression of PHB that markedly attenuated ceramide-, staurosporine- and serum withdrawal-induced apoptosis via the intrinsic apoptotic pathway. In contrast, over-expression of PHB in differentiated granulosa cells from antral follicles induces apoptosis. This latter response is mediated, in part, via the mitochondrial death pathway, and is suppressed by FSH at a post-mitochondrial level (our unpublished data). The mechanism of PHB-induced apoptosis in granulosa cells from antral follicles, and its protective effect in cells from preantral follicles will be the focus of future investigations.

A second form of prohibitin [PHB2; b-cell receptor associated protein (BAP37); repressor of estrogen activity (REA)] may also play a role in follicle development. In endometrial and breast cancer cells, PHB2 is recruited to hormone-occupied estrogen receptor leading to decreased transcriptional activity of ER, mediated by the recruitment of class I and II histone deacetylases (79-82). Similar to PHB1, PHB2 is expressed in all cells of the ovary (our unpublished data), however the role of PHB2 in the ovary is yet unknown.

5. FUTURE DIRECTIONS

The last decade has witnessed considerable advances in both our understanding of follicular development and atresia, and the tools available to further investigate these processes. As a consequence, these advances have led to important applications of gonadotropin preparations in the ovulation induction during assisted reproduction. In addition, the availability of techniques such as *in vitro* follicle culture now allows one to manipulate the expression of a gene(s) of interest in follicles at specific stages of development and in a more physiological environment, permitting more comprehensive studies to be performed. Despite the widespread use of gonadotropins in fertility treatment, there remains a subset of patients who either fail to respond or respond poorly to this hormonal induction (83). Understanding synthesis and actions/interactions of intra-ovarian regulators may allow one to finally unravel the mystery of follicle development and atresia, and ultimately offer the rational for design of better stimulation protocols in assisted reproduction.

While the pathophysiology of gonadotropin poor responders is not fully understood, it is possible that the lack of response of the follicle to gonadotropic stimulation may partly be due to suppressed preantral follicle development and insufficient ovarian expression of the gonadotropin receptor. Although the role of GDF9 in the growth of the primary follicle is well established (34), its importance in this process, particularly in the context of gonadotropin poor responders, is not clear. Moreover,

while the importance of GDF9 as a cell survival factor and in the control of follicular atresia is now beginning to be understood, many questions remain regarding GDF9 regulation and how it exerts its anti-apoptotic action on follicular cells. It is of interest that down-regulation of GDF9 results in decreased follicle cell expression of FSHR and preantral follicle growth (37), suggesting a basal level of GDF9 is required for FSHR expression and early follicular development. However, whether gonadotropin poor responders exhibit decreased GDF9 expression and action, and thus reduced gonadotropin receptor expression is not known. The regulation of GDF9 expression appears to depend on the stage of follicle development and the nature of its regulation appears to be species dependent (84, 85). We have observed that FSH and GDF9 act synergistically in regulating follicle growth *in vitro* (37), although precisely how these factors interact in this regulation, is unknown. Moreover, it is worth noting that ovaries of patients with premature ovarian failure still contain primordial follicles (86). Whether this pathology is in part due to dysregulated expression of GDF9 and if this could be ameliorated with a strategy which increases GDF9 expression/availability should be an area of future investigation. Further studies into the interaction between GDF9 and FSH will provide important information on how GDF9 and FSH co-operate to promote follicle growth and survival.

Polycystic ovarian syndrome (PCOS) affects up to 10% of women of reproductive age, and accounts for 75% of anovulatory infertility (87). Although TGFβ family members have been investigated extensively in the context of ovarian follicular development, their involvement of the pathophysiology of PCOS is unclear. GDF9 expression is reduced in ovarian follicles and oocytes in PCOS patients (88) although its significance in the regulation of early antral follicle growth remains to be determined. While no differences in BMP15 expression are detected in between these patients and normal subjects (88), information on the expression and role of Nodal/Alk7 in this pathology is lacking. Moreover, while recent studies indicate that the Nodal/Alk7 pathway is involved in the regulation of apoptosis in large antral follicles (45), its role in granulosa cell proliferation in early antral follicle growth is not known. Furthermore, since PCOS follicles are believed not to be associated with increased follicular apoptosis (89), whether this pathological condition is due to Nodal/Alk7-mediated suppression of granulosa cell proliferation and the mechanism thereof should be an area of future investigation.

We have recently demonstrated that Nodal is a physiological inducer of granulosa cell apoptosis (45). In healthy follicles, Nodal is found in the theca cell layer while its type I receptor is expressed in the granulosa cells (45). However, in atretic follicles, Nodal and ALK7 are co-localized, which may allow Nodal to serve as a marker to identify follicles destined to undergo atresia. Further investigation into the signaling pathways common to both GDF9 and Nodal is required, as both GDF9 and Nodal activate the Smad2/3 pathway, yet result in granulosa cell survival and apoptosis, respectively. As FSH is a determinant of follicle health, and regulates the function of other pro-apoptotic factors in the ovary, it will also be of interest to determine if and how FSH plays a role in regulating the pro-apoptotic role of Nodal. It is hypothesized that FSH, via PI3K/Akt, suppresses the release of mitochondrial death proteins (e.g. cytochrome C, Smac and Omi) and increases XIAP content (Figure 6). However, with declining FSH levels in mid/late follicular phase, Nodal and ALK7 are co-expressed in granulosa cells, resulting in the down-regulation of phospho-Akt and XIAP via the Smad signaling pathway, the release of mitochondrial death proteins and ultimately granulosa cell death and follicular atresia.

The role of prohibitin in the ovary is poorly understood. While PHB is pro-apoptotic in granulosa cells from antral but not preantral follicles (unpublished data), the molecular basis for this difference is not known. It has been suggested that PHB exists as both phosphorylated (acidic) and un-phosphorylated (basic) forms (69), which may partly explain its multifunctional nature. While the kinase responsible for this phosphorylation is unknown, analysis of the PHB protein sequence reveals the presence of a consensus Akt phosphorylation sequence. PHB2 also contains this sequence and physically interacts with Akt in muscle cells (90). Whether PHB and Akt interact physiologically and the functional significance of this phenomenon in granulosa cells remains to be investigated. As FSH activates the PI3K/Akt pathway (91), and this pathway is vital for cell survival in many cell types (92), it is possible that FSH may influence PHB expression and/or action via the PI3K/Akt pathway. Alternatively, it is also possible that PHB expression and/or activity could be regulated by intra-ovarian factors. Preliminary studies suggest that Nodal increases granulosa cell PHB content (unpublished data) and apoptosis *in vitro*, although the mechanism through which this occurs is unknown.

In addition to activation of the mitochondrial death pathway (unpublished data), the nuclear localization of PHB suggests it may also be capable of inducing apoptosis via altered gene transcription. As a nuclear factor, PHB is known to repress the transcriptional activity of E2F family members (60), while promoting p53 transcriptional activity (75), suggesting PHB may induce apoptosis via up-regulation of genes involved in the apoptotic response.

To further our understanding of the role of PHB in follicle development, it will be of importance to identify the factor(s) regulating its expression and/or function in the follicle. As it appears that PHB plays a different role at different stage of follicle development, understanding the role of PHB in controlling the fate of the follicular cells (ie pro- vs anti- apoptotic) and thus the destiny of the follicle (continual growth or atresia) may provide insight in developing strategies to artificially rescue or prevent atresia in follicles which do not respond to gonadotropin stimulation.

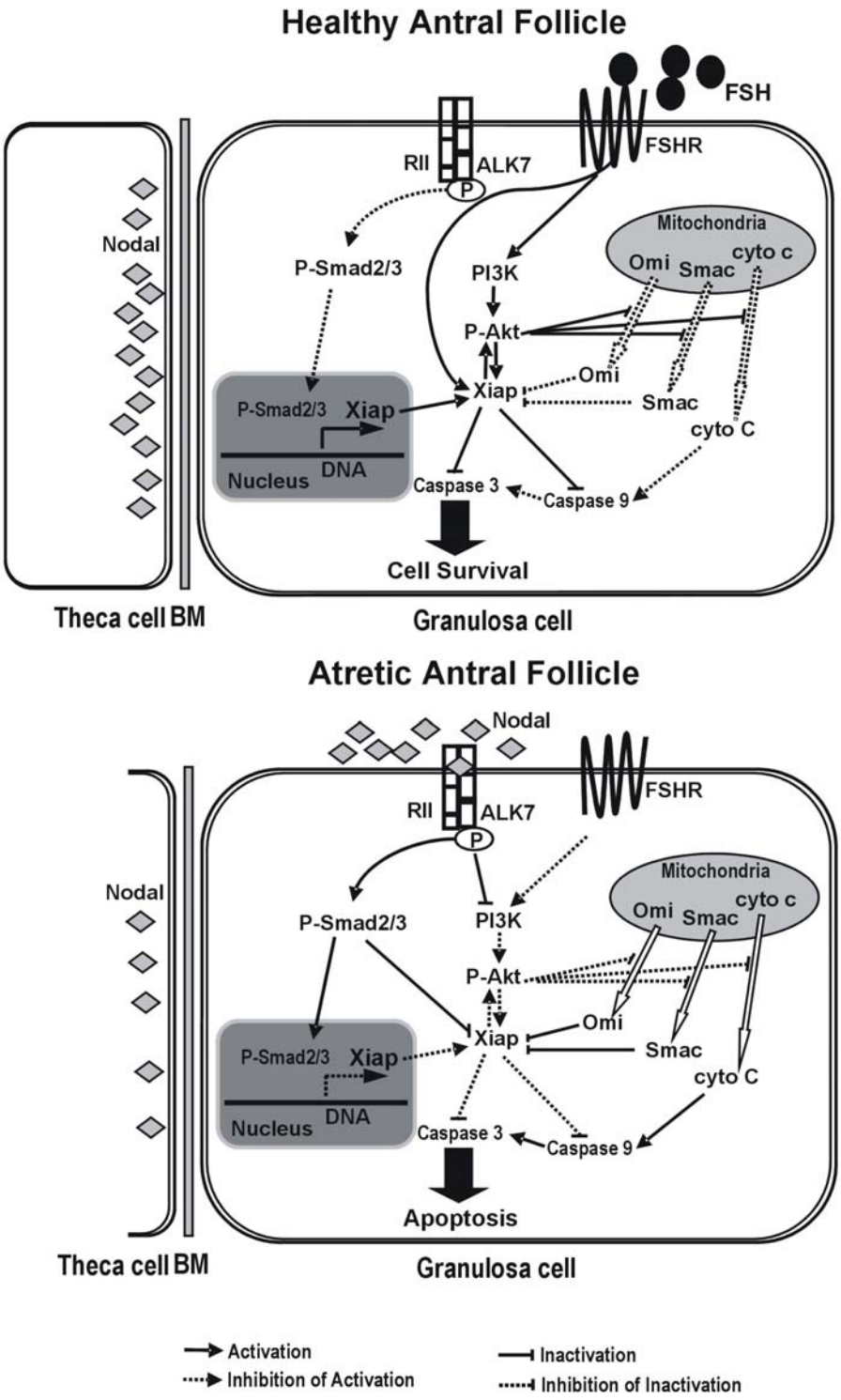

Figure 6. A hypothetical model illustrating the role and regulation of the Nodal-ALK7 in the control of follicular development and atresia. Reproduced with modifications from (45). Copyright 2006, The Endocrine Society.

6. CONCLUSIONS

Follicular growth and atresia are complex processes, governed by a host of endocrine, paracrine and autocrine signals. The fate of the follicle cell and ultimately of the follicle is determined by the balance between these factors. While we are beginning to identify what these factors are, the signaling pathways and the molecular

mechanisms which determine cell fate are poorly understood. Understanding the interplay between pro-apoptotic factors (e.g. Nodal) and anti-apoptotic intermediates (e.g. GDF9) at the subcellular level will allow one to better understand how granulosa cell fate (life versus death) is determined. As the transition of the follicle from the preantral to antral stage is most susceptible to follicular atresia, understanding these mechanisms is of clinical significance in providing germ cells for assisted reproduction. The challenge ahead is to understand not only how these factors work in isolation, but how they interact in the regulation of follicle destiny, and how dysregulation in these interactions may lead to ovarian pathology such as PCOS, premature ovarian failure and gonadotropin non-responsiveness. In addition, identification of the factor(s) that promote follicle growth from the preantral/small antral stage may provide important information for the identification of intra-follicular biomarkers for the selection of healthy oocytes and embryos in assisted reproduction.

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Abbreviations: FSH: follicle stimulating hormone, GDF9: growth differentiation factor 9, PI3K: phosphatidylinositol 3-OH-kinase, PHB: prohibitin 1, PHB2: prohibitin 2, TNF: tumour necrosis factor, FasL: Fas ligand, Smac: second mitochondrial-derived activator of caspase, FSHR: FSH receptor, IAP: inhibitor of apoptosis protein, XIAP: X-linked inhibitor of apoptosis protein, MAPK: mitogen activated protein kinase, ERK; extracellular signal-regulated kinase, PKC: protein kinase C, STAT: signal transducer and activator of transcription, TGF: transforming growth factor, IGF: insulin-like growth factor, EGF: epidermal growth factor, BMP15: bone morphogenic protein 15, phospho-: phosphorylated, ALK: Activin receptor-like kinase, ALK7-ca: ALK7-constitutively active.

Key Words: Ovary, Cytokine, Follicle, Development, Atresia, FSH, Nodal, Growth and differentiation factor, GDF9, Prohibitin, Reivew

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