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Ovarian Follicular Atresia

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1. Introduction

Throughout ovarian development and function in mammals, a highly orchestrated, periodic process known as follicular atresia occurs that destroys and eliminates follicles and oocytes from the ovary. Follicular atresia is pervasive. In humans, it is estimated to account for 99.9% of the loss of oocytes from development of the fetal ovary until reproductive senescence (Baker, 1963; Faddy et al., 1992). Overall, the process of follicular atresia eliminates all but 300-400 oocytes, some of which become available for selection, ovulation and potential fertilization. Similar phenomena of loss are observed in other mammals. The rationale for such extensive elimination of oocytes during fetal and adult life is unknown. However, in the case of larger, more mature follicles (i.e., antral follicles), the importance of follicular atresia is attributed to a finite lifespan of the oocyte. Hence, in the adult female, atresia ensures that only the healthiest follicles, containing oocytes of optimal quality for fertilization, remain available throughout the reproductive period. In this chapter, we provide a broad overview of the physiological process of follicular atresia, giving emphasis to the cellular and molecular mechanisms that influence the process in two monovulatory species, the cow and human female.

2. Follicular development and classification of follicles

Prior to the onset of follicular atresia, the ovary contains an abundance of non-dividing, primordial follicles, which contain the reserve of germ cells available for fertilization throughout the reproductive life of the adult. Primordial follicles consist of an immature oocyte surrounded by a single layer of granulosa cells. In many mammals the number of primordial follicles is established at the time of birth, whereas in the human female and in many domesticated livestock, including the cow, this number is determined during fetal development. In either scenario the transition from non-dividing/non-growing primordial follicles to growing follicles is a critical part of follicular development or "folliculogenesis". It is a process that begins gradually, almost imperceptibly after the formation of primordial follicles, and then continues throughout the reproductive life of the animal (Fortune et al., 1998; Oktem & Urman, 2010). However, the factors influencing the formation of primordial

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follicles and the mechanism(s) responsible for their activation are largely unknown and beyond the scope of this review. A recent article by Aerts and Bols (Aerts & Bols, 2010) provides insight about these topics as they pertain to the cow, which may be informative for the reader. Here, the discussion will focus on literature concerning folliculogenesis and follicular atresia beyond that of the primordial follicle. In addition, studies emphasizing bovine (cow) and human ovarian function will be highlighted because both species are principally monovulatory (i.e., release one oocyte for fertilization per estrous/menstrual cycle), and both entail similar follicular dynamics in which development, selection and dominance of a single follicle occurs from, and at the expense of, a cohort of growing follicles.

A general classification of follicles in the adult ovary consists of the following four categories: 1) Primordial follicles, 2) Primary follicles, 3) Secondary follicles, and 4) Tertiary follicles (General characteristics are summarized in Table 1). Primordial follicles consist of a single layer of granulosa cells (< 10 cells total) surrounding an immature oocyte (~30um) with no zona pellucida. Overall follicle diameter is <40 um. Primary follicles are comprised of one to two layers of cuboidal granulosa cells (10-40 cells) surrounding an immature, spherical oocyte (25-45 um). Sparse patches of zona pellucida are evident around the oocyte. Ultrastructurally, the mitochondria of the oocyte are "round" and located in the deep cortical region. Golgi complexes are also located in the deep cortical region with junctional adherins detectable between the oocyte and the surrounding granulosa cells. The oocyte also contains extensive rough and smooth endoplasmic reticulum, but no cortical granules or microtubules (Gougeon & Chainy, 1987; Fair et al., 1997; Kacinskis et al., 2005; Westergaard et al., 2007). Secondary follicles have two to six layers of cuboidal granulosa cells (40-250 cells) surrounding a maturing oocyte (35-70um). Small amounts of zona pellucida surround the oocyte in half the follicles. Ultrastructural features include erect microvilli of the oocyte peneterating the zona pellucida, gap junctions between the oocyte and surrounding granulosa cells, "elongated" mitochondria in the deep cortical region, extensive rough and smooth endoplasmic reticulum, clusters of cortical granules in a few oocytes, but again, no microtubules (Gougeon & Chainy, 1987; Fair et al., 1997; Kacinskis et al., 2005). Tertiary follicles have greater than six layers of granulosa cells (>250 cells), which contain a mature oocyte (100-150um) and a fully-formed zona pellucida surrounded by specialized granulosa cells called the cumulus oophorus. The oocyte of these follicles contain erect microvilli which traverse the zona pellucida, gap and adherens junctions, abundant round and elongated mitochondria, extensive rough and smooth endoplasmic reticulum, numerous lipid droplets and vesicles, increased numbers of Golgi, clusters of cortical granules in all oocytes, and vast arrays of microtubules (Fair *et al.*, 1997). Tertiary follicles are most notably distinguished by the presence of a fluid-filled antrum which, in the case of the bovine, results in follicles of 0.5 to 25mm in diameter. In addition to the above general characteristics and classification of follicles, specialized cells derived from the ovarian stroma, called theca cells, surround the basal lamina of primary follicles (Hirshfield, 1991), but later form distinct layers of theca interna and externa as the follicles transition from secondary to tertiary status. Throughout the reproductive life of the female, any and all of the above types of follicles are evident histologically within the ovary. For further review about the classifications and descriptions of ovarian follicles, the reader is referred to the following authors (Mossman & Duke, 1973; Gougeon & Chainy, 1987; Fair et al., 1997; Kacinskis et al., 2005; Westergaard et al., 2007).

Follicle Category	Granulosa Layer	Oocyte Characteristics	Other Characteristics
Primordial Follicles	Single layer, cuboidal cells (<10 cells)	Immature oocyte (~30 microns; no zona pellucida)	Follicle diameter <40 microns
Primary Follicles	One to two layers, cuboidal cells (10-40 cells)	Immature, spherical oocyte (25-45 microns; sparse zona pellucida)	"Round" mitochondria, golgi complexes in deep cortical region of oocyte
Secondary Follicles	Two to six layers, cuboidal cells (40-250 cells)	Maturing oocyte (35-70 microns; small amounts of zona pellucida)	Erect microvilli of oocyte penetrate the zona pellucida, "Elongated" mitochondria, extensive smooth and rough endoplasmic reticulum
Tertiary Follicles	More than six layers, squamous cells (>250 cells)	Mature oocyte (100-150 microns; fully formed zona pellucida)	Presence of cumulus oophorus, extensive organelle development within the oocyte, antrum formation

Table 1. General classification and characteristics of follicles of the adult ovary. Morphometric and ultrastructural characteristics of follicles are further described by Gougeon & Chainy, 1987; Fair et al., 1997; Kacinskis et al., 2005; and Westergaard et al., 2007.

3. Characteristics of growth of antral follicles

In the cow, growth of follicles is an ongoing process, beginning with the growth of primordial follicles around day 90 of fetal gestation (Fortune, 2003; Fortune et al., 2010, 2011). These follicles develop into primary, secondary, and early tertiary (preantral) follicles in the absence of gonadotropins, with late tertiary, antral follicles beginning to emerge near day 210 of gestation (Yang & Fortune, 2008). Thereafter, especially following puberty, pituitary gonadotropins and locally-secreted ovarian modulators prompt the growth of cohorts of tertiary follicles within both ovaries, and facilitate the selection of a single follicle suitable for ovulation and conception. Tertiary follicles develop into mature, preovulatory-size follicles within 42 days, encompassing approximately the period of two estrous cycles in cows. Among the many hormones that stimulate the growth and development of these follicles, follicle-stimulating hormone (FSH) is recognized as a major influence.

Systemic, pulsatile secretion of FSH triggers the synchronous development of a cohort of tertiary follicles in the ovary of the cow during the estrous cycle, which is often referred to as a "follicular wave". The emergence of these waves coincides temporally with a surge of FSH secretion (Adams *et al.*, 1992), during which one or two dominant follicles and several subordinate follicles develop (Savio et al., 1988; Sirois & Fortune, 1988; Knopf et al., 1989). Most estrous cycles of the cow consist of two or three waves of follicular growth preceding ovulation. A similar pattern of follicular growth during the menstrual cycle occurs in women (Baerwald *et al.*, 2003). Interestingly, granulosa cells of follicles express FSH receptors relatively early in follicular development, particularly in primary follicles (Oktay et al., 1997; Bao & Garverick, 1998; Findlay & Drummond, 1999; Webb et al., 1999).

Conceptually it is reasonable to suggest pulsatile FSH secretion might also influence the growth of primary and secondary follicles, but this possibility has not yet been adequately explored. Regardless, we know that during a follicular wave a cohort of tertiary follicles emerges as a result of FSH stimulation. One follicle is selected and becomes dominant; whereas the remaining follicles of the cohort become subordinates (Ginther *et al.*, 1997). Dominant follicles secrete hormones that play a prominent role in their continued growth while simultaneously limiting the growth and possibly triggering the regression of the subordinate follicles of the cohort. Inhibins, for instance, are secreted by dominant follicles, which then act systemically to diminish FSH secretion (Armstrong & Webb, 1997; Webb et al., 1999; Knight & Glister, 2006). Dominant follicles also maintain high levels of estradiol secretion, which further reduces FSH secretion and compromises the growth needs of the subordinate follicles (Ginther *et al.*, 2000). Insulin-like growth factor-1 (IGF-1), its binding proteins, and other growth factors are additional influences within the microenvironment of the ovary that impact the growth and dominance of follicles (Armstrong & Webb, 1997; Webb et al., 1999; Armstrong et al., 2000). Elevated concentrations of free insulin-like growth factor-1 (IGF-1) within the dominant follicle, for instance, support its continued development as the availability of FSH declines (Beg & Ginther, 2006). Concomitantly, dominant follicles acquire additional luteinizing hormone (LH) receptors to respond to increased LH availability and the preovulatory LH surge (Beg & Ginther, 2006). Although subordinate follicles also possess these developmental capabilities, they evidently lack sufficient time during the follicular wave to attain them and, hence, are destined to undergo regression in a process known as "follicular atresia".

4. Overview of follicular atresia

Based upon the etiomology of the word (from Greek: a= not, tresia=perforated), follicular atresia strictly refers to the failure of a follicle to rupture or ovulate. More broadly, follicular atresia encompasses the fate or demise of all follicles except those destined for ovulation. While most studies focus on follicular atresia in the adult ovary, the process also predominates in the fetal ovary and after birth. Before the time of follicle formation, and upon establishment within the developing ovary, the primordial germ cells become oogonia; while oogonia continue to proliferate, they are also subject to large-scale apoptotic demise. Around mid-gestation (about 20 weeks of fetal development in human), oogonia undergo transformation into oocytes that enter meiosis, but are later arrested at the dictyate stage. This is also the period when oocytes become surrounded by granulosa cells to form primordial follicles. In the human female fetus, the peak number of oocytes is reached at mid-gestation (~ 7 million cells), but during the last half of gestation at least two-thirds of these are lost, leaving a reserve of 1 to 2 million oocytes at birth. This massive loss of germ cells (named oocyte attrition) results from apoptosis of these cells at all developmental stages (Baker, 1963; Forabosco et al., 1991). Oocyte attrition also occurs prenatally before follicle formation. Of note is the observation in the bovine that any oocyte that fails to become part of a primordial follicle will be lost (Ohno & Smith, 1964). The loss of germ cells does not end at the time of birth; in the human female, there is an additional 75% loss of oocytes through puberty (with about 400,000 remaining within follicles) (Baker, 1963; Peters et al., 1978; Himelstein-Braw et al., 1976). In contrast to the prenatal situation, post-natal depletion of oocytes occurs by follicle atresia. Follicular development is characteristically dynamic throughout childhood, with the size of the follicle reserve at puberty being a

reflection of the dynamic outcomes of follicular quiescence, growth, or atresia (Tingen et al., 2009). Throughout reproductive life, about 400 follicles will attain ovulation with an estimated 250,000 follicles lost by atresia at a rate of about 1000 follicles per month. However, the rate of follicular atresia is accelerated in the years preceding menopause (Faddy et al., 1992).

Follicular atresia affects all stages of follicular development, but the proportion of follicles that become atretic is enhanced by increased follicle size. In natural cycles, small antral follicles are particularly prone to atresia (Gosden & Spears, 1997; Hirshfield, 1991; Kaipia & Hsueh, 1997). With no new oocytes or follicles forming after birth, the subject of follicular loss is particularly poignant. The adult female mammal has only a finite number of follicles and there is a very high rate of follicular atresia. This suggests follicular atresia is under tight control to ensure oocytes remain available for ovulation throughout the reproductive life of the female. The regulation of follicular atresia is a topic presented below.

5. Antral versus basal follicular atresia in the cow

Histological descriptions of follicular atresia in the bovine ovary date back nearly 50 years. Among these, two studies in particular established classifications of atresia which differed (Rajakoski, 1960; Marion et al., 1968), and since may have contributed to the misinterpretation of findings by authors of more recent investigations. Irving-Rodgers and coworkers (Irving-Rodgers *et al.*, 2001) re-visited this subject and provided evidence for two basic morphological forms of atresia in cattle: 1) Antral atresia, and 2) Basal atresia. The general histological features of these two forms atresia are summarized below. However, more importantly, Irving-Rodgers and co-workers (Irving-Rodgers *et al.*, 2001) also suggested that more recent studies in which the previous classifications had been implemented to correlate with biochemical or physiological parameters of follicle status should be re-evaluated.

Antral atresia is characterized by the initial elimination of granulosa cells proximal to the antrum. Numerous pyknotic nuclei are evident in these antral layers of the membrana granulosa, and sometimes within the antrum itself. Remnants of mitochondrial and plasma membranes are also seen associated with the pyknotic nuclei (Irving-Rodgers *et al.*, 2001). The basal granulosa cells (i.e., those aligning the basal lamina), conversely, remain intact and possess many ultrastructural characteristics of healthy cells (e.g. moderate numbers of mitochondria, lipid droplets, and moderate amounts of endoplasmic reticulum)(Irving-Rodgers *et al.*, 2001). Antral atresia is viewed as the classic and most widely-observed form of follicular atresia because it occurs at all stages of follicle development in most species, and it is universally seen in large follicles (> 5 mm in diameter), including the dominant follicle, of monovulatory species (Irving-Rodgers *et al.*, 2001).

Basal atresia entails the destruction of the most basal layer of the follicle, whereas the most antral layers remain intact and healthy (Irving-Rodgers *et al.*, 2001). The basal lamina is often penetrated by macrophages and invading capillaries, and the theca layer of the follicle has additional deposition of collagen. The middle layers of the membrana granulosa exhibit a progression of cellular morphology and ultrastructure from the fragmented, pyknotic cells typical of the basal layers to the healthy, intact cells found in the antral layers. In the

cow/heifer, this form of atresia occurs only in small follicles (< 5 mm in diameter)(Irving-Rodgers *et al*., 2001). Whether or not this form atresia is unique to the bovine is uncertain because, to date, there have been no other reports of its existence in other species.

6. Apoptosis as a mechanism of follicular atresia

Apoptosis is recognized as a hallmark and contributing factor of atresia of antral follicles (Tilly et al., 1991; Tilly, 1996; Chun & Hsueh, 1998; Johnson, 2003; Matsuda-Minehata et al., 2006; Inoue et al., 2011). It is a cell-specific mechanism of discrete elimination of cells during follicular atresia that ensures regression of the follicle without inciting an overt inflammatory response. During atresia the cells of the follicle undergoing apoptosis are generally scattered throughout the parenchyma, and may or may not include the oocyte (Kim et al., 1998; D'Haeseleer et al., 2006; Peluffo et al., 2007). Initiating mechanisms of apoptosis include extrinsic factors, such as the cytokines, and intrinsic factors including oxidative stress, irradiation, and the activation of tumor suppressor genes.

Cytokines are among the extrinsic factors of apoptosis because their effects are initiated extracellularly through receptor-mediated mechanisms. Members of the tumor necrosis factor (TNF) superfamily are among the most widely-recognized cytokines triggering apoptotic events in follicles. They include TNF (Basini et al., 2002; Sasson et al., 2002), Fas ligand (Porter *et al.*, 2000), and TNF-related apoptosis-inducing ligand (TRAIL)(Johnson et al., 2007; Jaaskelainen et al., 2009). Additional extrinsic factors that influence apoptosis of granulosa cells include interferon-gamma (Quirk et al., 2000; Vickers et al., 2000) and several types of growth factors (Quirk *et al.*, 2000). Intrinsic factors of apoptosis are those that are generally provoked by aspects of stress. For instance, nutrient deprivation, oxidative damage, and genetic impairment are all examples of cellular/molecular stress that can lead to the upregulation of intrinsic mechanisms of apoptosis.

Aspects of follicular growth, selection, and atresia are considered highly-orchestrated processes in which the ovarian microenvironment and the interplay between pro-apoptotic and anti-apoptotic molecules have a significant role. A fairly comprehensive review of many of these molecules, their actions, with accompanying references, has been described previously (Hussein, 2005). The complexity of the signaling pathways these molecules utilize, however, is not well understood, and the discovery of novel molecules and mechanisms which influence granulosa cell survival continues (Hennebold, 2010). For instance, the recent finding of a microRNA (*Mir21*), which blocks apoptosis of murine granulosa cells (Carletti *et al*., 2010), indicates there may be a vast array of other molecular mechanisms controlling granulosa cell fate, and hence follicular fate, which have yet to be investigated.

Members of the tumor necrosis superfamily are perhaps among the most readily identified pro-apoptotic molecules associated with granulosa cell death and follicular atresia. In the human, bovine, and other species, Fas ligand and the Fas-mediated pathway of apoptosis are considered prominent mechanisms of granulosa cell death during follicular atresia (Quirk et al., 1995; Hakuno et al., 1996; Kondo et al., 1996; Vickers et al., 2000). The targeted, cell-specific nature of granulosa cell death without any accompanying inflammatory response or collateral damage to adjacent cells is a unique feature of the Fas ligand-Fas system, consistent with its renowned role(s) in immune response, the establishment of

immune tolerance, and the activation-induced cell death of lymphocytes. The cytokines TNF, TRAIL, and their corresponding receptors are additional factors, similar to Fas ligand and Fas, that potentially influence cell fate and follicle status in certain species (Prange-Kiel et al., 2001; Xiao et al., 2002; Inoue et al., 2003). In general, cytokine binding and reception triggers the intracellular activation of initiator and effector caspases (Boone & Tsang, 1998; Johnson & Bridgham, 2002; Valdez et al., 2005; Hurst et al., 2006). Initiatior caspases include caspase-6, 8, 9 and 10, while effector caspases include caspase-2, 3, 6, 7 and 14 (McCarthy & Bennett, 2002). Caspases are constitutively expressed in their inactive zymogen form. Following proteolytic cleavage and activation, the caspases cleave target proteins at sites following aspartic residues (Muzio *et al.*, 1998). A representative model is the activation of the caspase cascade following Fas ligand binding to Fas, in which oligomerization of caspase-8 results in cleavage of the prodomain from its active subunit, and autoactivation occurs (Figure 1). The active domain of caspase-8 then cleaves the prodomain of the effector caspase, caspase-3. Active caspase-3 cleaves a vast number of proteins within the cell including the cell cycle regulators Cdc 27, Cyclin A, and Topoisomerase (Fischer *et al.*, 2003). In addition, caspase-3 cleaves various DNA-associated repair enzymes and cytoskeletal proteins, particularly the cytokeratin-containing intermediate filaments (McCarthy & Bennett, 2002; Fischer et al., 2003). As described later, cytokeratin intermediate filaments are a diverse family of proteins that generally exert protective effects within cells, preventing cell stress and apoptosis. Hence, their disassembly or loss renders cells vulnerable to a variety of insults and apoptotic processes. Beyond these considerations, intracellular pro-apoptotic proteins, such as Bax and p53, also increase in cells undergoing apoptosis. A variety of extracellular and intracellular signals stimulate Bax and p53 expression in granulosa cells of follicles (Tilly et al., 1995; Amsterdam et al., 1996; Kim et al., 1999; Zwain & Amato, 2001; Das et al., 2008; Salvetti et al., 2010), which result in apoptotic effects such as the release of cytochrome C from mitochondria, further activation of caspases, and ultimately fragmentation of nuclear DNA.

In a relatively recent *in vitro* study, transforming growth factor-beta1 (TGF-beta1) was identified as a pro-apoptotic signal to bovine granulosa cells (Zheng *et al.*, 2009). Essentially TGF-beta 1 prevented luteinization of the cells while maintaining an estrogenic phenotype. TGF-beta1 also induced apoptosis of the granulosa cells under control and FSH-stimulated conditions. Based upon these results, the authors suggested TGF-beta 1 influences selection of the dominant follicle during folliculogenesis in cattle by controlling the proliferation and the steroidogenic differentiation of granulosa cells. However, whether or not TGF-beta1 can be truly ascribed a pro-apoptotic role is debatable. It shares structural and functional properties with other members of the transforming growth factor-beta superfamily, many of which are essential for follicular growth and development in a variety of species (for an extensive review, see Juengel & McNatty, 2005), and thus possesses anti-apoptotic or pro-survival attributes.

Anti-apoptotic or pro-survival molecules in the context of follicular development and atresia are those that promote growth and proliferation of cells within the follicle, or counteract the actions of apoptotic molecules. Examples of extracellular factors include the gonadotropins, steroids, certain cytokines and growth factors. Intracellularly, there are a variety of molecules that directly counteract the actions of pro-apoptotic factors, while others utilize unique signaling pathways. Among the intracellular factors preventing apoptosis through

these means are the regulators of cell cycle progression (e.g., cyclins and cyclin-dependent kinases); the serine/threonine protein kinase, Akt; an anti-caspase 8 molecule known as cellular FLICE inhibitory protein (cFLIP); and the apoptosis regulator protein, Bcl-2.

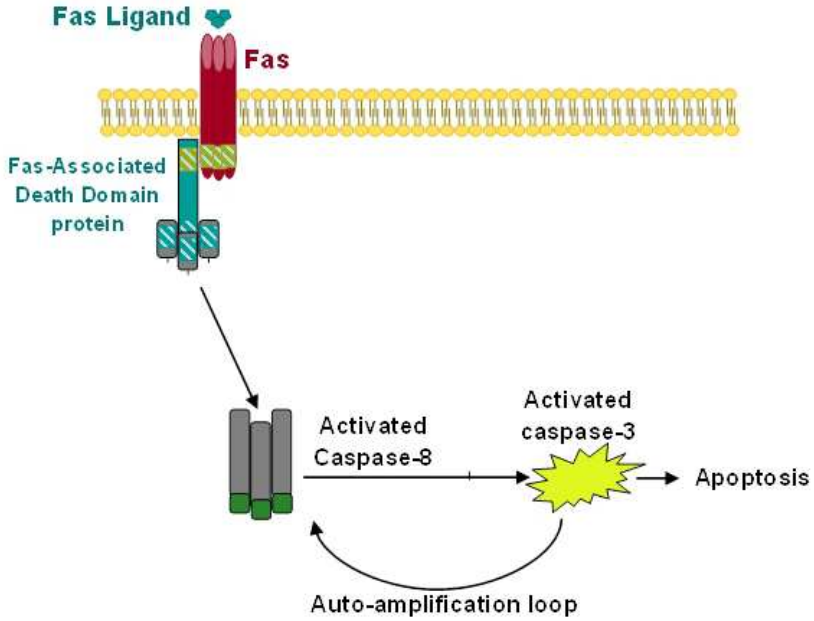

Fig. 1. Fas ligand-induced apoptosis through caspase -8 and caspase-3 activation. Fas ligand induces apoptosis by triggering aggregation and oligomerization of the Fas receptor on the cell surface. Once oligomerized, the receptor associates with an intracellular Fas-Associated Death Domain (FADD) protein. The FADD protein enzymatically cleaves the prodomain of caspase-8, triggering its activation, the activation of caspase-3, and then subsequent downstream events that result in apoptosis.

Acknowledging that growth of tertiary follicles during a follicular wave is gonadotropin-dependent, it is not surprising to learn that gonadotropins also impact apoptosis of granulosa cells during this process. In general, FSH suppresses apoptosis of granulosa cells in medium and large follices (Yang & Rajamahendran, 2000), whereas LH suppresses apoptosis of granulosa cells in large follicles only (Chun et al., 1996; Porter et al., 2001). The anti-apoptotic effects of the gonadotropins are likely attributable to their enhancement of steroidogenic enzymes and steroid synthesis within the follicle. In particular, increased estradiol synthesis is a tell-tale indication of a healthy follicle with viable granulosa cells. Quirk and coworkers (Quirk *et al*., 2006) were among the first to determine estradiol protects bovine granulosa cells from apoptosis by increasing cyclin D2 expression and stimulating the cells to progress from the G1 to S phase of the cell cycle. As follicles progress through the pre-ovulatory stages of growth, progesterone secretion prevails over estradiol secretion as granulosa cells become luteinized, prompted by the LH surge. During this period of steroidogenic transition the responsiveness of the granulosa cells to progesterone is enhanced, triggering their withdrawal from the cell cycle (Quirk *et al*., 2004), and anti-

apoptotic effects (Quirk et al., 2004; Peluso et al., 2009). Conversely, a high ratio of androgen to estradiol within follicles is generally viewed as an indication of follicular atresia (McNatty et al., 1976; Fortune & Hansel, 1985). Although androgens (i.e., androstenedione and testosterone) are admittedly produced by theca cells as precursors for aromatization to estradiol in growing follicles; at later stages of folliculogenesis androgens have the net effects of inhibiting FSH-stimulated LH receptor expression, enhancing granulosa cell apoptosis, and promoting follicular atresia within the ovary (Kaipia & Hsueh, 1997). In summary, the actions of estradiol and progesterone within tertiary follicles may be generally viewed as anti-apoptotic and promoting follicular growth, whereas the effects of androgens are primarily pro-apoptotic, enhancing the occurence of follicular atresia.

Historically, many of the initial *in vitro* studies of granulosa cell apoptosis invariably utilized serum-containing culture medium as part of their experimental methods. In fact for some studies, serum-withdrawal during the culture period was implemented to induce the onset of apoptosis (Quirk et al., 1995; Porter et al., 2000; Quirk et al., 2000; Vickers et al., 2000). We now know that the various growth factors contained within serum, specifically insulin-like growth factor (IGF), basic fibroblast growth factor (bFGF), and epidermal growth factor (EGF), are what provide the beneficial, anti-apoptotic effects (Quirk et al., 2000; Yang & Rajamahendran, 2000; Mani et al., 2010). In contrast, other growth factors, including keratinocyte growth factor, transforming growth factor, and platelet-derived growth factor have no anti-apoptotic effects (Quirk *et al.*, 2000). Many of the beneficial growth factors likely exert their anti-apoptotic actions via enhancement of intracellular signaling pathways (e.g., Akt)(Hu et al., 2004a), regulation of anti-apoptotic genes such as Bcl-2 (Ratts et al., 1995; Tilly et al., 1995; Kugu et al., 1998; Salvetti et al., 2010), and promoting cell cycle progression (Hu et al., 2004a; Quirk et al., 2006). Another intracellular molecule known to inhibit apoptosis of granulosa cells is cellular FLICE-like inhibitory protein, or cFLIP (Matsuda-Minehata et al., 2006; Matsuda-Minehata et al., 2007; Matsuda et al., 2008). This protein is structurally homologous to pro-caspase 8, but lacks the enzymatic domain to cleave effector caspases such as caspase 3. Essentially cFLIP is a "decoy" caspase, capable of oligomerizing with other caspase 8 molecules and cytokine receptors following ligand-receptor binding, but lacking the enzymatic activity to promote downstream activation of apoptotic pathways. Among the more recent molecules recognized for their anti-apoptotic effects are the bone morphogenetic proteins, BMP-4 and BMP-7 (Kayamori *et al.*, 2009). These proteins are members of the transforming growth factor-beta superfamily and, at present, their anti-apoptotic actions within granulosa cells are attributed to an inhibition of caspase-activated DNase enzymes (Kayamori *et al.*, 2009). Thus, we are only beginning to understand the intricacies of the various growth factors and their intracellular signaling pathways as they relate to granulosa cell apoptosis, and ultimately follicular atresia. Many of these mechanisms are well-suited to hypothesis testing through molecular manipulation, for instance the use of interfering RNAs. As these molecular approaches continue to evolve, an expectation would be that considerable insight will be gained about the intracellular regulation of apoptosis within granulosa cells.

7. Influence of the cytoskeleton on granulosa cell/oocyte viability and differentiation

Beyond the above-described secreted and intracellular influences on granulosa cell and oocyte viability within follicles, there are ultrastructural or cytoskeletal influences to

consider. In the last decade, for instance, a number of studies indicate the cytoskeletal elements (i.e., microtubules, microfilaments, and intermediate filaments) profoundly affect follicular growth, potentially resulting in anovulation and cystic follicles (Salvetti et al., 2004; Ortega et al., 2007; Salvetti et al., 2010). Microtubules are required for granulosa cell steroidogenesis (Chen *et al.*, 1994), but they also determine cell shape and affect cytoplasmic movement of organelles (Šutovský *et al.*, 1994). Within oocytes, microtubules promote organelle movement (e.g., mitochondria, endoplasmic reticulum, Golgi complex, cortical granules, etc.) during oocyte maturation and the segregation of chromosomes during meiotic and mitotic processes (Albertini, 1992; Ferreira et al., 2009). Hormone-induced oocyte maturation is accompanied by a surge of microtubule assembly within the cumulus cells, which constitute part of the oocyte-granulosa cell communication conduit (Allworth & Albertini, 1993). Thus it is conceivable microtubules have a similar role in regulating steroidogenesis and controlling organelle movement during granulosa/oocyte apoptosis and follicular atresia.

Microfilaments within oocytes are closely associated with the activities of microtubules, particularly the proper positioning of chromatin during meiosis (Kim *et al.*, 2000). They also help establish polarity of the oocyte, influence polar body extrusion during fertilization, and regulate cortical granule release as the block to polyspermy (Sun & Schatten, 2006). Microfilaments and other cytoskeletal components are considered essential in driving granulosa cells toward differentiation (i.e., luteinization) (Amsterdam and Rotmensch, 1987; Motta et al., 2002) or facilitating death (Amsterdam *et al.*, 1997). For instance, F-actin mediates LH-induced expansion of the cumulus cells surrounding bovine oocytes (Šutovský *et al.*, 1995), facilitating oocyte maturation and potential fertilization. Under apoptotic conditions, rearrangement of the microfilaments within granulosa cells compartmentalizes the steroidogenic machinery to the perinuclear region of the cells while directing proteolytic activities to the apoptotic bodies (Amsterdam *et al.*, 1997).

The intermediate filaments, including vimentin, the cytokeratins, and desmin, are thought to influence cell mitosis, follicular atresia, and de-differentiation of cells of the follicle (van den Hurk et al., 1995; Khan-Dawood et al., 1996; Loffler et al., 2000). These so-called "stress filaments" also participate in the maintenance of cell contact between the oocyte and cumulus cells, orchestrate distribution of organelles throughout the cytoplasm of the oocyte, and possibly control resumption of its meiotic division (Gall *et al.*, 1992), in part by influencing cumulus expansion (Šutovský *et al.*, 1995). Most recently, we have identified intermediate filaments, particularly cytokeratin 8/18 filaments, as a possible intrinsic influence of granulosa cell apoptosis during folliculogenesis (Townson et al., 2010).

The cytokeratins constitute a diverse class of intermediate filaments that derive from a family of approximately 65 homologous proteins, forming six classes of molecules (Moll *et al.*, 1982). The cytokeratins are obligate heterodimers composed of an acidic CK (Type I: numbered 9-20) paired with a basic CK (Type II: numbered 1-8). The cytokeratin 8/18 (CK8/18) filament is considered one the most abundant Type I: Type II filaments found in normal epithelia, cultured cell lines, and carcinomas. Functionally, CK8/18 filaments provide structural integrity to cells, but they also influence intracellular transport mechanisms and signaling (Singh et al., 1994; Eriksson et al., 2009). Recently, the expression of these filaments in certain types of epithelial cells has been implicated in the resistance of

these cells to apoptosis (Figure 2). Mechanisms of protection include impairing cytokine receptor trafficking and cell surface expression (Gilbert et al., 2001; Marceau et al., 2001; Ku et al., 2003), and the inhibition of cytokine-induced apoptotic intracellular signals (Caulin et al., 2000; Oshima, 2002; Ku et al., 2003; Gilbert et al., 2008). These observations are consistent with earlier suggestions that intermediate filaments regulate transport processes between the cell surface and nucleus, and influence nuclear events (Li et al., 1994; Singh et al., 1994). Hence, the proposition that intermediate filaments increase cell resistance (i.e., granulosa cell, theca cell, and oocyte) to apoptosis during the process of follicular atresia is conceptually plausible. To date, however, there has been very little exploration of this possibility.

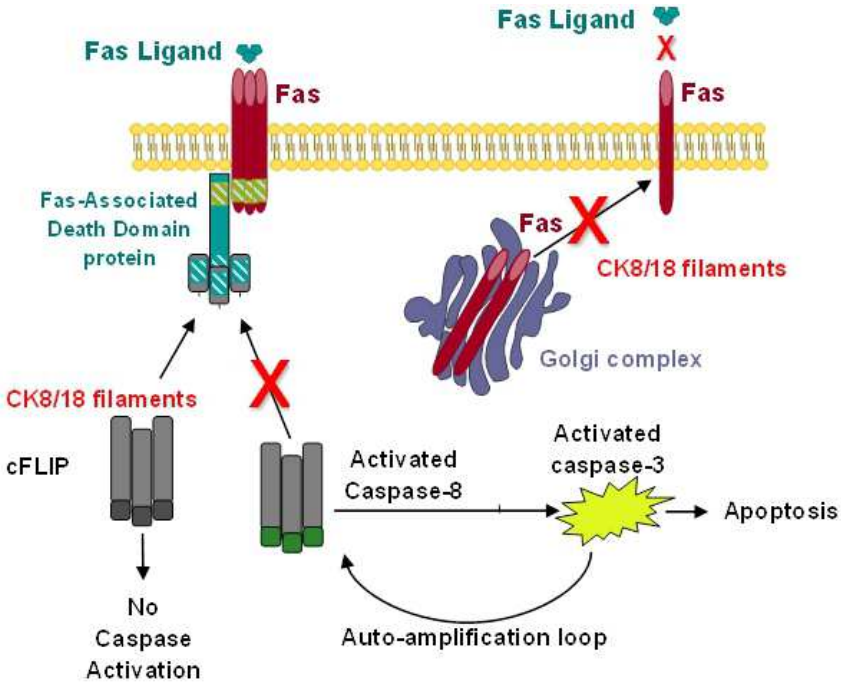

Fig. 2. Potential influences of cytokeratin 8/18 intermediate filaments on Fas ligand-induced apoptosis. Putative mechanisms by which cytokeratin 8/18 filaments prevent Fas ligand-induced apoptosis (designated by a red "X") include: 1) impeding Fas receptor trafficking from the Golgi complex to the cell surface; 2) impairing Fas receptor oligomerization and, hence, Fas ligand binding; and 3) inhibiting caspase-8 activation by enhancing the expression/binding of cellular FLICE inhibitory protein (cFLIP) with the Fas Associated Death Domain protein.

8. Role of oxidative stress during follicular atresia

Mechanistically, a balance between cell survival and apoptosis may be mediated by oxidative stress. Oxidative stress refers to a pathological state when pro-oxidants (reactive oxygen or nitrous species) are not neutralized adequately by antioxidant defenses. Cell

damage may occur, including irreparable aberrations in protein, DNA, and lipid structures and functions. Reactive oxygen species (ROS) are inevitable byproducts of any cellular system in which cell division and metabolism occur. Through its developmental journey, the follicle is thus a dominant source of ROS. Several investigators have established a role for oxidative stress in cell differentiation, proliferation, and death (Ott et al., 2007; Covarrubias et al., 2008; Circu & Aw, 2010). Notably, cumulative damage from excessive ROS and oxidative stress eventually leads to cell death, hence the unsurprising relationship between oxidative stress and cell fate. But under conditions of decreased amounts of ROS, there is an early and active role of oxidative stress in evoking apoptosis; for instance, the redox status of cells (itself controlled by the thioredoxin and glutathione systems) directly modulates the initiation and execution of apoptotic pathways (Carmody & Cotter, 2001; Ueda et al., 2002; Kwon et al., 2003; Circu & Aw, 2008). Specific to follicular atresia, a few studies have postulated the involvement of pro- and anti-oxidants in influencing the fate of follicles. With the paucity of studies in any one species, below is a review of all evidence to date that investigates a potential link between oxidative stress and follicular atresia.

Past studies in systems other than the ovary have demonstrated interactions between anti-apoptotic factors (*e.g.,* Bcl-2 family members) and oxidative stress pathways (reviewed by Voehringer, 1999; Voehringer & Meyn, 2000). Bcl-2 modulates the redox status of HeLa cells (specifically the non-protein thiol, glutathione, GSH), thereby preventing any redox-dependent changes characteristic of apoptosis (Voehringer et al., 1998). Thus, Bcl-2 increases antioxidant capacity, which mitigates the oxidative stress known to provoke early stages of apoptosis. Along these same lines, Tilly and Tilly (Tilly & Tilly, 1995), using an *in vitro* follicle culture model, determined that inhibitors of ROS production and action (i.e., free radical scavengers) reduce apoptosis in rat follicles. The effects of chemical exposure (*e.g.* methoxychlor, a pesticide) on reproductive function reveal impaired follicular development, including an increase in the rate of atresia of antral follicles. Methoxychlor (MXC) reduces mRNA expression of antioxidant enzymes (SOD1, GPx, and CAT) in mouse antral follicles, and treatment with an exogenous antioxidant (N-acetyl cysteine) significantly decreases the rate of atresia induced by MXC (Gupta et al., 2006). Although the current evidence does not resolve the precise pathway(s) by which MXC induces follicle atresia, it may do so by either inducing changes in Bcl-2 followed by OS or, conversely, by leading to oxidative stress that results in Bcl-2 changes. Other chemical toxicants (cyclophosphamide and 9,10-dimethyl-1,2-benzanthracene) also induce granulosa cell apoptosis, and of relevance is the protective role of GSH in these instances (Lopez & Luderer, 2004; Tsai-Turton et al., 2007a; Tsai-Turton et al., 2007b). In ovine antral follicles, a correlation exists between an increased incidence of atresia and a decrease in GSH and the enzyme, glucose-6-phosphate dehydrogenase (G6PD), an enzyme needed for NADPH-dependent recycling of GSH (Ortega-Camarillo et al., 2009). Moreover, these changes are accompanied by an increase in protein oxidation in granulosa cells and follicular fluid (Ortega-Camarillo et al., 2009). Thus, the relative balance between oxidative stress and antioxidant capacity within the follicle has relevance to the developmental potential of follicles during folliculogenesis.

One study exploring the role of oxidative stress during follicle atresia determined that bovine follicles express increased amounts of mRNA for antioxidant enzymes only during

advanced stages of atresia (Valdez et al., 2005). These findings contradict the perhaps more intuitive and widely-held perception that diminished antioxidant protection elicits apoptosis. However, an explanation for these seemingly contradictory views about antioxidants might include further consideration of the influence of follicle status (e.g., growing, dominant, subordinate), follicle size, and differing expression of each antioxidant during follicle development. For instance, in sheep, only large antral follicles (versus small) exhibit significant decreases in GSH with atresia; in contrast, another oxidative stress marker G6PD decreases with atresia in both small and large antral follicles (Ortega-Camarillo et al., 2009). In general, however, the vast majority of studies support the concept that compromised antioxidant protection is associated with the initiation and/or further progression of apoptosis.

The involvement of ROS in triggering and/or mediating follicular atresia has not been directly examined and offers fertile ground for further investigation. A recent correlative analysis failed to demonstrate a relationship between lipid peroxidation in follicular fluid and apoptosis in granulosa cells; in contrast, levels of hydrogen peroxide are increased in follicular fluid from follicles deemed non-atretic (with <10% apoptotic granulosa cells) when compared to fluid from atretic follicles (Combelles et al., 2011). At certain concentrations, ROS regulates intracellular signaling pathways, among which are the apoptotic signaling cascades. Thus, future studies focused on the involvement of ROS during follicular atresia would be informative.

The pro- and anti-oxidants described above may not represent the most upstream signals controlling follicular atresia. Rather, we suggest the aforementioned gonadotropins, steroids, cytokines and growth factors, all of which influence follicular atresia, do so via modulation of oxidative stress and, in turn, cell death pathways. Gonadotropins, for example, enhance the expression of antioxidant genes; an effect that accounts for the protective effects of gonadotropins (notably FSH) on the developing follicle. Exposure of rat ovaries to chorionic gonadotropin (with FSH and LH activity) *in vivo* increases the expression of some, but not all, antioxidants (Tilly & Tilly, 1995). The expression of glutathione S-transferase isoenzymes is increased in bovine follicles by *in vivo* exposure to gonadotropins (Rabahi et al., 1999). In the goat, granulosa cells exposed to FSH *in vitro* exhibit increased activity of catalase (Behl & Pandey, 2002). Further evidence supporting the role for gonadotropin-regulated antioxidant activity within the follicle stems from a series of rat studies. Gonadotropins enhance glutathione synthesis in the ovary (Luderer et al., 2001). FSH protects pre-ovulatory follicles from apoptosis via increases in GSH and decreases in ROS (Tsai-Turton & Luderer, 2006). Glutamate cysteine ligase (GCL), an enzyme essential for the *de novo* synthesis of GSH, is expressed only in granulosa cells of healthy follicles, and generally only following the gonadotropin surge *in vivo*, or after treatment with exogenous chorionic gonadotropin (Luderer et al., 2003; Tsai-Turton & Luderer, 2005). And lastly, stimulation of GSH synthesis and GCL expression in granulosa cells and small antral follicles are maximal following concomitant exposure to FSH and estradiol, rather than each hormone alone (Hoang et al., 2009). Overexpression of GCL in a line of human granulosa tumor cells increases GSH production and protects the cells from oxidative stress-induced cell death (Cortes-Wanstreet et al., 2009). For these reasons, we suggest gonadotropins influence granulosa cell apoptosis, and thus follicular atresia, through oxidative stress mechanisms (Figure 3).

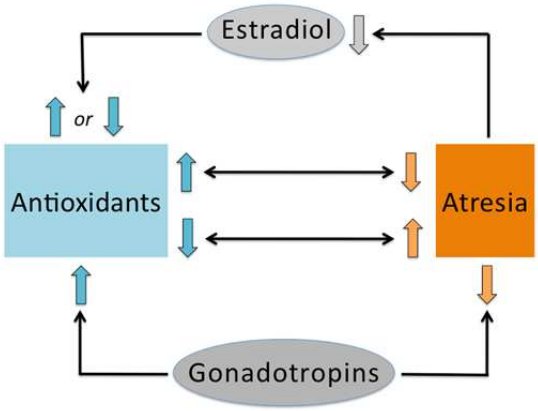

Fig. 3. Model of interactions between antioxidants, atresia, estradiol, and gonadotropins within the developing follicle. Double-headed arrows reflect correlations between antioxidants and atresia, with color-coded block arrows indicating increases or decreases in one of the parameters.

Estradiol is another hormone pertinent to the discussion of follicular atresia and oxidative stress pathways because it also influences the expression of antioxidants. In myocardiac cells, estradiol upregulates the GSH/glutaredoxin system, in turn, modulating the redox state. Consequently, the Akt/protein kinase B redox-sensitive enzyme is activated, thereby protecting cells from apoptosis (Murata et al., 2003; Urata et al., 2006). Other evidence in which estradiol favorably influences the expression of antioxidant enzymes include *in vitro* studies using cultured cells of various types (e.g., human endometrial stromal cells, vascular smooth muscle cells, mammary gland tumor cells, and ovine granulosa cells) wherein estradiol increases select antioxidant enzymes (Sugino et al., 2000; Strehlow et al., 2003; Borras et al., 2005; Basini et al., 2006). Of relevance to the ovary and follicular atresia, estradiol protects ovine and porcine granulosa cells from oxidative stress (hydrogen peroxide)-induced cell death (Lund et al., 1999; Murdoch, 1998).

As described previously, atretic follicles synthesize and contain less estradiol than their healthy counterparts. Considering the positive attributes of estradiol with respect to antioxidants and antioxidant activity, one would presume less estradiol equates with compromised antioxidant defense mechanisms of the follicle and possibly greater vulnerability to cell apoptosis and follicle atresia. However, the precise relationships between estradiol action and antioxidant activity within the follicle remain to be fully established. In bovine follicles that have established dominance, estradiol inhibits SOD activity (Valdez et al., 2005). In human follicles there is a positive correlation between estradiol concentration and total antioxidant capacity (Appasamy et al., 2008). However, estradiol is also associated with decreased, rather than increased, antioxidant expression in follicles in certain instances (Al-Gubory et al., 2008). Others have demonstrated both pro- and anti-oxidant effects (Nathan & Chaudhuri, 1998; Thibodeau et al., 2002). Collectively, these observations justify the need for further insight about the relationship between estradiol and antioxidants during the process of follicular atresia. The regulatory loops are likely complex considering antioxidants themselves may affect estrogen production within

the follicle. In at least one study, SOD exposure of granulosa cells decreased aromatase activity and estradiol production despite FSH stimulation (LaPolt & Hong, 1995). Thus, a conceptual map of the specific role(s) of estradiol, pro-oxidants, and antioxidants during follicle atresia *in vivo* awaits further study (Figure 3).

Similar to the postulated actions of estradiol, growth factors upregulate the expression of antioxidants and provide protection against oxidative stress-induced cell death in chondrocytes (Jallali et al., 2007) and placental endothelial cells (Liu et al., 2010). In light of the prominent involvement of growth factors in follicular fate (section 6 above), future studies focused on the modulation of oxidative stress by growth factors, notably during follicular atresia, are needed.

In conclusion, there is growing evidence indicating involvement of oxidative stress mechanisms in follicular atresia although the precise role(s) of these mechanisms remain unclear (Figure 3). Control of follicular atresia is clearly multi-faceted and complex. Further complicating our ability to decipher the effects of oxidative stress on atresia are the dual roles of ROS. For instance, ROS may either act positively or negatively on a cellular process, including cell proliferation and death (Hernandez-Garcia et al., 2010; Poli et al., 2004). Moreover, atresia is a developmental process occurring along a temporal continuum; it is not a sudden process, and the timing of when follicles are experimentally analyzed during atresia might impact the determination of oxidative status. Such dynamic relationships are observed following ovarian exposure to MXC: increases followed by decreases in antioxidant enzyme expression occur prior to the time follicular atresia is evident (Gupta et al., 2006). In an initial attempt for protection, the follicle may first respond to an insult or its atretic fate by upregulating antioxidants. However, beyond the point of no return, an eventual decrease in antioxidants may occur allowing follicular loss to follow.

9. Involvement of immune cells and immune mediators in follicular fate

Immune cells play a key role in both innate and acquired immunity throughout the body, but they also maintain tissue homeostasis in a variety of organs, including the ovary, through cytokine secretion and remodeling capabilities. In the context of folliculogenesis, macrophages will be the primary focus of discussion here because they are arguably the most abundant type of immune cell residing within the ovary (Best et al., 1996; Takaya et al., 1997). Their proximity to healthy follicles and distribution throughout ovarian function suggests they directly impact follicular growth and atresia (Wu *et al.*, 2004). Macrophages secrete many of the aforementioned cytokines and growth factors, including TNF, bFGF, IGF, and EGF, which stimulate granulosa cell proliferation, suppress apoptosis, and promote follicular development. Moreover, in a number of rodent models, experimental induction of a paucity of ovarian macrophages has detrimental effects on follicle development and ovulation rate (Van der Hoek *et al.*, 2000), ovarian vascularization (Turner *et al.*, 2011), and fertility (Cohen *et al.*, 1997). Others have noted there are no direct interactions between macrophages and primordial follicles during the earliest phases of follicular growth, indicating early growth of follicles occurs independently of macrophage influences (Wu *et al.*, 2004). Hence, there is little debate that macrophages impact the growth and development of follicles, but lingering questions remain as to what prompts macrophage recruitment to the larger growing follicles, and the relative importance of macrophages to follicular growth in monovulatory species such as the cow and the human female.

With respect to follicular atresia, macrophages are found within the granulosa cell layer of bovine follicles only in the most advanced stages of atresia, basal atresia, in which the basal lamina has been disrupted (Irving-Rodgers *et al.*, 2001). Similar observations have been made in rodents (Petrovska *et al.*, 1996). Considering macrophages are known to eliminate dying cells and cellular debris via phagocytosis, it is thought they participate in these same activities during granulosa cell apoptosis, once follicular atresia is underway (Takaya *et al.*, 1997). Conversely, recognizing that macrophages, once activated, have a repertoire of cytokines, growth factors, and destructive metabolites at their disposal to initiate cell death, it is plausible they may play a larger role in initiating apoptosis of granulosa cells, thus provoking follicular atresia. Whether macrophages play a role as instigator, facilitator, or follower of granulosa cell apoptosis is currently unclear, but offers opportunity for future investigation in understanding their influence on follicular fate.

10. Relationship between follicular atresia and the oocyte

The follicle nurtures and supports the development of the oocyte through all stages, and it is thus pertinent to consider the potential role of the oocyte in the control of atresia as well as the effects of atresia on the oocyte. The early stages of follicular growth (primordial and primary follicles) are when oocyte loss is likely responsible for follicular loss (Markstrom et al., 2002; Depalo et al., 2003). Throughout follicular development the support from somatic cells is essential to oocyte development, but likewise, the somatic cells are dependent upon support from the oocyte. Indeed, the oocyte controls many facets of granulosa cell activities (Mermillod et al., 2008), including, ostensibly, the process of apoptosis. One hypothesis is that oocytes retaining an intrinsically compromised differentiation state may fail to support follicular development or to protect cells from apoptosis adequately. The potential role of the oocyte in modulating the selection pressure placed on the growing follicle merits experimental exploration. Whether there exists any link between granulosa cell apoptosis and oocyte quality is a fundamental question with no clear answers to date. However, current evidence supports three potential scenarios for a relationship between granulosa cell fate and oocyte quality that vary depending on the level of atresia. All three of these scenarios are biologically relevant because atresia is not a sudden, coordinated process, but rather one that proceeds progressively. Firstly, there may be no detriments of granulosa cell apoptosis on oocyte quality. This was first demonstrated in the sheep, wherein oocytes from small atretric follicles retain the ability to yield blastocysts *in vitro* (Moor & Trounson, 1977). Conversely, there are detriments to the oocyte (as manifested by degenerative changes) in late stage atretic follicles with high levels of apoptotic granulosa cells (Blondin & Sirard, 1995; de Wit et al., 2000; Feng et al., 2007; Zeuner et al., 2003). Nevertheless, follicles with a high degree of atresia, as evidenced by apoptosis of the membrane granulosa and cumulus-oocyte-complex (COC), still have the capacity to result in embryonic development (Hagemann et al., 1999). It is conceivable that the COC may be affected last by cell death in these instances (Blondin & Sirard, 1995). Thirdly, a prescribed level of atresia may impart improvements in oocyte quality, as described in the following paragraph. This is evident for oocytes originating from follicles with some degree of granulosa cell apoptosis.

It is particularly interesting that follicles in the early stages of atresia contain oocytes of superior developmental competence (i.e., the ability to support embryonic development). Blondin and Sirard (Blondin & Sirard, 1995) showed that COCs from slightly atretic follicles yielded oocytes with greater developmental competence compared to COCs from nonatretic

follicles. In the case of bovine oocytes collected *in vivo* from subordinate follicles in growing, static, and regressing phases, oocytes from early atretic follicles (i.e., regressing) are of improved developmental competence compared to non-atretic follicles (i.e., growing) (Salamone et al., 1999). Furthermore, storage of bovine ovaries for 4 hours post-mortem (*versus* shorter or longer periods of time) yields oocytes with significantly greater developmental competence (Blondin et al., 1997). In this scenario, early atresia might have been induced in the follicles by storing the ovaries for 4 hours, in turn, permitting the penultimate phases of oocyte development to occur.

Pre-maturation refers to a set of cytoplasmic and molecular changes in the oocyte during the final pre-ovulatory stages of development of the dominant follicle (Dieleman et al., 2002; Sirard et al., 2006). Follicles in which some apoptosis has occurred may mimic changes that normally take place during pre-maturation, allowing the oocytes to complete their developmental program. In contrast, oocytes from non-atretic follicles may be hindered and not undergo final cytoplasmic maturation (Hendriksen et al., 2000). Several groups of investigators have documented additional evidence of a relationship between low levels of follicular atresia and increased developmental competency of the oocyte (some of which include the use of a powerful single *in vitro* production system) (Vassena et al., 2003; de Wit et al., 2000; Feng et al., 2007).

Although initially unexpected, the co-existence of an oocyte of superior quality in an early atretic environment can also be rationalized, especially in light of our current understanding of developmental competence. The acquisition of complete developmental competence occurs late during folliculogenesis, a time when apoptotic changes may have already occurred. There is evidence supporting the hypothesis that similar cellular and biochemical changes occur in the follicular microenvironment during both pre-ovulatory development and early atresia of follicles. For instance, a rapid decline in follicular estradiol, and increases in androgen, progesterone, and prostaglandin are all post-LH-surge events of pre-ovulatory follicles, which also occur in follicles undergoing follicular atresia (Kruip & Dieleman, 1982; Kruip & Dieleman, 1985; Kruip & Dieleman, 1989). Additional events common to both pre-ovulatory and early atretic follicles include the mucification of the cumulus cells (i.e., cumulus cell expansion), and structural changes in the oocyte (nuclear modifications and organelle rearrangements) (Assey et al., 1994; Blondin & Sirard, 1995). Collectively, these observations indicate there is relevance to considering the relationship between follicular atresia and oocyte quality together. Noteworthy is the difficulty of studies that aim to link atretic status of the follicle with the developmental competence of the enclosed oocyte. The atretic history of the follicle may be more appropriate to consider if some non-atretic follicles have actually encountered episodes of early atresia followed by a recovery period. In addition, there may be a threshold of follicular atresia above or below which the enclosed oocyte may be positively or negatively influenced. We acknowledge, however, that atresia is not the only factor influencing the developmental potential of oocytes. Additional considerations include the interactions between atresia and follicle size, stage of the estrous cycle, and morphological grades of the COC (Feng et al., 2007; Hagemann et al., 1999).

In conclusion, follicular atresia merits careful attention in the research arena, notably with respect to the influences it exerts on the developmental competence of oocytes. These

relationships, in turn, are of biological and economical significance because they impact our understanding of fertility as it relates to livestock and human embryo production. Future research should focus on the differentiation pathways shared by granulosa cells and oocytes during atresia and development of pre-ovulatory follicles. Once identified, they may hold the key to improvements in *in vitro* maturation of oocytes, but also the procurement of *in vivo* matured oocytes of high developmental potential.

11. Relevance

Given its scope, it is likely that follicular atresia has a variety of biological roles although our full understanding of the process remains elusive (Figure 4). Ovarian follicular atresia is likened to the several instances during normal embryonic development when apoptosis possesses physiological roles, such as regulation of development of the nervous system (Martimbeau & Tilly, 1997). The massive attrition of germ cells prior to adulthood may offer the best illustration of a quality control mechanism for fertility (Krysko et al., 2008). A very large prenatal loss of germ cells coincides with entry into meiosis and primordial follicle formation; atresia could thus be a mechanism to assure the number of oocytes matches the appropriate number of follicle cells. Alternatively, apoptosis could eliminate oocytes harboring any chromosomal defects. Lastly, germ cells may be lost by self-sacrifice or an altruistic pathway by which the surviving oocytes acquire cellular elements from neighboring dying germ cells, as described during invertebrate oogenesis.

In the adult, proposed functions for follicular atresia include the selection of follicles containing oocytes of the highest developmental potential. Another interesting hypothesis relates to the large number of atretic follicles that retain steroidogenic activity, thereby perhaps imparting on follicular atresia an endocrine function within the ovary (Hsueh et al., 1994). The further pursuit of a thorough understanding of follicular atresia should provide insights into the biological significance of this remarkable process.

Clinically, there are mechanistic correlates that exist between follicular atresia and the pathological conditions leading to infertility, namely premature ovarian failure (POF) and polycystic ovary syndrome (PCOS) (Figure 4). POF is defined as the cessation of ovarian function (with elevated gonadotropins and reduced estrogens, similar to menopause) prior to the age of 40 (Conway, 2000). Its etiology is typically unknown, and the known causes of POF present a complex picture (Cordts et al., 2011). When mutated, several genes may result in POF, including the androgen receptor (AR) and forkhead box family of transcription factors (FOXO3a, FOXL2). A cessation of ovarian activity with a depleted follicle pool may stem from a reduced size of the follicle pool and/or an accelerated rate of follicular loss by atresia. The AR knockout mouse exhibits a POF phenotype together with a significant increase in follicular atresia (Hu et al., 2004b; Shiina et al., 2006; Sen & Hammes, 2010). The *Foxo3a* and *Foxl2* mutations are characterized by an accelerated rate of follicular initiation, and a follicular loss that leads to POF (Castrillon et al., 2003; Schmidt et al., 2004). Besides a spontaneous onset, POF also occurs following cancer treatment (Stroud et al., 2009), and mastering the genes that regulate apoptosis offers promise in the development of therapeutic strategies to combat POF.

Follicular atresia is also pertinent to the pathogenesis of PCOS. Many defects arise in PCOS patients, primarily chronic anovulation and hyperandrogenism (Matalliotakis et al., 2006). The primary defect is at the level of the ovary, particularly at the level of folliculogenesis,

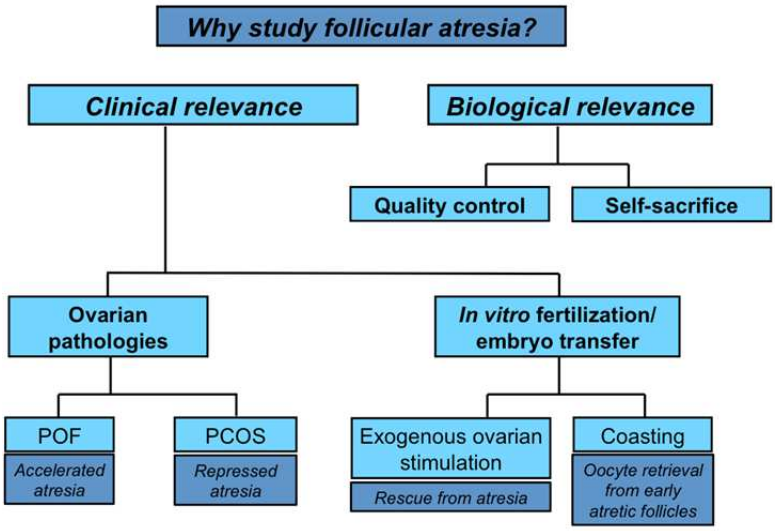

Fig. 4. The importance of studying follicular atresia.
Schematic summarizing the clinical and biological situations when follicle atresia appears involved. Further details and supporting evidence are presented in the text.

wherein the ovary contains many small antral follicles (at least more than 10 follicles between 2-8 mm in diameter). These follicles are arrested in development and do not show overt signs of atresia. The upregulation of anti-apoptotic and survival factors are hypothesized to account for the accumulation of small antral follicles (Homburg & Amsterdam, 1998); whereas the lack of developmental progression is explained by the abnormal endocrine environment in PCOS patients, notably FSH suppression (Matalliotakis et al., 2006). PCOS is thus a unique pathology deviating significantly from the normally concomitant occurrence of developmental arrest and atresia of the follicle. Indeed, PCOS is characterized by premature growth arrest without atresia. PCOS could then be considered a syndrome of repressed atresia because follicles persist despite low FSH concentrations. However, the specific aspects of apoptotic control in PCOS pathogenesis require further experimental support. Current lines of evidence support the role of a survival/apoptotic balance as one of likely multiple mechanisms explaining PCOS symptoms. Intrinsic abnormalities in folliculogenesis, notably in preantral follicles, characterize PCOS (Franks et al., 2008). A larger than typical reserve of small preantral follicles (primordial and primary) could be attributed to diminished follicle loss by atresia (Webber et al., 2003). In support of this scenario, *in vitro* cultured preantral follicles from polycystic ovaries have a decreased rate of follicle loss and an increased rate of follicle survival (Webber et al., 2007). In human granulosa cells obtained from 4-8 mm follicles, apoptotic rates are decreased (and proliferation rates increased) in granulosa cells from polycystic ovaries compared to normal ovaries. In these polycystic ovaries, the levels of apoptotic effectors and anti-apoptotic survival factors were decreased and increased, respectively (Das et al., 2008). Animal models of polycystic ovaries have facilitated our understanding of PCOS. For instance, studies of polycystic ovaries in an induced rat model show low levels of apoptosis with

enhanced protection at the molecular level, explaining the persistence of the follicles (Salvetti et al., 2009). Cystic ovarian disease affects cow fertility, although it is defined in this animal as the presence of one or more follicular cyst(s), at least 20 mm in diameter and remaining for more than 10 days. Still, of relevance to human PCOS are the suppressed proliferation and apoptotic rates that appear to undermine follicle persistence in cystic cow ovaries (Salvetti et al., 2010). Further understanding of the control of apoptosis at the molecular level will facilitate the identification of any defect(s) in the apoptotic pathway during PCOS; this could then open doors to future targeted therapies.

During routine *in vitro* fertilization and embryo transfer (in humans and animals), ovarian stimulation is often employed. Ovarian stimulation protocols include stimulation with FSH, thereby reducing the FSH threshold needed for continued development and permitting the effective rescue of follicles normally destined for atresia. Consequently, more ovulatory follicles can be aspirated and multiple mature oocytes obtained following LH stimulation. Undeniably, many of the rescued follicles give rise to viable embryos and pregnancies; however, the quality of a retrieved oocyte cohort is characteristically heterogeneous. With hormonal stimulation interfering with the physiological selection pressures placed on the follicle, it is conceivable that oocytes of diminished potential are retained. Perhaps such follicles, and in turn oocytes, are compromised beyond a point of no return. Very little consideration has been given to this issue, suggesting it is a critical area for future study and improvements. A goal should be to optimize conditions for obtaining a whole cohort of superior quality embryos, which would assist in the treatment of human infertility and animal embryo production. For instance, there is a dire need to determine the effects of gonadotropin stimulation protocols on follicular atresia, particularly in the human female, with the cow serving as an extremely useful and relevant animal model.

There is little evidence to indicate that follicles are destined and irreversibly committed to atresia from the start of their developmental journey since most follicles remain capable of continued development if stimulated properly. For instance, although the dominant follicle of the first wave of the cow estrous cycle is theoretically destined for atresia, it can be rescued as long as the stimulus is provided in advance of the regression phase (Fortune et al., 1991). Atretic follicles can also be rescued by exogenous exposure to gonadotropins (Hsueh et al., 1994; Monniaux et al., 1984; Blondin et al., 1996). Currently, however, we hold little knowledge about how long the rescue period may last, or how long atretic fate can be manipulated. While diminishing the rate follicular atresia and increasing oocyte yield, superovulation procedures alone do not yield more embryos than those of non-stimulated cycles (Blondin et al., 1996). These outcomes justify the need for further refinement of gonadotropin stimulation protocols.

An ovarian stimulation protocol with particular promise in the cow involves the use of gonadotropins together with a so-called "coasting" phase. In essence, coasting protocols are aimed at collecting oocytes from follicles in stages of early atresia (Sirard et al., 1999; Blondin et al., 2002). The approach is based upon the premise that early atretic follicles contain oocytes of quality comparable to pre-ovulatory follicles as alluded to previously (see above discussion in section 10). Coasting protocols thus target oocyte collection from follicles in a plateau phase of follicular growth, during which follicular atresia may be already under way and an optimal follicular microenvironment exists. Essentially the protocols entail stimulation with FSH (in constant or decreasing dosages) followed by a

period of coasting for 24-72 hours with no further gonadotropin exposure and prior to oocyte aspiration. When collected at the optimal time post-FSH withdrawal (and presumably in the throes of early atresia), the oocytes possess the highest developmental potential (Sirard et al., 1999; Blondin et al., 2002). More recently, the optimal timing for oocyte collection has been narrowed to 54 hours +/- 7 hours, post-FSH withdrawal (Bunel et al., 2011). Bovine studies thus provide promise for future efforts to manipulate the atretic status of follicles *in vivo*, coupled with obtaining oocytes of optimal developmental quality (Figure 4). These approaches stand in stark contrast to current stimulation protocols in both livestock and in women, in which oocytes are collected from follicles during the growing phase, final differentiation of the oocytes has not occurred, and oocyte developmental competence is often compromised.

12. Conclusions

In conclusion, our understanding of the cellular factors influencing the onset of follicular atresia within the ovary is only beginning to emerge. Future research focusing on the mechanisms shared by granulosa cells and oocytes that dictate cell fate (i.e., growth, differentiation, death) and, ultimately, follicular fate (i.e., growth, dominance, atresia), including the molecular, cytoskeletal, and metabolic influences, should provide considerable insight. Examples of these influences include microRNA regulation, cytokeratin filament expression, and oxidative stress control, respectively. These factors, in turn, are of biological and economic significance because they impact other aspects of fertility in both livestock and humans. Once identified, they may hold the key to therapeutic improvements in treating infertility and poor reproductive performance in animals.

13. Acknowledgement

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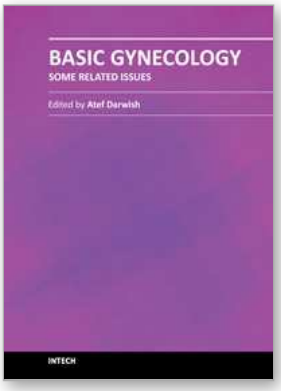

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