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Keratin 8/18 filaments: Potential modulators of death signaling in ovarian granulosa cells

Sarah E. Kinsman
University of New Hampshire, Durham

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Abstract
Granulosa cell apoptosis is associated with follicular atresia; but the cellular mechanisms that drive this process, especially its cell specificity, are relatively unknown. Here, we determined that cultured granulosa cells abundantly express K8/K18 filaments and inhibition of protein synthesis enhances Fas-induced apoptosis. In this context, the roles of cFLIP, ERK1/2 and Akt are minimal but conversely, K8/K18 filaments have a prominent role in granulosa cell resistance to Fas-induced apoptosis. Keratin 8/18 filaments in granulosa cells provide a plausible mechanism to avoid Fas-induced apoptosis and this mechanism potentially involves the synthesis of labile proteins. The existence of K8/K18 filaments in granulosa cells has relevance to follicular atresia and the selection of follicles for ovulation. These insights may have bearing on future therapeutic strategies to improve female fertility.

Keywords
Biology, Cell, Biology, Physiology, Biology, Molecular
KERATIN 8/18 FILAMENTS: POTENTIAL MODULATORS OF DEATH SIGNALING IN OVARIAN GRANULOSA CELLS

BY

Sarah E. Kinsman
B.S., University of New Hampshire, 2009

THESIS

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Thesis Director, David H. Townson, Ph.D.
Associate Professor of Molecular, Cellular and Biomedical Sciences

John S. Davis, Ph.D.
Professor of Obstetrics and Gynecology
University of Nebraska Medical Center

W. Kelley Thomas, Ph.D.
Professor of Molecular, Cellular and Biomedical Sciences

Paul C. Tsang, Ph.D.
Professor of Molecular, Cellular and Biomedical Sciences

Date 12/10/12
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ABSTRACT

KERATIN 8/18 FILAMENTS: POTENTIAL MODULATORS OF DEATH SIGNALING IN OVARIAN GRANULOSA CELLS

BY

Sarah E. Kinsman

University of New Hampshire, Durham, December 2012

Granulosa cell apoptosis is associated with follicular atresia; but the cellular mechanisms that drive this process, especially its cell specificity, are relatively unknown. Here, we determined that cultured granulosa cells abundantly express K8/K18 filaments and inhibition of protein synthesis enhances Fas-induced apoptosis. In this context, the roles of cFLIP, ERK1/2 and Akt are minimal but conversely, K8/K18 filaments have a prominent role in granulosa cell resistance to Fas-induced apoptosis. Keratin 8/18 filaments in granulosa cells provide a plausible mechanism to avoid Fas-induced apoptosis and this mechanism potentially involves the synthesis of labile proteins. The existence of K8/K18 filaments in granulosa cells has relevance
to follicular atresia and the selection of follicles for ovulation. These insights may have bearing on future therapeutic strategies to improve female fertility.
INTRODUCTION

Implications of Dairy Infertility

Animal infertility is an economic concern of today’s agricultural industry. The modern dairy cow is now producing more milk than ever before, essentially doubling output from 1951 to 1996 (Butler, 1998), but this increase in milk production has been accompanied by an equally-striking decline in fertility (Butler and Smith, 1989; Lucy, 2001). To make matters worse, farmers are now faced with the challenge of declining cow numbers and agricultural land (Dobson et al., 2007). Annual milk production per cow in the United States is projected to reach over 14 tons in 2050 (Santos et al., 2010), placing greater pressure on the metabolic needs (especially the reproductive needs) of the animal. Expanding our understanding of fertility at the cellular level will benefit the cow, the dairy farmer and ultimately, the dairy industry, if it leads to improvement in reproductive efficiency.

Negative Energy Balance and Infertility

At calving, high-milk producing cows enter a metabolic state referred to as negative energy balance (NEBAL) because nutritional intake of the cow is
unable to meet the energy requirements for milk production. The occurrence of NEBAL mobilizes the cows' body reserves (i.e., fat and muscle), which collectively has a negative impact on body condition, health and reproductive function of the cow. These metabolic changes disrupt the endocrine system as a whole, ultimately hindering ovarian activity (Beam and Butler, 1997; Lucy, 2001). During NEBAL, serum levels of luteinizing hormone (LH), insulin and insulin-like growth factor (IGF-I) are depleted, which impairs follicle maturation and delays ovulation (Beam and Butler, 1997; Butler, 2000; Lucy, 2002). In addition, the metabolic by-products of NEBAL (e.g., non-essential fatty acids, ketones and urea) infiltrate the follicular fluid of ovarian follicles, adversely affecting egg (oocyte) quality (Leroy, 2004) and, thus, further compromising animal fertility. For the high-milk producing cow, NEBAL and a decline in body condition are directly associated with a higher incidence of delayed ovulation, reduced conception rates, and a greater occurrence of embryonic loss (Lucy, 2002).

The Ovary

Ovarian Structures

The ovary in females is the primary reproductive structure responsible for influencing reproductive cyclicity through its secretion of steroids and for producing gametes (oocytes) for purposes of conception and pregnancy. The ovary consists of two notable endocrine structures; the follicle and the corpus
luteum. Both of these structures develop, mature and regress during the course of a single reproductive cycle.

The Ovarian Follicle

Within each ovarian follicle there is the female gamete, the oocyte (egg), which is surrounded by two other somatic cell types, the granulosa and the theca cells (reviewed by Aerts and Bols, 2010). Granulosa cells are characterized as either membrana granulosa cells, which constitute the majority of cells within the interior of the follicle, or the cells that surround the oocyte in a mature follicle, which are known as the cumulus oophorus (Figure 1). Theca cells comprise the supportive cells surrounding the follicle, separated from the granulosa cells by a basement membrane. The theca cells can be further subdivided into theca interna and theca externa based upon their vascularity and relative proximity to the basement membrane of the follicle (Young and McNeilly, 2010) (Figure 1). In response to systemic luteinizing hormone (LH), theca interna cells synthesize androgens, such as androstenedione, derived from the precursor molecule cholesterol. Theca-derived androgens diffuse across the basement membrane of the follicle to the granulosa cells, where follicle-stimulating hormone (FSH), also secreted systemically, directs the conversion of androgens to estradiol (Fortune, 1994). This "shared synthesis" of steroids between the theca and granulosa layers of maturing follicles is referred to as the "two-cell theory" and was first described by Roger Short and colleagues (Short, 1962).
Figure 1 Representative diagram of ovarian follicle structure and morphology.

**Folliculogenesis**

Anatomically, ovarian follicles are divided into four classifications according to relative developmental stage: primordial, primary, secondary and tertiary follicles (Figure 2). Primordial follicles contain a non-growing oocyte surrounded by squamous epithelial granulosa cells, which range from 25-50 μm in diameter (Figure 2a). Primary follicles contain a growing oocyte surrounded by cuboidal granulosa cells, and range in diameter from 35-70 μm (Figure 2b). Secondary follicles are roughly doubled in size (70-120 μm), and contain multiple layers of granulosa cells (Figure 2c). Lastly, tertiary follicles are largest follicles (≥ 5 mm in diameter) with a defined cumulus oophorus surrounding the mature oocyte and antral space enclosed by membrana granulosa and vascularized theca cells (Figure 2d-f) (Pedersen and Peters, 1968; Gougeon and Chainy, 1987; Fair et al., 1997; Myers et al., 2004;
Kacinskis et al., 2005; Hunzicker-Dunn and Maizels, 2006; Rodgers and Irving-Rodgers, 2010).

Figure 2 Follicle Classification. (a) primordial, (b) primary, (c) secondary, (d) early tertiary, (e) tertiary, and (f) late tertiary follicles. Bar = 20µm. (Myers et al., 2004)

The Corpus Luteum

Another structure characteristic of the mammalian ovary is the corpus luteum. The corpus luteum (CL; Latin for yellow body) is a transient endocrine structure responsible for the maintenance of pregnancy through its
secretion of progesterone (Niswender et al., 1994; Niswender et al., 2000; Davis and Rueda, 2002). Briefly, following the process of ovulation, the remaining granulosa and theca cells of the recently ovulated follicle differentiate to form the so-called “luteal cells” comprising the CL. In some species, these luteal cells retain size characteristics reminiscent of their precursor theca and granulosa cells, but they are generally referred to as simply small (<20 μm) and large (20-30 μm) steroidogenic luteal cells based upon their relative diameter (Alilia and Hansel, 1984; McCracken et al., 1999). There are distinguishing physiological characteristics of the small and large luteal cells, though, such as progesterone synthetic capability, sensitivity to LH stimulation, etc., but these aspects of distinction are beyond the scope of the current literature review. Suffice-it-to-say that progesterone secretion by both types of luteal steroidogenic cells facilitates the maintenance of pregnancy by preparing the uterus for implantation and preventing subsequent ovulations (Kasa-Vubu et al., 1992; Davis and Rueda, 2002). In the event that conception fails, or a pregnancy is lost mid-gestation, the CL promptly undergoes regression, progesterone secretion declines, and the resumption of follicular development and ovulation for the next reproductive cycle occurs.

**The Estrous Cycle**

In the cow, the duration of the reproductive cycle (estrous cycle) is typically 21 days, but may range from 17-28 days. Each estrous cycle begins with the onset of ovulation (d=0), followed by an extended luteal phase (days
1-17) and a brief follicular phase (days 18-21) in non-pregnant animals. Similar to horses and humans, cows typically ovulate only a single oocyte per cycle. However, the process leading to ovulation in these species is distinguished by successive, temporal waves of follicular development, defined as "follicular waves" (Ginther et al., 1989), in which groups of follicles grow to yield a single, mature follicle for ovulation. Typically two to three waves of follicular growth occur per cycle, in which each wave consists of a group or cohort of follicles selected to mature, or undergo atresia, during the wave. This process of follicular waves ultimately results in the selection of a single follicle for ovulation (Fortune et al., 1991; Lucy et al., 1992).

Follicular waves during the bovine estrous cycle are characterized by the recruitment, selection and dominance, or atresia, of ovarian follicles (Hogden, 1982). Recruitment and growth of the cohort of follicles within a wave occurs in response to follicle stimulating hormone (FSH) from the anterior pituitary gland (Walters and Schallenberger, 1984). Generally, one follicle of the cohort is ultimately selected to continue to grow and becomes the dominant follicle, whereas the remaining follicles of the cohort die off in a process referred to as follicular atresia. The onset of dominance is identified as the first day the dominant follicle is 1-2 mm larger than the next largest follicle in the cohort, and all other follicles in the cohort (subordinate follicles) cease growth, as determined by ultrasonographic measures (Hodgen, 1982).

The dominant follicle of the first follicular wave may ovulate, but typically undergoes atresia because of high systemic concentrations of
progesterone attributed to the presence of the recently formed CL. A second or third wave of follicular development ensues, also yielding a dominant follicle which eventually becomes the preovulatory follicle (Savio et al., 1988). Increased plasma estradiol produced by the preovulatory follicle generates a positive feedback loop to the anterior pituitary, triggering a robust surge of luteinizing hormone (LH) secretion and ovulation. Following ovulation, remnants of the ruptured follicle (i.e., granulosa and theca cells) undergo differentiation to form the corpus luteum (CL). In the absence of fertilization, the CL persists for only 16-17 days after ovulation, undergoes regression (luteolysis), which then triggers the onset of the next estrous cycle.

As described above, dairy cows typically have two to three follicular waves per estrous cycle (Rajakoski, 1960; Ireland and Roche, 1983). Conversely, the loss of follicles (follicular atresia), the absence of ovulation, and premature regression of the CL are all issues contributing to infertility in dairy cows. For purposes of this review, we will focus only on those mechanisms directly pertaining to follicular atresia.

**Follicular Atresia and Apoptosis**

Throughout the lifespan of most female mammals, over 99% of ovarian follicles are lost by degenerative and hormonally controlled processes collectively called follicular atresia (Erickson, 1966). Tilly and Hughes (1991) were among the first investigators to propose apoptosis (programmed cell death) of granulosa cells as a direct cellular mechanism contributing to
follicular atresia (Hughes et al., 1991; Tilly et al., 1991). Programmed cell death is specific, but the external factors controlling this specificity are relatively unknown. It is generally accepted that crosstalk within cells between cellular survival and death signals determine the fate of ovarian follicles (Amsterdam et al., 2003). However, these survival and death signals can be induced by a variety of external endocrine, paracrine, and autocrine factors, or any combination of the three (Amsterdam et al., 1999). Here we provide a general overview of the survival signals and death signals that affect granulosa cell fate within follicles, and some of the factors that influence these signals.

**Survival Signals**

The growth of follicles, or folliculogenesis, depends upon hormonal signals from the anterior pituitary to direct theca and granulosa cell growth while inhibiting apoptosis (Quirk et al., 2004). The gonadotropins, LH and FSH, are required at the antral stage of folliculogenesis to direct selection of tertiary follicles. During this time, one follicle of the cohort establishes its presence and becomes dominant, while subordinate follicles undergo atresia (Webb et al., 2007). One aspect influencing selection of the dominant follicle is the fact that FSH stimulates estradiol production by granulosa cells within this follicle, establishing its further growth and dominance (reviewed by Matsuda et al., 2012). For their part, the granulosa cells of selected follicles also secrete survival signals that promote growth (Matsuda et al., 2012). These signals include insulin-growth factor I (IGF-I) (Hirshfield, 1991; Webb et
al., 2007). IGF-I promotes mitosis of granulosa cells, estradiol production and responsiveness to further FSH and LH stimulation by increasing gonadotropin receptor expression (Guthrie et al., 1998; Quirk et al., 2004). Paracrine and autocrine effects of estradiol secretion by granulosa cells, in concert with IGF-I secretion, further enhance mitosis and differentiation of granulosa cells, while preventing apoptosis (Quirk et al., 2004; reviewed by Palter, 2011). In subordinate follicles undergoing atresia these survival signals diminish, the granulosa cells lose functionality, and ultimately undergo apoptosis. A loss of estradiol and IGF-I secretion in genetic knockout mice, for instance, triggers granulosa cell apoptosis, abnormal follicular development, and infertility (Baker et al., 1996; Britt et al., 2000; Dupont et al., 2000; Zhou et al., 1997).

Other factors influencing granulosa cell fate include epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), interleukin-6 (IL-6) and IL-1β (Chun et al., 1995; Guthrie et al., 1998; Lynch et al., 2000; Tilly et al., 1992), which stimulate granulosa cell growth and proliferation, enhance folliculogenesis, and promote follicle selection and dominance.

**Death Signals (Apoptosis)**

Apoptosis is a biological process responsible for controlling cell numbers and tissue size, while concurrently providing protection from invasive cells (Tschopp et al., 1998; reviewed by Hengartner, 2000; Peter, 2004). The term "apoptosis" was first coined by John Foxton Ross Kerr in the 1970's who defined the process as consisting of two stages: 1) the formation
of apoptotic bodies, and 2) the phagocytosis of apoptotic bodies (Kerr et al., 1972).

All cells are equipped with the machinery to trigger apoptosis but most often the process is avoided by the actions of survival signals (Alberts et al., 2002; Kanamaru et al., 2012; Peter and Krammer, 1998). Apoptosis is principally executed by a class of intracellular cysteine proteases known as caspases, which become activated by a variety of intracellular (intrinsic pathway) or extracellular (extrinsic pathway) influences (Fernandes-Alnemri et al., 1995). All caspases are initially translated as inactive proteins containing a pro-domain (zymogens). Most caspases are commonly activated by: 1) other nearby caspases (caspase-8) or 2) autocatalytic cleavage by another active caspase (caspase cascade; caspase-3, -6 and -7) (Thornberry et al., 1997; Hengartner, 2000). Active caspases are proteolytic and cleave proteins having aspartate residues, inhibiting their biological function (Hengartner, 2000).

**Intrinsic Pathway**

The intrinsic (mitochondrial) apoptotic pathway integrates pro-apoptotic and anti-apoptotic signals within the target cell by apoptotic regulator proteins of the bcl-2 family (Quirk et al., 2004). The bcl-2 family includes pro-apoptotic proteins (Bax, Bad, Bim, Bid, Bok, Bcl-2-short) and anti-apoptotic proteins (Bcl-2, Bcl-2-long, Bcl-w) (Antonsson and Martinou, 2000; Quirk et al., 2004). In response to an insult or stress, the intrinsic mitochondrial pathway
becomes activated, releasing cytochrome c into the cytoplasm. Cytochrome c, apoptosis-activating factor (Apaf)-1 and pro-caspase 9 form an apoptosome complex (Li et al., 1997). This complex cleaves pro-caspase-9 to its activate form, which then activates caspase-3 and other downstream caspases (Hengartner, 2000). Other mitochondrial pro-apoptotic proteins include Smac-diablo and apoptosis inducing factor (AIF) (Susin et al., 1999s; Du et al., 2000; Verhagen et al., 2000).

**Extrinsic Pathway**

Signaling molecules responsible for inducing apoptosis via the extrinsic pathway are mediated by transmembrane death receptors (Barnhart et al., 2003; Thornburn, 2004). Several types of cytokines induce the extrinsic pathway in a variety of cells (e.g., Fas ligand, tumor necrosis factor, TRAIL, etc.), but for purposes of the current discussion about granulosa cells, we will focus only on the Fas ligand-Fas-mediated pathway. The Fas receptor is a 42-52 kDa member of the tumor necrosis factor (TNF) super family and is thought to trigger the apoptotic death of granulosa cells during follicular atresia (Matsuda-Minehata et al., 2006; Porter et al., 2000; Vickers et al., 2000).

The Fas-induced apoptotic pathway is characterized by the expression of the cytokine, Fas ligand (FasL; CD95), which binds to Fas receptor, causing trimerization of the ligand-receptor complex. Within the target cell, an adaptor protein known as the Fas-Associated Death Domain (FADD) protein
binds to the cytoplasmic region of the Fas receptor, specifically the "death domain" region of Fas. This interaction between Fas and FADD results in the binding of pro-caspase 8, forming the Death-Inducing Signaling Complex (DISC). Formation of the DISC results in cleavage of pro-caspase 8 to activated caspase 8, initiating a signaling cascade that leads to apoptosis of the cell (Thornburn, 2004).

Counteracting the effects of pro-caspase-8 activation and the downstream stimulation of apoptotic mechanisms is the molecule known as cellular flice inhibitory protein (cFLIP) (Hu et al., 1997). Originally discovered as a viral apoptotic inhibitor (Thome et al., 1997), cFLIP is a labile, anti-apoptotic protein that competes with procaspase-8 for binding to FADD and the formation of the DISC. The structure of cFLIP is identical to pro-caspase-8 but it lacks the enzymatic region to trigger downstream death effects (Micheau, 2003; Öztürk et al., 2012). The structural similarities between cFLIP and pro-caspase-8 enable cFLIP to competitively bind to FADD, preventing pro-caspase-8 binding, and thus inhibiting pro-caspase-8 cleavage, activation and downstream apoptotic signaling.

**Survival Signaling Pathways**

Growth of ovarian follicles depends upon survival signaling pathways within granulosa cells to override potential death signals and prevent the occurrence of apoptosis. Two survival pathways of particular interest in
granulosa cells include the Ras/mitogen-activated protein kinase (MAPK) and the phosphoinositide 3'-OH kinase (PI3K)/Akt pathways.

The MAPK/ERK Pathway

The MAPK pathway contributes to cell survival, proliferation, and differentiation of granulosa cells (Chang and Karin, 2001; Pearson et al., 2001). Mammalian MAPK are divided into four classes- the extracellular-signal regulated kinases (ERK1/2), Jun amino-terminal kinases (JNK1/2/3), p38 proteins and ERK5 (Peter and Dhanasekaran, 2003; Shiota et al., 2003). ERK1/2 and ERK5 are survival kinases (Nishimoto and Nishida, 2006), whereas p38 and JNK are implicated as apoptotic kinases (Fey et al., 2012).

The MAPK pathways are activated in response to extracellular stimuli; the ERK pathways are activated by growth factors, while the JNK and p38 pathways are activated by cytokines and cellular stress (Ip and Davis, 1998). All of these signaling cascades involve activation of intracellular MAP kinase kinases that then phosphorylate either the MAP kinases ERK1/2, JNK, p38 or ERK5. Phosphorylated MAPK act as second messengers and translocate to the nucleus where they regulate gene expression of survival and apoptotic proteins (Zeng et al., 2005). Ultimately, the fate of the cell is determined by the balance between survival and apoptotic signals; i.e. if survival signals outweigh apoptotic signals, cell survival prevails (Nagata, 1997; Jarpe et al., 1998).
An illustration of this delicate balance between survival and apoptosis is seen in porcine granulosa cells, wherein treatment with growth factors, such as FSH or fetal bovine serum (FBS), actually results in apoptosis if the phosphorylation of ERK1/2 (a survival signal) is inhibited (Shiota et al., 2003). Conversely, inhibition of the p38 pro-apoptotic pathway prevents peroxide-induced apoptosis in this same model (Shiota et al., 2003). Phosphorylation of JNK prompts apoptosis of granulosa cells, implicating JNK signaling in follicular atresia (Peter and Dhanasekaran, 2003). ERK5 enhances progesterone secretion by granulosa cells, but the effect of ERK5 phosphorylation on granulosa cell fate is not known (Gao et al., 2011). Overall, ERK1/2 phosphorylation generally prevents apoptosis of granulosa cells, but phosphorylation of JNK and p38 promote apoptosis and possibly contribute to follicular atresia.

The PI3K/Akt Pathway

The serine/threonine kinase Akt (also known as protein kinase B; PKB) was originally discovered as v-Akt, a retrovirus-associated oncogene from the AKT8 murine retrovirus (Staal, 1977). The molecule Akt is a key player in cell survival, metabolism, motility and gene expression associated with the PI3K pathway (Cantley, 2002). This pathway is activated in response to: 1) phosphorylation of receptor tyrosine kinases (RTKs) by growth factors, 2) stimulation of G-coupled receptors, and 3) activation of integrins (Fayard et al., 2005). Ligand-receptor interaction results in the activation of second messenger PI3K by phosphorylation. Phosphorylation of PI3K can also be
achieved by interacting with the protein Ras, which is also associated with the MAPK pathway (Datta et al., 1999). The activation of PI3Ks further stimulates second messenger phosphoinositides by phosphorylation (i.e. converting phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3) (Rameh and Cantley, 1999). The formation of PIP3 then facilitates recruitment of Akt to the plasma membrane and activates it by phosphorylation (Fayard et al., 2005).

This pathway and its interaction with the MAPK pathway are relevant to the fate of granulosa cells and follicular development because within bovine ovaries, levels of Akt, phosphorylated Akt (p-Akt), ERK and phosphorylated ERK (p-ERK) are all elevated in dominant follicles compared to subordinate follicles (Ryan et al., 2007). In addition, inhibition of the PI3K pathway impairs granulosa cell responsiveness to FSH and IGF stimulation (Ryan et al., 2008). This suggests both the MAPK and PI3K pathways have a critical role in the fate of granulosa cells within the follicle, and most likely influence the selection of dominant versus subordinate follicles during folliculogenesis.

**Fas and the Follicle**

A physiologic mechanism of atresia in follicles is the effect of Fas-induced apoptosis of granulosa cells (Hughes et al., 1991; Tilly et al., 1991). Both Fas and FasL are expressed in ovaries of mammals across most species, including humans, domesticated livestock and rodents (Dharma et
Differential expression of FasL and/or Fas among granulosa cells of follicles might influence the occurrence of apoptosis and follicular atresia.

In granulosa cells, Fas expression increases following pretreatment with the cytokines interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα), causing the cells to be vulnerable to Fas-induced apoptosis (Quirk et al., 1998; Porter et al., 2000; Vickers et al., 2000). Inhibition of protein synthesis (via cycloheximide) also enhances granulosa cell sensitivity to Fas-induced apoptosis (Quirk et al., 1995; Quirk et al., 1998), implying that labile, anti-apoptotic proteins exist within granulosa cells to prevent apoptosis.

The expression of Fas has been evaluated in bovine follicles across the estrous cycle; expression of Fas mRNA is highest in granulosa cells of atretic, subordinate follicles and lowest in preovulatory follicles (Porter et al., 2000). This suggests one aspect of follicular atresia is the upregulation of Fas mRNA in granulosa cells. However, it is noteworthy that granulosa cells of these same atretic follicles in vitro are generally resistant to Fas-induced apoptosis unless pretreated with cytokines such as IFNγ or TNFα (Quirk et al., 1998; Porter et al., 2001). Additional factors must exist either within the granulosa cells, or within their follicular environment (i.e., in vivo) to affect sensitivity to Fas-induced apoptosis.
Mechanisms that influence Fas expression

In vitro versus in vivo environment could have profound effects on the expression of the Fas receptor by granulosa cells. Fas expression and trimerization in response to FasL are both necessary for induction of the Fas-induced apoptotic pathway, but it is currently unclear what cellular mechanisms regulate Fas expression on the surface of granulosa cells. In liver epithelial cells (hepatocytes), cytoskeletal intermediate filaments influence Fas expression and Fas-induced cell signaling (Coulombe and Omary, 2002). Genetic knockout of the intermediate filament protein, keratin 8 (K8), increases Fas expression and enhances the sensitivity of hepatocytes to Fas-induced apoptosis (Gilbert et al., 2001). Similarly, our laboratory has determined that the absence of keratin 18 (K18)-containing intermediate filaments makes HeLa cells more sensitive to Fas-induced apoptosis (Sullivan et al., 2010). Interestingly, hepatocytes of K8-null mice also express less ERK1/2 and cFLIP compared to hepatocytes of wild-type mice, suggesting the expression of keratin intermediate filaments, the presence of cFLIP and the activation of ERK1/2 may together protect the cells from Fas-induced apoptosis (Gilbert et al., 2004).

Keratin Intermediate Filaments

Intermediate filaments (IFs) were first described by Ishikawa et al. in 1968 as cytoskeletal scaffolds in the nucleus and cytoplasm in metazoans (Ishikawa et al., 1968; Kim et al., 2007). Together with microtubules and
microfilaments, IFs comprise the third component of the cytoskeleton of most cells. Structurally, all IFs are comprised of a central helical rod flanked by N-terminal and C-terminal head and tail domains, respectively; the rod domains are conserved among subclasses, but terminal domains are conserved across all IFs (Figure 3) (Fuchs and Weber, 1994).

![Figure 3 Tripartite structure of intermediate filaments (Coulombe and Omary 2002).](image)

The generally understood function of IFs is to provide structural integrity, but in recent years a more dynamic role for these structures has been suggested, including the modulation of apoptosis, responsiveness to physiologic stress, wound healing, facilitating cell growth and mitosis, regulating tissue polarity and influencing tissue remodeling (Moll et al., 2008).

The keratins are a subclass of IFs occurring as heterodimers composed of a type I (acidic; numbered 9-20) and type II (basic; numbered 1-8) filament. Keratin 8/18 (K8/K18) IFs are considered characteristic of simple epithelia (including granulosa and theca cells of follicles), immortalized cell
lines and carcinomas; and they are the first keratin proteins expressed during embryonic development (Moll et al., 1982; Jackson et al., 1980).

In recent years, investigators have discovered many novel functions for keratin IFs beyond their well-accepted role in providing structural support to the cell. For instance, there is current thinking that K8/K18 IFs sequester the activity of intracellular kinases, especially following cellular stress. Essentially the filaments provide an abundance of serine residues within their structure, serving as phosphorylation substrates or "phosphate sponges" for stress-activated kinases (e.g., Akt). This action prevents the activation of downstream pathways by these stress kinases that might otherwise lead to apoptosis (Ku and Omary, 2006). The solubilization and polymerization activities of the keratins are directly influenced by this serine phosphorylation in the head and tail domains of K8 (Ser-23/Ser-73/Ser-431) and K18 (Ser-33/Ser-52) (Omary et al., 1998; Coulombe and Omary 2002; Owens and Lane, 2003; Omary et al., 2006). In this manner, the sheer abundance of the filaments with their multiple phosphorylation sites re-direct the activities of apoptotic kinases away from downstream death-signaling mechanisms (Ku and Omary, 2001). Interestingly, K8 and K18 also have phosphorylation sites that are recognized by MAPK (Galarneau et al., 2006) and cell cycle progression 14-3-3 proteins (Liao and Omary, 1996) The Ser74 and Ser431 phosphorylation sites on K8 are associated with ERK1/2 (Ku and Omary, 1997) and p38 (Ku et al., 2002). Liao and Omary demonstrated that K8/K18 hyperphosphorylation during the S and G2/M phases of the cell cycle result in
14-3-3 protein binding, that in turn, activate PI3K and MAPK pathways (Liao and Omary, 1997). Thus K8/K18 IFs within cells may activate cell proliferation pathways in response to stress signals as a protective mechanism.

Beyond these measures, K8/K18 filaments protect cells from apoptosis, stress and injury by impairing the cell surface expression of Fas and the downstream activation of caspases (Gilbert et al., 2001). Inhibition of caspase activation occurs, in part, by enhancing the expression and activation of the anti-apoptotic proteins cFLIP and ERK1/2 (Gilbert et al., 2004). Genetic knock-down of K8 reduces endogenous c-FLIP expression and decreases phosphorylation of ERK1/2, making mouse hepatocytes three to four times more sensitive to FAS-induced apoptosis than their wild-type counterparts (Gilbert et al., 2001; Gilbert et al. 2004). Thus, K8/K18 filaments are thought to provide resistance to Fas-induced apoptosis by suppressing Fas expression and caspase activation, while concomitantly activating survival pathways and promoting cell proliferation.
K8/K18 Filaments and Granulosa Cells

Keratins are expressed in granulosa cells of bovine follicles across the developmental stages, but their role in folliculogenesis is unknown (Czernobilsky et al., 1985; Gall et al., 1992; Santini et al., 1993; Townson et al., 2010; van den Hurk et al., 1995). The K8/K18 filaments are expressed in granulosa cells of the follicle, but their expression in atretic follicles is less evident than growing or recently ovulated follicles (Townson et al., 2010). Additionally, the relative expression of K8/K18 filament expression in granulosa cells is inversely proportional to the number of cells undergoing apoptosis as observed by Cyto-DEATH immunodetection (Townson et al., 2010). This suggests the onset of apoptosis of granulosa cells and the occurrence of follicular atresia are associated with a loss of K8/K18 filaments. Others have shown Fas-induced apoptosis in granulosa cells is augmented by inhibition of cFLIP and inactivation of ERK1/2 (Matsuda et al., 2008; Shiota et al., 2003). At present, however, no one has determined whether K8/K18 filaments modulate Fas-induced apoptosis of granulosa cells of follicles through cFLIP expression and/or ERK1/2 phosphorylation as has been described in simple epithelial cells of other tissues (Gilbert et al., 2001; Gilbert et al., 2004).
Objectives and Hypotheses

The present study proposes there is a functional connection between K8/K18 filament expression, apoptosis-inhibition and survival signaling in granulosa cells influencing their vulnerability to apoptosis. The current study investigated the above-described relationships in the context of Fas-induced apoptosis and follicular atresia.

The objectives of the study were to 1) determine the relative expression of K8/K18 filaments in granulosa cells of human and bovine origin and 2) identify putative cellular mechanisms by which granulosa cells resist Fas-induced apoptosis, specifically determining whether or not K8/K18 filaments have a role in this protection.

We postulated K8/K18 expression, in part, accounts for the relative resistance of granulosa cells to Fas-induced apoptosis by: 1) decreasing the cell surface expression of Fas, 2) enhancing apoptosis-inhibiting proteins, and 3) activating intracellular pro-survival pathways.
CHAPTER I

INVESTIGATION OF THE ROLE OF KERATIN 8/18 INTERMEDIATE FILAMENTS ON THE FATE OF GRANULOSA CELLS

Introduction

Infertility is an ever-growing concern of today's agricultural industry (Lucy, 2001). For dairy operations, profitability depends upon the ability of the cow to give birth to a live calf each year. Follicular atresia is one aspect of ovarian function that contributes to poor reproductive performance and may result in infertility (Lucy, 2002).

Apoptosis of granulosa cells is contributing factor to follicular atresia within the ovary, preventing follicle maturation and ovulation, and possibly impairing fertility in females (Hughes and Gorospe, 1991; Tilly et al., 1991).
The extrinsic pathway of apoptosis, activated by cytokines and other influences external to the targeted cell, is thought to contribute to the process of follicular atresia (Townson and Combelles, 2012). The cytokine Fas ligand (FasL) and its corresponding receptor, Fas, for instance, are elevated in atretic follicles compared to healthy follicles (Kim et al., 1998; Vickers et al., 2000; Porter et al., 2001; Inoue et al., 2006). However, cultured granulosa cells are typically resistant to Fas-induced apoptosis unless first pretreated with tumor necrosis factor (TNF) and interferon-gamma (IFN) (Quirk et al., 1995), which is thought to increase the surface expression of Fas (Quirk et al., 1998). It is therefore conceivable that Fas-induced apoptosis of granulosa cells contributes to follicular atresia, and that cytokines such as TNF and IFN facilitate this process.

The targeted and selective loss of granulosa cells by apoptosis without accompanying collateral damage to adjacent cells is a characteristic aspect of follicular atresia. Apoptosis is also a hallmark of the FasL-Fas system, by which the immune system establishes immune tolerance and the elimination of lymphocytes targeted against self-antigens. The cytokines TNF, TRAIL, and their corresponding receptors are additional influences, similar to FasL and Fas, which potentially trigger apoptosis and induce follicular atresia in certain species (Prange-Kiel et al., 2001; Xiao et al., 2002; Inoue et al., 2003). However, beyond these hormonal influences on granulosa cell viability within follicles, there are structural, cytoskeletal influences to consider. In the last decade, for instance, a number of studies have implicated the cytoskeletal
elements (i.e., microtubules, microfilaments, and intermediate filaments) as profoundly affecting follicular growth, potentially impairing ovulation, and causing cystic follicles (Salvetti et al., 2004; Ortega et al., 2007; Salvetti et al., 2010).

Microtubules, microfilaments, and intermediate filaments collectively influence the activities of most cells, including the granulosa cells within follicles. Microtubules regulate steroidogenesis (Chen et al., 1994), but they also determine cell shape and affect cytoplasmic movement of organelles within granulosa cells (Šutovský et al., 1994). Microfilaments drive granulosa cells toward differentiation (i.e., luteinization) (Amsterdam and Rotmensch, 1987) and facilitate death (Amsterdam et al., 1997). Under apoptotic conditions, for example, microfilaments within granulosa cells undergo rearrangement to compartmentalize the steroidogenic machinery to the perinuclear region while directing other proteolytic activities to the apoptotic bodies (Amsterdam et al., 1997). The intermediate filaments, including vimentin, the cytokeratins, and desmin, 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). Most recently, our laboratory has identified intermediate filaments, particularly keratin 8 and keratin 18 (K8/K18) filaments, as possible intrinsic modulators of granulosa cell apoptosis during folliculogenesis (Townson et al., 2010).

The K8/K18 filaments are considered “stress filaments” characteristic of most simple epithelia, immortal cell lines and carcinomas. They provide
structural integrity and mechanical stability to cells via cell-cell junctions (Moll et al., 2008; Waschke, 2008). Recently, dynamic features of these filaments have become evident, suggesting the K8/K18 filaments also modulate death signaling by regulating cytokine receptors, enhancing anti-apoptotic proteins and activating intracellular survival pathways (Gilbert et al., 2001; Marceau et al., 2001; Eriksson et al., 2009; Sullivan et al., 2010). Genetic knockdown of K8 in mouse hepatocytes, for instance, increases the cell surface expression of Fas and up-regulates caspase activation (Gilbert et al., 2001). The expression of the anti-apoptotic protein cellular flice inhibitory protein (cFLIP; also known as CFLAR) and the phosphorylation of extracellular regulated kinases 1 and 2 (ERK1/2) are also impaired in these hepatocytes (Gilbert et al., 2004). As a result, the K8-null hepatocytes are three to four times more sensitive to Fas-induced apoptosis (Gilbert et al., 2001; Gilbert et al., 2004). The K8/K18 filaments within granulosa cells of follicles might orchestrate similar aspects of cellular fate, possibly enabling the cells to evade Fas-induced apoptosis by impairing Fas expression, augmenting cFLIP expression and/or ERK1/2 phosphorylation.

The objectives of the current study were to investigate the above-described possibilities by: 1) determining the relative expression of K8/K18 intermediate filaments within granulosa cells, 2) identifying cellular mechanisms by which granulosa cells resist Fas-induced apoptosis, and 3) determining if K8/K18 filaments have a role in this resistance. For the majority of this work, immortal cells established from a granulosa cell tumor,
the KGN cell line, were utilized. The cells retain many physiological attributes of granulosa cells, including responsiveness to FSH through a functional FSH receptor (Nishi et al., 2001). Additional experiments with primary cultures of granulosa cells derived from bovine ovarian follicles were then initiated for comparative purposes.
Materials and Methods

Animal Care and Use

Follicles from bovine ovaries were obtained from local slaughterhouse facilities or research cows at the Fairchild Dairy Teaching and Research Center. A protocol for this purpose was approved by the UNH Animal Care and Use Committee (IACUC) # 120104.

Cells and culture conditions

KGN cell line

The human granulosa cell tumor line, KGN, was generously provided by Dr. Fukuzawa (RIKEN Cell Bank, Koyadai, Japan) through our collaborator, John S. Davis (University of Nebraska Medical Center, Omaha, NE). The KGN cells were maintained in DMEM/F12 (1:1; Life Technologies, Grand Island, NY) supplemented with 10% FBS (JRH Biosciences, Lenexa, KS) at 37°C with 5% CO₂ and 95% air, with 95% humidity.

Primary bovine granulosa cells

The bovine ovaries obtained from slaughterhouse facilities and the Fairchild Dairy were transported to the laboratory at 25°C in sterile saline, and then rinsed in ethanol, Betadine solution and PBS before dissecting all follicles ≥ 10 mm in DMEM/F12 + 2 μL/mL gentamicin. Follicular fluid from each of the follicles was removed by aspiration, the follicles were bisected
and then gently scraped to release the granulosa cells. The granulosa cells were washed three times for 10 minutes at 25 °C at 500, 250 and 175 x g to remove red blood cells. Once washed, the cells were counted using the Trypan Blue exclusion method then seeded in plastic cultureware and maintained in DMEM/F12 (1:1; Life Technologies, Grand Island, NY) supplemented with 10% FBS (JRH Biosciences, Lenexa, KS) at 37°C with 5% CO₂ and 95% air, with 95% humidity.

**Culture conditions**

The granulosa cells (KGN and bovine) were either seeded in 96-well plates (BD Biosciences, San Jose, CA) at 1x10⁴ cells/well for Caspase-Glo 3/7, CellTiter 96 and In-Cell Western assays, seeded in 6-well plates (Corning, Corning, NY) at 3x10⁵ cells/well for immunoblotting experiments or seeded in microchamber slides (Nunc, Rochester, NY) at 1x10⁴ cells/well and T75 or T150 vented flasks (BD Biosciences, San Jose, CA) at 5x10⁵ cells and 1x10⁶ cells/flask, respectively, for immunofluorescence and flow cytometry analysis. In all cases, the granulosa cells were grown to 70% confluency prior to the onset of treatments. For siRNA experiments, conditioned culture medium was exchanged for antibiotic-free DMEM/F12 medium + 10% FBS, and immediately prior to cytokine treatments, exchanged for again for serum-free DMEM/F12 medium.
Immunofluorescent visualization of K8/K18 intermediate filaments

Following culture, granulosa cells grown in microchamber slides were rinsed twice with PBS and then fixed for 20 minutes at room temperature with 2% paraformaldehyde (PFA) in a microtubule-stabilizing buffer (100 mM HEPES, pH 7.31; 1 mM MgSO₄·7H₂O; 1 mM EGTA; 0.2% Triton X-100). Subsequently, the fixed cells were permeabilized for 20 minutes in ice-cold 100% methanol. After permeabilization, the cells were washed three times with PBS + 0.1% BSA and labeled with mouse anti-human K18-FITC-conjugated antibody (CY90; Sigma-Aldrich, St. Louis, MO) diluted 1:100 in PBS + 1% BSA and Rhodamine Phalloidin (Life Technologies; Grand Island, NY) diluted 1:400 in PBS + 1% BSA for 1 hour at 37°C in a humidified chamber. In previous work, the dimerization of K18 protein with K8 protein to form a K8/K18 heterodimeric filament bovine granulosa cells and luteal cells was verified (Townson et al., 2010; Duncan et al., 2012). Thus, immunological targeting of K18 for the detection of K8/K18 filaments in the granulosa cells was considered adequate. For a negative control, the cells were exposed to a mouse anti-human IgG-FITC-conjugated antibody (Sigma-Aldrich, St. Louis, MO) diluted 1:100 in PBS + 1% BSA as a substitute for the primary antibody. The slides were washed three times with PBS + 0.1% BSA, then mounted on coverslips with ProLong® Gold Antifade reagent containing DAPI (Life Technologies, Grand Island, NY).

Photographic Images of the K8/K18 filament immunostaining were collected using an Olympus BH2-RFC upright fluorescent microscope (Center Valley,
PA), Qimaging QICAM monochrome digital camera (Surrey, BC, Canada) and ImagePro Insight software (Media Cybernetics, Bethesda, MD).

**Flow cytometric quantification of Fas surface and K18 expression**

Cultured cells were trypsinized using Cellgro 0.25% Trypsin-EDTA (Corning, Corning, NY) and fixed for 20 minutes at room temperature with 2% paraformaldehyde in a microtubule-stabilizing buffer (100 mM HEPES, pH 7.31; 1 mM MgSO4-7H2O; 1 mM EGTA; 0.2% Triton X-100). For Fas surface staining, the cells were stored in paraformaldehyde; for K18 staining, the cells were fixed with paraformaldehyde, permeabilized in ice-cold 70% ethanol for 20 minutes and then stored at -20°C. For antibody labeling, both types of cell preparations were washed three times with PBS + 0.1% BSA, and then either labeled overnight at 4°C with mouse anti-human Fas (CH11; EMD Millipore, Darmstadt, Germany) antibody diluted to 20 μg/mL in 10% normal goat serum + PBS + 1% BSA or labeled for 1 hour at 37°C with mouse anti-human K18-FITC-conjugated antibody (Sigma-Aldrich, St. Louis, MO) diluted 1:100 in PBS + 1% BSA. Separate preparations labeled with mouse anti-human IgG-FITC-conjugated antibody (Sigma-Aldrich, St. Louis, MO) diluted 1:100 in PBS + 1% BSA served as negative controls. For cells labeled with Fas antibody, the cells were subsequently washed three times with PBS + 0.1% BSA, and then incubated for 1 hour at 37°C with Alexa Fluor® 488 (Life Technologies, Grand Island, NY) diluted 1:200 in 10% NGS + PBS + 1% BSA. For both Fas- and K18-labeled cells, the cells were washed three times with PBS + 0.1% BSA and then analyzed using a 4 color, dual laser FACS
Calibur Flow Cytometer (BD Biosciences, San Jose, CA), quantifying 10,000 cells. Data were collected using CellQuest software (BD Biosciences; San Jose, CA) and then analyzed using WinMDI (Joe Trotter; Purdue University, West Lafayette, IN) to quantify the cell surface expression of Fas and K8/K18 filament expression.

**Induction of Fas-mediated apoptosis**

Granulosa cells were cultured and exposed to pretreatments of cycloheximide (CHX; 0.25 μg/mL; Sigma-Aldrich, St. Louis, MO), the MEK1/2 inhibitor PD98059 (30 μM; Cell Signaling Technologies, Danvers, MA) or the Akt inhibitor Wortmannin (100 nM; EMD Millipore, Darmstadt, Germany) for 2 hours in serum-free culture medium. After pretreatment, the cells were exposed to a Fas-activating antibody (1 μg/mL; clone CH11; EMD Millipore, Darmstadt, Germany) or Staurosporine (1 μM; MP Biomedical, Santa Ana, CA) as a positive control, to induce apoptosis. The cells were exposed to the above treatments for 8 and 24 hours, at which time the incidence of apoptosis was measured by caspase 3/7 activity using a Caspase-Glo® 3/7 Assay or a cell viability Cell Titer 96 (MTS) assay. These assay were conducted according to the manufacturer’s instructions (Promega, Madison, WI).

**Immunoblot analysis for Fas, cFLIP, cleaved PARP and β-Actin**

Nearly confluent cells from the above-described experiments were washed twice with ice-cold PBS and harvested in lysis buffer (10mM Tris-HCL; 1mM EDTA; 1mM EGTA; 100mM NaCl; 1% Triton X-100; 0.5% Nonidet
P-40, pH 7.4) containing kinase, protease and phosphatase inhibitor cocktails (Sigma-Aldrich, St. Louis, MO). The cells were scraped, collected, and sonicated for 3 seconds then resuspended in 2X SDS loading buffer (100 mM Tris-Cl, pH 6.8 + 4% SDS, 0.2% bromophenol blue, 20% glycerol, 200 mM DTT) and denatured at 95°C for 5 minutes. Total cellular proteins were separated by 12.5% SDS-PAGE and then transferred to polyvinylidene difluoride (PVDF) membranes (EMD Millipore, Darmstadt, Germany). Immunoblotting was performed using antibodies to detect human cFLIP (also known as CFLAR) (rabbit anti-human CFLAR; Sigma Aldrich, St. Louis, MO) and human Fas (clone C-20; Santa Cruz Biotechnology, Santa Cruz, CA) to determine the effect of cytokine and inhibitors on expression of cFLIP and Fas. Membranes were stripped and reprobed for cleaved human poly ADP ribose polymerase (PARP) (# 9542, Cell Signaling Technology, Danvers, MA), involved in DNA depletion and DNA repair during cell death, to validate apoptotic activity and β-actin (clone AC-15; Sigma Aldrich, St. Louis, MO) for normalization of protein loading.

**Short interfering RNA (siRNA) knockdown of K8/K18**

Granulosa cells were transiently transfected with 6-10 pmol KRT8 and KRT18 siRNA constructs according to RefSeq numbers: NM_001033610.1 and NM_001192095.1 (siRNA ID s444557 and s444560; Silencer® Select siRNA, Ambion Inc., Foster City, CA). Transfection was achieved using Lipofectamine™ RNAiMAX in OptiMEM® Reduced Serum Media for final 60-100 nM RNAi duplexes according to the manufacturer’s instructions (Life
Technologies, Grand Island, NY). Briefly, cells were grown to 70% confluency then switched to antibiotic-free DMEM/12 + 10% FBS before siRNA-Lipofectamine duplexes were introduced. The cells were also exposed to a non-targeting siRNA (Silencer® Select Negative Control #1; Ambion Inc., Foster City, CA) and Lipofectamine™ alone as negative controls. Optimal siRNA and Lipofectamine concentrations were established and validated using an In-Cell Western assay according to the manufacturer’s instructions described briefly below (Cell Signaling Technology, Danvers, MA; LI-COR®, Lincoln, NE).

Validation of K8/K18 knockdown (In-Cell Western Assay)

After 72 hours of transfection, the siRNA duplexes were removed from the culture medium, the cells were washed and then fixed and permeabilized for 10 minutes in ice-cold 100% methanol. Following permeabilization, the cells were washed three times in PBS, then exposed to blocking for one hour at room temperature (Blocking Buffer: PBS + 5% normal goat serum + 0.3% Triton X-100). Subsequently, the cells were incubated overnight at 4°C with a primary antibody cocktail of mouse anti-human K18 (CY90; Sigma-Aldrich, St. Louis, MO) and rabbit anti-human β-Actin (13E5, Cell Signaling Technology, Danvers, MA) diluted 1:800 and 1:200, respectively, in antibody dilution buffer (PBS / 1% BSA / 0.3% Triton X-100), respectively. After washing three times for five minutes each in PBS, the granulosa cells were incubated for 1 hour at room temperature in the dark with a cocktail of fluorochrome-conjugated secondary antibodies (goat anti-mouse IgG H+L
DyLight 800 and goat anti-rabbit IgG H+L DyLight 680, Cell Signaling Technology, Danvers MA) diluted 1:2000 and 1:1000, respectively, in antibody dilution buffer. After washing three times for five minutes each, plates containing the cells were scanned on the LI-COR® Odyssey® Classic Infrared Imaging scanner (LI-COR, Lincoln, NE) at 680 and 800 nm in the infrared color spectrum. Staining intensity for K18 was normalized to staining intensity for β-actin using the provided scanning (LI-COR, Lincoln, NE) (protocol adapted from In-Cell Immunofluorescence protocol, Cell Signaling Technology, Danvers, MA). Expression of K18 (relative to β-actin) provided a method to normalize staining intensity across treatments to account for differences in seeding density, possibly as a result of cytotoxicity.

**Statistical analysis**

All experiments were repeated three to six times, using a fresh aliquot of cells (KGN) or bovine follicles for each experiment. Data were analyzed initially by one-way or two way analysis of variance (ANOVA), followed by a Tukey's post-test for multiple comparisons. Differences among means at a value of P<0.05 were considered statistically significant.
Results

K18 and β-Actin expressed in KGN cells

Immunofluorescence for K18 and β-actin indicated KGN cells express abundant K8/K18 and β-actin filaments (Figure 4).

Figure 4 Representative image of immunofluorescent staining in cultured KGN cells. (A) DAPI, (B) K18 filaments stained with FITC (green), (C) β-Actin stained with Phalloidin (red) and (D) merged image (200X, bars represent 20 microns).
**High K18 expression but low Fas surface expression on KGN Cells**

Flow cytometry analysis confirmed ~91% of the KGN cells express K18 protein, yet only 24% of the cells express Fas on the cell surface (Figure 5). Mean fluorescent intensity measures (an indication of fluorescence per cell) were similarly high for K18 expression, but low for Fas expression (Fig. 5D).

![Figure 5](image1.png)

**Figure 5** Representative flow cytometric analysis of K18 and surface Fas staining in cultured KGN cells (A,B). Percentage of cell population K18+ and surface Fas+ (C) and mean fluorescence intensity (D) in KGN cells. Representative histograms and dot plots of fluorescence intensity vs. number of recorded events for (A) K18 and (B) surface Fas expression. (C) Percentage (%) of KGN cell population (±SEM) stained positively for K18 and surface Fas. (D) Mean fluorescence intensity (±SEM) of K18 and surface Fas staining in KGN cells. (n=3 experiments).
Inhibition of protein synthesis sensitizes KGN cells to Fas-induced apoptosis

KGN cells exposed to the protein synthesis inhibitor, CHX, prior to treatment with the Fas activating antibody, CH11, augmented Fas-induced apoptosis (Figure 6). Treatment with CH11 or CHX alone, however, had no effect. Inhibition of de novo protein synthesis with CHX provoked a 10-fold increase in Fas-induced apoptosis, similar to the chemotherapeutic agent Staurosporine.

Figure 6  Fas-mediated apoptosis in cultured KGN cells, quantified by caspase 3/7 activity (RLU; ±SEM) following protein synthesis inhibition. The cultures were exposed to protein synthesis inhibitor CHX (0.25 µg/mL) for 10 hours and Fas (1 µg/mL activating antibody CH11) for a period of 8 hours (n=6 experiments; different letters denote differences, p< 0.05).
Fas and cFLIP expression following Fas-induced apoptosis of KGN cells

No overt changes in the expression of Fas or cFLIP protein were observed following exposure of cultured KGN cells to CHX or CH11 (Fas activating antibody) (Figure 7A). Conversely, Fas-induced apoptosis by these treatments was confirmed by detection of PARP and cleaved PARP (Figures 7A &B). Confirming the Caspase-Glo 3/7 assay results above, the combined treatment of CHX+CH11 caused a 2-fold increase in cleaved PARP compared to the control cultures.

Figure 7 Immunodetection of Fas, cFLIP, cleaved PARP and β-Actin following exposure to the protein synthesis inhibitor CHX (0.25 μg/mL) and Fas stimulation (1 μg/mL activating antibody CH11) in KGN cells. A. Representative immunodetection of Fas, cFLIP, cleaved PARP and β-Actin following treatments. B. Average expression of Fas, cFLIP and cleaved PARP (±SEM) relative to β-Actin following treatment (n=3 experiments, different letters denote differences, p<0.05).
Inhibition of ERK1/2 and Akt fails to sensitize KGN cells to Fas-induced apoptosis

Inhibition of the MAPK pathway had no effect on Fas-induced apoptosis (Figure 8). Conversely, inhibition of the PI3K pathway provided modest protection from Fas-induced apoptosis (Figure 9).

![Figure 8](image1.png)

**Figure 8** Induction of Fas-mediated apoptosis in cultured KGN cells, quantified by caspase 3/7 activity (RLU; ±SEM) following ERK1/2 inhibition. The cultured cells were exposed to ERK1/2 inhibitor PD98059 (30 µM) and CHX for 10 hours and Fas activating antibody for 8 hours (n=3 experiments; different letters denote differences, P<0.05).

![Figure 9](image2.png)

**Figure 9** Induction of Fas-mediated apoptosis in cultured KGN cells, quantified by metabolic activity (MTS assay; ±SEM). The cultured cells were exposed to Akt inhibitor Wortmannin (100 nM) and CHX for 26 hours and Fas activating antibody for 24 hours (n=3 experiments; different letters denote differences among treatments, apostrophes denote differences between without and with Wortmannin within treatment groups; P<0.05).
Genetic downregulation of K8/K18 filaments (siRNA) enhances Fas-induced apoptosis of granulosa cells

For the KGN cells, optimized treatment conditions after 72 hours of transfection were 10 pmol KRT8 and KRT18 siRNA combined with 0.3 μL Lipofectamine RNAiMAX (100 nM siRNA duplex) (Figure 10A), which resulted in 30% downregulation of K18 expression compared negative controls (Figure 10B). For primary cultures of bovine granulosa cells, optimal transfection conditions consisted of 6 pmol KRT18 siRNA combined with 0.3 μL Lipofectamine RNAiMAX (60 nM siRNA duplex), which reduced K18 expression by 65% expression (Figure 10B).

![Figure 10 Immunodetection of K18 expression in granulosa cells mock transfected or transfected with 100 nM (KGN cells) or 60 nM (bovine granulosa cells) siRNA to KRT8 and18. A. Representative In-Cell Western dual detection of K18 protein (green) and β-Actin protein (red) following KRT8/18 siRNA. Negative controls consisted of cells transfected with equimolar concentrations of a non-targeting siRNA or Lipofectamine™ alone B. Quantification of K18 relative to β-actin expression following KRT8/18-siRNA transfection. Apostrophes denotes differences within cell types (P<0.05; KGN, n=3 experiments; bGC, n=1 experiment).
Genetic knock-down of the K8/K18 filaments with siRNA enhanced Fas-induced apoptosis compared to negative control (i.e. scrambler siRNA) in KGN cells (Figure 11).

Figure 11 Induction of Fas-mediated apoptosis measured by caspase 3/7 activity (RLU; ±SEM) following siRNA transfection with a negative control siRNA and KRT8 and 18 siRNA in cultured KGN cells (n=3 experiments; different letters denote differences, P< 0.05).
Discussion

During follicular atresia, discrete populations of granulosa cells within the follicle undergo apoptosis. However, the mechanisms regulating the cell-specificity of this process are unclear (Tilly et al., 1991). Previously, we suggested the prevalence and the cell-specificity of K8/K18 filaments within the granulosa cells might influence their vulnerability to apoptosis (Townson et al., 2010). Here, we demonstrated, for the first time, that granulosa cells are indeed more sensitive to Fas-induced apoptosis when K8/K18 filament expression is impaired. The reduction of K8/K18 filament expression in the granulosa cells was achieved using siRNA transfection, but similar effects have been observed in other types of non-ovarian epithelial cells in knockout mouse models or immortal cell lines (Caulin et al., 2000; Gilbert et al., 2001; Gilbert et al., 2004; Sullivan et al., 2010). In the K8 knockout mouse, for example, hepatocytes lacking K8/K18 filaments express more Fas on the cell surface, but also express less cFLIP, ERK1/2 and Akt intracellularly, which make them two times more sensitive to Fas-induced apoptosis than hepatocytes from wild-type mice. Essentially the K8/K18 filaments are thought to orchestrate the fate of epithelial cells by suppressing death signaling, while enhancing survival signals (Gilbert et al., 2001; Gilbert et al., 2004). The K8/K18 filaments within granulosa cells of follicles might similarly dictate cellular fate, possibly by enabling the cells to evade Fas-induced apoptosis through similar mechanisms.
The objectives of the current study were to determine the expression of K8/K18 filaments in granulosa cells, identify possible protective mechanisms by which granulosa cells resist apoptosis, and determine if K8/K18 filaments have a role in such protection. The results of this study support the concept that K8/K18 filaments influence granulosa cell fate, but whether they augment cFLIP expression and/or downstream survival signaling remains unclear. Both KGN cells and primary cultures of bovine granulosa cells abundantly express K8/K18 filaments, and this abundance of filament expression was accompanied by a corresponding lack of Fas expression, at least in KGN cells. Whether or not these factors alone prevented Fas-induced apoptosis is uncertain, but our overall observations confirm those of previous studies. Namely, others have shown that cultured granulosa cells are extremely resistant to Fas-induced apoptosis (Quirk et al., 1998; Mezzanzanica et al., 2004; Quirk et al., 2004; Matsuda et al., 2008). The current study also confirmed reports of others (Quirk et al., 1998; Matsuda et al., 2008) that cultured granulosa cells express labile protein(s), which in part provide protection against Fas-induced apoptosis. The cells are resistant to Fas-induced apoptosis unless pretreated with a protein synthesis inhibitor such as CHX (Quirk et al., 1998), or exposed to cytokines in combination with Fas ligand, such as TNF and IFN (Quirk et al., 1998; Porter et al., 2001). In the current work in which CHX augmented Fas-induced apoptosis, there was no effect of CHX on the anti-apoptotic protein, cFLIP, or the expression of total Fas as detected by immunoblot analysis. This implies that other labile
survival protein(s), besides cFLIP and Fas, are expressed by granulosa cells to protect them from Fas-induced apoptosis.

Using KGN cells, we explored the possibility that signaling through MAPK and PI3K pathways (growth and stress activated pathways, respectively) might prevent Fas-induced apoptosis. Inhibition of the MAPK pathway, using the ERK1/2 inhibitor, had no clear effect on Fas-induced apoptosis. Surprisingly, inhibition of the PI3K pathway, using the Akt inhibitor, Wortmannin, actually enhanced the metabolic activity of the KGN cells, suggesting that although this pathway had no effect on Fas-induced apoptosis, other pathways affecting cellular metabolism become activated in response to Akt inhibition. Nevertheless, granulosa cell susceptibility to Fas-induced apoptosis is evidently not governed by the ERK1/2 or Akt pathways.

To determine overall whether K8/K18 filaments protect granulosa cells from Fas-induced apoptosis, the expression of the filaments was experimentally impaired using siRNA constructs. Knockdown of K8/K18 expression was evident in both KGN cells and primary cultures of bovine granulosa cells, although the KGN cells required a higher concentration of siRNA to achieve measurable knockdown. These cells also appeared to be more tolerant of siRNA transfection than the primary cultures of bovine granulosa cells. For the bovine granulosa cells, the In-Cell Western assays revealed cytotoxicity effects, evident from lower cell density following KRT18 siRNA transfection compared to those cells transfected with the non-targeting (i.e. scrambler) siRNA. Basal caspase activity (a measure of non-targeting
siRNA-induced apoptosis) was also higher in the primary cultures of granulosa cells compared to KGN cell cultures. However, both the KGN cells and bovine granulosa cells became vulnerable to Fas-induced apoptosis following siRNA knockdown, providing the first evidence that K8/K18 filaments influence granulosa cell resistance to apoptosis. The cellular mechanisms responsible for this protection (i.e., Fas reception and trafficking, activation of intracellular survival signals, etc.), however, remain unknown.

In conclusion, the abundance of K8/K18 intermediate filaments in granulosa cells provide a plausible cellular mechanism to prevent Fas-induced apoptosis, possibly by impairing Fas trafficking and/or activating downstream, intracellular survival signals. Here, we have provided evidence that labile protein(s), potentially associated with the K8/K18 filaments, provide resistance to apoptosis, while other downstream protective mechanisms await additional investigation. Similar mechanisms have been observed in epithelial cells of non-reproductive origin, notably hepatocytes (Gilbert et al., 2001; Gilbert et al., 2004) and HeLa cells (Sullivan et al., 2010), suggesting the influence of cytoskeletal structure on Fas-induced apoptosis may be more universal than previously thought. In the context of the current work, the existence of K8/K18 filaments within granulosa cells of ovarian follicles have relevance to aspects of follicular atresia and the selection of follicles for ovulation. These insights have some bearing on therapeutic strategies that might enhance follicular growth and health within the ovary, thus leading to overall improvement in female fertility.
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University of New Hampshire
Research Integrity Services, Service Building
51 College Road, Durham, NH 03824-3585
Fax: 603-862-3564

02-Feb-2012

Townson, David H
Molecular, Cellular & Biomedical Sciences, Kendall Hall
Durham, NH 03824

IACUC #: 120104
Project: Control of Oxidative Stress During Bovine Folliculogenesis
Category: D
Approval Date: 01-Feb-2012

The Institutional Animal Care and Use Committee (IACUC) reviewed and approved the protocol submitted for this study under Category D on Page 5 of the Application for Review of Vertebrate Animal Use in Research or Instruction - Animal use activities that involve accompanying pain or distress to the animals for which appropriate anesthetic, analgesic, tranquilizing drugs or other methods for relieving pain or distress are used. The IACUC made the following comment(s) on this protocol:

1. The researcher cannot use any animals that have previously undergone major surgery (e.g., cannulation).
2. In Section V, B of the application, the IACUC changed the drug to butorphanol, the dose to 20 mg per cow, and the route to IV.

Approval is granted for a period of three years from the approval date above. Continued approval throughout the three year period is contingent upon completion of annual reports on the use of animals. At the end of the three year approval period you may submit a new application and request for extension to continue this project. Requests for extension must be filed prior to the expiration of the original approval.

Please Note:
1. All cage, pen, or other animal identification records must include your IACUC # listed above.
2. Use of animals in research and instruction is approved contingent upon participation in the UNH Occupational Health Program for persons handling animals. Participation is mandatory for all principal investigators and their affiliated personnel, employees of the University and students alike. A Medical History Questionnaire accompanies this approval; please copy and distribute to all listed project staff who have not completed this form already. Completed questionnaires should be sent to Dr. Gladi Porsche, UNH Health Services.

If you have any questions, please contact either Dean Elder at 862-4629 or Julie Simpson at 862-2003.

For the IACUC,

Robert C. Drugan, Ph.D.
Chair

cc: File