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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

Submitted to the University of New Hampshire
In Partial Fulfillment of
The Requirement for the Degree of

Master of Science
In
Animal Science

December, 2012

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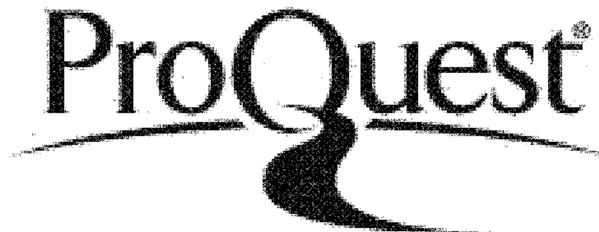

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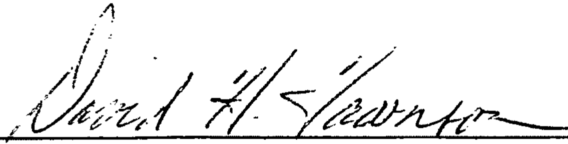

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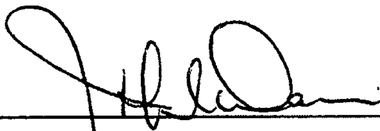

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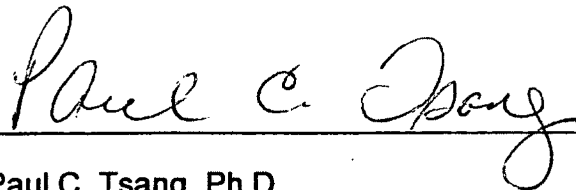

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS....iii

LIST OF FIGURES....vi

ABSTRACT....viii

CHAPTER PAGE

INTRODUCTION....1

Implications of Dairy Infertility....1

Negative Energy Balance and Infertility....1

The Ovary....2

The Estrous Cycle....6

Follicular Atresia and Apoptosis....8

Survival Signals....9

Death Signals (Apoptosis)....10

Survival Signaling Pathways....13

Fas and the Follicle....16

Keratin Intermediate Filaments....18

K8/K18 Filaments and Granulosa Cells....22

Objectives and Hypotheses....23

I. INVESTIGATION OF THE ROLE OF KERATIN 8/18 INTERMEDIATE FILAMENTS ON FATE OF GRANULOSA CELLS....24

Introduction....24

Materials and Methods....29

Results..37

Discussion..44

LITERATURE CITED...48

APPENDIX...67

Institutional Care and Use Committee Approval...................................68

LIST OF FIGURES

FIGURE PAGE

Figure 1. Representative diagram of ovarian follicle structure and morphology....4

Figure 2. Follicle Classification (Myers et al., 2004)....5

Figure 3. Tripartite structure of intermediate filaments (Coulombe and Omary, 2002)....19

Figure 4. Representative images of immunofluorescent staining in cultured KGN cells....37

Figure 5. Representative flow cytometry analysis of K18 and surface Fas staining in cultured KGN cells....38

Figure 6. Fas-mediated apoptosis in cultured KGN cells, quantified by Caspase 3/7 activity following protein synthesis inhibition....39

Figure 7. Immunodetection of Fas, cFLIP, cleaved PARP and β-Actin following exposure to the protein synthesis inhibitor CHX and Fas stimulation in KGN cells....40

Figure 8. Induction of Fas-mediated apoptosis in cultured KGN cells, quantified by caspase 3/7 activity following ERK1/2 inhibition....41

Figure 9. Induction of Fas-mediated apoptosis in cultured KGN cells, quantified by metabolic activity (MTS assay) following Akt inhibition....41

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 and KRT 18....42

Figure 11. Induction of Fas-mediated apoptosis measured by caspase 3/7 activity following siRNA transfection with a negative control siRNA and KRT8 and 18 siRNA in cultured KGN cells..43

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).

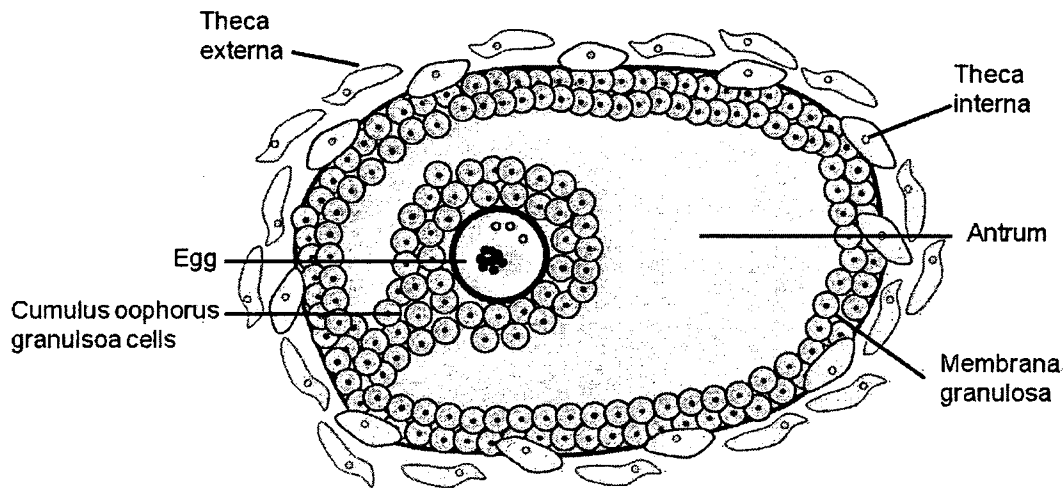

Figure 1 Representative diagram of ovarian follicle structure and morphology.

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).

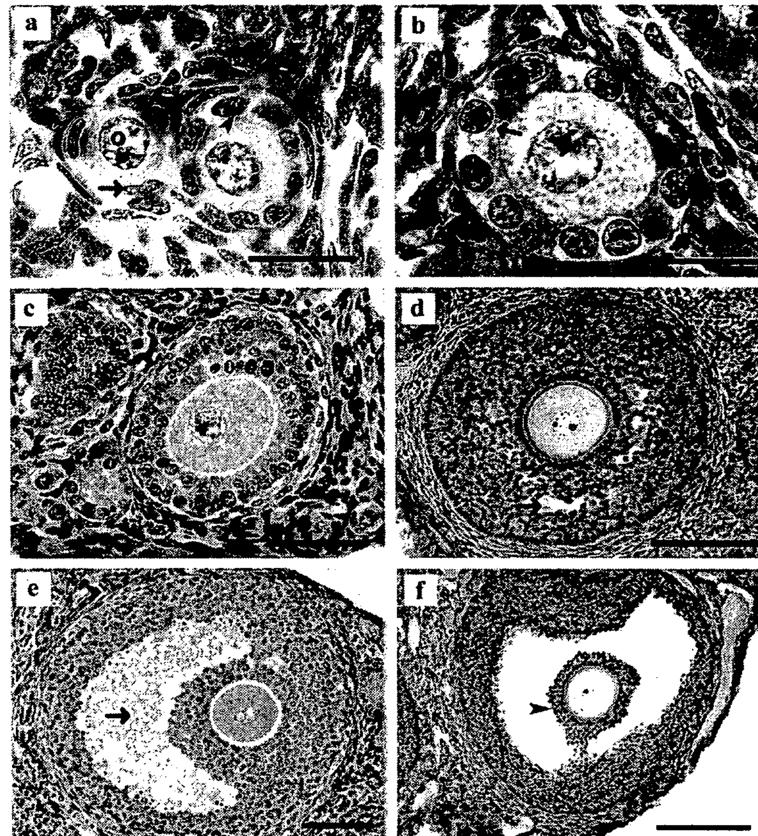

Figure2 Follicle Classification. **(a)** primordial, **(b)** primary, **(c)** secondary, **(d)** early tertiary, **(e)** tertiary, and **(f)** late tertiary follicles. Bar = 20µm. (Myers et al., 2004)

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, wé 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 seceretion 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).

<u>Intrinsic Pathway</u>

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., 19992; 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; Oztürk 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 stimulate second messenger phosphoinosotides by phosphorylation (i.e. converting phosphatidylinosotide-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

al., 2003; Inoue et al., 2006; Kim et al., 1998; Quirk et al., 1995; Quirk et al., 1998; Vickers et al., 2000). 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 sensitve 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).

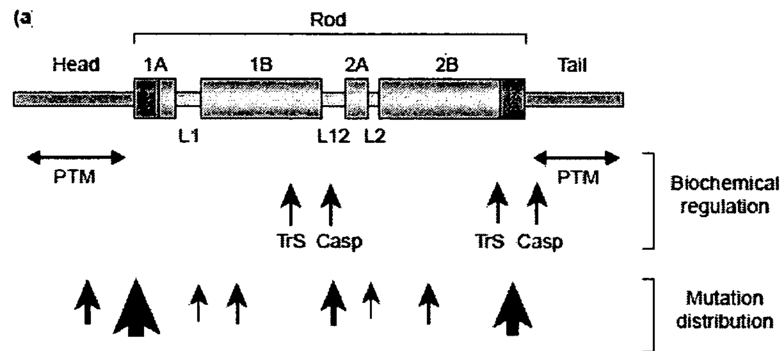

Figure 3 Tripartite structure of intermediate filaments (Coulombe and Omary 2002).

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% CO_2 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% CO_2 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 $1x10^4$ cells/well for Caspase-Glo 3/7, CellTiter 96 and In-Cell Western assays, seeded in 6-well plates (Corning, Corning, NY) at $3x10^5$ cells/well for immunoblotting experiments or seeded in microchamber slides (Nunc, Rochester, NY) at $1x10^4$ cells/well and T75 or T150 vented flasks (BD Biosciences, San Jose, CA) at $5x10^5$ cells and $1x10^6$ 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 MgSO4-7H_2O; 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 MgSO4-7H_2O; 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-Alrich, 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

Technoloies, 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 Technolgy, 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 Immunofluoresence 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).

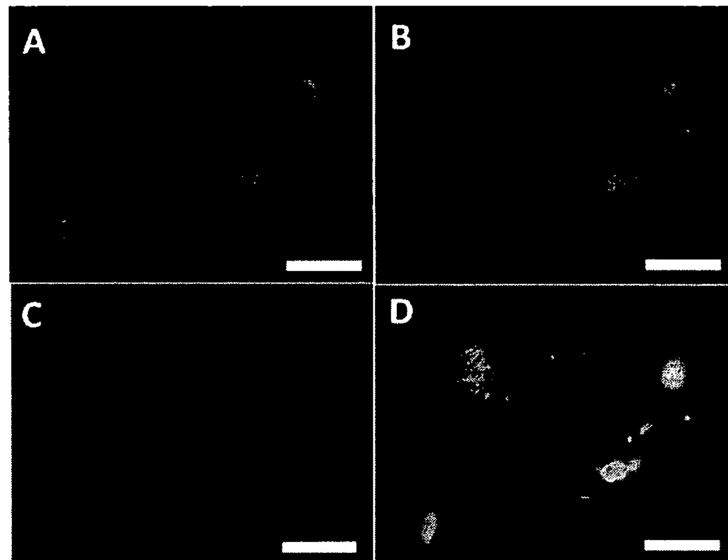

Figure 4 Representative image of immunofluorescent staining in cultured KGN cells. **(A)** DAPI, **(B)** K18 filaments stained with FITC (green), **(C)** β-Actin stained with Phalloidin (red) and **(D)** merged image (200X, bars represent 20 microns).

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).

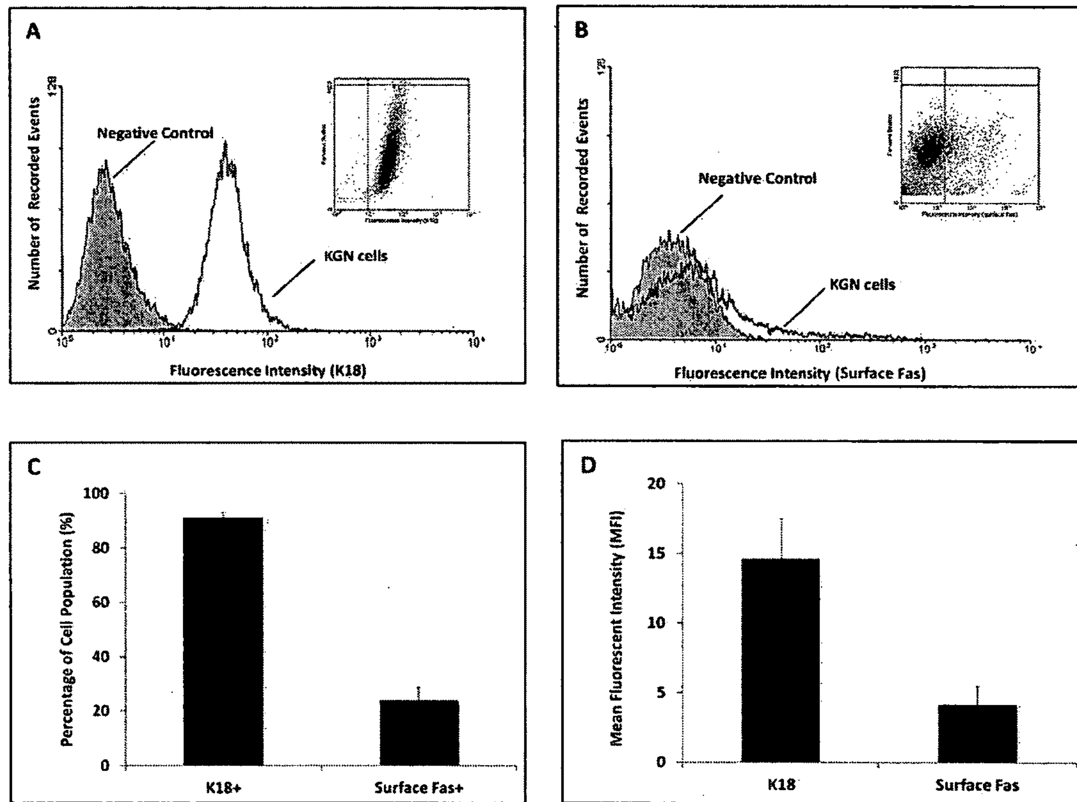

Figure 5 Representative flow cytometric analysis of K18 and surface Fas staining in cultured KGN cells **(A,B)**, Percentage of cell population K18+ and surface Fas+ **(C)** and mean fluorescence intensity **(D)** in KGN cells. Representative histograms and dot plots of fluorescence intensity vs. number 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.

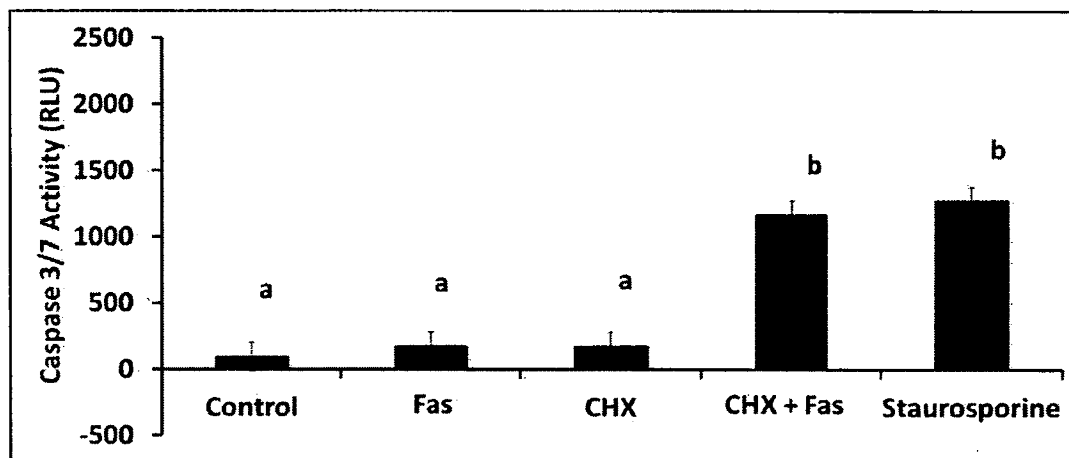

Figure 6 Fas-mediated apoptosis in cultured KGN cells, quantified by caspase 3/7 activity (RLU; ±SEM) following protein synthesis inhibition. The cultures were exposed to protein synthesis inhibitor CHX (0.25 µg/mL) for 10 hours and Fas (1 µg/mL activating antibody CH11) for a period of 8 hours (n=6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

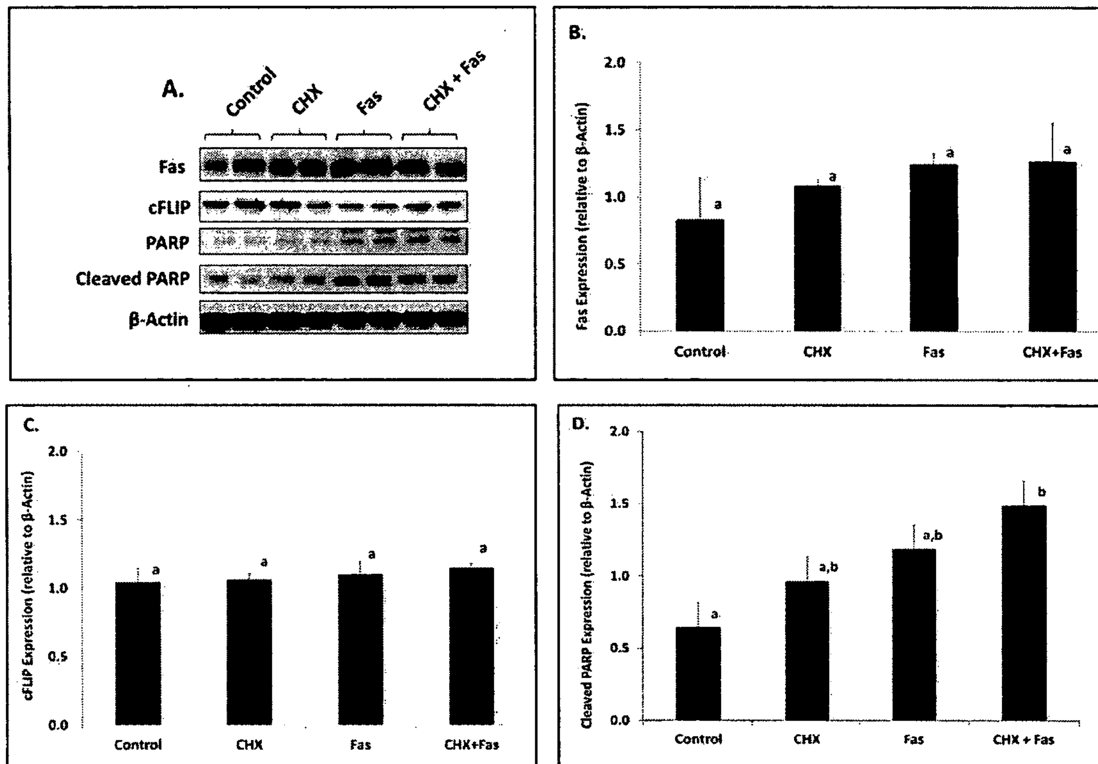

Figure 7 Immunodetection of Fas, cFLIP, cleaved PARP and β-Actin following exposure to the protein synthesis inhibitor CHX (0.25 µg/mL) and Fas stimulation (1 µg/mL activating antibody CH11) in KGN cells. **A.** Representative immunodetection of Fas, cFLIP, cleaved PARP and β-Actin following treatments. **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).

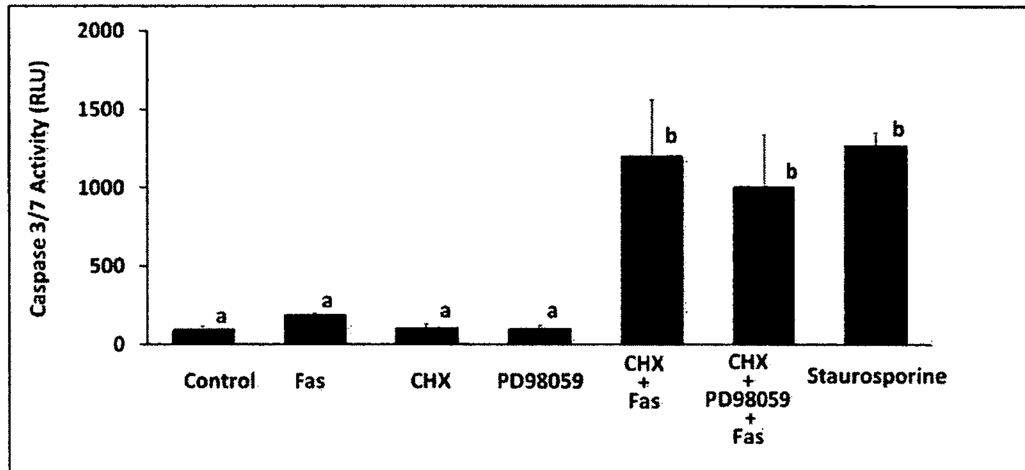

Figure 8 Induction of Fas-mediated apoptosis in cultured KGN cells, quantified by caspase 3/7 activity (RLU; ±SEM) following ERK1/2 inhibition. The cultured cells were exposed to ERK1/2 inhibitor PD98059 (30 μM) and CHX for 10 hours and Fas activating antibody for 8 hours (n=3 experiments; different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

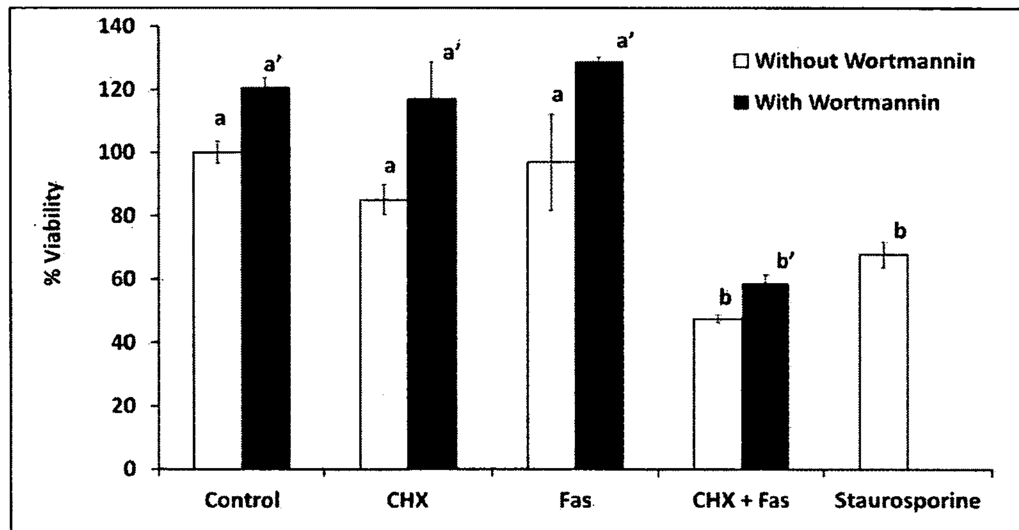

Figure 9 Induction of Fas-mediated apoptosis in cultured KGN cells, quantified by metabolic activity (MTS assay; ±SEM). The cultured cells were exposed to Akt inhibitor Wortmannin (100 nM) and CHX for 26 hours and Fas activating antibody for 24 hours (n=3 experiments; different letters denote differences 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).

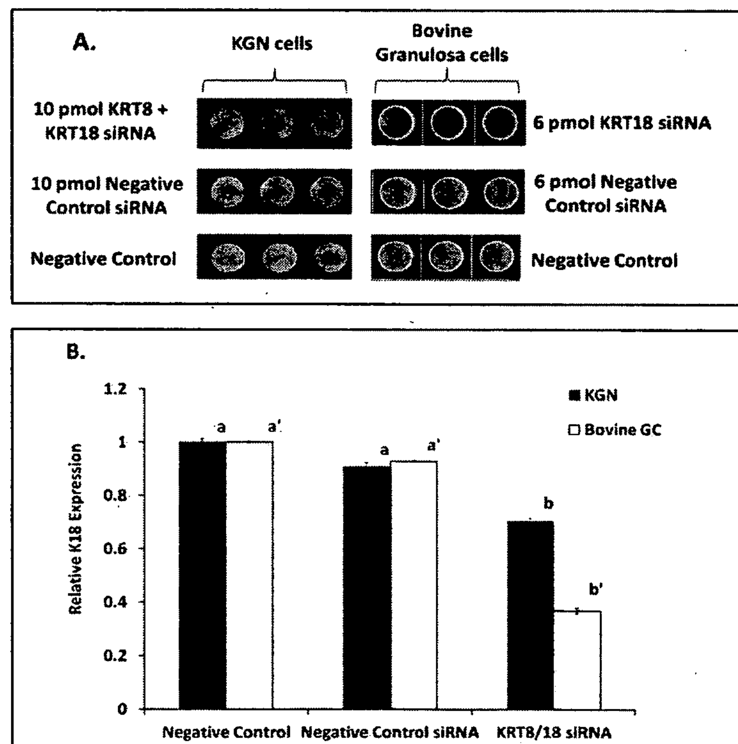

Figure 10 Immunodetection of K18 expression in granulosa cells mock transfected or transfected with 100 nM (KGN cells) or 60 nM (bovine granulosa cells) siRNA to KRT8 and18. **A.** Representative In-Cell Western dual detection of K18 protein (green) and β-Actin protein (red) following KRT8/18 siRNA. 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).

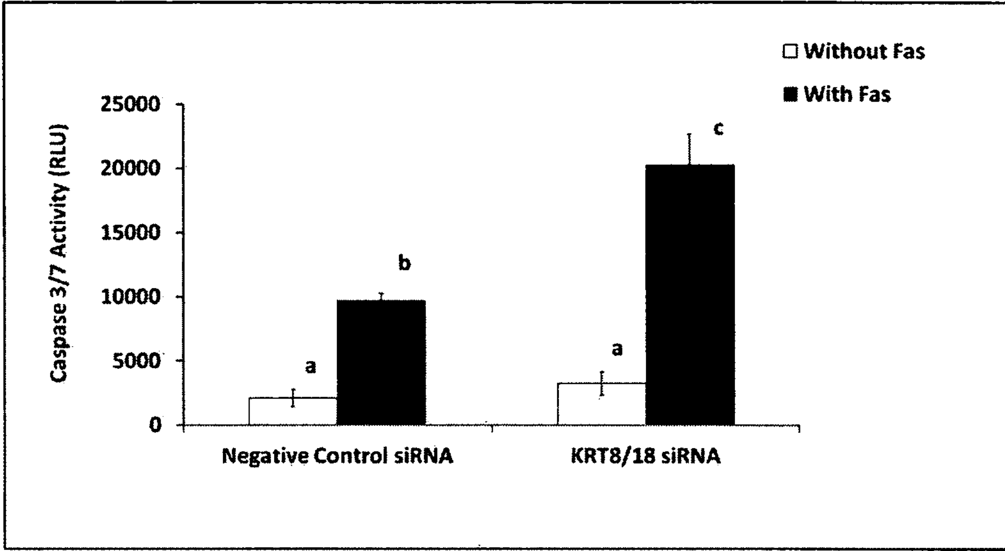

Figure 11 Induction of Fas-mediated apoptosis measured by caspase 3/7 activity (RLU; ±SEM) following siRNA transfection with a negative control siRNA and KRT8 and 18 siRNA in cultured KGN cells (n=3 experiments; different letters denote differences, $P < 0.05$).

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.

LITERATURE CITED

1. Adashi EY, Resnick CE, Brodie AM, Svoboda ME and Van Wyk JJ. Somatomedin-C-mediated potentiation of follicle-stimulating hormone-induced aromatase activity of cultured rat granulosa cells. Endocrinology 1985;117(6):2313-2320.

2. Aerts JMJ and Bols PEJ. Ovarian follicular dynamics: A review with emphasis on the bovine species. Part I: folliculogenesis and pre-antral follicle development. Reprod Dom Anim 2010;45:171-179.

3. Alberts B, Johnson A, Lewis J, et al. Molecular Biology of the Cell. 4th edition. New York: Garland Science; 2002. Programmed Cell Death (Apoptosis).

4. Alila HW and Hansel W. Origin of different cell types in the bovine corpus luteum as characterized by specific monoclonal antibodies. Biol Reprod 1984;31(5):1015-1025.

5. Amsterdam A and Rotmensch S. Structure-function relationships during granulosa cell differentiation. Endocr Rev 1987;8(3):309-337.

6. Amsterdam A and Selvaraj N. Control of differentiation, transformation, and apoptosis in granulosa cells by oncogenes, oncoviruses, and tumor suppressor genes. Endocr Rev 1997;18(4):435-461.

7. Amsterdam A, Gold R, Hosokawa K, Yoshida Y, Sasson R, Jung Y and Kotsuji F. Crosstalk among multiple signaling pathways controlling ovarian cell death. Trends Endocrinol Metab 1999;10:255-262.

8. Amsterdam A, Sasson R, Keren-Tal I, Aharoni D, Dantes A, Rimon E, Land A, Cohen T, Dor Y and Hirsh L. Alternative pathways of ovarian apoptosis: death for life. Biochem Pharm 2003;66:1355-1362.

9. Antonsson B and Martinou JC. The Bcl-2 protein family. Exp Cell Res 2000;256(1):50-57.

10. Baker J, Hardy MP, Zhou J, Bondy C, Lupu F, Bellve AR and Efstratiadis A. Effects of an Igf1 gene null mutation on mouse reproduction. Mol Endocrinol 1996;10:903-918.

11. Barnhart BC, Alappat EC and Peters ME. The CD95 type 1/type II model. Semin Immunol 2003;15(3):185-193.

12. Beam SW and Butler WR. Energy balance and ovarian follicular development prior to the first ovulation postpartum in dairy cows receiving three levels of dietary fat. Biol Reprod 1997;56:133-142.

13. Braw RH and Tsafriri A. Effect of PMSG on follicular atresia in the mmature rat ovary. J Reprod Fertil 1980;59(2):267-272.

14. Braw RH, Bar-Ami S and Tsafriri A. Effect of hypophysectomy on atresia of rat preovulatory follicles. Biol Reprod 1981;25:989-996.

15. Britt KL, Drummond AE, Cox VA, Dyson M, Wreford NG, Jones MEE, Simpson ER and Findlay JK. An age-related ovarian phenotype in mice with targeted disruption of Cyp 19 (aromatase) gene. Endocrinology 2000;141:2614-2623.

16. Butler WR and Smith RD. Interrelationships between energy balance and postpartum reproductive function in dairy cattle. J Dairy Sci 1989;72:767-783.

17. Butler WR. Effect of protein nutrition on ovarian and uterine physiology in dairy cattle. J Dairy Sci 1998;81:2533-2539.

18. Butler WR. Nutritional interactions with reproductive performance in dairy cattle. Anim Reprod Sci 2000;60-61:449-457.

19. Cantley LW. The phosphoinositide 3-kinase pathway. Science 2002;296:1655-1657.

20. Caulin C, Ware CE, Magin TM and Oshima RG. Keratin-dependent, epithelial resistance to tumor necrosis factor-induced apoptosis. J Cell Biol 2000;149(1):17-22.

21. Chang L and Karin M. Mammalina MAP kinase signaling cascades. Nature 2001;410:37-40.

22. Chen TT, Massey PJ and Caudle MR. The inhibitory action of taxol on granulosa cell steroidogenesis is reversible. Endocrinology 1994;134(5):2178-2183.

23. Chun SY, Billig H, Tilly JL, Furuta I, Tsafriri A and Hsueh AJ. Gonadotropin suppression of apoptosis in cultured preovulatory follicles: mediatory role of endogenous insulin-like growth factor I. Endocrinology 1994;135(5):1845-1853.

24. Chun SY, Eisenhauer KM, Kubo M and Hsueh AJW. Interleukin-1-beta suppresses apoptosis in rat ovarian follicles by increasing nitric-oxide production. Endocrinology 1995;135:3120-3127.

25. Coulombe PA and Omary MB. 'Hard' and 'soft' principles defining the structure, function and regulation of keratin intermediate filaments. Curr Opin Cell Biol 2002;14:110-122.

26. Czernobilsky B, Moll R, Levy R and Franke WW. Co-expression of cytokeratin and vimentin filaments in mesothelial, granulosa and rete ovarii cells of the human ovary. Eur J Cell Biol 1985;37:175-190.

27. Datta SR, Brunet A and Greenberg ME. Cellular survival: a play of three Akts. Genes Dev 1999;13:2905-2927.

28. Davis JS and Rueda BR. The corpus luteum: an ovarian structure with maternal instincts and suicidal tendencies. Front Biosci 2002;7:1949-1978.

29. Dharma SJ, Kelkar RL and Nandedkar TD. Fas and Fas ligand protein and mRNA in normal and atretic mouse ovarian follicles. Reproduction 2003;126(6):783-789.

30. Dobson H, Smith RF, Royal MD, Knight CH and Sheldon IM. The high-producing dairy cow and its reproductive performance. Reprod Dom Anim Suppl 2007;42:17-23.

31. Du C, Fang M, Li Y, Li L and Wang X. Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. Cell 2000;102(1):33-42.

32. Duncan A, Forcina J, Birt A and Townson D. Estrous cycle-dependent changes of Fas expression in the bovine corpus luteum: influence of keratin 8/18 intermediate filaments and cytokines. Reprod Biol Endocrinol 2012:10(1):90.

33. Dupont S, Krust A, Gansmuller A, Dierich A, Chambon P and Mark M. Effect of single and compound knockouts of estrogen receptors alpha (ER alpha) and beta (ER beta) on mouse reproductive phenotypes. Development 2000;127:4277-4291.

34. Erickson BH. Development and senescence of the postnatal bovine ovary. Anim Sci 1966;25:800–805.

35. Eriksson JE, Dechat T, Grin B, Helfand B, Mendez M, Pallari HM and Goldman RD. Introducing intermediate filaments: from discovery to disease. J Clin Invest 2009;119(7):1763-1771.

36. Fair T, Hulshof SC, Hyttel P, Greve T and Boland M. Nuclear ultrastructure and transcriptional activity of bovine oocytes in preantral and early antral follicles. Mol Reprod Dev 1997;46(2):208-215.

37. Fayard E, Tintignac LA, Baudry A and Hemmings BA. Protein kinase B/Akt at a glance. Cell Sci 2005;118:5675-5678.

38. Fernandes-Alnemri T and Takahasi A, Armstrong R, Krebs J, Fritz L, Tomaselli KJ, Wang L, Yu Z, Croce CM, Salveson G. Mch3, a novel human apoptotic cysteine protease highly related to CPP32. Cancer Res 1995;55(24):6045-6052.

39. Fey D, Croucher DR, Kolch W and Kholodenko BN. Crosstalk and signaling switches in mitogen-activated protein kinase cascades. Front Physiol 2012;3:355.

40. Fortune JE, Sirois J, Turzillo AM and Lavoir M. Follicle selection in domestic ruminants. J Reprod Fert Suppl 1991;43:187-198.

41. Fortune JE. Ovarian follicular growth and development in mammals. Biol Reprod 1994;50(2):225-232.

42. Fuchs E and Weber K. Intermediate filaments: Structure, dynamics, function, and disease. Annu Rev Biochem 1994;63:345-382.

43. Galarneau L, Loranger A, Gilbert S and Marceau N. Keratins modulate hepatic cell adhesion, size and G1/S transition. Exp Cell Res 2007;313(1):179-194.

44. Gall L. Intermediate filaments in oocytes. Bull Assoc Anat (Nancy) 1991;75(228):63-65.

45. Gao XB, Yao N, Ma X, Lu CL, Yang Bq and Peng XZ. Role of extracellular signal-regulated protein kinase 5 in the biosynthesis of follicle-stimulating hormone-stimulated progesterone in primary granulosa cells. Acta Academiae Medicinae Sinicae 2011;33(6):615-619.

46. Gilbert S, Loranger A and Marceau N. Keratins modulate c-Flip/extraceullar signal-regulated kinase 1 and 2 antiapoptotic signaling in simple epithelial cells. Mol Cell Biol 2004;24(16):7072-7081.

47. Gilbert S, Loranger A, Daigle N and Marceau N. Simple epithelium keratins 8 and 18 provide resistance to Fas-mediated apoptosis. The protection occurs through a receptor-targeting modulation. J Cell Biol 2001;154(4):763-773.

48. Ginther OJ, Wiltbank MC, Fricke PM, Gibbons JR and Kot K. Selection of the dominant follicle in cattle. Biol Reprod 1996;55:1187-1194.

49. Gougeon A and Chainy GBN. Morphometric studies of small follicles in ovaries of women at different ages. J Reprod Fert 1987;81:433-442.

50. Guthrie HD, Garrett WM and Cooper BS. Follicle-stimulating hormone and insulin-like growth factor-1 attenuate apoptosis in cultured porcine granulosa cells. Biol Reprod 1998;58:380-396.

51. Hengartner MO. The biochemistry of apoptosis. Nature 2000;47:770-776.

52. Hirshfield AN. Development of follicles in the mammalian ovary. Int Rev Cytol 1991;124:43-101.

53. Hogden GD. The dominant ovarian follicle. Fertil Steril 1982;38:281-300.

54. Hu S, Vincenz C, Ni J, Gentz R and Dixit VM. I-FLICE, a novel inhibitor of tumor necrosis factor receptor-1- and CD-95-induced apoptosis. J Biol Chem 1997;272(28):17255-17257.

55. Hughes FM and Gorospe WC. Biochemical identification of apoptosis (programmed cell death) in granulosa cells: evidence for a potential mechanism underlying follicular atresia. Endocrinology 1991;129(5):2415–2422.

56. Hunzicker-Dunn M and Maizels ET. FSH signaling pathways in immature granulosa cells that regulate target gene expression: Branching out from protein kinase A. Cell Signal 2006;18:1351-1359.

57. Inoue N, Maeda A, Matusda-Minehata F, Fukuta K and Manabe N. Expression and localization of Fas ligand and Fas during atresia in porcine ovarian follicles. J Reprod Dev 2006;52(6):723-730.

58. Inoue N, Manabe N, Satsui T, Maeda A, Nakagawa S, Wada S and Miyamoto H. Role of tumor necrosis factor-related apoptosis-inducing ligand signaling pathway in granulosa cell apoptosis during atresia in pig ovaries. J Reprod Dev 2003;49(4):313-321.

59. Ip YT and Davis RJ. Signal transduction by the c-Jun N-terminal kinase (JNK)—from inflammation to development. Curr Opin Cell Biol 1998;10(2):205-219.

60. Ireland JJ and Roche JF. Development of nonovulatory antral follicles in heifers: changes in steroids in follicular fluid and receptors for gonadotropins. Endocrinol 1983;112:150-156.

61. Ishikawa H, Bischoff R and Holtzer H. Mitosis and intermediate-sized filaments in developing skeletal muscle. J Cell Biol 1968;38(3):538-555.

62. Jackson BW, Grund C, Winter S, Franke WW and Illmensee K. Epithelial differentiation and intermediate-sized filaments in early postimplantation embryos. Differentiation 1981;20(1-3):203-216.

63. Jarpe MB, Widmann C, Knall C, Schlesinger TK, Gibson S, Yujiri T, Fanger GR, Gelfand EW and Johnson GL. Anti-apoptotic versus pro-apoptotic signal transduction: checkpoints and stop signs along the road to death. Oncogene 1998;17:1475-1482.

64. Kacinskis MA, Lucci CM, Luque MCA and Báyo SN. Morphometric and ultrastructural characterization of *Bos indicus* preantral follicles. Anim Reprod Sci 2005;87:45-57.

65. Kanamaru Y, Sekine S, Ichijo H and Takeda K. The phosphorylation-dependent regulation of mitochondrial proteins in stress responses. J Signal Transduct 2012;2012:931215.

66. Kasa-Vuvu JZ, Dahl GE, Thrun LA, Moenter SM, Padmanabhan V and Karsch FJ. Progesterone blocks the estradiol-induced gonadotropin discharge in the ewe by inhibiting the surge of gonadotropin-releasing hormone. Endocrinol 1992;131(1):208-212.

67. Kerr JFR, Wyllie AH and Currie AR. Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 1972;26:239-257.

68. Khan-Dawood FS, Yusoff Dawood M and Tabibzadeh S. Immunohistochemical analysis of the microanatomy of primate ovary. Biol Reprod 1996;54(3):734-742.

69. Kim JM, Boone DL, Auyeung A and Tsang BK. Granulosa cell apoptosis induced at the penultimate stage of follicular

development is associated with increased level of Fas and Fas ligand in the rat ovary. Biol Reprod 1998;58:1170-1176.

70. Kim S and Coulombe PA. Intermediate filament scaffolds fulfill mechanical, organizational, and signaling functions in the cytoplasm. Genes Dev 2007;21(13):1581-1597.

71. Ku NO and Omary MB. A disease- and phosphorylation-related nonmechanical function for keratin 8. J Cell Biol 2006;174(1):115-125.

72. Ku NO and Omary MB. Effect of mutation and phosphorylation of type I keratins on their caspase-mediated degredation. J Biol Chem 2001;276(29):26792-26798.

73. Ku NO and Omary MB. Phosphorylation of human keratin 8 in vivo at conserved head domain serine 23 and at epidermal growth factor-stimulated tail domain serine 431. J Biol Chem 1997;272(11):7556-7564.

74. Ku NO, Azhar S and Omary MB. Keratin 8 phosphorylation by p38 kinase regulates cellular keratin filament reorganization: modulation by a keratin 1-like disease causing mutation. J Biol Chem 2002;277(13):10775-10782.

75. Ku NO, Michie S, Resurreccion EZ, Broome RL and Omary MB. Keratin binding to 14-3-3 proteins modulates keratin filaments and hepatocytes mitotic progression. Proc Natl Acad Sci USA 2002;99(7):4373-4378.

76. Ku NO, Soetikno RM and Omary MB. Keratin mutation in transgenic mice predisposes to Fas but not TNF-induced apoptosis and massive liver injury. Hepatology 2003;37(5):1006-1014.

77. Leifeld L, Kothe S, Söhl G, Hesse M, Sauerbrunch T, Magin TM and Spengler U. Keratin 18 provides resistance to Fas-mediated liver failure in mice. Eur J Clin Invest 2009;39(6):481-488.

78. Leroy JLMR, Opsomer G, Soom AV, Goovaerts IGF and Bols PEJ. Reduced fertility in high-yielding dairy cows: Are the oocyte and embryo in danger? Part I. Reprod Dom Anim 2008;43:612-622.

79. Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES and Wang X. Cytochrome c and dATP-dependent formation of Apaf-1/Caspase-9 complex initiates an apoptotic protease cascade. Cell 1997;91(4):479-489.

80. Li S, Zhao Y, He X, Kim TH, Kuharsky DK, Rabinowich H, Chen J, Du C and Yin XM. Relief of extrinsic pathway inhibition by the Bid-dependent mitochondrial release of Smac in Fas-mediated hepatocte apoptosis. J Biol Chem 2002;277(30):26912-26920.

81. Liao J and Omary MB. 14-3-3 proteins associate with phosphorylated simple epithelial keratins during cell cycle progression and act as a solubility cofactor. J Cell Biol 1996;133(2):345-357.

82. Löffler S, Horn LC, Weber W and Spanel-Borowski K. The transient disappearance of cytokeratin in human fetal and adult ovaries. Anat Embryol (Berl) 2000;201(3):207-215.

83. Lucy MC, Savio JD, Badinga L, De La Sota RL and Thatcher WW. Factors that affect ovarian follicular dynamics in cattle. J Anim Sci 1992;70:3615-3626.

84. Lucy MC. Reproductive loss in high-producing dairy cattle: where will it end? J Dairy Sci 2001;84:1277-1293.

85. Lucy MC. The bovine dominant ovarian follicle. J Anim Sci 2007;85:E89-99.

86. Lucy MC. The future of dairy reproductive management. Advances in Dairy Technology 2002;14:161-173.

87. Lynch K, Fernandez G, Pappalardo A and Peluso JJ. Basic fibroblast growth factor inhibits apoptosis of spontaneously immortalized granulosa cells by regulating intracellular free calcium

levels through a protein kinase C delta-dependent pathway. Endocrinology 2000;141:4209-4217.

88. Marceau N, Loranger A, Gilbert S, Daigle N and Champetier S. Keratin-mediated resistance to stress and apoptosis in simple epithelial cells in relation to health and disease. Biochem Cell Biol 2001;79:543-555.

89. Matsuda F, Inoue N, Goto Y, Maeda A, Cheng Y, Sakamaki K and Manabe N. cFLIP regulates death receptor-mediated apoptosis in an ovarian granulosa cell line by inhibiting procaspase-8 cleavage. J Reprod Dev 2008;54(5):314-320.

90. Matsuda F. Inoue N, Manabe N and Ohkura S. Follicular growth and atresia in mammalian ovaries: regulation by survival and death of granulosa cells. J Reprod Dev 2012;58(1):44-50.

91. Matsuda-Minehata F, Inoue N, Goto Y and Manabe N. The regulation of ovarian granulosa cell death by pro- and anti-apoptotic molecules. J Reprod Dev 2006;52:695-705.

92. McCracken JA, Custer EE and Lamsa JC. Luteolysis: a neuroendocrine-mediated event. Physiol Rev 1999;79(2):262-323.

93. Mezzanzanica D, Balladore E, Turatti F, Luison E, Alberti P, Bagnoli M, Figini M, Mazzoni A, Raspagliesi F, Oggionni M, Pilotti S and Canevari S. CD95-mediated apoptosis is impaired at receptor level by cellular FLICE-inhibitory protein (long form) in wild-type p53 human ovarian carcinoma. Clin Cancer Res 2004;10:5202-5214.

94. Micheau O. Cellular FLICE-inhibitory protein: an attractive therapeutic target? Expert Opin Ther Targets 2003;7(4):559-573.

95. Moll R, Divo M and Langbein L. The human keratins: biology and pathology. Histochem Cell Biol 2008;129:705-733.

96. Moll R, Franke WW, Schiller DL, Geiger B and Krepler R. The catalog of human cytokeratins: Patterns of expression in normal epithelia, tumors and cultured cells. Cell 1982;31:11-24.

97. Myers M, Britt KL, Wreford NGM, Ebling FJP and Kerr JB. Methods for quantifying follicular numbers within the mouse ovary. Reprod 2004;127:569-580.

98. Nagata S. Apoptosis by death factor. Cell 1997;88(3):355-365.

99. Nishi Y, Yanase T, Mu Y, Oba K, Ichino I, Saito M, Nomura M, Mukasa C, Okabe T, Goto K, Takayanagi R, Kashimura Y, Haji M, Nawata H. Establishment and characterization of a steroidogenic human granulosa-like tumor cell line, KGN, that expresses functional follicle-stimulating hormone receptor. Endocrinology 2001;142(1):437-445.

100. Nishi Y,Yanase T, Mu YM, Oba K, Ichino I, Saito M, Nomura M, Mukasa C, Okabe T, Goto K, Takayanagi R, Kashimura Y, Haji M and Nawata H. Establishment and characterization of a steroidogenic human granulosa-like tumor cell line, KGN, that expresses functional follicle-stimulating hormone receptor. Endocrinol 2001;142(1):437-445.

101. Nishimoto S and Nishida E. MAPK signaling: ERK5 versus ERK1/2. EMBO Rep 2006;7(8):782-786.

102. Niswender GD, Juengel JL, McGuire WJ, Belfiore CJ and Wiltbank MC. Luteal function: the estrous cycle and early pregnancy. Biol Reprod 1994;50(2):239-247.

103. Niswender GD, Juengel JL, Silva PJ, Rollyson MK and McIntush EW. Mechanisms controlling the function and life span of the corpus luteum. Physiol Rev 2000;80(1):1-29.

104. Omary MB, Ku NO, Liao J and Price D. Keratin modifications and solubility properties in epithelial cells and in vitro. Subcell Biochem 1998;31:105-140.

105. Omary MB, Ku NO, Tao GZ, Toivola DM and Liao J. "Heads and tails" of intermediate filament phosphorylation: multiple sites and functional insights. Trends Biochem Sci 2006;31(7):383-394.

106. Ortega HH, Salvetti NR, Müller LA, Amable P, Lorente JA, Barbeito CG and Gimeno EJ. Characterization of cytoskeletal proteins in follicular structures of cows with cystic ovarian disease. J Comp PAthol 2007;136(4):222-230.

107. Owens DW and Lane EB. The quest for the function of simple epithelial keratins. Bioessays 2003;25(8):748-758.

108. Oztürk S, Schleich K and Lavrik IN. Cellular FLICE-like inhibitory proteins (c-FLIPs): fine-tuners of life and death decisions. Exp Cell Res 2012;318(11):1324-1331.

109. Palter SF, Tavares AB, Hourvitz A, Velduis JD and Adashi EY. Are estrogens of import to primate/human ovarian folliculogenesis? Endocr Rev 2001;22:389-424.

110. Pearson G, Robinson F, Beers Gibson T, Xu BE, Karandikar M, Berman K and Cobb MH. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endor Rev 2001;22(2):153-183.

111. Pedersen T and Peters H. Proposal for a classification of occytes and follicles in the mouse ovary. J Reprod Fert 1968;17:555-557.

112. Peter AT and Dhanasekaran N. Apoptosis of granulosa cells: a review on the role of MAPK-signalling modules. Reprod Domest Anim 2003;38(3):209-213.

113. Peter ME and Krammer PH. Mechanisms of CD95 (APO-1/Fas)-mediated apoptosis. Curr Opin Immunol 1998;10(5):545-551.

114. Peter ME. The flip side of FLIP. Biochem J 2004;382(Pt 2):e1-3.

115. Porter DA, Harman RM, Cowan RG and Quirk SM. Relationship of Fas ligand expression and atresia during bovine follicle development. Reprod 2001;121:561-566.

116. Porter DA, Vickers SL, Cowan RG, Huber SC and Quirk SM. Expression and function of Fas antigen vary in bovine granulosa

and theca cells during ovarian follicular development and atresia. Biol Reprod 2000;62(1):62-66.

117. Prange-Kiel J, Kreutzkamm C, Wehrenberg U and Rune GM. Role of tumor necrosis factor in preovulatory follicles of swine. Biol Reprod 2001;65(3):928-935.

118. Quirk SM, Cowan G, Harman RM, Hu CL and Porter DA. Ovarian follicular growth and atresia: The relationship between cell proliferation and survival. J Anim Sci 2004;82:E40-52.

119. Quirk SM, Cowan RG, Joshi SG and Henrikson KP. Fas antigen-mediated apoptosis in human granulosa/luteal cells. Biol Reprod 1995;52:279-287.

120. Quirk SM, Porter DA, Huber SC and Cowan RG. Potentiation of Fas-mediated apoptosis in murine granulosa cells by interferon-{gamma}, tumor necrosis factor-{alpha}, and cycloheximide. Endocrinol 1998;139:4860-4869.

121. Quirk SM, Cowan RG, Harman RM. Progesterone receptor and the cell cycle modulate apoptosis in granulosa cells. Endocrinology 2004;145(11):5033-5043.

122. Rajakoski E. The ovarian follicular system in sexually mature heifers with special reference to seasonal, cyclic, and left-right variations. Acta Endocrinol Suppl 1960;34(Suppl 52):1-68.

123. Rameh LE and Cantley LC. The role of phosphoinositide 3-kinase lipid products in cell function. Biol Chem 1999;274(13):8347-8350.

124. Roche JR, Friggens NC, Kay JK, Fisher MW, Stafford KJ and Berry DP. Body condition score and its assocatiom with dairy cow productivity, health and welfare. J Dairy Sci 2009;92:5769-5801.

125. Rodgers R and Irving-Rodgers HF. Formation of the ovarian follicular antrum and follicular fluid. Biol Reprod 2010;82:1021-1029.

126. Rosenfeld CS, Wagner JS, Roberts RM and Lubahn DB. Intraovarian actions of oestrogen. Reproduction 2001;122:215-226.

127. Ryan KE, Casey SM, Canty MJ, Crowe MA, Martin F and Evans ACO. Akt and Erk signal transduction pathways are early markers of differentiation in dominant and subordinate ovarian follicles in cattle. Reproduction 2007;133:617-626.

128. Ryan KE, Glister C, Lonergan P, Martin F, Knight PG and Evans ACO. Functional significance of the signal transduction pathways Akt and Erk in ovarian follicles: in vitro and in vivo studies in cattle and sheep. J Ovarian Res 2008;1(1):2.

129. Salvetti NR, Gimeno EJ, Lorente JA and Ortega HH. Expression of cytoskeletal proteins in the follicular wall of induced ovarian cysts. Cells Tissues Organs 2004;178(2):117-125.

130. Salvetti NR, Stangaferro ML, Palomar MM, Alfaro NS, Rey F, Gimeno EJ and Ortega HH. Cell proliferation and survival mechanisms underlying the abnormal persistence of follicular cysts in bovines with cystic ovarian disease induced by ACTH. Anim Reprod Sci 2010;122(1-2):98-110.

131. Santini D, Ceccarelli C, Mazzoleni G, Pasquinelli G, Jasonni VM and Martinelli GN. Demonstration of cytokeratin intermediate filaments in occytes of the developing and adult human ovary. Histochemistry 1993;99(4):311-319.

132. Santos JE, Bisinotto RS, Ribeiro ES, Lima FS, Greco LF, Staples CR and Thatcher WW. Applying nutrition and physiology to improve reproduction in dairy cattle. Soc Reprod Fertil Suppl 2010;67:387-403.

133. Savoi JD, Keenan L, Boland MP and Roche JF. Pattern of growth of dominant follicles during the oestrous cycle in heifers. J Reprod Ferti 1988;83:663-671.

134. Shiota M, Sugai N, Tamua M, Yamaguchi R, Fukushima N, Miyano T and Miyazaki H. Correlation of mitogen-activated protein kinase activities with cell survival and apoptosis in porcine granulosa cells. Zool Sci 2003;20(2):193-202.

135. Shiota M, Sugai N, Tamura M, Yamaguchi R, Fukushima N, Miyano T and Miyazaki H. Correlation of mitogen-activated protein kinase activities with cell survival and apoptosis in porcine granulosa cells. Zoolog Sci 2003;20(2):193-201.

136. Short RV. Steroids in the follicular fluid and the corpus luteum of the mare. A 'two cell type' theory of ovarian steroid synthesis. J Endocrinol 1962:24;59-63.

137. Staal SP, Hartley JW and Rowe WP. Isolation of transforming murine leukemia viruses from mice with a high incidence of spontaneous lymphoma. Proc Natl Acad Sci 1977;74(7):3065-3067.

138. Sullivan BT, Cherry JA, Sakamoto H, Henkes LE, Townson DH and Rueda BR. Cytokeratin 18 expression inhibits cytokine-induced death of cervical cancer cells. Int J Gynecol Cancer 2010;20:1474-1481.

139. Susin SA, Lorenzo HK, Zamzami N, Marzo I, Snow BE, Brothers GM, Mangion J, Jacotot E, Costantini P, Loeffler M, Larochette N, Goodlett DR, Aebersold R, Siderovski DP, Penninger JM and Kroemer G. Molecular characterization of mitochondrial apoptosis-inducing factor. Nature 1999;397(6718):441-446.

140. Sutovský P, Fléchon JE and Pavlok A. Microfilaments, microtubules and intermediate filaments fulfil differential roles during gonadotropin-induced expansion of bovine cumulus oophorus. Reprod Nutr Dev 1994;34(5):415-425.

141. Thome M, Schneider P, Hofmann K, Fickenscher H, Meinl E, Neipel F, Mattmann C, Burns K, Bodmer JL, Schröter M, Scaffidi C, Krammer PH, Peter ME and Tschopp J. Viral FLICE-inhibitory proteins (FLIPs) prevent apoptosis induced by death receptors. Nature 1997;386(6624):517-521.

142. Thorburn A. Death receptor-induced cell killing. Cell Signal 2004;16:139-144.

143. Thornberry NA, Rano TA, Peterson EP, Rasper DM, Timkey T, Garcia-Calvo M, Houtzager VM, Nordstrom PA, Roy S, Vaillancourt JP, Chapman KT and Nicholson DW. A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis. J Biol Chem 1997;272(29(:17907-17911.

144. Tilly JL, Billig H, Howalski KI and Hsueh AJ. Epidermal growth factor and basic fibroblast growth factor suppress the spontaneous onset of apoptosis in cultured rat ovarian granulosa cellsand follicles by a tyrosine kinase-dependent mechanism. Mol Endocrinol 1992;6(11):1942-1950.

145. Tilly JL, Kowalski KI, Johnson A and Hsueh AJW. Involvement of apoptosis in ovarian follicular atresia and postovulatory regression. Endocrinol 1991;91:1295-2799.

146. Townson DH and Combelles CMH. Ovarian follicular atresia. Basic Gynecology: Some Related Issues 2012:43-76.

147. Townson DH, Putnam AN, Sullivan BT, Guo L and Irving-Rodgers HF. Expression and distribution of cytokeratin 8/18 intermediate filaments in bovine antral follicles and corpus luteum: an intrinsic mechanism of resistance to apoptosis? Histol Histopathol 2010;25:889-900.

148. Tschopp J, Irmler M and Thome M. Inhibition of fas death signals by FLIPs. Curr Opin Immunol 1998;10(5):552-558.

149. Turzillo AM and Fortune JE. Effects of suppressing plasma FSH on ovarian follicular dominance in cattle. J Reprod Fertil 1993;98:113-119.

150. van den Hurk R, Dijkstra G, van Mil FN, Hulshof SC and van den Ingh TS. Distribution of the intermediate filament proteins vimentin, keratin, and desmin in the bovine ovary. Mol Reprod Dev 1995;41(4):459-467.

151. Verhagen AM, Ekert PG, Pakusch M, Silke J, Connolly LM, Reid GE, Moritz RL, Simpson RJ and Vaux DL. Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. Cell 2000;102(1):43-53.

152. Verhagen AM, Ekert PG, Pakusch M, Silke J, Connolly LM, Reid GE, Moritz RL, Simpson RJ and Vaux DL. Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. Cell 2000;102(1):43-53.

153. Vickers SL, Cowan RG, Harman RM, Porter DA and Quirk SM. Expression and activity of the Fas antigen in bovine ovarian follicle cells. Biol Reprod 2000;62:54-61.

154. Walsh SW, Williams EJ and Evans ACO. A review of the causes of poor fertility in high milk producing dairy cows. Anim Reprod Sci 2011;123:127-138.

155. Walters DL and Schallenberger E. Pulsatile secretion of gonadotropins, ovarian steroids and ovarian oxytocin during the periovulatory phase of the oestrous cycle in the cow. J Reprod Fert 1984;71:503-512.

156. Waschke J. The desmosome and pemphigus. Histochem Cell Biol 2008;130(1):21-54.

157. Webb R, Garnsworthy PC, Campbell BK and Hunter MG. Intra-ovarian regulation of follicular development and oocyte competence in farm animals. Theriogenology 2007;68:S22-29.

158. Xiao CW, Asselin E and Tsang BK. Nuclear factor κB-mediated induction of flice-like inhibitory protein prevents tumor necrosis factor α-induced apoptosis in rat granulosa cells. Biol of Reprod 2002;67:436-441.

159. Young JM and McNeilly AS. Theca: the forgotten cell of the ovarian follicle. Reproduction 2010;140(4):489-504.

160. Zeng P, Wagoner HA, Pescovitz OH and Steinmetz R. RNA interference (RNAi) for extraceullar signal-regulated kinase 1

(ERK1) alone is sufficient to suppress cell viability in ovarian cancer cells. Cancer Biol Ther 2005;4(9):961-967.

161. Zhou J, Chin E and Bondy C. Cellular-pattern of insulin-like growth factor-I (IGF-I) and IGF-I receptor gene-expression in the developing and mature ovarian follicle. Endocrinology 1991;129:3281-3288.

APPENDIX

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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