

DEVELOPMENTAL ACQUISITION OF APOPTOSIS IN THE PREIMPLANTATION  
BOVINE EMBRYO: RETURNING TO THE BALANCE OF LIFE AND DEATH

By

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To Laura M. Neumann, for all of her love, help, and understanding and to my parents;  
Karen M. and Jerry J. Fear for their love, support, and the many opportunities they have  
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## LIST OF ABBREVIATIONS

Abbreviation	Term (Alias)
AZA	5-Aza-2'-Deoxycytidine
ANT	Adenine-Nuclotide Translocator
ADP	Adenosine Diphosphate
ATP	Adenosine Triphosphate
AIF	Apoptosis Inducing Factor 1
ART	Apoptosis Regulating and Targeting Sequence
APAF1	Apoptotic Peptidase Activating Factor 1
BIR	Baculoviral IAP Repeat
BCL2	B-cell CLL/Lymphoma 2
BBC3	BCL2 Binding Component 3 (PUMA)
BAK1	BCL2-Antagonist/Killer 1 (BAK)
BAD	BCL2-Associated Agonist of Cell Death
BAX	BCL2-Associated X Protein
BCL2L1	BCL2-Like 1 (BCL-xL)
BCL211	BCL2-Like 11 (BIM)
BOK	BCL2-Related Ovarian Killer
BID	BH3 Interacting Domain Death Agonist
BEND cell	Bovine Endometrial Cell Line
CCCP	Carbonyl Cyanide 3-Chlorophenyl Hydrazone
CARD	Caspase-Recruitment Domain
CAPK	Ceramide-Activated Protein Kinase
cDNA	Complementary DNA
COCs	Cumulus-Oocyte Complexes

Ct	Cycle Threshold
DD	Death Domain
DED	Death Effector Domains
DISC	Death Inducing Signaling Complex
Diablo	Diablo Homolog (SMAC/DIABLO)
DEPC	Diethyl Pyrocarbonate
DFF	DNA Fragmentation Factor
DFFA	DNA Fragmentation Factor A (DFF45, ICAD)
DFFB	DNA Fragmentation Factor B (DFF40, CAD)
DNA	Deoxyribonucleic Acid
DNAJB1	DNAJ Homolog 1
DRP1	Dynamin Related Protein 1
EGA	Embryonic Genome Activation
FASLG	Fas Ligand (FASL)
FADD	Fas-Associated Protein with Death Domain
FIS1	Fission 1
FPA	Fluid Processing Apparatus
GLM	General Linear Model
GAP	Group Activation Pack
HSPA1A	Heat-Shock Protein 70 kDa 1A (HSP70.1)
HPM	High-Polarized Mitochondria
HIST1H2A	Histone Cluser 1, H2a (H2A.1)
hpi	Hours Post-Insemination
HTRA2	HtrA Serine Peptidase 2 (OMI)
IVF	In Vitro Fertilization

IAP	Inhibitors of Apoptosis
ICM	Inncer Cell Mass
KSR1	Kinase Supressor of RAS (KSR)
KSOM-BE2	KSOM-Bovine Embryo 2
MII	Metaphase 2
MOM	Mitochondrial Outer Membrane
MOMP	Mitochondrial Outer Membrane Permeabilization
MFN1	Mitofusin 1
MAPK	Mitogen-Activated Protein Kinase
MAPK8	Mitogen-Activated Protein Kinase 8 (JNK)
MAPK9	Mitogen-Activated Protein Kinase 9 (SAPK)
MAP3K1	Mitogen-Activated Protein Kinase Kinase Kinase 1 (MEKK1)
MCL1	Myeloid Cell Leukemia Sequence 1
NLS	Nuclear Localization Signal
NB-ARC	Nucleotide Binding and Oligomerization
OPA1	Optic atrophy 1 Homolog
PBS-PVP	PBS + Polyvinylpyrrolidone
PHE	Penicillamine Hypotaurin Epinephrine
PTP	Permeability Transition Pore
PBS	Phophate Buffered Saline
PMAIP1	Phorbol-12-Myristate-13-Acetate-Induced protein 1 (NOXA)
PARP1	Poly (ADP-ribose) Polymerase 1
PCR	Polymerase Chain Reaction
KSOM	Potassium Simplex Optomized Medium
PCD	Programmed Cell Death

qPCR	Quantitative Real-Time RT-PCR
ROS	Reactive Oxygen Species
RT-PCR	Reverse Transcription PCR
RNA	Ribonucleic Acid
RNAi	RNA Interference
SDS	Sodium Dodecyl Sulfate
SMPD	Sphingomyelin Phosphodiesterase (SMase)
SMPD1	Sphingomyelin Phosphodiesterase 1 (aSMase)
SMPD2	Sphingomyelin Phosphodiesterase 2 (nSMase)
SAS	Statistical Analysis System
TBS-T	TBS + Tween
TBS-TM	TBS + Tween + Non-Fat Dry Milk
TUNEL	Terminal Deoxynucleotidyl Transferase Deoxyuridine Triphosphate Nick End Labeling
TCM-199	Tissue Culture Medium 199
TIMM8A	Translocase of Inner Mitochondrial Membrane 8 Homolog A
TSA	Trichostatin-A
TBS	Tris Buffered Saline
TE	Trophectoderm
tBID	Truncated BID
TNFSF10	Tumor Necrosis Factor (Ligand) Superfamily, Member 10 (TRAIL)
TNF	Tumor Necrosis Factor- $\alpha$ (TNF- $\alpha$ )
TALP	Tyroses Albumin Lactate Pyruvate
TL	Tyroses Lactate
YWHAQ	Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Proteins (14-3-3)

AKT1	v-akt Murine Thymoma Viral Oncogene Homolog 1 (AKT/PKB)
VDAC	Voltage-Dependent Anion Channel
VDAC1	Voltage-Dependent Anion Channel 1
VDAC2	Voltage-Dependent Anion Channel 2
XIAP	X-Linked Inhibitor of Apoptosis
$\alpha$ -PFPs	$\alpha$ -Pore Forming Proteins

Abstract of Thesis Presented to the Graduate School  
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The developmental acquisition of apoptosis occurs in a stage dependent manner in the bovine preimplantation embryo. Two-cell embryos lack the capacity for apoptosis, and the embryo remains refractory to apoptotic stimuli until the 8- to 16-cell stage. The apoptotic machinery is present in the 2-cell embryo but the mitochondria are resistant to depolarization following apoptotic stimuli. This suggests that there is a developmental regulation of apoptosis at the level of the mitochondria.

Propagation of an apoptotic signal requires mitochondrial outer membrane depolarization, which is dependent upon the complex balance of anti- versus pro-apoptotic proteins. It was hypothesized that 2-cell embryos have higher amounts of anti-apoptotic proteins and lower amounts of pro-apoptotic proteins, thereby shifting the balance towards life and preventing the capacity for apoptosis. After embryonic genome activation, at the 8- to 16-cell stage, there is a decrease in anti-apoptotic proteins and an increase in pro-apoptotic proteins causing the embryo to adjust the balance of life and death and acquire the capacity for apoptosis in  $\geq 16$ -cell embryos. Accordingly, a series of experiments were conducted to test variations in expression of anti- and pro-apoptotic genes early during stages of preimplantation development.

Expression of anti-apoptotic genes *BCL2* and *HSPA1A* were higher in oocytes, 2-cell embryos, and 2-cell embryos treated with a transcription inhibitor compared to  $\geq 16$ -cell embryos. In contrast, expression of pro-apoptotic gene *BAD* was higher in  $\geq 16$ -cell embryos when compared to oocytes, 2-cell embryos, and 2-cell embryos treated with a transcription inhibitor. Steady-state mRNA for *BCL2L1*, *BAX*, *DFFA*, and *HIST1H2A* was not affected by stage of development.

Protein concentrations also varied between the 2-cell embryo and the  $\geq 16$ -cell embryo. The 2-cell embryo had higher immunoreactive amounts of the anti-apoptotic protein BCL2 compared to the  $\geq 16$ -cell embryo. In contrast, the  $\geq 16$ -cell embryo had higher immunoreactive amounts of BAX when compared to the 2-cell embryo. Immunoreactive amounts of BCL2L1, HSPA1A and BAD were not significantly affected by stage of development.

These results suggest that the loss of capacity for apoptosis in the 2-cell embryo is due at least in part, to higher amounts of the anti-apoptotic protein BCL2. Developmental acquisition of apoptosis is dependent upon a decrease in expression of BCL2 along with an increase in expression of the pro-apoptotic protein BAX and possibly increased availability of BAD after embryonic genome activation.

## CHAPTER 1 LITERATURE REVIEW

The term apoptosis is proposed for a hitherto little recognized mechanism of controlled cell deletion, which appears to play a complementary but opposite role to mitosis in the regulation of animal cell populations.

Kerr et al. 1972

### **Overview of the Role of Apoptosis During Preimplantation Development**

The developmental success of a preimplantation embryo is dependent on its ability to adapt to the surrounding environment and protect itself against intrinsically or extrinsically-induced cellular damage. Upon damage to DNA or organelles, somatic cells respond by arresting the cell cycle and activating repair mechanisms (Friedberg, 2003). If damage is repaired, the cell cycle resumes; otherwise the cell undergoes programmed cell death (PCD), also termed apoptosis (Friedberg, 2003). Apoptosis is a cascade of events leading to cytoplasmic, nuclear, and DNA fragmentation. The cell fragments into membrane-bound apoptotic bodies which are either dispersed or phagocytized by neighboring cells (Kerr et al., 1972; Wyllie et al., 1980). Apoptosis is heavily regulated to prevent unwanted cell death. Regulation ranges from genetic controls (Wyllie, 1995) to complex protein interactions (Hanada et al., 1995; Yang et al., 1995; Wang et al., 1996; Zha et al., 1996; Garland and Rudin, 1998).

Apoptosis plays a variety of roles in the preimplantation embryo including removal of cells with chromosomal defects (aneuploidy) (Hardy, 1999; Liu et al., 2002) or those with inappropriate developmental potential such as cells of the inner cell mass (ICM) that fail to lose the potential to form trophectoderm (TE) (Handyside and Hunter, 1986; Pierce et al., 1989; Parchment, 1993; Hardy, 1997). Apoptosis is also involved in elimination of damaged cells in the preimplantation embryo as has been shown

experimentally for embryos exposed to reactive oxygen species (ROS) (Yang et al., 1998), menadione (Moss et al., 2009), ultraviolet radiation (Herrler et al., 1998), heat-shock (Paula-Lopes and Hansen, 2002a), and arsenic (Krininger et al., 2002). Indeed, clearance of damaged cells in a compromised blastocyst by apoptosis can enhance the probability that an embryo can survive stress (Paula-Lopes and Hansen, 2002; Jousan and Hansen, 2007).

One characteristic of apoptosis responses during the preimplantation period is that the capacity for apoptosis is developmentally acquired. In the bovine preimplantation embryo, it is between the 8- to 16-cell stage, that signals such as heat-shock (Paula-Lopes and Hansen, 2002; Brad et al., 2007), ceramide (de Castro e Paula and Hansen, 2008), arsenic (Krininger et al., 2002), or tumor necrosis factor- $\alpha$  (TNF) (Soto et al., 2003a) first are capable of inducing apoptosis. Earlier in development, apoptosis is not possible.

Apoptosis responses may be inhibited early in development to prevent accidental triggering of apoptosis by signals generated during fertilization and preimplantation development. Fertilization causes a complex set of intracellular  $[Ca^{2+}]$  oscillations which allow the oocyte to complete the second meiotic division (Whitaker, 2006). These oscillations may have the potential to induce an apoptotic response given that increases in intracellular  $[Ca^{2+}]$  have been associated with apoptosis (Kaiser and Edelman, 1977; McConkey et al., 1989). In the bovine, calcium activated cysteine proteinase,  $\mu$ -calpain, is activated in oocytes, morulae and blastocysts. Inhibition of  $\mu$ -calpain reduced apoptotic indices (Sergeev and Norman, 2003) showing it is involved in apoptosis in the early preimplantation embryo.

It may also be that, when cell number is low, removal of cells by apoptosis in response to stress is harmful rather than beneficial. For example, the elimination of 10 damaged cells from a 150-cell blastocyst may be an effective strategy for facilitating continued development but removal of 1 damaged cell from a 2-cell embryo may accentuate reduction in developmental potential caused by stress. Although a bisected 2-cell embryo can develop into a blastocyst (Loskutoff et al., 1993), insults to the embryo are likely to affect both blastomeres and loss of one cell to apoptosis may make further development problematic.

### **Pathways of Apoptosis**

There are two well-known pathways of apoptosis - the extrinsic or receptor mediated-pathway and the intrinsic or mitochondrial pathway. Both of these pathways result in the activation of caspases and DNases that cause cytoplasmic, nuclear, and DNA fragmentation and lead to the classic morphological signs of apoptosis, membrane blebbing and formation of apoptotic bodies. The major difference between the two pathways is the involvement of a receptor-mediated signal versus an internally-generated, mitochondrial signal.

#### **The Extrinsic Pathway**

The extrinsic pathway, illustrated in [Figure 1-1](#), utilizes specialized membrane-bound 'death receptors' that are members of the tumor necrosis factor superfamily (Ashkenazi and Dixit, 1998). These receptors share a homologous domain (about 80 amino acids) in their cytoplasmic region called the death domain (DD) (Nagata, 1999). Binding of a variety of extra-cellular signaling molecules such as Fas ligand (FASLG) (Nagata, 1997), TNF-related apoptosis inducing ligand (TNFSF10, previously TRAIL) (MacFarlane, 2003), and TNF (Ding and Yin, 2004), activates these receptors. An

adaptor molecule, Fas-associated protein with death domain (FADD), is recruited by the receptor and interacts with the DD. FADD then interacts with procaspase-8. Two death effector domains (DED) on the N-terminal region of procaspase-8 combine to form the death-inducing signaling complex (DISC). Procaspase-8 contains a weak proteinase activity that is activated upon DISC formation, allowing self-cleavage to produce active caspase-8 (Nagata, 1997). Caspase-8 preferentially cleaves procaspase-3 and -7 into caspase-3 and -7 which in turn activate other caspases and cleave a variety of cellular proteins.

There are two cell types with regards to the extrinsic pathway based on whether the cell has sufficient quantities of caspase-8, -3, and -7 for induction of apoptosis. For cells with insufficient quantities, the intrinsic pathway (see below) is activated through cleavage of BH3 interactive domain death agonist (BID) into truncated BID (tBID) by caspase-8, allowing for the apoptosis signal to be amplified (Scaffidi et al., 1998).

Caspase-3 and -7 then cleave DNA fragmentation factor A (DFFA), also known as the inhibitor of caspase-activated DNase. Cleavage of DFFA leads to its release from the DNA fragmentation factor B (DFFB) or caspase-activated DNase. As a result, DFFB becomes an active DNase and cleaves internucleosomal regions of chromosomal DNA (Nagata, 1999).

### **The Intrinsic Pathway**

The intrinsic pathway, illustrated in [Figure 1-2](#), is the stress induced pathway of apoptosis. This pathway does not utilize membrane-bound receptors like the extrinsic pathway but instead depends upon activation for a stress-induced signal such as the activation of sphingomyelin phosphodiesterase (SMPD) by heat-shock causing the hydrolysis of sphingomyelin to ceramide. Ceramide, in turn activates BH3-only

members of the BCL2 family through mitogen-activated protein kinase (MAPK9; previously SAPK) or mitogen-activated protein kinase 8 (MAPK8; previously JNK). BH3-only proteins inhibit anti-apoptotic actions of B-cell CLL/lymphoma 2 (BCL2) and BCL2-like 1 (BCL2L1, previously BCL-xL) by binding to the anti-apoptotic BCL2 and BCL2L1 causing release of the pro-apoptotic proteins BCL2-associated X protein (BAX) and BCL2-antagonist/killer 1 (BAK1). In addition to the inhibition of BCL2 and BCL2L1, some BH3-only proteins bind to BAX and BAK1 causing a conformational change allowing targeting to the mitochondrial outer membrane and formation of multimers. BAX and BAK1 then form pores in the mitochondrial outer membrane leading to mitochondrial depolarization. Consequently, cytochrome c is released into the cytosol. Cytochrome c binds to apoptosis inducing factor 1 (APAF1) and causes a conformational change allowing it to form a 7 member unit called the apoptosome. The apoptosome recruits multiple units of the zymogen procaspase-9. Once procaspase-9 concentrations are high enough, there is a conformational change in procaspase-9 exposing the enzymatically active site of caspase-9. Caspase-9 in turn cleaves and activates the executioner or group II caspases (-3, -6, and -7). The caspase cascade terminates with cleavage of DFFA leading to its disassociation from DFFB activating the DNase. DFFB then cuts DNA into the internucleosomal fragments which are the hallmark of apoptosis (Wyllie et al., 1980).

### **Molecular Events Controlling Apoptosis in the Intrinsic Pathway**

Mitochondria are membrane-enclosed organelles found in the majority of eukaryotic cells. It was incorporated during eukaryotic evolution from symbiotic bacteria. The mitochondrion has retained its own genome that encodes 37 genes but it also requires nuclear encoded genes. The major function of the mitochondria is the

production of adenosine tri-phosphate (ATP). As part of the process of energy production, the outer mitochondrial membrane becomes highly polarized with respect to  $H^+$ . Polarization is also key to the participation of mitochondria in signal transduction in several cellular pathways including apoptosis.

Mitochondria have the ability to form complex and dynamic networks. These networks allow for mitochondrial communication, cellular signal transduction and signal amplification. Perhaps the clearest example of this is the signal transduction event at fertilization. During oocyte maturation there is a formation of a ring of high-polarized mitochondria (HPM) in the subplasmalemmal region. This ring of HPM is required for sperm penetration and cortical granule exocytosis (van Blerkom and Davis, 2007). The authors suggested that the HPM establish a continuous circumferential circuit, capable to reacting to and propagating a signal across the subplasmalemmal cytoplasm. Apoptosis leads to fragmentation of the mitochondrial network communication both upstream and downstream of apoptotic stimuli (Gao et al., 2001; Arnoult et al., 2005a; Youle and Karbowski, 2005). In the initial stages of apoptosis, however, the mitochondrial network is important for propagation of the apoptotic signal because early fragmentation of the mitochondrial network prevents cell death (Perfettini et al., 2005).

The key event in induction of apoptosis by the intrinsic pathway is permeabilization of the mitochondrial outer membrane (MOM) (termed mitochondrial outer membrane permeabilization or MOMP). Sensitivity of the mitochondrion to outer-membrane permeabilization may be affected by mitochondrial shape which is derived from equilibrium of fusion and fission events affecting membrane integrity and structural proteins. The mitochondria can undergo fusion with other mitochondria, or fission

leading to the production of two daughter organelles. Both mitochondrial fusion and fission depend upon GTPases; mitofusin 1 (MFN1), optic atrophy 1 (OPA1) and dynamin related protein 1 (DRP1) (Smirnova et al., 2001; Santel et al., 2003; Cipolat et al., 2004). While early fragmentation of the mitochondrial network may prevent apoptotic signaling from propagation, there is evidence showing the induction of network fragmentation downstream of BAX/BAK1 signaling events (Arnoult et al., 2005a). BAX has been shown to co-localize with DRP1 at mitochondrial fission sites suggesting its involvement (Karbowski et al., 2002). BAX and BAK1 also induce sumoylation of DRP1 during apoptosis, prior to pore formation, which leads to the stabilization and prevents lysosomal degradation of DRP1 (Wasiak et al., 2007). Finally as a result of MOMP there is a release of the mitochondrial factor, translocase of inner mitochondrial membrane 8 homolog A (TIMM8a), which leads to further activation of DRP1 (Arnoult et al., 2005b). Even though mitochondrial network breakdown is part of apoptosis, its inhibition does not prevent BAX/BAK1-dependent apoptosis (Parone et al., 2006).

Mitochondrial outer-membrane depolarization leads to the release of many mitochondrial factors including cytochrome c, endonuclease g and Diablo homolog (DIABLO; previously SMAC/DIABLO). These factors are involved in further signal transduction by the creation of the apoptosome and initiation of the caspase signaling cascade. Indeed, MOMP and cytochrome c release is the "point of no return" in the apoptotic signal transduction system so that once activated the cell is committed to destruction. It is the irreversibility of events contingent on MOMP that has led to development of tight regulatory control mechanisms for MOMP.

## **Pore Formation and Permeabilization**

At the level of the mitochondria, the first step in signal propagation is pore formation and permeabilization of the MOM. Currently there are several competing models to explain this mechanism. The first model suggests that there is an opening of the permeability transition pore (PTP). The PTP spans both the outer and inner mitochondrial membranes with a voltage-dependent anion channel (VDAC) on the outer membrane and the adenine-nucleotide translocator (ANT) channel or a similar channel on the inner-membrane (Green and Kroemer, 2004). In addition, there is possible involvement of several other proteins including cyclophilin D and hexokinase-II (Kumarswamy and Chandna, 2009). In this model, VDAC is semipermeable to allow passage of molecules up to 5 kDa, while the ANT is nearly impermeable. Differential permeability is essential for the generation of the electrochemical proton gradient used for oxidative phosphorylation (Bernardi, 1999). In response to an apoptotic stimulus, mitochondrial calcium concentrations increase, which leads to the opening of the PTP allowing calcium, water, and other low molecular weight molecules (~1.5 kDa) to pass through the inner-membrane (Green and Kroemer, 2004). With the influx of water, there is a swelling of the mitochondrial matrix which is sufficient enough to lead to rupture of the outer membrane and release of cytochrome c.

There are two major issues with this model. First, mitochondrial function and structure are preserved after cytochrome c release suggesting that there is not a rupture of the outer membrane (Ashen and Goff, 2000). Also, cells lacking BAX and BAK1 fail to undergo MOMP in response to a wide range of apoptotic stimuli including staurosporine, ultraviolet radiation, growth factor deprivation, and tBID-induced

cytochrome c release (Wei et al., 2001). Thus, the two major pro-apoptotic members of the BCL2 family, BAX and BAK1, are necessary for initiation of pore formation.

BAX is primarily localized to the cytosol and translocates to the mitochondrial membrane when activated by the binding of certain BH3-only domain proteins like BID and BCL2-like 11 (BCL2L11, previously BIM) (Lovell et al., 2008). BAK1 is localized to the mitochondrial outer membrane associated with VDAC2, myeloid cell leukemia sequence 1 (MCL1) and BCL2L1 (Cheng et al., 2003; Willis et al., 2005; Li et al., 2008). Like BAX, BAK1 is also activated by binding of BH3-only proteins (e.g. BID).

BAX and BAK1 belong to a class of amphipathic proteins called  $\alpha$ -pore-forming proteins ( $\alpha$ -PFPs) that contain several  $\alpha$ -helices that can bind to the water-lipid interface of the mitochondrial lipid bi-layer. BAX and BAK1 then form multimer units in the mitochondrial membrane resulting in the interfacial area increasing and stretching the hydrocarbon core of the bi-layer. This strain on the membrane increases with protein concentration, when the concentration is such that it exceeds a threshold, a stable pore is formed with a defined size (Lee et al., 2008). The  $\alpha$ 5-helix of BAX forms a type of pore referred to as toroidal or lipidic pore which is made of both protein and lipids (Qian et al., 2008). When the pore is formed, it relieves some of the interfacial strain and becomes stable.

One interesting player that appears to be involved in both models of mitochondrial outer membrane permeabilization are VDACS. There are three isoforms of VDACS and each have different functions with regards to pore formation (reviewed Blachly-Dyson and Forte, 2001). VDAC2 has been shown to be inhibitory of BAK1 (Cheng et al., 2003), while VDAC1 appears to be a contributing factor to pore formation in ROS

stimulated apoptosis, through its binding with active BAX or BAK1. The VDACs may function to group small amounts of cardiolipin and related lipids present in the MOM into microdomains. When active BAX and BAK1 associate with VDAC1, these lipid domains may aid in the lipidic pore formation and permeabilization.

### **Cytochrome C Release**

Regardless of how MOMP occurs, cytochrome c release is "the point of no return". Cytochrome c is a molecule that has been shown to be involved in electron transportation between the mitochondrial inner and outer membranes (Bernardi and Azzone, 1981). Beyond its role in respiration, cytochrome c is a major signaling factor in the apoptotic cascade (Liu et al., 1996). Release of cytochrome c occurs within minutes following an apoptotic stimulus (Goldstein et al., 2000) leading to the propagation of the apoptotic signal.

Cytochrome c resides in the mitochondrial inter-membrane space. Approximately 10-15% of cytochrome c is available in the mitochondrial inter-membrane space while the rest is compartmentalized within pockets formed by the cristae or inner-membrane folds (Bernardi and Azzone, 1981). One implication of the localization of cytochrome c is that apoptosis requires not only formation of pores in the MOM but also remodeling of the cristae (Scorrano et al., 2002) to allow complete cytochrome c releases as it occurs in programmed cell death (Goldstein et al., 2000). Like in mitochondrial network plasticity, OPA1 is involved in cristae remodeling. OPA1 is the major factor involved in maintenance and formation of the cristae folds (Olichon et al., 2003). Depletion of OPA1 using RNA interference (RNAi) results in restructuring the cristae allowing complete release of cytochrome c (Olichon et al., 2003; Griparic et al., 2004; Zhang et al., 2007). With an apoptotic stimulus, OPA1 is also depleted by its release from the

mitochondria at the same rate as cytochrome c (Arnoult et al., 2005; Yamaguchi et al., 2008). In addition to OPA1, active BAX and BAK1 have been shown to be involved with cristae restructuring during apoptosis. Yamaguchi et al. (2008) evaluated the role of BH3-only proteins BID, BCL2L11, and BCL2-associated agonist of cell death (BAD) along with the multi-domain proteins BAX and BAK1, in cristae remodeling and cytochrome c release. Active forms of BID and BCL2L11 showed a loss of OPA1 complexes and a complete release of cytochrome c. BAD had no effect on loss of OPA1 or cytochrome c release. Using different BAX and BAK1 knockdown or knockout models, they concluded that BID-induced cristae remodeling was dependent on either BAX or BAK1. BAX/BAK1-dependent events on the mitochondrial inner-membrane (cristae remodeling) and on the mitochondrial outer membrane (pore formation) could experimentally separated (Yamaguchi et al., 2008).

### **Other Apoptogenic Factors**

Besides cytochrome c, there are other apoptogenic factors that are released at the time of mitochondrial permeabilization including DIABLO, HTRA serine peptidase 2 (HTRA2; previously OMI), endonuclease g, and apoptosis-inducing factor 1 (AIF1).

DIABLO is a nuclear encoded protein that is imported into the mitochondria and co-localizes with cytochrome c (Du et al., 2000). Like cytochrome c DIABLO's complete release from mitochondria requires cristae remodeling (Yamaguchi et al., 2008). When released into the cytosol, DIABLO binds to inhibitors of apoptosis (IAPs) such as X-linked IAP (XIAP) and survivin (Du et al., 2000; Verhagen et al., 2000; Srinivasula et al., 2001). IAPs contain three baculoviral IAP repeat (BIR) domains. These domains function together to bind both procaspases and activated caspases and thereby inhibit

their enzymatic activity (Srinivasula et al., 2001). DIABLO binds to the BIR2 and BIR3 domains and blocks the action of IAPs enhancing caspase activity.

HTRA2 is an evolutionarily conserved nuclear encoded protein with serine proteinase activity (Hu et al., 1998) it is found in the endoplasmic reticulum, nucleus, and mitochondria. HTRA2 is released from the mitochondria upon apoptotic stimuli (anti-FAS antibodies, ultraviolet irradiation, or tBID) (Verhagen et al., 2002; van Loo et al., 2002). The N-terminus of HTRA2 is almost identical to DIABLO and has been shown to have similar binding and inhibitory properties towards IAPs (Suzuki et al., 2001). In addition to the inhibition of IAPs, the serine proteinase also has a pro-apoptotic function. Over-expression of HTRA2 outside of the mitochondria leads to a caspase-independent cell death (Suzuki et al., 2001; Verhagen et al., 2002).

Endonuclease g is a nuclear encoded protein that is imported into the mitochondria and is involved in mitochondrial DNA processing and generation of RNA primers required for DNA synthesis (Côté and Ruiz-Carrillo, 1993). Endonuclease g is also released from the mitochondria upon apoptotic stimuli such as ultraviolet radiation or treatment with tBID coincident with release of cytochrome c (Li et al., 2001). Release of endonuclease g is independent of release of mitochondrial heat-shock protein 70 (HSPA1A), which is localized to the inner-membrane matrix, suggesting that there are substantial amounts of endonuclease g in the inter-membrane space and not in the inner-membrane matrix where DNA processing occurs (Li et al., 2001). Like HTRA2, the release of endonuclease g can be blocked by the over-expression of BCL2 (Li et al., 2001; van Loo et al., 2001). Once in the cytosol, endonuclease g translocates to the nucleus where it cleaves chromatin DNA into fragments (Li et al., 2001; van Loo et al.,

2001). This activity is similar to DFFB except that endonuclease g has been shown to act as a caspase-independent DNase (van Loo et al., 2001). Endonuclease g also has RNase activity that has been suggested may play a role as an apoptotic RNase (Kalinowska et al., 2005).

Apoptosis-inducing factor 1 is a flavoprotein with homology to a bacterial oxidoreductase; it is encoded in the nucleus and imported into the mitochondria where it is anchored to the inner-membrane by an amino-terminal transmembrane segment (Otera et al., 2005) and plays a role in oxidative phosphorylation and redox control (Modjtahedi et al., 2006). During MOMP, the transmembrane portion of AIF1 is cleaved by calpain I, a class of cysteine proteinases, and is released into the cytosol (Otera et al., 2005; Polster et al., 2005). Once in the cytosol, AIF1 translocates to the nucleus and causes large-scale (~50 kb) DNA fragmentation and chromatin condensation (Susin et al., 1999). Like for endonuclease g, this apoptogenic function is caspase-independent (Susin et al., 1999). AIF1 has been shown to play a central role in several caspase-independent apoptotic signaling mechanisms such as response to DNA damage (Yu et al., 2002) and by interacting poly(ADP-ribose) polymerase 1 (PARP1). PARP1 is a nuclear enzyme involved in DNA repair by transferring ADP-ribose to an acceptor protein such as histone (Hong et al., 2004). AIF1 also stimulates the DNase activity of endonuclease g (Joza et al., 2009).

### **The Apoptosome**

As mentioned previously, the point of no return for cell death is the release of cytochrome c and its translation into an irreversible death signaling cascade. Several signaling modules are required to translate and propagate this signal. The first is apoptotic peptidase activating factor 1 (APAF1), a monomeric cytosolic protein (Zou et

al., 1997), that senses mitochondrial depolarization by binding to cytochrome c leading to its oligomerization and formation of a wheel-shaped signaling platform with seven spokes, the apoptosome (Acehan et al., 2002). Given the importance of regulating this signal, the formation of the apoptosome is a multi-step process requiring either 2'-deoxy ATP (dATP) or ATP (Riedl et al., 2005), for simplicity will refer to them as (d)ATP.

To understand the regulation of the apoptosome, it is necessary to first understand the structure and function of APAF1. APAF1 is a multi-domain protein consisting of a N-terminal caspase-recruitment domain (CARD), two C-terminal domains consisting of a string of WD40 repeats, and three central domains called a nucleotide-binding and oligomerization (NB-ARC) region that contain an ATPase domain (Hanson and Whiteheart, 2005). In the monomeric form, both the CARD and NB-ARC domains are in a locked conformational state with the WD40 domain folded over (Bao and Shi, 2007; Riedl and Salvesen, 2007). Associated with the NB-ARC region is a (d)ATP that is hydrolyzed to (d)ADP with binding of cytochrome c to the WD40 domain. This leads to a conformational change removing the WD40 domains from inhibiting the CARD and NB-ARC domains (Li et al., 1997; Riedl et al., 2005).

The CARD and NB-ARC domains are still in an inhibitory conformation and require the exchange of (d)ADP for (d)ATP to become activated (Kim et al., 2005; Riedl et al., 2005; Yu et al., 2005). Therefore, the ADP to ATP exchange represent another level of control in apoptosome formation. With the exchange of ADP for ATP, the CARD and NB-ARC domains undergo a conformational change that removes their inhibition. The active NB-ARC domain can then oligomerize to form the 7 member holoenzyme that is

arranged in a wheel shape with the WD40 domains extending out like spokes and the CARD domain sitting in on top of the NB-ARC domain in the center.

After apoptosome formation, the final step is for this cytosolic receptor to bind and activate procaspase-9. The CARD domain of procaspase-9 binds to the CARD domain of the apoptosome. Caspase-9 is an initiator caspase and functions upstream in the intrinsic pathway. Caspase-9 is present as a monomeric zymogen (procaspase-9) in the cytosol. Like other initiator caspases, caspase-9 is sensitive to apoptotic stimuli but is not easily activated so that the caspase cascade is not accidentally triggered. The monomeric form of caspase-9 has very little enzymatic activity (Renatus et al., 2001), but activity increases upon dimerization suggesting that caspase-9 is activated by a conformational change caused by dimerization instead of a cleavage event like the group II caspases (Riedl and Salvesen, 2007). Procaspase-8 also has a similar activation mechanism and both require being physically brought together onto an activation platform (Boatright et al., 2003). This model, called the induced proximity model, suggests that the role of the apoptosome is to bring multiple units of procaspase-9 together not to cause a cleavage event, but to induce dimer formation leading to a conformational change activating caspase-9 (Muzio et al., 1998; Boatright et al., 2003).

### **The Caspase Cascade**

Caspases are cysteine proteinases that cleave peptide bonds on the carboxyl side of aspartic acid residues (Cohen, 1997). Caspases are synthesized as zymogens or procaspases with little to no enzymatic activity. Procaspases are structurally organized into three regions; a prodomain, a large subunit, and a small subunit. Caspases involved in apoptosis (-2, -3, -6, -7, -8, -9, -10, -12) can be split into two groups based

on the size of their prodomains; initiator caspases with long prodomains (-2, -8, -9, -10) and executioner caspases with short prodomains (-3, -6, -7, -12). As previously alluded to, it is hypothesized that the long prodomains on initiator caspases can be activated by a dimerization event that causes a conformational change allowing for exposure of an enzymatic active site, while the short prodomain of executioner caspases requires a cleavage event for activation (Muzio et al., 1998; Boatright et al., 2003).

In the intrinsic apoptotic pathway, caspase-9 is activated by its recruitment to the apoptosome and dimerization as previously discussed. The executioner caspases -3 and -7 exist at physiological concentrations as dimers with no detectable activity. Active caspase-9 acts enzymatically on caspase-7 and caspase-3 to cleave the Asp-X bond between the prodomain and the large subunit and then cleave the Asp-X bond between the large and small subunits to allow the formation of a tetramer with two large and two small subunits (Muzio et al., 1998; Slee et al., 1999; Boatright et al., 2003). Activated caspase-3 has a variety of cellular targets including BCL2 family proteins, a variety of IAPs, various cytoskeletal proteins (e.g. gelsolin, fodrin, actin, keratin), other caspases (-2 and -6), and even a feedback amplification loop that further processes caspase-9 (Srinivasula et al., 1998; Slee et al., 1999).

Activation of caspase-2 and -6 is through a similar mechanism as caspase-3 activation, with cleavage of the prodomain and large and small subunits. Caspase-2 and -6 then activate caspase-8 and -10. The caspase cascade continues to disassemble the cell with cleavage of various proteins and finally terminates with the activation of DNA fragmentation factor.

DNA fragmentation factor (DFF) is a heterodimer consisting of DFFA and DFFB. DFFA and DFFB each contain their own nuclear localization signal (NLS) on the C-terminus. As a result, DFF is localized to the nucleus in non-apoptotic cells (Liu et al., 1998; Lechardeur et al., 2000). In addition to the normal 45 kDa form of DFFA, there is a splice variant that is 35 kDa (DFFA-short) that missing the C-terminal NLS and is therefore localized to the cytosol (Sakahira et al., 1999; Samejima and Earnshaw, 2000).

DFFB contains an endonuclease active site that is inhibited by its dimerization with DFFA (Enari et al., 1998; Sakahira et al., 1998). DFFA binds to DFFB during translation, allowing for the proper folding of DFFB into an enzymatically capable protein (Enari et al., 1998; Liu et al., 1998) with the possible involvement of HSPA1A and DNAJ homolog 1 (DNAJB1, previously HSP40) (Sakahira and Nagata, 2002). With activation of the caspase cascade, DFFA is cleaved at two specific sites by caspase-2, -3, or -7 (Liu et al., 1997; Widłak et al., 2003; Woo et al., 2004; Dahal et al., 2007). Cleavage of DFFA causes it to disassociate from DFFB, activating DFFB's endonuclease site (reviewed Widłak and Garrard, 2005).

After removal of DFFA, DFFB forms a homo-oligomer that increases its endonuclease activity (Liu et al., 1999; Woo et al., 2004), however this large unit can still be inhibited by binding of either DFFA or DFFA-short (Widłak et al., 2003). Multiple layers of activation and inhibition suggest that there are multiple fail-safes in non-apoptotic cells to prevent the accidental activation of DFFB (Widłak and Garrard, 2005).

DFFB cleaves DNA leaving double strand breaks with 5'-phosphate and 3'-hydroxyl groups exposed (Liu et al., 1999; Widłak and Garrard, 2005). Cleavage occurs

in two stages. The first stage is the cleavage of the chromatin loop domains in  $\geq 50$  kb intervals (Oberhammer et al., 1993) possibly due to DFFB interacting with other nuclear proteins such as histone H1 (Liu et al., 1999) and topoisomerase II $\alpha$  (Durrieu et al.). The second stage is the preferential cleavage of DNA into internucleosomal fragments (Wyllie et al., 1980).

### **The Role of Ceramide as a Signaling Molecule Triggering Apoptosis**

The early signaling mechanism for stressed-induced MOMP is not well understood. One possible pathway is through sphingomyelin signaling which involves hydrolysis of the phospholipid sphingomyelin (N-acylsphingosine-1-phosphocholine) into the sphingolipid ceramide. Ceramide is a second messenger in the stress-induced pathway caused by heat-shock, ultra-violet radiation, and ROS (Basu and Kolesnick, 1998; Chung et al., 2003; Moulin et al., 2007; de Castro e Paula and Hansen, 2008). Sphingomyelin is a ceramide linked to a phosphocholine with a phosphodiester bond (Kolesnick, 1991). Hydrolysis is performed by a sphingomyelin-specific phospholipase C termed sphingomyelin phosphodiesterase (SMPD; previously SMase). There are two specific forms of SMPD, an acidic form that functions at a pH around 5 (SMPD1) and neutral form that functions at a pH around 7.4 (SMPD2), both yield ceramide and phosphocholine (Kolesnick, 1991). Ceramide can have a variety of chain lengths ranging from a short-chain C(2)-ceramide to a very long-chain C(24)-ceramide. Chain lengths may affect how ceramide is involved in cellular responses (Kroesen et al., 2003; Senkal et al., 2007).

There are two potential ways ceramide may interact with mitochondrial signaling, one of which involves BCL2 family proteins. The first signaling mechanism is through the stimulation or inactivation of various kinase leading to the phosphorylation or

dephosphorylation of various members of the BCL2 family. Kinase pathways implicated in ceramide signaling include MAPK9/MAPK8 and kinase suppressor of RAS/ceramide-activated protein kinase (KSR1/CAPK) (Basu and Kolesnick, 1998; Basu et al., 1998). For example, the phosphorylation of the BH3-only pro-apoptotic protein BAD by v-akt murine thymoma viral oncogene homolog 1 (AKT1; previously AKT/PKB) on serine 112 and 136 prevents BAD from heterodimerizing with BCL2 or BCL2L1. Instead BAD is targeted for binding with members of the tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation proteins (YWHAQ; previously 14-3-3) that sequester and inactivate BAD (Zha et al., 1996). Stimulation of ceramide leads to activation of various kinase that cause prolonged deactivation of AKT1, resulting in the dephosphorylation of BAD on serine 112 and 136 allowing BAD to form heterodimers with BCL2 and BCL2L1 (Basu et al., 1998). Heat-shock induced ceramide signaling, has been shown to stimulate MAPK9/MAPK8, triggering apoptosis in human monoblastic leukemia cells and bovine aorta endothelial cells (Verheij et al., 1996). The addition of C(2)-ceramide to cells has also been shown activate MAPK9 in a concentration dependent manner and induce apoptosis (Verheij et al., 1996).

Besides ceramide's signaling interactions with BCL2 family proteins, ceramide may also affect mitochondrial biophysics either in cooperation or separately from involvement of the BCL2 family pathway. Ceramides has been shown modify membrane curvature in liposomes leading to their fragmentation (Holopainen et al., 2000) a similar action may occur in the mitochondria (Siskind et al., 2010). Addition of C(2)-ceramide altered the shape of the mitochondria, this was associated with a transient increase of DRP1 and fission 1 (FIS1), which are involved with mitochondrial

fission (Parra et al., 2008). In addition to of ceramide affecting mitochondrial membrane structure, ceramide has been shown to induce MOMP through the formation of ceramide channels in the absence of pro-apoptotic BCL2 family members BAX and BAK1 or synergistically in cooperation with them (Ganesan et al., 2010). BCL2 and BCL2L1 have also been shown to prevent the formation of ceramide channels probably by the inhibition of ceramide accumulation, and BCL2L1 has been shown to disassemble ceramide channels with an unknown mechanism (Siskind et al., 2008; Ganesan and Colombini, 2010).

### **BCL2 Family Proteins**

BCL2 was first identified as a proto-oncogene in human follicular lymphoma (Tsujimoto et al., 1984; Bakhshi et al., 1985; Cleary and Sklar, 1985). Whereas most oncogenes promote proliferation, BCL2 promoted cellular survival (Vaux et al., 1988; Núñez et al., 1990). Since the initial discovery of BCL2, other proteins in the family have been identified (BCL2L1, BCL2L2, BCL2L3, BCL2L10, BCL2L11, BCL2L12, BCL2L15, BAX, BAK1, BAD, BID, BMF, BOK, PMAIP1, BBC2). BCL2 family proteins are evolutionarily conserved, and a BCL2 homolog is encoded by a number of viruses, including the majority of gamma herpes viruses and African swine fever virus (Hardwick, 1998; Huang et al., 2002).

### **Structure**

BCL2 family proteins exist as a globular structure consisting of 5-7 amphipathic  $\alpha$ -helices surrounding two central hydrophobic helices (Muchmore et al., 1996; Petros et al., 2001). Similar structures have been characterized in the membrane-translocation domains of pore-forming bacterial toxins (Parker and Pattus, 1993; Petros et al., 2004). The C-terminal domain of the some of the BCL2 proteins (e.g. BCL2, BCL2L1, BAX,

BAK1) contain a single membrane spanning region consisting of hydrophobic amino acids that allows insertion into the membrane of the mitochondria, endoplasmic reticulum, and the nuclear envelope (Adams and Cory, 1998; O'Connor et al., 1998; Wang et al., 1998).

BCL2 family proteins also contain up to 4 BCL2 homology (BH) domains. These domains are important for protein-protein interactions, signal transduction, and regulation of cytosolic solubility (Figure 1-3). Anti-apoptotic family members (e.g. BCL2, BCL2L1, BCL2L2, and MCL1) contain all 4 BH domains (Yin et al., 1994; Chittenden et al., 1995; Hunter et al., 1996; Huang et al., 1998). It is the BH4 domain that allows the anti-apoptotic proteins to heterodimerize with other members of the BCL2 family.

Multi-domain pro-apoptotic family members contain either 3 BH domains (BH 1, 2, 3), e.g. BAX, BAK1, BCL2L1-short, and BCL2-related ovarian killer (BOK) (Yin et al., 1994; Chittenden et al., 1995). The BH3-only pro-apoptotic family members contain only 1 BH domain (BH3) (e.g. BID, BAD, BCL2L11, BIK, BMF, BLK, BCL-GS, PMAIP1, and BBC2) (Wang et al., 1996; Kelekar et al., 1997; Adams and Cory, 1998; Hsu et al., 1998; O'Connor et al., 1998; Oda, 2000; Wu and Deng, 2002).

The BH1, BH2, and BH3 domains form hydrophobic pockets which allow proteins existing in the cytoplasm, for example BAX and BCL2L1, to be soluble (Muchmore et al., 1996). The hydrophobic C-terminal transmembrane domain region inserts into the hydrophobic pocket created by the BH1 and BH2 domains (Nechushtan et al., 1999; Suzuki et al., 2000; Petros et al., 2004). Interactions of BH3-only proteins with multi-domain proteins are a result of the BH3 domain of the BH3-only protein acting as a

“donor,” and the multi-domain protein's hydrophobic pocket (BH 1, 2, 3) acting as an “acceptor” (Eskes et al., 2000; Wei et al., 2000; Letai et al., 2002; Cartron et al., 2004).

### **Membrane Permeabilization and Pore Formation**

The outer mitochondrial membrane is permeabilized by active BAX or BAK1. The N-terminus of BAX contains a localization sequence, termed apoptosis regulating and targeting sequence (ART), which allows the specific targeting of BAX to the MOM (Goping et al., 1998). The C-terminal contains the transmembrane domain which is inserted into the MOM (Eskes et al., 2000; Marani et al., 2002; Lucken-Ardjomande and Martinou, 2005; Youle and Strasser, 2008). Activation occurs with binding of certain BH3-only proteins (e.g. BID and BCL2L1) in response to apoptotic stimuli that in turn leads to several structural changes. The hydrophobic C-terminal is removed from the hydrophobic pocket, and the N-terminus undergoes a conformational change. Also, some BH3-only proteins recruit BAX to the MOM. For example, tBID is rapidly targeted to the MOM following an apoptotic signal (Lovell et al., 2008) even though it lacks a transmembrane domain (Wang et al., 1996). Once associated with the membrane, tBID recruits BAX, inducing a conformational change allowing hairpin formed by the  $\alpha 5$ - $\alpha 6$  helices of BAX to be inserted into the outer-membrane (Veresov and Davidovskii, 2009). Thereafter, BAX forms oligomers via interactions of BH3-hydrophobic pockets to form pores that allow components of the mitochondrial inter-membrane space proteins such as DIABLO, cytochrome c, AIF1, and endonuclease G to be released and trigger apoptosis (Antonsson et al., 2000; Eskes et al., 2000; Wei et al., 2000). This process can be prevented by the addition of BCL2L1, which can bind tBID and to a lesser extent with BAX (Billen et al., 2008). The BH3 protein, BAD, in turn, can neutralize BCL2L1 to allow BAX activation and pore formation (Lovell et al., 2008).

In contrast to BAX, BAK1 is constitutively inserted into the MOM where, in the absence of apoptotic signals, it is inhibited by binding with VDAC2, MCL1, and BCL2L1 (Cheng et al., 2003; Willis et al., 2005; Li et al., 2008). Like BAX, BAK1 activation is dependent on BH3-only proteins such as tBID, where binding causes a conformational change that exposes the BH3 domain of BAK1 allowing it to associate with the hydrophobic pocket of another BAK1 protein and form oligomers (Wei et al., 2000; Dewson et al., 2008). BAX and BAK1 are amphipathic proteins that contain several  $\alpha$ -helices that can bind to the water-lipid interface of the mitochondrial lipid bi-layer. BAX and BAK1 form multimer units in the mitochondrial membrane resulting in stretching the hydrocarbon core of the bi-layer in a concentration-dependent manner that leads eventually to the formation of a stable pore that is made up of both proteins and lipids (Lee et al., 2008; Qian et al., 2008).

### **Protein Interactions and Signal Transduction**

Many death stimuli are propagated by BH3-only proteins by signal transduction to the multi-domain BCL2 family members. Two different models for signal transduction have been proposed. The “direct activation” model suggests that certain BH3-only proteins (e.g. BID, BCL211, and BBC3) bind directly to the hydrophobic pocket of BAX or BAK1 activating them. The truncated form of BID, tBID, helps recruit BAX to the MOM (Lovell et al., 2008) and participates in the oligomerization and pore-formation with BAX (Eskes et al., 2000; Tan et al., 2001; Marani et al., 2002; Cartron et al., 2004). BAK1, on the other hand, resides in complexes in the mitochondrial outer-membrane and endoplasmic reticulum (Wei et al., 2000). Binding of BH3-only proteins causes a conformational change that allows for BAK1 oligomerization (Wei et al., 2000; Cheng et al., 2003; Willis et al., 2005; Dewson et al., 2008).

In addition to activation of pro-apoptotic BCL2 family members (BAX and BAK1) by “activator” BH3-only protein, the “direct activation” model also proposes a second group of BH3-only proteins that function in an “inhibitory” capacity. These “inhibitory” BH3-only proteins (BAD and BIK) bind the anti-apoptotic proteins (BCL2 and BCL2L1) preventing them from binding to the “activator” BH3-only proteins (tBID, BCL2L11, and BBC3). As a result, the “activator” BH3-only proteins are available to bind and activate BAX and BAK1 (Letai et al., 2002).

As is apparent from the previous paragraph, the role of the anti-apoptotic family members in the “direct activation” model of apoptosis is to bind and neutralize the pro-apoptotic “activator” BH3-only proteins preventing their action on BAX/BAK1. In the second model, termed “indirect activation,” the BH3-only proteins activate BAX/BAK1 by removing the direct inhibition of the anti-apoptotic proteins on BAX and BAK1 (Adams and Cory, 1998). In this model, BAX and BAK1 are constitutively bound to BCL2 and BCL2L1, thereby they are prevented from acting on the MOM. Upon signal transduction, the BH3-only proteins bind to BCL2 and BCL2L1 via the the BH3 domain interacting with the hydrophobic pocket of BCL2 and BCL2L1 freeing BAX and BAK1 (Kelekar et al., 1997; Sattler et al., 1997). Free BAX and BAK1 then can form multimers within the MOM causing MOMP without the need for further activation.

Current evidence suggests that signal transduction involves a combination of events described in both models. The activation of BAX and BAK1 by “activator” BH3-only proteins has been demonstrated often (Wang et al., 1996; Desagher et al., 1999; Eskes et al., 2000; Wei et al., 2000; Letai et al., 2002; Cheng et al., 2003; Cartron et al., 2004; Lovell et al., 2008; Kim et al., 2009). There is also evidence showing inhibitory

function of anti-apoptotic proteins directly on the BAX and BAK1. Interestingly, BCL2, which is expressed only in the mitochondrial outer-membrane, binds to and inhibits BAX localized in the cytosol (Zha and Reed, 1997; Wang et al., 1998). BCL2L1, which is membrane bound and cytosolic, also binds and inhibits BAX (Billen et al., 2008). Finally, BCL2L1/MCL1, but not BCL2/BCL2L2, sequester and inhibit BAK1 until BH3-only activation (Willis et al., 2005).

### **Regulation of BCL2 Family Members**

Tight regulatory controls of BCL2 family proteins are necessary in order to preserve the balance of life and death. Like the apoptotic cascade itself, there are multiple layers of regulation. The first layer is at the level of gene expression. While some of the BCL2 family proteins are expressed ubiquitously, certain members, especially the pro-apoptotic BH3-only members, are transcriptionally upregulated upon an apoptotic stimulus. One example is the p53-dependent upregulation of the BH3-only pro-apoptotic proteins phorbol-12-myristate-13-acetate-induced protein 1 (*PMAIP1*; previously NOXA) and BCL2 binding component 3 (*BBC3*; previously PUMA) upon DNA damage (Oda, 2000; Nakano and Vousden, 2001). Another example is the upregulation of expression of the BH3-only pro-apoptotic gene *BCL2L11-EL* by serum or growth factor starvation (Chalmers et al., 2003; Biswas et al., 2007). Interestingly, upregulation of *BCL2L11-EL* expression could be blocked by addition of the serine proteinase thrombin (Chalmers et al., 2003).

Survival factors, like apoptotic signals, can also effect gene expression of BCL2 family members. For example, insulin-like growth factor-1 (IGF1) causes reduced expression of the pro-apoptotic gene *BCL2L11* (De Bruyne et al., 2010), while the

addition of granulocyte-macrophage stimulating factor (CSF2) leads to increased expression of the anti-apoptotic gene *MCL1* (Chao et al., 1998).

Besides inducible changes in gene expression by various factors, there is also temporal and cell/tissue specific variation in gene expression of BCL2 family members. An example is the reciprocal expression of *BCL2* and *BCL2L1* in various lymphocyte populations (Chao and Korsmeyer, 1998). *BCL2* is highly expressed in pro-B-cells and mature B-cells, but there is low expression in pre-B-cells. In contrast, *BCL2L1* has low expression in pro-B-cells and high expression in pre-B-cells (Chao and Korsmeyer, 1998). Finally, *BOK*, *BCL2L10* and *BCL2L11* are only expressed in the reproductive tissues (Hsu et al., 1997; Song et al., 1999).

Alternative splicing is another form of regulation for BCL2 family members. Leading to multiple isoforms in the majority of the BCL2 family members. Some variants have no apparent effect on the resulting protein such as the anti-apoptotic protein BCL2L1 (Ko et al., 2003), or the pro-apoptotic protein BAD (Hamnér et al., 2001; Seo et al., 2004). For other splice variants, certain isoforms have greater potency. BCL2 has a long and short isoform (BCL2-L and BCL2-S) of which the long form is more potent (Hockenbery et al., 1993). The pro-apoptotic protein BCL2L11 has three isoforms, BCL2L11-S, BCL2L11-L and BCL2L11-EL. The shortest of these, BCL2L11-S, is the most potent (O'Connor et al., 1998).

Alternative splicing can also affect the functional properties of the protein. BAX has two isoforms, BAX $\alpha$  and BAX $\beta$ , which share BH1-3 domains but differ in their C-terminal. BAX $\beta$ 's unique C-terminal targets it for immediate proteasomal degradation. With apoptotic stimuli, BAX $\beta$  is upregulated by inhibition of its ubiquitination. Moreover,

changes in the C-terminal make BAX $\beta$  constitutively-active so that it can be inserted into the MOM without need for further activation (Fu et al., 2009). Splice variants can even result in ordinarily anti-apoptotic proteins acting as pro-apoptotic proteins as is seen with BCL2L1 (Boise et al., 1993) and MCL1 (Bingle et al., 2000). Similarly, some isoforms of the pro-apoptotic BID can act as an anti-apoptotic protein (Renshaw et al., 2004).

Some splice variants only occur in specific cell types or tissues. BCL2L14 is a pro-apoptotic protein that has two isoforms, BCL2L14-L and BCL2L14-S. BCL2L14-L is widely expressed while BCL2L14-S is only expressed in the testis (Guo et al., 2001). Another example is the fourth isoform of BCL2L11 called BCL2L11- $\gamma$  which is mostly expressed in the small intestine and colon (Liu et al., 2002). The final and perhaps most complex example is a splice variant of BAK1 called N-BAK1, which has cell-specific expression and function. BAK1 is expressed ubiquitously except in central and peripheral neurons, which is the only location where N-BAK1 is expressed. *N-BAK1* includes a novel 20-base pair exon that changes BAK1 from a pro-apoptotic multi-domain (BH 1, 2, 3) protein to a BH3-only protein. As a BH3-only protein, N-BAK1 functions in neurons as an anti-apoptotic protein, but if *N-BAK1* is experimentally expressed in a non-neuronal cell type it functions as a pro-apoptotic BH3-only protein (Sun et al., 2001).

The final layer of regulation of BCL2 family members involves post-translational modifications. Post-translational modifications activate BCL2 by phosphorylating the serine residual Ser70 (Haldar et al., 1997; Ito et al., 1997; Maundrell et al., 1997). In addition to activation events, post-translation modifications can also lead to the

inactivation of anti-apoptotic proteins. BCL2 and BCL2L1 inhibit pro-apoptotic proteins by inserting their BH4 domain into the pro-apoptotic proteins' hydrophobic pocket created by the BH2 and BH3 domains. In response to apoptotic stimuli BCL2 and BCL2L1 are phosphorylated on serine residuals in the loop region between their BH4 and BH3 domains (Figure 1-3) (Fang et al., 1998; Poruchynsky et al., 1998) possibly by activated MAPK8 (Chang et al., 1997). This phosphorylation event prevents the binding of BCL2 and BCL2L1 to their pro-apoptotic targets (Yamamoto et al., 1999). Other post-translational modifications convert BCL2 and BCL2L1 from anti-apoptotic to pro-apoptotic proteins. Caspase-3 can cleave BCL2 and BCL2L1 in the loop region resulting in the production of C-terminal truncations (tBCL2 and tBCL2L1) that are missing their BH4 domain changing the function protein to pro-apoptotic (Chang et al., 1997).

Post-translation regulation is also used on pro-apoptotic proteins to maintain them in an inactive state until apoptotic stimuli. MAPK8 and phosphatidylinositol 3-kinase can phosphorylate BAX at Ser184, leading to its insertion into the mitochondrial membrane (Kolliputi and Waxman, 2009). BAK1 is also regulated by mitogen-activated protein kinase kinase kinase 1 (MAP3K1; previously MEKK1) and MAPK8 (Ihrlund et al., 2006). MAP3K1 is involved in the conformational change caused by binding to proteins like tBID and may also prevent deconvolution of BAK1 by cross linking several residues. After unfolding and activating BAK1, it has been proposed that MAPK8 induces the formation of BAK1 complexes of 80-170 kDa (Ihrlund et al., 2006).

The pro-apoptotic BH3-only proteins are another group of BCL2 family members regulated by post-translational modifications. This group of proteins uses

phosphorylation, dephosphorylation, and cleavage events for regulation, resulting in changes in cellular localization and binding potential. BAD is phosphorylated at Ser112 by cAMP-dependent kinase (Zha et al., 1996; Harada et al., 1999) and Ser136 by AKT1 (Datta et al., 1997). The phosphorylation of these two serine causes BAD to be sequestered in the cytosol by YWHAQ proteins, preventing its interaction with anti-apoptotic proteins (Zha et al., 1996; Datta et al., 1997). Dephosphorylation occurs in response to an apoptotic stimulus leading to its release from YWHAQ and allowing BAD to contribute to cell death (Zha et al., 1996).

BID is activated by caspase-8 dependent cleavage (Li et al., 1998). This cleavage event occurs in different parts of the loop region leading to the formation of a truncated form of BID (tBID) (Li et al., 1998). This truncation event leads to the exposure of a hydrophobic pocket that was blocked by the  $\alpha 1$  and  $\alpha 2$  helices allowing tBID to translocate to the MOM (Lovell et al., 2008).

### **Apoptosis in the Preimplantation Embryo**

#### **Occurrence of Apoptosis in the Oocyte and the Preimplantation Embryo**

Spontaneous apoptosis is used throughout life as a means to deplete ovarian pools of oocytes (Morita and Tilly, 1999; Perez et al., 1999; Tilly, 2001), signal cell death in ovulated and cultured oocytes (van Blerkom and Davis, 1998; Perez et al., 1999), and as a mechanism responsible for stress-induced infertility. For example, dairy cattle show a decrease in fertility rates during summer as a result of heat-stress (Badinga et al., 1985; Cavestany et al., 1985; al-Katanani et al., 1999) that may be due to heat-shock induced apoptosis in the oocyte (Roth and Hansen, 2004). In addition to the oocyte, spontaneous apoptosis has also been well documented in preimplantation embryo for variety of species including; mouse, rabbit (Fabian et al., 2007), human

(Hardy et al., 1993; Jurisicova et al., 1996), bovine (Byrne et al., 1999; Matwee et al., 2000), and porcine (Long et al., 1998). Several comparative studies also shown that there was higher incidence of apoptosis for embryos produced *in vitro* as compared embryos produced *in vivo* (Long et al., 1998; Gjørret et al., 2003). It is because of the importance of apoptosis for determining developmental potential of the oocyte and embryo that it is important to understand mechanisms involved in activation of apoptosis in the oocyte and early cleavage embryo.

To understand apoptosis in the preimplantation embryo, a variety of studies have used various stimuli to induce an apoptotic response: pro-oxidants (Yang et al., 1998; Feugang et al., 2003; Feugang, 2004; Moss et al., 2009), ultraviolet radiation (Herrler et al., 1998), heat-shock (Paula-Lopes and Hansen, 2002a), ceramide (de Castro e Paula and Hansen, 2008), arsenic (Krininger et al., 2002), and TNF (Soto et al., 2003; Loureiro et al., 2007). In these experiments, the hallmark of apoptosis has been DNA fragmentation measured by terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling (TUNEL) (Gavrieli et al., 1992).

Stress induced apoptosis is mediated through a caspase-dependent pathway in oocytes and preimplantation embryos. Stress leads to activation of the caspase cascade beginning with the initiator caspase, caspase-9 (Brad et al., 2007; Loureiro et al., 2007; de Castro e Paula and Hansen, 2008) which leads to the induction of group II caspases, including caspase-2, -3, -7 which are responsible for the activation of DFFB leading to DNA fragmentation and TUNEL (Krininger et al., 2002; Paula-Lopes and Hansen, 2002; Paula-Lopes and Hansen, 2002; Roth and Hansen, 2004; Brad et al., 2007). One of the best studied stress stimuli in preimplantation embryos is heat-shock.

Not only does heat-shock induce caspase-9 activity (Brad et al., 2007; Loureiro et al., 2007) and group II caspase activity (Paula-Lopes and Hansen, 2002; Paula-Lopes and Hansen, 2002), but inhibition of caspases with z-LEHD-fmk, a specific inhibitor of caspase-9, or z-DEVD-fmk, a specific inhibitor of group II caspases, blocks heat-shock induced TUNEL in the oocyte and preimplantation embryo (Paula-Lopes and Hansen, 2002; Roth and Hansen, 2004; Loureiro et al., 2007)

### **Apoptosis as a Protective Mechanism**

One of the most interesting features of heat-shock induced apoptosis, in the preimplantation embryo, is the protective role that apoptosis plays. When a preimplantation embryo is exposed to a stress signal, upregulation of apoptosis can help the embryo survive. This has been shown experimentally in several studies. Paula-Lopes and Hansen (2002a) pre-treated bovine embryos that were  $\geq 16$ -cells on day 4 of culture with 200  $\mu\text{M}$  z-DEVD-fmk or vehicle for 15 h at 38.5°C. After pre-treatment embryos were exposed to heat-shock for 9 h at 41°C and then cultured continuously at 38.5°C until day 8 when blastocyst rate was measured. Addition of z-DEVD-fmk blocked the induction of group II caspases and DNA fragmentation caused by heat-shock. Control embryos, which were continuously cultured at 38.5°C in either vehicle or z-DEVD-fmk, had 20% blastocyst development. Embryos that were exposed to heat-shock for 9 h had a significant decrease blastocyst development to 10%. Pre-treatment of heat-shock embryos with z-DEVD-fmk significantly lowered percent blastocyst development to 3%. Showing that while heat-shock has a negative effect on development, if apoptosis is also inhibited by a caspase inhibitor development is further reduced. In another study, embryos collected at Day 5 of development, and treated with z-DEVD-fmk did not modify embryo survival after heat-shock (Jousan and Hansen,

2007). However, when embryos were also treated with IGF1, which also reduced apoptosis, effects of heat-shock were greater when embryos were also treated with z-DEVD-fmk.

### **Developmental Regulation of Apoptosis in Preimplantation Embryos**

Experiments evaluating effects of stress on the bovine preimplantation embryo can be interpreted as indicating that there is a developmental acquisition of resistance to cellular stresses as the embryo advances in development. For example, embryos become more resistant to the effects of heat-shock as they develop, with the 2-cell embryo being most susceptible (Edwards and Hansen, 1997; Krininger et al., 2002; Sakatani et al., 2004). In fact, the oocyte, is more resistant to effects of heat-shock on development than the 2-cell embryo (Edwards and Hansen, 1997). One mechanism that could explain this developmental acquisition of resistance is apoptosis.

Apoptosis occurs in the oocyte where it is involved in cell death in ovulated and cultured oocytes (van Blerkom and Davis, 1998; Morita et al., 1999; Perez et al., 1999; Tilly, 2001). For example, Roth and Hansen (2004) matured bovine oocytes at 38.5°C, 40°C, and 41°C. Maturation under heat-shock conditions reduced the number of oocytes that cleaved and the percentage that became blastocysts. Moreover, treatment of oocytes with heat-shock increased group II caspase activity along with TUNEL. Blocking apoptosis with z-DEVD-fmk blocked the effect of heat-shock on oocyte competence for development (Roth and Hansen, 2004).

During or after fertilization, apoptosis becomes blocked. Apparently spontaneous apoptosis is first observed at different time points in early preimplantation development: the late 1-cell in mouse (Jurisicova et al., 1998), 2-cell to uncompact morulae in human (Jurisicova et al., 1996), 16-cell in rabbit (Fabian et al., 2007), and 8-16 cells in

porcine and bovine (Long et al., 1998; Byrne et al., 1999; Matwee et al., 2000).

Developmental acquisition of apoptosis has also been shown in response to apoptotic stimuli. Paula-Lopes and Hansen (2002b) treated 2-cell and  $\geq 16$ -cell embryos with heat-shock at 41°C for 9 h. In both heat-shock and control 2-cell embryos there was no TUNEL, while in  $\geq 16$ -cell embryo there was a significant increase in heat-shock induced TUNEL. Both control and heat-shocked 2-cell embryos had very low amounts of group II caspase activity, where  $\geq 16$ -cell embryos had higher amounts of groups II caspase activity in control embryos, compared to the 2-cell, and there was significant heat-shock induced increase of group II caspase activity.

Due to the temporal coincidence of early signs of apoptosis and embryonic genome activation (EGA), it has been hypothesized that apoptotic regulation in the preimplantation embryo is dependent on EGA (Jurisicova et al., 1998; Byrne et al., 1999). Early cleavage embryos are in a transcriptionally quiescent state where the majority of the proteins produced during this period are translated from maternal stores of mRNA in the oocyte (van Blerkom, 1981). There is a transition over time from maternal stores to new transcripts produced by the embryo (Flach et al., 1982), with minor amounts of transcription occurring followed by a major wave transcription that occurs again in a species specific manner; late 1-cell to early 2-cell in the mouse (Aoki et al., 1997; Aoki et al., 2003), 4- to 8-cell stage in human, porcine, equine, and feline: (Braude et al., 1988; Tománek et al., 1989; Brinsko et al., 1995; Hoffert et al., 1997) and 8-16 cell stage in rabbit, ovine, and bovine (Crosby et al., 1988; Kopečný et al., 1989; Memili et al., 1998).

## Possible Causes for Developmental Regulation

A series of studies have looked at various aspects of the apoptotic cascade and its developmental regulation to try to elucidate why there is a need for EGA and transcription. As previously discussed, heat-shock induced apoptosis begins with the conversion of sphingomyelin into ceramide (Chung et al., 2003). To investigate if the developmental regulation of apoptosis was due to a failure to initiate this signaling cascade, de Castro e Paula and Hansen (2008) compared 2-cell and  $\geq 16$ -cell embryos treated with 50  $\mu\text{M}$  ceramide. Embryos treated at  $\geq 16$ -cells with ceramide had an increase in caspase-9 activity, a decrease in number of nuclei, an increase in TUNEL, and a decrease in further development. Treatment of 2-cell embryos with ceramide also decreased developmental competence, but ceramide did not increase caspase-9 activity or TUNEL. Thus, there is a block to apoptosis in the 2-cell embryos at one or more regulatory events downstream of ceramide signaling.

Another approach to studying developmental regulation of apoptosis has been to treat embryos with staurosporine. This protein kinase inhibitor can activate apoptosis through a caspase-dependent or caspase-independent pathway (Belmokhtar et al., 2001; Nicolier et al., 2009). Caspase-dependent effects of staurosporine cause DNA cleavage in less than 3 h, where the addition of a caspase inhibitor or use of a cell line with defective caspases delays DNA cleavage until after 12 h of treatment (Belmokhtar et al., 2001). Caspase-dependent effects of staurosporine involve regulation of the BCL2 family of proteins such as the activation of BAD by inhibition of its hyperphosphorylation (Zha et al., 1996) and through the cleavage of BAD-L to BAD-S by caspase (Seo et al., 2004). The caspase-independent effects of staurosporine involve either AIF1 (Nicolier et al., 2009) or endonuclease G (Zhang et al., 2003).

Matwee et al. (2000) treated bovine embryos ranging from 1- to 16-cells with 10  $\mu$ M staurosporine for 30 hours. Treated embryos did not cleave during the 30 h of culture and 56 of 59 embryos (95%) displayed TUNEL. The authors concluded that cell death could be induced with staurosporine in the preimplantation embryo, but there may be specific upstream mechanisms necessary for the induction of apoptosis by other common environmental stressors. Matwee et al. (2000) observed no cell cycle events during staurosporine treatment of 30 h. The lack of cell cycle is unusual for this stage of development, suggesting an interaction of staurosporine with cell cycle events may also have an effect on DNA fragmentation.

In a second study, Gjørret et al. (2007) treated various stages of bovine preimplantation embryos with 10  $\mu$ M staurosporine for 24 h. There was no induction of TUNEL in 2-cell embryos and only small amounts of TUNEL in 4- and 8-cell embryos. However, most morulae and blastocyst had several blastomeres with TUNEL. Similarly, there was no activated caspase-3 in 2-cell embryos, only small amounts of activated caspase-3 in 4- and 8-cell embryos. Again like TUNEL, the majority of morulae and blastocysts had several blastomeres with activated caspase-3. Taken together these data suggest that the 2-cell embryo may have the capacity for staurosporine induced DNA fragmentation, however due to the extended period of treatment (24-30 h) and the lack of a caspase inhibition study it is unclear if DNA fragmentation is through a caspase-dependent mechanism.

Another model to study developmental regulation of apoptosis has been to evaluate effects of the chemical depolarization of the mitochondria using carbonyl cyanide 3-chlorophenylhydrazone (CCCP). CCCP bypasses the control of the BCL2

family of proteins to chemically depolarize the mitochondrial membrane and sets into motion the caspase cascade. Brad et al. (2007) found that CCCP led to the activation of caspase-9 and group II caspases in both the 2-cell and  $\geq 16$ -cell embryos. Thus, an intact system leading to activation of executioner caspase is present in the 2-cell embryo. However, CCCP did not induce TUNEL in 2-cell embryos, although it did in  $\geq 16$ -cell embryos, suggesting that there is block at the level of the nucleus preventing caspase-dependent DNA fragmentation.

One possible explanation for the lack of DNA fragmentation could be the structure of the DNA in the early preimplantation embryo. Prior to the EGA, the DNA is highly methylated (Jung Sun Park et al., 2007). As the embryo approaches EGA, the DNA becomes demethylated with each cell cycle (Dean et al., 2001). Carambula et al. (2009) found that treatment of 2-cell embryos with 100  $\mu$ M of 5-aza-2'-deoxycytidine (AZA) to reduce DNA methylation or 100nM trichostatin-A (TSA) to inhibit histone deacetylation allowed a CCCP dependent increase of TUNEL positive nuclei in 2-cell embryos. These data suggest that that apoptosis is blocked by the inaccessibility of DNA to DFFB due to extensive chromatin structure caused by DNA methylation and histone acetylation.

### **Thesis**

In addition to the nuclear block, Brad et al. (2007) identified a second developmental block of apoptosis in the bovine preimplantation embryo; at the level of the mitochondria. Bypassing the upstream intrinsic signaling mechanism, CCCP induces mitochondrial depolarization and caspase activation (Brad et al., 2007). However, induction of the upstream signaling mechanism, specifically the BCL2 family of proteins, with either heat-shock or ceramide fails to induce mitochondrial

depolarization and caspase activation in 2-cell embryo (Paula-Lopes and Hansen, 2002; de Castro e Paula and Hansen, 2008). Indicating that anti- and pro-apoptotic proteins involved in this upstream signaling mechanism may be developmentally regulated.

To further investigate the developmental acquisition of apoptosis in the bovine embryo, we hypothesize that the block at the level of the mitochondria involves an upregulation of anti-apoptotic genes (BCL2, BCL2L1, HSPA1A, DFFA) and a down regulation of pro-apoptotic genes (BAX, BAD, DFFB) in the 2-cell bovine preimplantation embryo.

Objective 1 measure steady state mRNA using quantitative real-time RT-PCR to identify transcriptional regulation in the oocyte, 2-cell embryo, 2-cell embryo treated with a transcription inhibitor  $\alpha$ -amanitin, and embryos that have undergone genome activation ( $\geq 16$ -cell).

Objective 2 determine the presence and quantify immunoreactive concentrations of anti-apoptotic proteins (BCL2, BCL2L1, HSPA1A) and pro-apoptotic proteins (BAX, BAD) using immunocytochemistry to identify protein expression differences between 2-cell and  $\geq 16$ -cell bovine embryos.

Objective 3 verify significant differences found by immunocytochemical results by Western blotting.

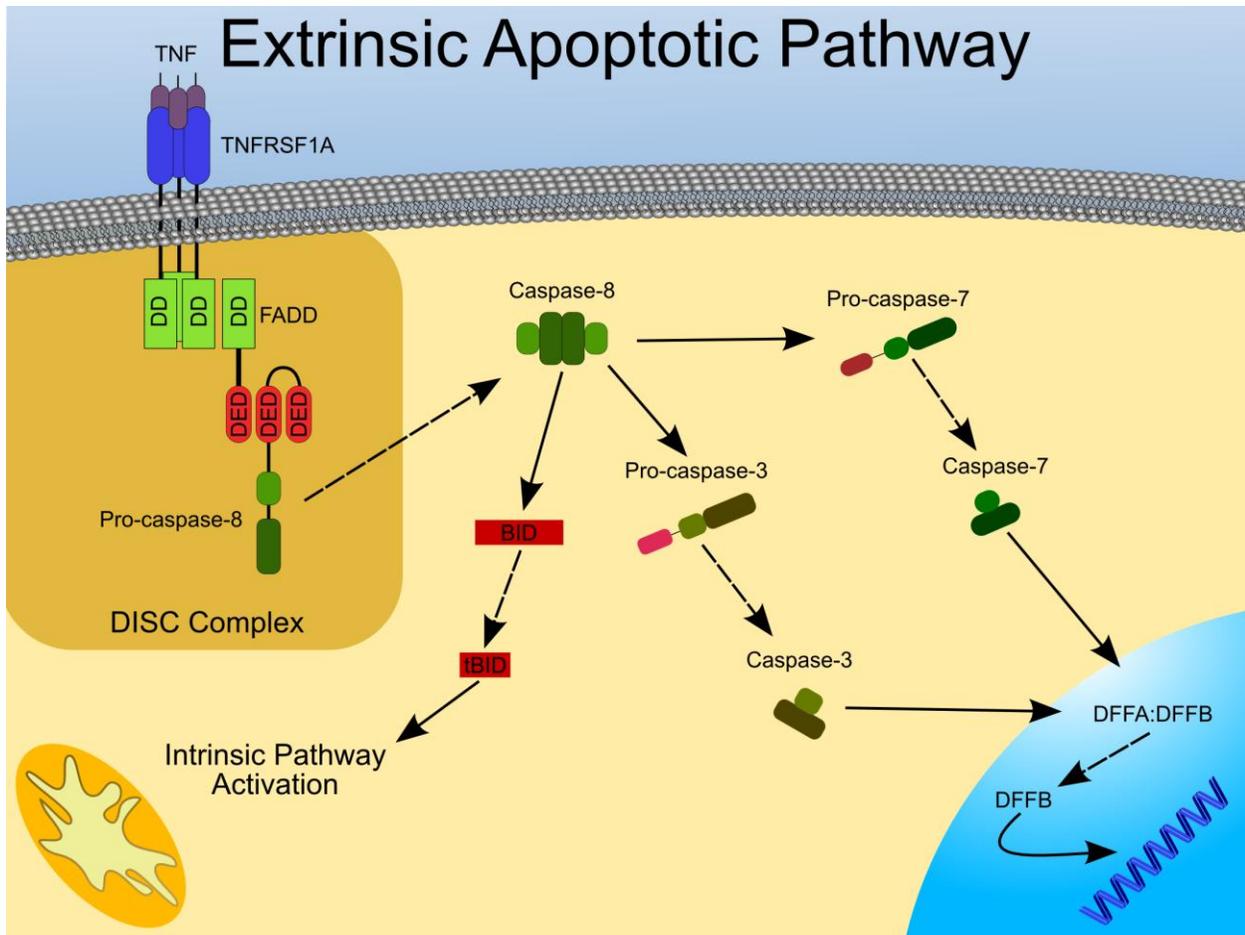


Figure 1-1. Schematic of the extrinsic or receptor-mediated apoptotic pathway. Dashed lines represent a cleavage event changing a protein from an inactive to active form. Solid lines represent the protein acting upon another molecule. Adapted from MacFarlane (2003).

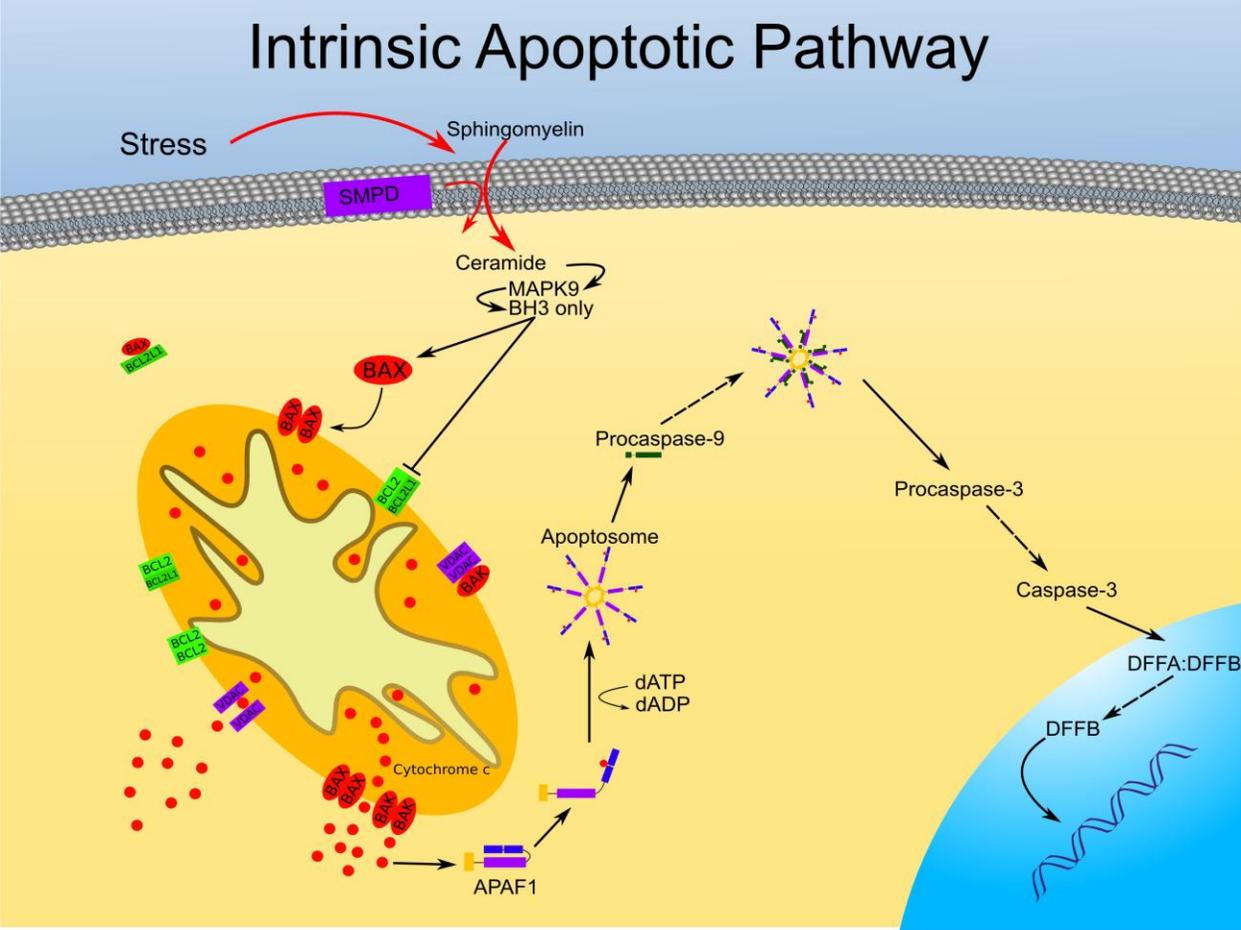
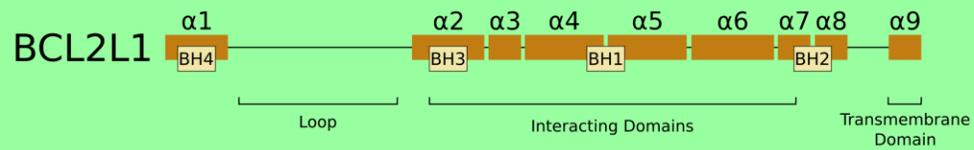


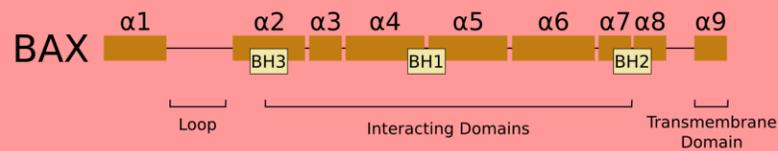
Figure 1-2. Schematic of the intrinsic or mitochondrial apoptotic pathway. Dashed lines represent a cleavage event changing a protein from an inactive to active form. Solid lines represent the protein acting upon another molecule.

## Anti-Apoptotic



## Pro-Apoptotic

### Multi-domain



### BH3-only

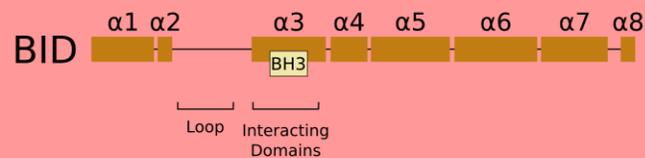


Figure 1-3. Schematic drawing of the BCL2 family proteins. There are two major forms of BCL2 family proteins, anti-apoptotic and pro-apoptotic. BCL2L1 (green) represents the anti-apoptotic proteins including BCL2, BCL2L2, and MCL1. The pro-apoptotic BCL2 family members (red) are further separated into those that contain multiple BH domains (multi-domain) and those that contain only the BH3 domain (BH3-only). BAX represents the multi-domain pro-apoptotic proteins including BAK1 and BOK. BID represents BH3-only proteins including BAD and BCL2L11. Adapted from Yin et al. (2003).

## CHAPTER 2 DEVELOPMENTAL ACQUISITION OF APOPTOSIS IN THE BOVINE PREIMPLANTATION EMBRYO

### **Introduction**

Exposure of preimplantation embryos to a variety of cellular stresses can induce apoptosis in all or a fraction of blastomeres. Among the conditions that induce apoptosis in the bovine embryo are heat-shock (Paula-Lopes and Hansen, 2002; Paula-Lopes and Hansen, 2002), arsenic (Krininger et al., 2002), pro-oxidants (Feugang et al., 2003; Feugang, 2004; Moss et al., 2009), and tumor necrosis factor- $\alpha$  (TNF) (Soto et al., 2003; Loureiro et al., 2007). The consequences of apoptosis on the developmental competence of the embryo are dependent on the extent of its induction. Induction of apoptosis in up to 30% of blastomeres, as for example occurs after TNF administration (Soto et al., 2003a), has no effect on development to the blastocyst stage. In fact, apoptosis may allow the embryo to survive stress by removal of damaged cells. Inhibition of apoptosis by the addition of the group II caspase inhibitor z-DEVD-fmk exacerbated the deleterious effect of heat-shock on development to the blastocyst stage (Paula-Lopes and Hansen, 2002; Jousan and Hansen, 2007). Extensive apoptosis, as it occurs after inhibition of survivin synthesis through RNA interference (SY Park et al., 2007), leads to a block in development.

Induction of apoptosis is developmentally regulated. In cultured bovine embryos, TUNEL-positive cells are first seen between the six- and eight-cell stage (Matwee et al., 2000; Gjørret et al., 2003). Similarly, acquisition of an apoptotic response to heat-shock (Paula-Lopes and Hansen, 2002b) and TNF (Soto et al., 2003a) also occurs after the eight-cell stage. Heat shock induces apoptosis through the mitochondrial or intrinsic pathway by activation of caspase-9 and group II caspases such as caspase-3 (Krininger

et al., 2002; Paula-Lopes and Hansen, 2002; Paula-Lopes and Hansen, 2002; Brad et al., 2007; Loureiro et al., 2007). The induction of apoptosis by heat-shock, as shown by TUNEL, can be blocked by the addition of caspase-9 or group II caspase inhibitors (Paula-Lopes and Hansen, 2002; Loureiro et al., 2007). Interestingly, TNF also acts in the bovine preimplantation embryo through a caspase-9 dependent pathway (Loureiro et al., 2007). The inhibition of apoptosis in early-cleavage stage embryos involves at least two blocks in the intrinsic pathway. The mitochondria itself is resistant to depolarization since neither heat-shock (Paula-Lopes and Hansen, 2002; Brad et al., 2007) nor ceramide (a putative signaling molecule for activation of the intrinsic pathway) (de Castro e Paula and Hansen, 2008) induce caspase-9 or group II caspase activation in the 2-cell embryo. In addition, the nucleus is resistant to caspase-mediated TUNEL. Artificial activation of the intrinsic pathway by carbonyl cyanide 3-chlorophenylhydrazone (CCCP), a chemical inducer of mitochondrial depolarization, activated caspase-9 and group II caspases in 2-cell embryos but did not result in DNA fragmentation (Brad et al., 2007). Resistance to caspase-activated DNases is caused, at least in part, by epigenetic modifications that can be reversed by interfering with DNA methylation or histone deacetylation (Carambula et al., 2009).

The objective of the present study was to determine the molecular basis for the developmental changes in mitochondrial and nuclear responses to pro-apoptotic signals. We hypothesized that the 2-cell embryo contains more anti-apoptotic proteins and less pro-apoptotic proteins as compared to the  $\geq 16$ -cell embryo. The focus was on several members of the BCL2 family of proteins regulating mitochondrial permeability in response to pro-apoptotic signals (Youle and Strasser, 2008), heat shock protein 70

(HSPA1A), which can inhibit mitochondrial depolarization by interrupting the BCL2 family signaling cascade (Stankiewicz et al., 2005), inhibit caspase activation, and caspase-3 activity (Garrido et al., 2001), and DFFA (also called inhibitor of caspase-activated DNase), which is cleaved during apoptosis to activate caspase-activated DNase (DFFB) (Liu et al., 1997).

## **Materials and Methods**

### **Materials**

Media for in vitro production of embryos were obtained as follows. HEPES-Tyrodes Lactate (TL) and IVF-TL were purchased from Millipore (Billerica, MA, USA) or Caisson Laboratories, Inc. (North Logan, UT, USA) and used to prepare HEPES-Tyrodes albumin lactate pyruvate (TALP) and IVF-TALP as described by Parrish et al. (1986). Oocyte collection medium was Tissue Culture Medium 199 (TCM-199) with Hank's salts without phenol red (Atlanta Biologicals, Lawrenceville, GA, USA) supplemented with 2% (v/v) bovine steer serum (Pel-Freez Biologicals, Rogers, AR, USA) containing 2 U/mL heparin, 100 U/mL penicillin-G, 0.1 mg/mL streptomycin, and 1 mM glutamine. Oocyte maturation medium was TCM-199 (Gibco®, Invitrogen, Carlsbad, CA, USA) with Earle's salts supplemented with 10% (v/v) bovine steer serum, 2 µg/mL estradiol 17-β, 20 µg/mL bovine FSH (Folltropin-V; Agtech Inc., Manhattan, KS, USA), 22 µg/mL sodium pyruvate, 50 µg/mL gentamicin sulfate (Sigma-Aldrich, St. Louis, MO, USA), and 1 mM L-glutamine or alanyl-glutamine. Percoll was from GE Healthcare (Uppsala, Sweden). Frozen semen from various bulls was donated by Southeastern Semen Services (Wellborn, FL, USA). Potassium simplex optimized medium (KSOM), containing 1 mg/mL bovine serum albumin, was obtained from Millipore or Caisson Laboratories Inc. KSOM was modified with 3 mg/mL essentially

fatty-acid free BSA (Sigma-Aldrich), 2.5 mg/mL gentamicin, 100 U/mL penicillin-G, and 1x nonessential amino acids (Sigma-Aldrich), to produce KSOM-Bovine Embryo 2 (BE2) as described elsewhere (Soto et al., 2003b).

Medium for culture of BEND cells was prepared using powdered Eagle's Minimum Essential Medium and Ham's F12 from Sigma-Aldrich. Fetal bovine serum and heat-inactivated horse serum were from Atlanta Biologicals. Penicillin-streptomycin was purchased from Millipore, human recombinant insulin was purchased from Invitrogen, and D-valine and sodium bicarbonate were from Sigma-Aldrich. BEND cell culture medium, as previously described (Staggs et al., 1998), was 4.81 g/L powdered Eagle's Minimum Essential Medium and 5.35 g/L powdered Ham's F-12 with 10% (v/v) fetal bovine serum, 10% (v/v) heat-inactivated horse serum, 0.2 U/mL insulin, 0.034 mg/mL D-valine, 1.685 mg/mL sodium bicarbonate, 100 U/mL penicillin-G, and 0.1 mg/mL streptomycin.

### **In Vitro Production of Embryos**

Embryo production was performed as previously described (Soto et al., 2003b). Briefly, a mixture of beef and dairy cattle ovaries were obtained from a local abattoir (Central Beef Packing Co., Center Hill, FL, USA) and cumulus-oocyte complexes (COCs) were collected by slicing follicles that were 2 to 10 mm follicles in diameter on the surface of ovaries. Cumulus-oocyte complexes containing at least one layer of compact cumulus cells were selected for subsequent steps. These COCs were washed twice in oocyte collection medium and placed in groups of 10 in 50  $\mu$ L drops of oocyte maturation medium with a mineral oil overlay and matured for 20 to 22 h at 38.5°C and 5% CO<sub>2</sub> in humidified air. Matured oocytes were then washed twice with HEPES-TALP and transferred in groups of 200 to a 35 mm x 10 mm petri dish containing; 1.7 mL IVF-

TALP, 80  $\mu$ L PHE (0.5 mM penicillamine, 0.25 mM hypotaurine, and 25  $\mu$ M epinephrine in 0.9% (w/v) NaCl), and 120  $\mu$ L Percoll-purified spermatozoa [ $17 \times 10^6$  sperm/mL] from a pool of frozen-thawed semen (3 to 4 bulls; a separate pool of semen was used for each replicate) for a final concentration of approximately  $1.2 \times 10^6$  sperm/mL at fertilization. After 8 to 10 h of co-incubation at 38.5°C, 5% CO<sub>2</sub> in humidified air, putative zygotes were removed from fertilization plate and denuded of cumulus cells by vortexing in 100  $\mu$ L hyaluronidase (1000 U/mL in HEPES-TALP). Denuded putative zygotes were cultured in groups of 25 to 30 in 50- $\mu$ L drops of KSOM-BE2 or KSOM-BE2 +  $\alpha$ -amanitin [50  $\mu$ M] with a mineral oil overlay at 38.5°C, 5% CO<sub>2</sub>, 5% O<sub>2</sub>, ~90% N<sub>2</sub>, with humidified air.

### **BEND Cell Culture**

The BEND cell line, derived from bovine endometrium, was purchased from American Type Culture Collection (Manassas, VA, USA). Cells were grown in BEND cell culture medium at 38.5°C and 5% CO<sub>2</sub> with humidified air. At confluence, cells were trypsinized, centrifuged for 10 min at 300 x g and re-suspended in fresh complete medium. Cells were then either collected or propagated.

### **RNA Extraction**

Oocytes were collected after 22 h of maturation, 2-cell and 2-cell embryos treated with  $\alpha$ -amanitin were collected between 28 to 29 h post-insemination (hpi) and  $\geq 16$ -cell embryos were collected at Day 5 post-insemination. Total RNA was extracted using the PicoPure® RNA isolation kit (Molecular Devices, Sunnyvale, CA, USA) following the manufacturer's instructions. Briefly, 100  $\mu$ L of extraction buffer was added to 20 oocytes or embryos and the mixture incubated at 42°C for 30 min. Thereafter, 100  $\mu$ L of 70% (v/v) ethanol was added and the mixture added to a pre-conditioned RNA

purification column. After a series of 3 washes using two different wash buffers, RNA was eluted using 15  $\mu$ L of elution buffer into a clean 1.5 mL microcentrifuge tube. RNA concentration was determined using a NanoDrop 2000 (Thermo Fisher Scientific Inc., Waltham, MA, USA) and samples were stored at  $-80^{\circ}\text{C}$  until analysis.

Total RNA was extracted from BEND cells using the RNeasy® Plus Micro Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. Briefly,  $5 \times 10^5$  BEND cells were lysed in a 1.5 mL microcentrifuge tubes by repeat pipetting with a 20 gauge needle in 350  $\mu$ L lysis buffer. Cell lysate was placed into gDNA eliminator columns to remove genomic DNA. After mixing with 70% (v/v) ethanol, samples were transferred to the RNeasy® MiniElute spin columns. Samples were then washed with a series of buffers and 80% ethanol and total RNA was eluted with 14  $\mu$ L RNase-free water into a clean 1.5 mL microcentrifuge tube. The RNA concentration was determined using a NanoDrop 2000 and samples were stored at  $-80^{\circ}\text{C}$ .

### **Quantitative Real-Time RT-PCR (qPCR)**

Primers used for qPCR were manufactured by Integrated DNA technologies and are described in [Table 2-1](#). To remove contaminating DNA, RNA samples were treated with DNase I (New England Biolabs, Ipswich, MA, USA) according to manufacturer's specifications. Briefly, 10  $\mu$ L RNA sample was treated with 10  $\mu$ L DNase mix (1  $\mu$ L DNase I, 2  $\mu$ L DNase Buffer, and 7  $\mu$ L 0.1% (v/v) Diethyl Pyrocarbonate treated  $\text{H}_2\text{O}$  (DEPC) at  $37^{\circ}\text{C}$  for 30 min followed by  $75^{\circ}\text{C}$  for 15 min. cDNA was produced using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's directions. DNase treated samples (20  $\mu$ L) were diluted to a final volume of 35  $\mu$ L with DEPC  $\text{H}_2\text{O}$ . Reverse transcription was performed in 15  $\mu$ L reactions by adding 7.5  $\mu$ L diluted DNase treated sample to either 7.5  $\mu$ L of

RT(+) master mix (3.15  $\mu$ L DEPC H<sub>2</sub>O, 1.5  $\mu$ L 10X reaction buffer, 1.5  $\mu$ L 10X random primers, 0.6  $\mu$ L 25X dNTPs, and 0.75  $\mu$ L 20X RTase) or 7.5  $\mu$ L of RT(-) master mix (3.9  $\mu$ L DEPC H<sub>2</sub>O, 1.5  $\mu$ L 10X reaction buffer, 1.5  $\mu$ L 10X random primers, and 0.6  $\mu$ L 25X dNTPs). Reaction conditions were as follows; 25°C for 10 min, 37°C for 2 h, and 85°C for 5 min.

cDNA from BEND cell samples was used to produce a standard curve to test efficiency of each primer. After reverse transcription, samples were diluted using fivefold dilutions to create a five point standard curve. Standard curves were run in a 25  $\mu$ L reaction volume [12.5  $\mu$ L 2x SYBR® Geen PCR master mix (Applied Biosystems), 0.75  $\mu$ L each of 10 mM forward and reverse primers ([Table 2-1](#)) and 8.5  $\mu$ L DEPC H<sub>2</sub>O] using an ABI 7300 instrument (Applied Biosystems). The conditions for amplification were as follows: 1 cycle for 10 min at 95°C followed by 50 cycles of 15 sec at 95°C and 1 min at 60°C. Cycle threshold (Ct) values were plotted against the log<sub>10</sub> of the template concentration and primers used if the slope was between -2.9 to -3.7 with an R<sup>2</sup> >0.95. In addition, agarose gel electrophoresis was performed to verify synthesis of a single product at the appropriate size (data no shown).

cDNA from multiple RT(+) reactions, from the same pool 20 of oocytes or embryos, were pooled together to ensure adequate cDNA. Each real-time plate consisted of one replicate of all genes in all sample groups (oocyte, 2-cell, 2-cell +  $\alpha$ -amanitin, and  $\geq$ 16-cell embryos). Samples were analyzed in duplicate in 25  $\mu$ L reactions [12.5  $\mu$ L 2x SYBR® Geen PCR master mix, 0.75  $\mu$ L each 10 mM forward and reverse primers ([Table 2-1](#)), and 8.5 $\mu$ L DEPC H<sub>2</sub>O] using an ABI 7300 PCR machine

(Applied Biosystems). Amplification proceeded for 1 cycle for 10 min at 95°C followed by 50 cycles of 15 sec at 95°C and 1 min at 60°C.

### **Immunocytochemistry**

Two-cell embryos were collected between 28 to 29 hpi and  $\geq 16$ -cell embryos were collected at Day 5 post-insemination. Two-cell embryos and  $\geq 16$ -cell embryos were washed four times in 50  $\mu$ L drops of 10 mM  $KPO_4$ , pH 7.4 containing 0.9% (w/v) NaCl (PBS) and 1 mg/mL polyvinylpyrrolidone (PBS-PVP) by transferring from drop to drop. Embryos were fixed for 15 min in PBS containing 4% (v/v) paraformaldehyde. After fixation, embryos were washed in PBS-PVP and placed in 500  $\mu$ L of PBS-PVP and stored at 4°C for up to a week.

Antibodies were affinity purified rabbit polyclonal antibodies for BCL2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), BCL2L1 (AbCam Cambridge, MA, USA), BAX (Santa Cruz Biotechnology) and BAD (Assay Designs, Ann Arbor, MI, USA) or a purified mouse monoclonal antibody for HSPA1A (Chemicon International) and either fraction purified rabbit IgG or mouse IgG (Sigma-Aldrich) was used as a negative control. Each antibody was used at a concentration determined to be optimal in preliminary experiments (BCL2: 4  $\mu$ g/mL, BCL2L1: 10  $\mu$ g/mL, HSPA1A: 5  $\mu$ g/mL, BAX: 2  $\mu$ g/mL, BAD: 10  $\mu$ g/mL). Antibodies were labeled using the Zenon® 488 labeling kit (Invitrogen) following the manufacturer's instructions For every 1  $\mu$ g antibody or non-specific IgG being labeled, 5  $\mu$ L of the Zenon® Fab fragment mixture was added and incubated for 5 min in the dark. An equal mass of Zenon® nonspecific IgG was then added to block unbound Fab fragments and incubated for 5 min in the dark.

Two-cell and  $\geq 16$ -cell embryos were permeabilized in groups of 30-50 in 500  $\mu$ L PBS + 0.2% (v/v) Triton-x (Sigma-Aldrich) for 30 min at room temperature. Embryos

were washed four times in PBS-PVP and placed into 50  $\mu$ L PBS containing 20% (v/v) normal goat serum (Pel-Freez Biologicals, Rogers, AR, USA ) for at least 1 h at room temperature. After blocking, embryos were briefly washed in 1 drop of PBS-PVP and then transferred to a drop of Zenon® labeled antibody or labeled IgG and incubated for 1 h at room temperature. Embryos were then washed 4 times, fixed for 15 min in 4% (v/v) paraformaldehyde and nuclei labeled with Hoescht 33342 (1  $\mu$ g/mL, Sigma-Aldrich) for 15 min. Embryos were washed 4 to 5 times, mounted on a microscope slide using ProLong® Gold Anti-Fade mounting medium (Thermo Fisher Scientific Inc), and fluorescence visualized using the a Zeiss Axioplan microscope (Zeiss, Göttingen, Germany) with a 40x objective and the FITC, DAPI, and DIC filter sets. Digital images were acquired using the AxioVision software and a high-resolution black and white AxioCam MRm digital camera (Zeiss).

### **Image Analysis**

Using ImageJ (National Institute of Health, Bethesda, MA, USA), individual embryos were outlined by hand with the polygon selection tool. After selection, additional exclusion was made for areas that did not correspond to the embryo as well as areas within the embryo between cells and, in cases where it occurred, in regions of the embryo that had broken through the zona pellucida. In the latter case, extrusion through the zona pellucida was a fixation artifact and extruded areas were consistently associated with lower fluorescent intensity. Mean gray pixel intensity of the selected area was measured using the FITC channel. Background and non-specific binding was removed by averaging mean pixel for IgG controls and subtracting these values from individual mean pixel intensities of embryos in corresponding stages. Adjusted mean pixel intensities that were below zero were set to zero.

## Western Blotting

Oocytes were collected after 22 h of maturation, 2-cell and 2-cell embryos treated with  $\alpha$ -amanitin were collected between 28 to 29 hpi and  $\geq 16$ -cell embryos were collected at Day 5 post-insemination. Before collecting oocytes, cumulus-oocyte complexes were washed twice with HEPES-TALP and then denuded of cumulus cells by vortexing in 100  $\mu$ L hyaluronidase (1000 U/mL in HEPES-TALP). Denuded oocytes and embryos were washed four times in 50  $\mu$ L drops of PBS-PVP by transferring from drop to drop. Groups of 25 to 100 oocytes/embryos were placed in a pre-warmed 50  $\mu$ L drop pronase for 2-5 min on a slide warmer to digest the zona pellucida. Groups of oocytes or embryos up to 200 in number were then placed into 1.5 mL microcentrifuge tubes containing 1 mL PBS-PVP. Samples were centrifuged at 13,600 $\times$ g, supernatant removed and samples were stored at  $-80^{\circ}\text{C}$ .

BEND cells were used as a positive control. They were collected after trypsinization, washed once with 1 mL PBS, adjusted to  $1 \times 10^6$ /mL and then aliquoted in groups of  $10^5$  cells into 1.5 mL microcentrifuge tubes. Tubes were then centrifuged for 10 min at 13,600 $\times$ g and supernatant was removed. Cells were stored at  $-20^{\circ}\text{C}$ .

Oocyte and embryo samples were pooled to create groups of 200 in 20  $\mu$ L loading buffer [0.125 M Tris-HCl pH 6.8 containing 20% (v/v) sucrose, 10% (w/v) sodium dodecyl sulfate (SDS), trace amounts of bromophenol blue, and 5% (v/v) 2-mercaptoethanol]. Similarly, BEND cell samples were resuspended in 20  $\mu$ L loading buffer. Samples were boiled at  $95^{\circ}\text{C}$  for 5 min and cooled on ice for 1 min before performing one-dimensional, discontinuous SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a Ready Gel® 4-15% (w/v) Tris-HCl (Bio-Rad, Hercules, CA, USA) gel. Conditions for electrophoresis were 125 V, 40 mA for 1 to 1.5 h at room

temperature. Proteins were then transferred electrophoretically to a Hybond ECL 0.2  $\mu\text{m}$  nitrocellulose membrane (GE Healthcare). Conditions for transfer were 30 V, 90 mA for 12 h at 4°C using a degassed buffer of 25 mM Tris, 193 mM glycine, and 20% (v/v) methanol. Membranes were blocked overnight in TBS-T [10 mM Tris pH 7.6, 0.87% (w/v) NaCl, and 0.3% (v/v) Tween-20] that also contained 5% (w/v) non-fat dry milk (TBS-TM). Membranes were rinsed 2 times with TBS-T and incubated for 2 h at 4°C with either a polyclonal antibody (BCL2 or BAX: Santa Cruz Biotechnology) or rabbit IgG at 1  $\mu\text{g}/\text{mL}$  in TBS-TM. Membranes were washed 4 times with TBS-T and then incubated for 1.5 h at 4°C with horseradish peroxidase conjugated to goat anti-rabbit IgG (0.04  $\mu\text{g}/\text{mL}$ , Santa Cruz) in TBS-TM and then washed as above. Blots were exposed to the ECL Plus Western blotting chemiluminescence substrate kit (GE Healthcare) for 5 min and then exposed to film for 3 to 15 min.

To allow re-probing, membranes were stripped using a stripping buffer [62.5 mM Tris-HCl, 2% (w/v) SDS, 100 mM 2-mercaptoethanol] for 30 min at 50°C. Membranes were washed and re-probed as described before. Peptide neutralization was performed to verify the specificity of the two antibodies. Briefly, 5  $\mu\text{g}/\text{mL}$  of the peptide used to produce  $\alpha\text{-BCL2}$  or  $\alpha\text{-Bax}$  (Santa Cruz) was pre-incubated with 1  $\mu\text{g}/\text{mL}$  of the respective antibody for 1 h and the antibody mixture was used to probe blots as described above.

### **Experimental Design**

For all experiments, control drops were set aside to assess cleavage at Day 3 post-insemination and development to the blastocyst stage at Day 8 post-insemination. Only replicates with characteristic cleavage and blastocyst development rates were used for molecular or immunochemical analysis.

**qPCR:** Treatments included MII oocytes, 2-cell embryos, 2-cell embryos treated with 50  $\mu$ M  $\alpha$ -amanitin, and  $\geq$ 16-cell embryos. Oocytes and embryos were collected and analyzed in groups of 20. Eight genes were analyzed for each treatment four anti-apoptotic (*BCL2*, *BCL2L1*, *HSPA1A*, and *DFFA*), three pro-apoptotic (*DFFB*, *BAX*, and *BAD*), and *HIST1H2A* as a housekeeping gene (Robert et al., 2002). The qPCR experiments were replicated 5 times.

**Immunocytochemistry:** For each antibody, treatments included 2-cell embryos and  $\geq$ 16-cell embryos. Each replicate consisted of at least 10 2-cell and 10  $\geq$ 16-cell embryos for IgG negative controls and at least 10 2-cell and 10  $\geq$ 16-cell embryos for antibody labeling (*BCL2*, *BCL2L1*, *HSPA1A*, *BAX*, *BAD*). Experiments were replicated 4 to 8 times with (*BCL2*, n= 111; *BCL2L1*, n= 183; *HSPA1A*, n= 239; *BAX*, n= 142; *BAD*, n= 186).

**Western blotting:** A total of 4 Western blots were performed. Treatments included on all blots were MII oocytes, 2-cell embryos,  $\geq$ 16-cell embryos. In addition, 2-cell embryos treated with 50  $\mu$ M  $\alpha$ -amanitin and BEND cells were included on one blot. Except for BEND cells or where noted, each lane contained 200 oocytes or embryos. Membranes were stripped to allow for blotting with different antibodies.

### **Statistical Analysis**

Data on mRNA abundance were subjected to least-squares analysis of variance using the General Linear Models procedure (GLM) of the Statistical Analysis System (SAS for Linux, Release 9.2, SAS Institute Inc., Cary, NC, USA). PCR data were analyzed using  $\Delta$ Ct. In preliminary analysis using cycle threshold (Ct) values, *HIST1H2A* had significant variation between treatments, accordingly *DFFB* was used instead as a housekeeping gene because it was similarly expressed between

treatments. The p-diff procedure with Tukey adjustment was used as a means separation test. The  $\Delta\Delta\text{Ct}$  value for each gene was calculated by the difference between the  $\Delta\text{Ct}$  of the embryo groups and the  $\Delta\text{Ct}$  of MII oocytes. Data are reported as fold change calculated by  $2^{-\Delta\Delta\text{Ct}}$ .

Data on adjusted pixel intensity from immunocytochemical analysis were also analyzed by least-squares analysis of variance using PROC GLM. Replicate was considered as a fixed effect and the p-diff procedure was used as a means separation test.

## Results

### Quantitative Real-Time RT-PCR

A total of 8 genes were analyzed by qPCR including anti-apoptotic genes (*BCL2*, *BCL2L1*, *HSPA1A* and *DFFA*), pro-apoptotic genes (*BAX*, *BAD*, and *DFFB*), and *HIST1H2A*. *DFFB* did not vary between stages and was used as a housekeeping gene for calculation of  $\Delta\text{Ct}$ . Differences in steady state concentrations of mRNA for each gene are shown in [Figure 2-1](#). Of the anti-apoptotic genes, *BCL2* steady state mRNA did not differ significantly different between MII oocytes, 2-cell embryos and  $\alpha$ -amanitin treated 2-cells, but was lower in  $\geq 16$ -cell embryos compared to the earlier stages (panel A;  $P < 0.0001$ ). There was no significant effect of stage of development on steady state mRNA concentrations of *BCL2L1* (panel B). Patterns of mRNA concentrations for *HSPA1A* (panel C) and *DFFA* (panel D) were similar to those for *BCL2*, with a reduction in concentration in embryos  $\geq 16$  cells. This difference was significant for *HSPA1A* ( $P = 0.0005$ ) but not for *DFFA*. For the pro-apoptotic genes, steady state concentrations of *BAX* were not affected by stage (panel E). However, steady-state

concentrations of *BAD* had a distinct temporal pattern with a significant increase in mRNA levels in embryos  $\geq 16$ -cells (panel F;  $P < 0.0001$ ).

### **Immunocytochemistry and Western Blotting**

As shown in [Figure 2-2](#), immunoreactive BCL2 detected by immunofluorescence was greater for 2-cell embryos than for embryos  $\geq 16$  cells (compare [Figure 2-2](#) B with [Figure 2-2](#) panel E). Adjusted mean pixel intensity of immunofluorescence was greater ( $P = 0.001$ ) for 2-cell embryos ([Figure 2-2](#) panel G). These results were confirmed by Western blotting in three separate replicates. In the first (panel H), the BCL2 band was more intense for 2-cell embryos (lane 3) than  $\geq 16$ -cell embryos (lane 4) despite fewer embryos being loaded per lane. In the second blot (panel J), equal numbers of oocytes or embryos were loaded per lane and there was higher amounts of immunoreactive BCL2 in 2-cell embryos (lane 2) compared to  $\geq 16$ -cell embryos (lane 3). In the final blot (panel J), with equal numbers per lane, a membrane defect partially obscured the lane with 2-cell embryos (lane 2). However, amounts of immunoreactive BCL2 were lowest for  $\geq 16$ -cell embryos (lane 4). Immunolabeling of BCL2 was eliminated when antibody was co-incubated with a BCL2 peptide (results not shown).

Shown in [Figure 2-3](#) are immunoreactive amounts of BAX using immunocytochemistry (panels A-G) and Western blotting (panels H-J). Immunoreactive BAX detected by immunofluorescence was lower for 2-cell embryos than for embryos  $\geq 16$  cells (compare [Figure 2-3](#) B with [Figure 2-3](#) panel E). Pixel intensity of immunofluorescence was lower ( $P < 0.0001$ ) for 2-cell embryos ([Figure 2-3](#) panel G). Three Western blots were performed to further evaluate developmental changes in immunoreactive BAX. In the first blot, immunoreactive BAX was detected at the expected molecular weight (23 kDa) and there was slightly less BAX in embryos  $\geq 16$

cells than for MII oocytes and 2-cell embryos (panel H). In the second (panel I) and third blots (panel J), a different pattern was present. Three immunoreactive bands were detected, all of which could be eliminated by co-incubation of antibody with BAX peptide (data not shown). One band was of expected size (23 kDa), another larger band of 46 kDa was present that presumably represents BAX dimers, and a third, low molecular weight band that could represent proteolytic cleavage products was present.

Differences between stages in amounts of the 23 kDa BAX were variable. The higher molecular weight 46 kDa form was present in higher amounts in 2-cell embryos than in MII oocytes and yet higher amounts in  $\geq 16$  cell embryos.

There were no effects of stage of development on amounts of immunoreactive BCL2L1, HSPA1A or BAD as determined by immunofluorescence ([Figure 2-4](#)).

### **Discussion**

The ability of the preimplantation bovine embryo to undergo an apoptotic response is developmentally regulated with stress-induced apoptosis being blocked prior to embryonic genome activation, around the 8- to 16-cell stage in the bovine, (Krininger et al., 2002; Paula-Lopes and Hansen, 2002; Soto et al., 2003; Brad et al., 2007; Carambula et al., 2009). The block to apoptosis is not caused by a lack of apoptotic machinery because chemical depolarization of the mitochondria using CCCP leads to the activation of both caspase-9 and group II caspases (Brad et al., 2007). These data indicate that one of the developmental blocks to the apoptotic pathway is at the level of the mitochondria.

Life and death of a cell is dictated by a delicate balance of anti- and pro-apoptotic BCL2 family proteins (Adams and Cory, 1998). Anti-apoptotic BCL2 family members (BCL2 and BCL2L1) inhibit apoptosis by heterodimerizing with pro-apoptotic BCL2

family members (BAX, BAK, BOK). Heterodimerization prevents the pro-apoptotic BCL2 family proteins from forming homodimers which are required for mitochondrial pore formation and depolarization. Present results indicate that there is a switch from an abundance of BCL2 in early cleavage-stage embryos, which are not capable of apoptosis, to increased amounts of BAX protein and *BAD* mRNA in later stage embryos, which possess the capacity for apoptosis.

Quantitative real-time RT-PCR indicated higher amounts of *BCL2* in oocytes and 2-cell embryos compared to  $\geq 16$ -cell embryos. *BCL2* has also been shown to significantly decrease from the zygote to the 2-cell embryo in the mouse (Exley et al., 1999), which is also coincident with mouse genome activation (Flach et al., 1982). In the human preimplantation embryo *BCL2* is present at all stages however, it is highly expressed in the 2-cell embryo and expression decreases in the 4- to 6-cell embryo again coincident with genome activation (Spanos et al., 2002).

Immunoreactive amounts of BCL2 protein is also higher in the 2-cell embryo than in the  $\geq 16$ -cell embryo. While BCL2 is higher in the 2-cell embryo, it is not the result of active transcription since treatment with  $\alpha$ -amanitin, a transcription inhibitor, has no effect on steady-state amounts of *BCL2* mRNA or protein expression. Therefore, most likely BCL2 in the early cleavage embryo is maternally derived.

Prior to embryonic genome activation the embryo is transcriptionally quiescent with the majority of its mRNA and proteins being derived from maternal stores in the oocyte (van Blerkom, 1981; Flach et al., 1982). Total RNA, mRNA, and protein concentration decrease throughout preimplantation development until after embryonic genome activation. This temporal expression pattern has been seen in bovine with

RNA and protein expression decreasing from the 2-cell to the 8-cell stage and then slowly increasing from the morula to the blastocyst stage (Gilbert et al., 2009). Data presented show the same expression pattern for BCL2. The higher concentration of BCL2 in 2-cell embryos contributes to making the mitochondria refractory to stimuli leading to apoptosis.

Steady-state mRNA of *HSPA1A* is also higher in the oocyte and 2-cell embryo than the  $\geq 16$  cell embryo. *HSPA1A* mRNA follows the same temporal decrease as *BCL2*, but immunoreactive concentration of HSPA1A protein remained constant through the  $\geq 16$ -cell stage. One possible explanation for this could be post-transcriptional regulation of *HSPA1A* by RNA-binding proteins. RNA-binding proteins influence RNA localization, stability and translation. They serve an important role in the oocyte and preimplantation embryo maintaining mRNA stores throughout development until embryonic genome activation. The bovine embryo has been shown to have a variety of these RNA binding proteins including staufen 1 and 2 and ELAVL1 (Calder et al., 2008). ELAVL1 has been shown to bind to *HSPA1A* mRNA in the brain, and post-transcriptionally regulate HSPA1A expression (Amadio et al., 2008). The stability of HSPA1A protein concentration may be related to the regulatory control of ELAVL1 or other RNA binding proteins present in the preimplantation embryo.

BCL2L1, another anti-apoptotic member of the BCL2 family, does not show signs of developmental regulation in either mRNA or protein expression. *BCL2L1* mRNA and BCL2L1 protein concentration is extremely stable during preimplantation development confirming previous results in the bovine (Knijn et al., 2005) and mouse (Exley et al., 1999). However, BCL2L1 expression pattern may be extremely important in

preimplantation development. Unlike, BCL2 and HSPA1A where there is a temporal decrease of mRNA and protein, BCL2L1 expression remains constant. This suggests that BCL2L1 may be actively transcribed prior to embryonic genome activation, or there may be tight post-transcriptional controls to maintain BCL2L1 protein concentration.

Acquisition of capacity for apoptosis at the  $\geq 16$  cell stage is accompanied not only with a decrease in amounts of BCL2 but also by changes in the pro-apoptotic BCL2 family members. There are two major classes of the pro-apoptotic BCL2 family members; multi-domain proteins (BAX, BAK, BOK) and BH3-only domain proteins (BAD, BID, BIK, etc.). The multi-domain members form heterodimers with the anti-apoptotic proteins in the cytosol and mitochondrial membrane. Upon apoptotic stimuli, BH3-only members are activated and either heterodimerize with anti-apoptotic proteins (BCL2, BCL2L1, MCL1) to inhibit their function or bind to the multi-domain pro-apoptotic proteins leading to mitochondrial localization and pore formation (Lucken-Ardjomande and Martinou, 2005). In the current study we investigated the multi-domain member BAX and the BH3-only member BAD. There are complex developmental changes for both molecules.

Steady state mRNA for *BAX* does not vary between developmental stages. Similar results have been described in the mouse (Exley et al., 1999) and human (Metcalf et al., 2004). In contrast, immunocytochemical analysis indicates not only an increase in immunoreactive amounts BAX at the  $\geq 16$ -cell stage, but also an increased amount of high molecular weight BAX as detected in some of the Western blot results. Such results suggest post-translational regulation of BAX increasing its capacity for dimerization and therefore activity.

In SDS-PAGE under reducing conditions, proteins are denatured by the addition of a reducing agent such as 2-mercaptoethanol or dithiothreitol, which removes disulfide bonds resulting in the loss of oligomers and tertiary structure. As a result, BAX should not form multimers under these conditions, unlike our Western blotting results. However, there is evidence that  $\alpha$ -helical pore forming proteins can and do form multimer units under reducing conditions (Lemmon et al., 1992a, 1992b). BAX and BAK1 belong to a class of amphipathic proteins called  $\alpha$ -pore-forming proteins that contain several  $\alpha$ -helices that are involved in their pore forming properties (Qian et al., 2008). It is these  $\alpha$ -helical transmembrane domains and the extreme hydrophobic pockets that allow pore forming proteins to produce stable multimers even under reducing conditions (Lemmon et al., 1992a). Not only are the BAX multimer bands specific, bands are removed with peptide neutralization, but because of its pore forming properties structural domains, multimer formation under reducing conditions has previously been demonstrated.

*BAD* has the most interesting pattern of mRNA expression and protein concentration. The steady-state mRNA for *BAD* is very low through the 2-cell stage and then increases almost 10 fold at the  $\geq 16$ -cell stage. This same pattern is seen in the human preimplantation embryo, with very low levels of *BAD* expression until compaction when there is an increase in expression (Spanos et al., 2002; Metcalfe et al., 2004). Despite the increase of *BAD* mRNA at the  $\geq 16$  cell stage, there is no change in immunoreactive amounts of *BAD* as determined by immunohistochemistry.

BH3-only proteins have been shown to be regulated transcriptionally and post-translationally. For example *BBC3* (previously *PUMA*) is shown to be transcriptionally

upregulated by both p53 (Nakano and Vousden, 2001) and forkhead box O3 (FOXO3) (You et al., 2006) upon DNA damage or growth factor deprivation. BAD is shown to be post-translationally regulated undergoing two phosphorylation events at Ser112 (Zha et al., 1996; Harada et al., 1999) and Ser136 (Datta et al., 1997) leading to BAD being sequestered by YWHAQ proteins (Zha et al., 1996; Datta et al., 1997). Upon apoptotic stimuli these serine become dephosphorylated and BAD is released from YWHAQ proteins and contributes to cell death (Zha et al., 1996). Neither of these forms of regulation would contribute to our increase in mRNA after  $\geq 16$ -cell stage without a change in protein concentration.

A novel post-transcriptional mechanism has recently been proposed by Lam et al. (2009). Using RNAi they identified a novel kinase, MAP4K3. They show that MAP4K3 plays a role in DNA damaged induced cell death and its suppression results in a significant resistance to DNA damage. They also found a MAP4K3 dependent induction of BAD and BBC3 independent of p53.

MAP4K3 functions by activating the mechanistic target of rapamycin (mTOR) pathway. mTOR modulates the activity of eukaryotic translation initiation factors (eIF4B, eIF4E, eIF4F) which are involved in CAP-binding and mRNA stability (Ramirez, 2002). The authors suggest that this MAP4K3 stimulation of mTOR leads to the stabilization of *BBC3* and *BAD* and may contribute to and enhancement of *BBC3* and *BAD* translation.

This novel post-transcriptional mechanism of regulation can explain the pattern of BAD expression. After the  $\geq 16$ -cell stage a 10 fold increase of *BAD* mRNA can result in an increased availability of transcript that will be translated upon apoptotic stimuli.

While in the 2-cell embryo, the lack of pre-formed *BAD* mRNA will blunt the magnitude of a pro-apoptotic signal.

These data indicate that there is a developmental transition of anti- versus pro-apoptotic genes from the 2-cell to the  $\geq 16$ -cell embryo. This is complicated by the fact that the oocyte can undergo heat-shock induced apoptosis (Roth and Hansen, 2004) despite having similar expression of *BCL2*, *BCL2L1*, *HSPA1A*, *BAX* and *BAD* when compared to the 2-cell embryo. So why does the oocyte undergo apoptosis when the 2-cell embryo cannot?

Oocytes are extremely variable in both quality and development potential. Along with these variations in quality there is evidence for changes in certain protein concentrations. *BAX* is equally expressed in low and high quality bovine oocytes, but there is a higher amount of immunoreactive *BCL2* in high quality oocytes compared to low quality oocytes (Yang and Rajamahendran, 2002). This variation in *BCL2* concentration, similar to what is seen between the 2-cell and  $\geq 16$ -cell embryo, is a possible mechanism by which apoptosis could occur in the oocyte. For this study only high quality oocytes were selected, possibly explaining the lack of differences between our *BCL2* expression in oocytes and 2-cell embryo. In addition to inherent variation among oocytes there could also be other molecular mechanisms at work such as oocyte ageing.

Post-ovulatory oocyte ageing has been implicated with an increased incidence of apoptosis (Fissore et al., 2002). Like oocyte quality in bovine, oocyte ageing in mouse shows a stable expression of *BAX* mRNA, but an age dependent decrease in both *BCL2* mRNA and *BCL2* protein expression (Gordo et al., 2002). Again it is a change in

BCL2 expression that is related to the capacity for apoptosis. Although it is not fully understood how the oocyte undergoes heat-shock induced apoptosis, one potential mechanism is the pre-mature ageing of the oocyte resulting in the degradation of BCL2 allowing for apoptotic signal propagation.

Apoptosis is a tightly regulated pathway with multiple checks and balances to prevent accidental activation of cellular death without an appropriate signal. While caspases and DNases are the cellular executioners, it is the mitochondria in concordance with BCL2 family of proteins that serve as judge and jury. The data presented here show that developmental regulation of mRNA and immunoreactive amounts of protein, in particular BCL2, BAX and BAD, are key factors in preventing mitochondrial depolarization and apoptosis in the early preimplantation embryo and for establishing the capacity for apoptosis at the  $\geq 16$ -cell stage.

Table 2-1. Primer sets used for qRT-PCR.

Gene	Accession number	Sequence	T <sub>m</sub> (°C)	Product size (bp)	Reference
<i>BCL2</i>	XM_586976.4	5'-TCGTGGCCTTCTTTGAGTTC-3'	60	109	
		5'-CGGTTCAGGTACTIONCGGTCAT-3'			
<i>BCL2L1</i>	NM_001077486.2	5'-CTCAGAGTAACCGGGAGCTG-3'	60	142	
		5'-CTGGGGGTTTCCATATCTGA-3'			
<i>HSPA1A</i>	NM_174550.1	5'-GGGGAGGACTTCGACAACAGG-3'	60	245	(Block et al., 2008)
		5'-CGGAACAGGTCGGAGCACAGC-3'			
<i>DFFA</i>	NM_001075342.1	5'-GGTGCTTGACCAGAGAGAGG-3'	60	120	
		5'-CCAAGGTCAGCTCTGGACTC-3'			
<i>BAX</i>	NM_173894.1	5'-CTCCCCGAGAGGTCTTTTTTC-3'	60	176	
		5'-TCGAAGGAAGTCCAATGTCC-3'			
<i>BAD</i>	NM_001035459.1	5'-CTTTTCTGCAGGCCTTATGC-3'	59	151	
		5'-GGTAAGGGCGGAAAACTTC-3'			
<i>DFFB</i>	NM_001035109.1	5'-CCCTGCATAGCGAGAAGAAG-3'	60	129	
		5'-ATTCCGTGCCATCCTCATAG-3'			
<i>HIST1H2A</i>	U62674	5'-GTCGTGGCAAGCAAGGAG-3'	57	182	(Robert et al., 2002)
		5'-GATCTCGGCCGTTAGGTACTIONC-3'			

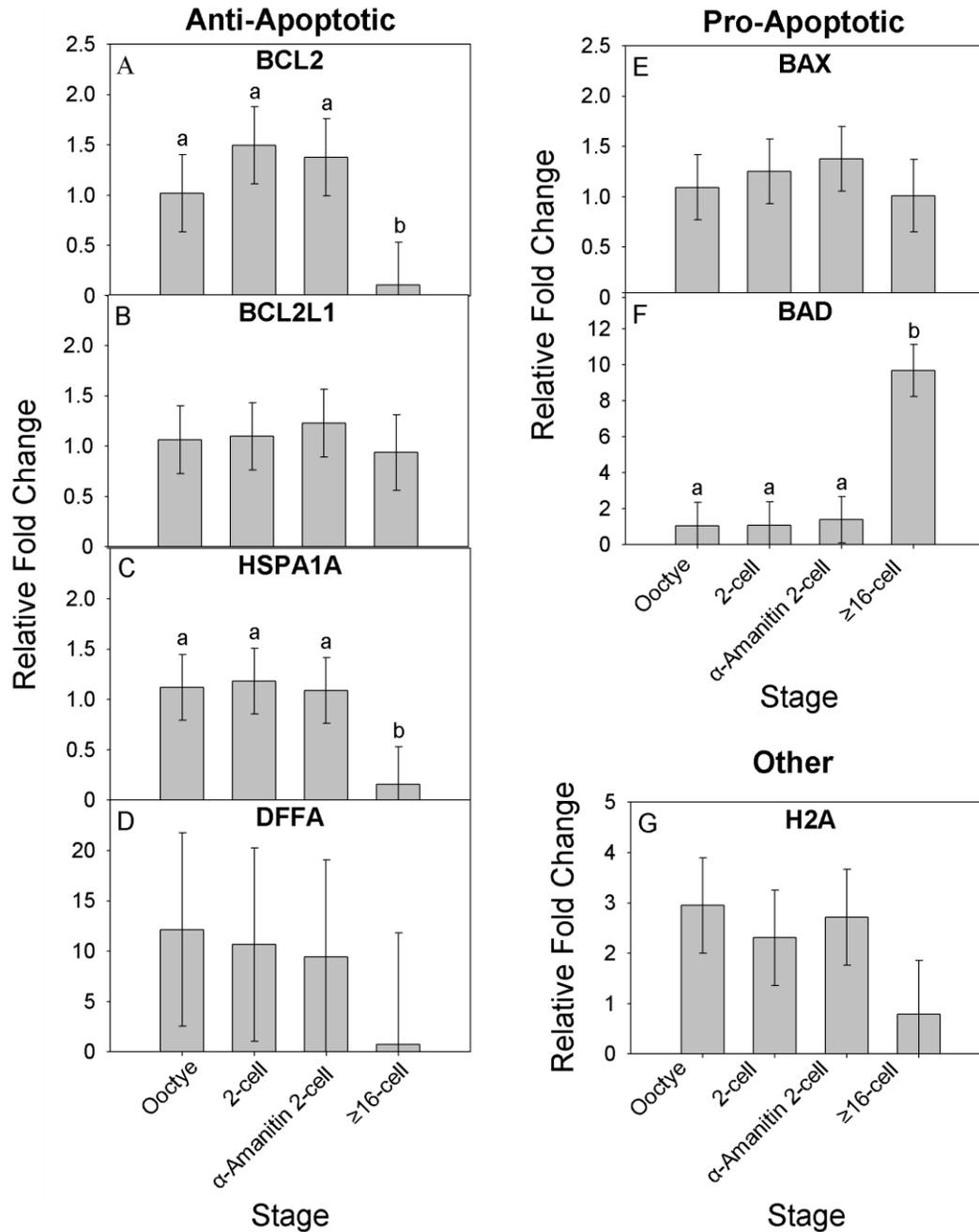


Figure 2-1. Quantitative real-time RT-PCR for anti-apoptotic genes A) *BCL2*, B) *BCL2L1*, C) *HSPA1A*, and D) *DFFA* and pro-apoptotic genes E) *BAX* and F) *BAD* and the housekeeping gene G) *HIST1H2A*. Data on steady state mRNA concentrations for bovine MII oocytes, 2-cell embryos, 2-cell embryos treated with  $\alpha$ -amanitin, and  $\geq 16$ -cell embryos are expressed as fold change relative to MII oocytes (least-squares means  $\pm$  SEM). Reactions were performed with cDNA samples (n=5) from individual groups of 20 oocytes or embryos. Different letters indicate a significant difference in relative mRNA abundance ( $P < 0.05$ ).

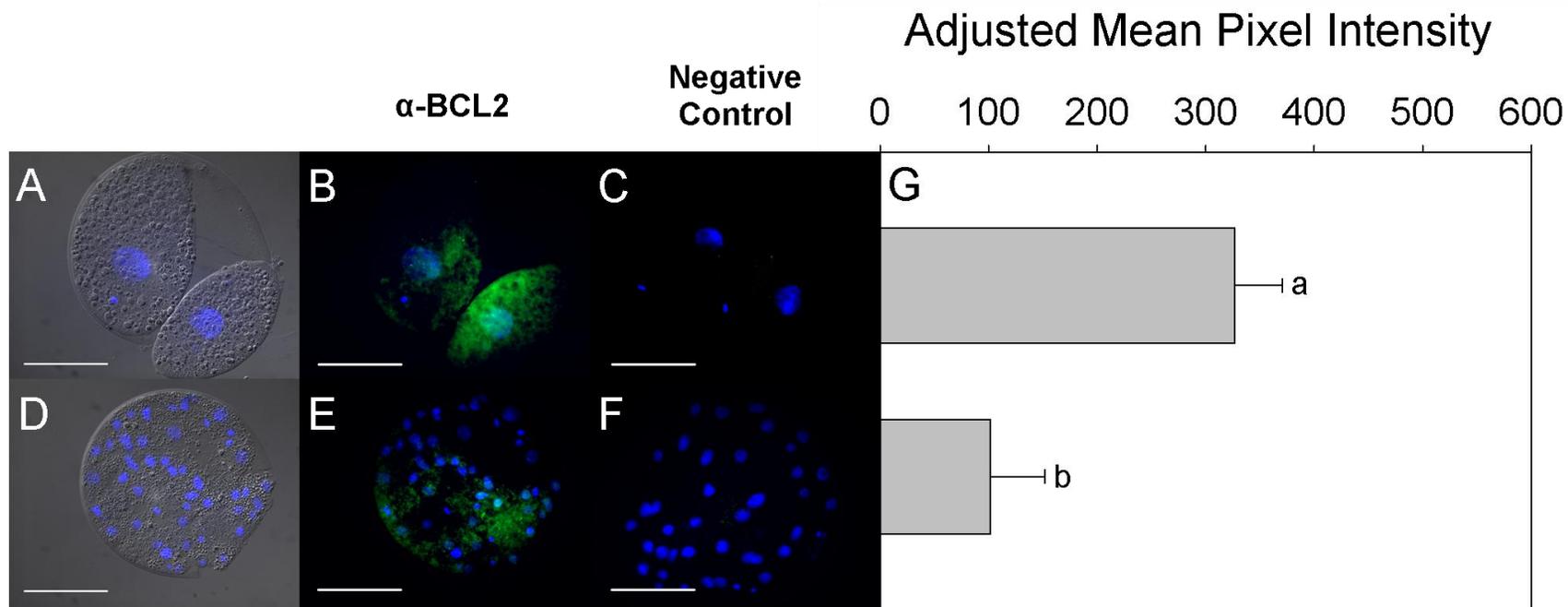


Figure 2-2. Immunoreactive amounts of BCL2 in 2-cell and  $\geq 16$ -cell *in vitro* produced embryos as determined by immunocytochemistry (A-G) and Western blotting (H-J). Immunocytochemical results are representative images of 2-cell embryos (A-C) and  $\geq 16$ -cell embryos (D-F). Shown are merged fluorescent images of Zenon 488 labeled anti-BCL2 (green) and nuclei labeled with Hoescht 33342 (blue) (B,C, E, F), or merged images of fluorescent labeling with differential interference contrast (A,D). Images of anti-BCL2 labeling are in panels (A,B,D,E) and labeling with the negative control (non-specific IgG labeled with Zenon 488) are in panels (C,F). The average pixel intensity of fluorescence associated with labeling with anti-BCL2, after correction for non-specific labeling, is shown in panel G (least-squares means  $\pm$  S.E.M.). Intensity of fluorescence was higher for 2-cell embryos as indicated by a different letter ( $P=0.001$ ). Results of Western blotting for BCL2 in MII oocytes, 2-cell embryos,  $\alpha$ -amanitin treated 2-cell embryos,  $\geq 16$ -cell embryos, and BEND cells (positive control) are shown in panels (H-J). The arrow indicates the band of interest at 26 kDa. All lanes of oocytes and embryos represent 200 oocytes or embryos except for panel H where 200 MII oocytes, 110 2-cell embryos and 150  $\geq 16$ -cell embryos were loaded.

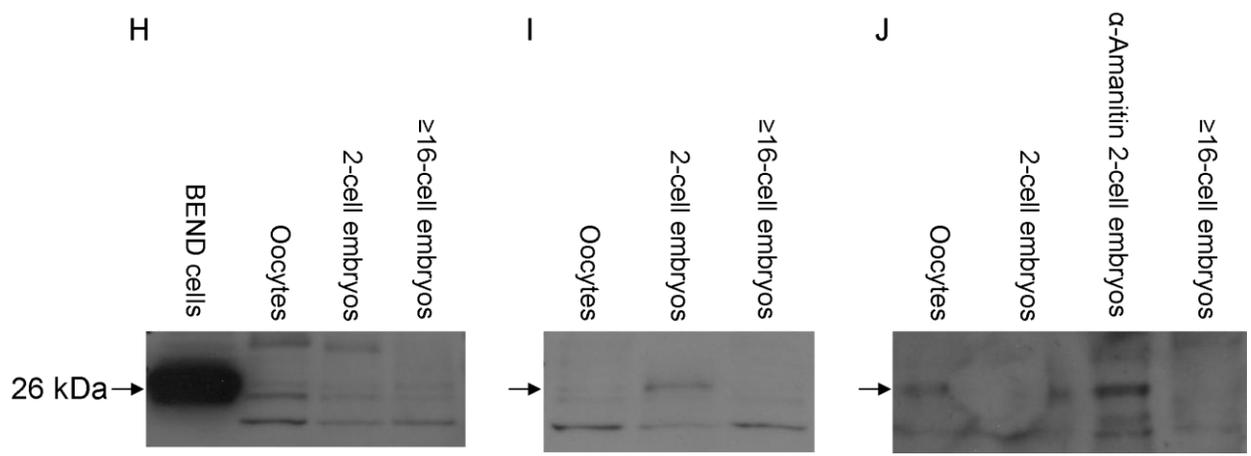


Figure 2-2. Continued

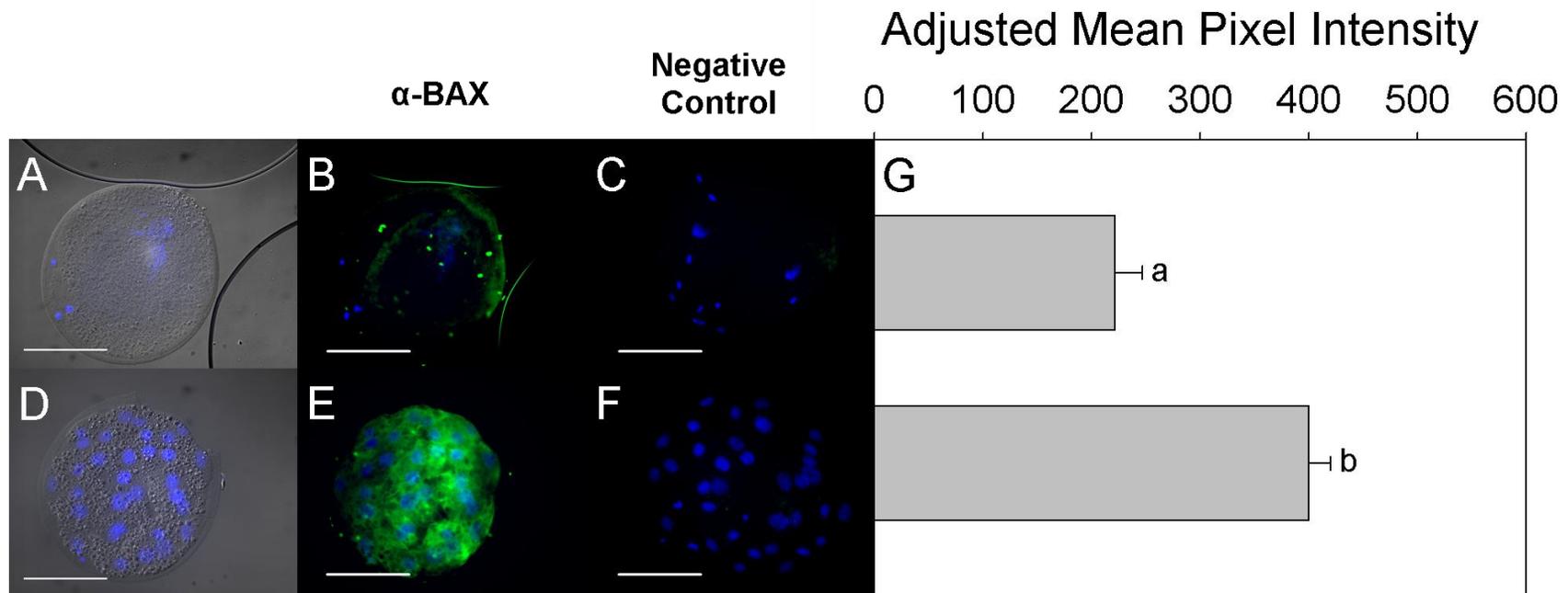


Figure 2-3. Immunoreactive amounts of BAX in 2-cell and  $\geq 16$ -cell *in vitro* produced embryos as determined by immunocytochemistry (A-G) and Western blotting (H-J). Immunocytochemical results are representative images of 2-cell embryos (A-C) and  $\geq 16$ -cell embryos (D-F). Shown are merged fluorescent images of Zenon 488 labeled anti-BAX (green) and nuclei labeled with Hoescht 33342 (blue) (B,C, E, F), or merged images of fluorescent labeling with differential interference contrast (A,D). Images of anti-BAX labeling are in panels (A,B,D,E) and labeling with the negative control (non-specific IgG labeled with Zenon 488) are in panels (C,F). The average pixel intensity of fluorescence associated with labeling with anti-BAX, after correction for non-specific labeling, is shown in panel G (least-squares means  $\pm$  S.E.M.). Intensity of fluorescence was lower for 2-cell embryos as indicated by a different letter ( $P < 0.0001$ ). Results of Western blotting for BAX in MII oocytes, 2-cell embryos,  $\alpha$ -amanitin treated 2-cell embryos,  $\geq 16$ -cell embryos, and BEND cells (positive control) are shown in panels (H-J). The arrow indicates the bands of interest at 46 and 23 kDa. All lanes represent 200 oocytes or embryos.

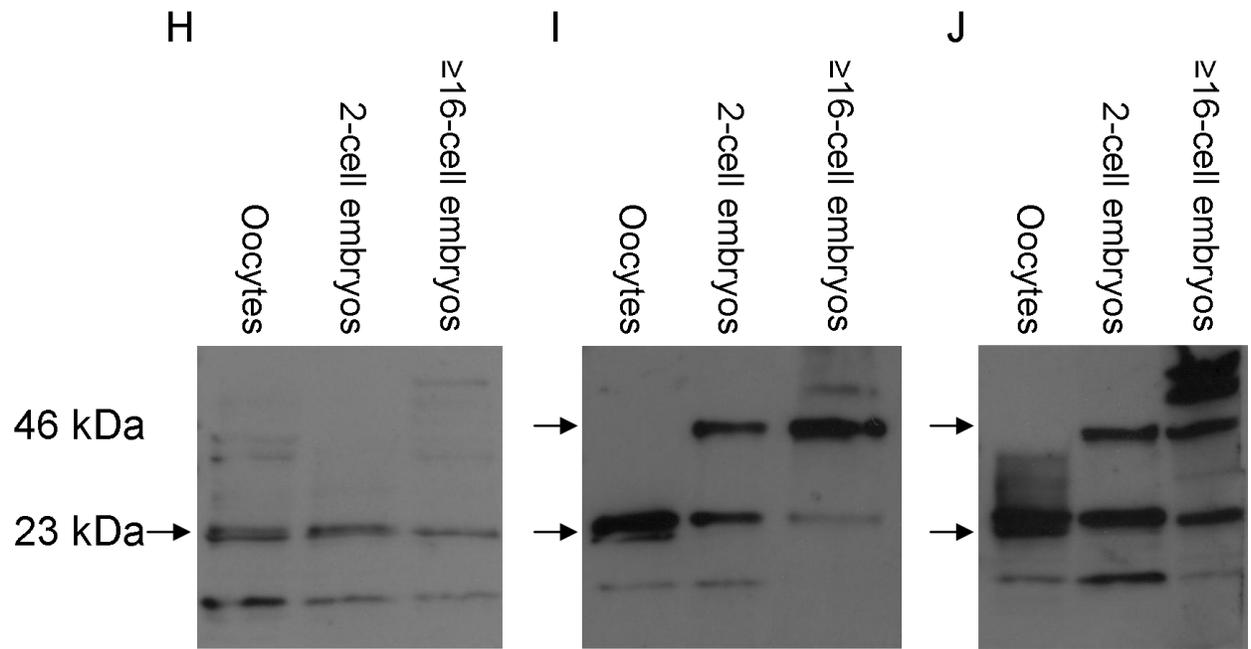


Figure 2-3. Continued

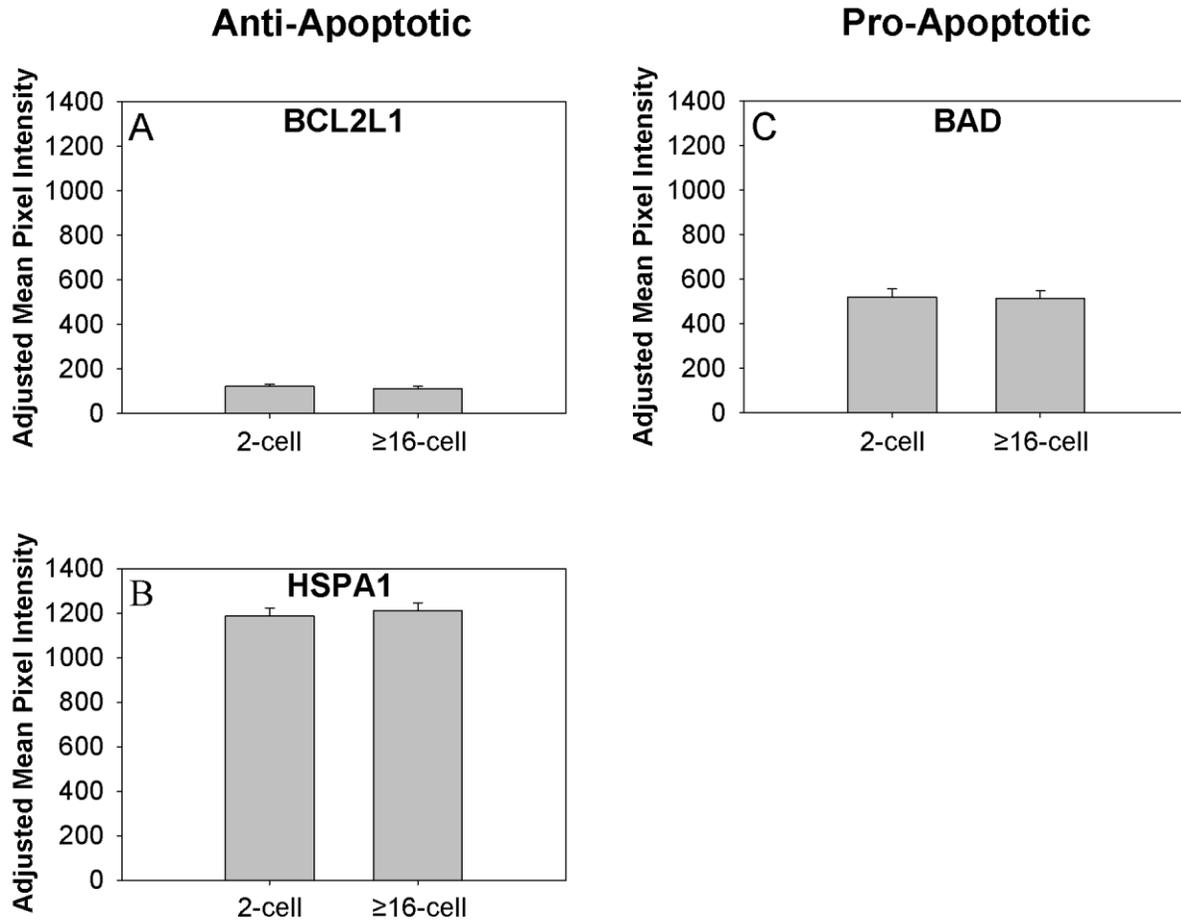


Figure 2-4. Immunoreactive amounts of A) BCL2L1, B) HSPA1A, and C) BAD in 2-cell and  $\geq 16$ -cell *in vitro* produced embryos. Data are the average pixel intensity of fluorescence associated with labeling with antibody, after correction for non-specific labeling (least-squares means  $\pm$  S.E.M.). There were no significant differences between 2-cell embryos and embryos  $\geq 16$  cells.

APPENDIX  
COWS IN SPACE: A PRELIMINARY INVESTIGATION TO DETERMINE EFFECTS OF  
SPACE FLIGHT ON BOVINE PREIMPLANTATION EMBRYO DEVELOPMENT

**Introduction**

The effects of space flight have been studied for a variety of cell types in different organisms. Despite the variation of cell types studied one common consequence of space flight are changes in the cell's physical structure as a result of disruption of cytoskeletal organization. Microtubule formation is disrupted leading to diffuse and shortened microtubules with poorly defined microtubule organizing centers (Lewis et al., 1998). Perinuclear cyokeratin networks are also disrupted and become diffuse (Vassy et al., 2003). Disruption of these cytoskeletal networks has a profound effect on the cell resulting in architectural changes, loss of morphological phenotypes (Gaboyard et al., 2002), changes in mitochondrial distribution (Schatten et al., 2001), and disruption of cellular function.

One major function of cytoskeletal structure is its involvement in cellular proliferation and mitosis, which is reduced as a result of space travel. This reduction could be seen in osteoblasts after as little as 3 days of exposure to space (Kacena et al., 2003) the number of MCF-7 cells undergoing progression through the cell cycle as measured by Ki-67 staining was similar between flight and ground controls but the duration of mitosis was significantly longer in the flight group (Vassy et al., 2003). Jurkat cells exposed to space flight actively progressed through the cell cycle and metabolized glucose, but cell numbers did not increase (Lewis et al., 1998).

In addition to decreases in proliferation, a reduction in size of a cellular population could be due to removal of cells by apoptosis. As apoptosis is a common mechanism used to remove damaged cells (Friedberg, 2003), cells with aneuploidy (Hardy, 1999;

Liu et al., 2002), and cells that fail to become properly polarized (Zahir and Weaver, 2004). Space travel leads to an increase in cellular damage due to ionizing radiation (Jones et al., 2007), the extreme g-forces during launch (Vassy et al., 2003), and microgravity effects on cellular polarization and cytoskeletal structure. As a result, one would expect an increase in apoptotic index among cells exposed to space. Lewis et al. (1998) showed that 4 h of space flight increased DNA condensation from 17% in ground controls to 30% in flight cells. They also found that there was a time dependent increase in expression of FAS, a member of the TNF receptor superfamily involved in the extrinsic apoptotic pathway.

Given the difficulty and expense of space travel, various conditions encountered in space have been partially replicated here on Earth. Microgravity can be simulated in the lab using clinostatic rotation in a rotating cell culture system. Clinostat rotation simulates microgravity by rotating the culture vessel on the horizontal axis so that cells or embryos within the vessel experience a gravitational pull from multiple directions leading to the cancellation of gravitational forces over time. Clinostat rotation has been used in a variety of culture environments to provide preliminary data to the effects of microgravity.

Simulated microgravity has been used to allow more in-depth investigation of changes in cytoskeletal structure (Uva et al., 2002) and apoptosis (Schatten et al., 2001). While fertilization and embryogenesis in space have also been studied, typical models include amphibians (Souza et al., 1995; Dournon et al., 2001; Gualandris-Parisot et al., 2002), fish (Ijiri, 1995), and pregnant rats (Ronca and Alberts, 2000). Studies of this type in space are few in number and are technically limited thus,

simulated microgravity has been used for a more in-depth examination of the effects of microgravity on fertilization and embryonic development.

Unfortunately, the effects of simulated microgravity on mammalian fertilization and embryonic development are not consistent. In the mouse, fertilization was not affected by clinostat rotation (Kojima et al., 2000). In a recent study in the bovine, however, clinostat rotation resulted in complete inhibition of fertilization compared to controls which had a 77% fertilization rate (Jung et al., 2009). Developmental competence has also been looked at under microgravity conditions. Using the mouse as a model there was a significant reduction in the number of embryos reaching the morulae or blastocyst stage (70% controls versus 40% clinostat rotation) (Kojima et al., 2000). In the bovine, none of the embryos under simulated microgravity reached the morulae or blastocyst stage while in the controls only 11% became morulae and only 3.6% developed to the blastocyst stage (Jung et al., 2009). While results do vary, they indicate that there is an effect of simulated microgravity on embryogenesis and development potential.

Both simulated microgravity and space flight have a wide variety of effects that could cause a decrease in development potential. As previously discussed, these effects most likely involve disruption of cytoskeletal structure, which would lead to disruption of fertilization, pronuclear formation, and retardation of mitosis. Cellular polarization could also be disrupted affecting; differentiation, gene expression, and tight junction formation. Finally, apoptotic index could increase due to up-regulation of pro-apoptotic genes (Lewis et al., 1998), or by disruption of mitochondrial networks due to cytoskeletal changes (Schatten et al., 2001). The objective of this study was to evaluate effects of space flight on development of bovine preimplantation embryos.

Given previous evidence, we hypothesized that space flight would inhibit development, possibly because of interference with tight junction formation, blastocoele formation or induction of apoptosis.

## **Materials and Methods**

### **Materials**

Media for in vitro production of embryos were obtained as follows. HEPES-TL and IVF-TL were purchased from Millipore (Billerica, MA, USA) or Caisson Laboratories, Inc. (North Logan, UT, USA) and used to prepare HEPES-TALP and IVF-TALP as described by Parrish et al. (1986). Oocyte collection medium was TCM-199 with Hank's salts without phenol red (Atlanta Biologicals, Lawrenceville, GA, USA) supplemented with 2% (v/v) bovine steer serum (Pel-Freez Biologicals, Rogers, AR, USA) containing 2 U/mL heparin, 100 U/mL penicillin-G, 0.1 mg/mL streptomycin, and 1 mM glutamine. Oocyte maturation medium was TCM-199 (Gibco®, Invitrogen, Carlsbad, CA, USA) with Earle's salts supplemented with 10% (v/v) bovine steer serum, 2 µg/mL estradiol 17-β, 20 µg/mL bovine FSH (Folltropin-V; Agtech Inc., Manhattan, KS, USA), 22 µg/mL sodium pyruvate, 50 µg/mL gentamicin sulfate (Sigma-Aldrich, St. Louis, MO, USA), and 1 mM L-glutamine or alanyl-glutamine. Percoll was from GE Healthcare (Uppsala, Sweden). Frozen semen from various bulls was donated by Southeastern Semen Services (Wellborn, FL, USA). Modified BBH7 was provided by Cooley Biotech LLC. (Gainesville, FL, USA). All flight hardware was provided by BioServe Space Technologies (Boulder, CO, USA)

### **In Vitro Production of Embryos**

Embryo production was performed as previously described (Soto et al., 2003b). A mixture of beef and dairy cattle ovaries were obtained from a local abattoir (Central Beef

Packing Co., Center Hill, FL, USA) and cumulus-oocyte complexes (COCs) were collected by slicing follicles that were 2 to 10 mm follicles in diameter on the surface of ovaries. Cumulus-oocyte complexes containing at least one layer of compact cumulus cells were selected for subsequent steps. These COCs were washed twice in oocyte collection medium and placed in groups of 10 in 50  $\mu$ L drops of oocyte maturation medium with a mineral oil overlay and matured for 23 h at 38.5°C and 5% CO<sub>2</sub> in humidified air. Matured oocytes were then washed twice with HEPES-TALP and transferred in groups of 50 to a 4-well plate containing;  $\sim 1 \times 10^6$  percoll-purified spermatozoa from a pool of frozen-thawed semen from three bulls (Dove IRL 95/068 Hochschwab SIM HWB, 90HH407 1922240 L1 Domino 910382 11-24-92, and 153LM46 NPM-1293502) 425  $\mu$ L IVF-TALP, and 20  $\mu$ L PHE (0.5 mM penicillamine, 0.25 mM hypotaurine, and 25  $\mu$ M epinephrine in 0.9% (w/v) NaCl). After 16 h of co-incubation at 38.5°C, 5% CO<sub>2</sub> in humidified air, putative zygotes were removed from fertilization plate and denuded of cumulus cells by vortexing in 100  $\mu$ L hyaluronidase (1000 U/mL in HEPES-TALP). Denuded putative zygotes were placed in groups of 25 in 50  $\mu$ L drops of modified BBH7 with a mineral oil overlay at 38.5°C, 5% CO<sub>2</sub>, 5% O<sub>2</sub>,  $\sim$ 90% N<sub>2</sub>, with humidified air for 2 h.

### **Fluid Processing Apparatus**

Fluid Processing Apparatuses (FPAs; [Figure A-1](#)) were provided by BioServe Space Technologies. FPA glass barrels and rubber septa were coated with Sigmacote<sup>®</sup> (Sigma-Aldrich) and autoclaved along with 3-well culture insert and gas exchange insert. Each well of a 3-well culture insert was loaded with 45  $\mu$ L of pre-equilibrated modified BBH7 and 25 embryos. Three-well inserts were then loaded into the FPA culture chamber with 0.7 mL of pre-equilibrated modified BBH7 [Figure A-1](#). Two rubber

septa sealed the culture chamber. A total of 1 mL of fixative, either 3.4% (v/v) glutaraldehyde or 6.8% (v/v) paraformaldehyde, was loaded into the fixative chamber and sealed with rubber septa. For FPAs that were to be fixed on day 9 of culture, a 1 mL displacement block was added with another rubber septa (Figure A-1). FPAs were returned to culture at 38.5°C, 5% CO<sub>2</sub>, 5% O<sub>2</sub>, ~90% N<sub>2</sub>, with humidified air.

### **Group Activation Pack Preparation and Handoff**

When contacted by BioServe staff, FPAs were removed from culture and loaded into the plunger apparatus. Plunger apparatuses were gassed with 5% CO<sub>2</sub>, 5% O<sub>2</sub>, ~90% N<sub>2</sub> for 30 seconds and sealed (Figure A-2). Plunger apparatuses were then loaded into Group Activation Packs (GAPs; Figure A-3) and turned over to the BioServe staff. BioServe had approximately 24 h pre-launch to perform a series of tests on all FPAs and GAPs and loaded them into flight incubators which were maintained at 37°C (Figure A-4).

### **Launch, Fixation, and Recovery**

Space shuttle *Endeavour* launched on November 14, 2008 at 1955 H or at approximately 52 hpi. While in orbit, on Day 9 of culture or Day 16 of culture, a crew member of STS-126 activated the GAP by mixing the fixative chamber with the culture chamber. STS-126 returned to Earth on November 30, 2008 at 1625. FPAs were received and embryos removed on December 2, 2008.

### **TUNEL and Hoescht 33342 Labeling**

TUNEL was used to detect DNA fragmentation that is associated with the late stages of apoptosis. The enzyme terminal deoxynucleotidyl transferase was used to catalyze the transfer of a fluorescein isothiocyanate-conjugated dUTP nucleotide to the free 3' hydroxyl group that is exposed after DNA cleavage. Embryos were washed four

times in 50  $\mu$ L drops of 10 mM  $KPO_4$ , pH 7.4 containing 0.9% (w/v) NaCl (PBS) and 1 mg/mL polyvinylpyrrolidone (PBS-PVP) by transferring from drop to drop. Embryos were then permeabilized in groups of 30-50 in 500  $\mu$ L PBS + 0.1% (v/v) Triton-x (Sigma-Aldrich) for 15 min at room temperature. After permeabilization, embryos were washed four times in 50  $\mu$ L drops of PBS-PVP. Embryos cultured under standard conditions were used as positive and negative controls for the TUNEL procedure. Positive and negative controls were treated in 50  $\mu$ L drops of RQ1-RNase-free DNase (50 U/mL; Promega, Madison, WI, USA) at 37°C in the dark for 1 h. Positive controls and experimental embryos were washed in PBS-PVP and incubated with 25  $\mu$ L of TUNEL reaction mixture (In Situ Cell Death Detection Kit, Fluorescein: Roche Diagnostics Corporation, Indianapolis, IN, USA) (containing fluorescein- conjugated dUTP and the enzyme terminal deoxynucleotidyl transferase) for 1 h at 37°C in the dark. Negative controls were incubated in the absence of terminal deoxynucleotidyl transferase. Embryos were washed four times in PBS-PVP and incubated in a 25  $\mu$ L drop of Hoechst 33342 (1  $\mu$ g/mL) for 15 min at room temperature. Embryos were washed four times, mounted on a microscope slide using ProLong® Gold Anti-Fade mounting medium (Thermo Fisher Scientific Inc), and fluorescence was visualized using a Zeiss Axioplan microscope (Zeiss, Göttingen, Germany) with a 20x objective and the FITC, DAPI, and DIC filter sets. Digital images were acquired using the AxioVision software and a high-resolution black and white AxioCam MRm digital camera (Zeiss).

### **Experimental Design**

A total of 1200 embryos were used in this experiment. Eight FPAs or 600 embryos were kept in an incubator at 37°C at Kennedy Space Center, Space Life Science Laboratory as ground control (GROUND). Another 8 FPAs or 600 embryos

were loaded onto the space shuttle *Endeavour* (FLIGHT). Within each group of 8 FPAs, 3 FPAs were fixed with 4% (v/v) paraformaldehyde on Day 9 of culture, 3 FPAs were fixed with 2% (v/v) glutaraldehyde on Day 9 of culture, and 2 FPAs were fixed with 2% (v/v) glutaraldehyde on Day 16 of culture.

Scanning electron microscopy was to be performed by the USDA on blastocysts recovered from embryos fixed with 2% (v/v) glutaraldehyde on Day 9 or Day 16 of culture. Development along with apoptotic index (TUNEL) was assessed for recovered embryos that were fixed in 4% paraformaldehyde on Day 9 of culture.

### **Statistical Analysis**

Data were subjected to least-squares analysis of variance using the General Linear Models procedure (GLM) with p-diff as means separation test. Binomial data (cleaved embryos greater than six cells) was analyzed using a chi square with PROC FREQ from the Statistical Analysis System (SAS for Linux, Release 9.2, SAS Institute Inc., Cary, NC, USA).

### **Results**

Of the 1200 embryos loaded (600 FLIGHT and 600 GROUND), 1067 were recovered (547 FLIGHT and 520 GROUND). No blastocysts were obtained from either group. Cleavage rate was not significantly different between FLIGHT (9.98%) and GROUND (11.84%). Of those embryos that cleaved, the majority were at the 2-4 cell stage (78% FLIGHT and 89% GROUND) ([Table A-1](#)), which would coincide with the approximate time of development the embryos would have been at the time launch (52 hpi). The percent of cleaved embryos that were  $\geq 6$  cells (i.e., embryos that developed at a time typically coincident with space flight) tended to be greater for FLIGHT (22% vs

11% GROUND) but differences were not significant ( $\chi^2 = 2.4681$ ;  $P=0.1162$ ) (Table A-2).

In addition to development data, embryos were analyzed for apoptotic index using TUNEL. A total of 367 embryos were analyzed using TUNEL (189 FLIGHT and 178 GROUND). In addition, an external set of embryos were used as a positive and negative controls for the TUNEL reaction. There was no evidence of TUNEL in FLIGHT or GROUND embryos (Figure A-5).

### **Discussion**

There was an inadequate amount of development in both FLIGHT and GROUND. Initially the USDA was going to perform scanning electron microscopy of any blastocysts recovered to examine morphological differences between FLIGHT and GROUND but this was not done because no blastocyst were recovered. TUNEL analysis to compare variations in apoptotic index was also performed. There were no TUNEL positive blastomeres from the embryos recovered from this experiment. This is not unexpected because all of the embryos recovered were  $\leq 16$ -cells, embryos are refractory to apoptosis (Krininger et al., 2002; Paula-Lopes and Hansen, 2002; Soto et al., 2003; Brad et al., 2007; Jousan and Hansen, 2007; Carambula et al., 2009).

The lack of development is due to a combination of the culture conditions along with the handling procedure required for these types of experiments. As their names suggest, FPAs were designed for fluid processing and not as a culture environment. Due to the tight regulation of materials that are allowed on board of the Space Shuttle, they have been adapted to be used as a culture system. Besides not being designed for embryo culture, FPAs are coated with a silicon compound (Sigmacote) to aid in the

insertion and removal of the rubber septa used in this system. We are unaware of the effects, if any, this compound may have had on the embryos.

In the bovine *in vitro* production system, like other *in vitro* production systems, embryos are cultured in groups in 10-50  $\mu\text{L}$  volumes. The FPA is designed to use a culture volume of 1 mL. To compensate for the larger volume, a 3-well insert was used, where each well held a volume of  $\sim 45 \mu\text{L}$ . While the majority of the 3-well insert was made from plastic, there was a metal piece and a screw as part of the assembly. The plastic or metal parts of the 3-well insert could also conceivably affect embryonic development.

Bovine embryos are also typically cultured in low oxygen at  $38.5^\circ\text{C}$  (Soto et al., 2003b). Individual plunger devices were gassed with a low oxygen mixture (5%  $\text{CO}_2$ , 5%  $\text{O}_2$ ,  $\sim 90\%$   $\text{N}_2$ ), but the incubator was maintained at  $37^\circ\text{C}$ . This could have a negative effect on embryonic development. In addition, working with FPAs is a more time consuming process compared to standard IVF culture systems. This fact, along with the required quality control leak testing, kept the embryos at room temperature longer than in a typical embryo culture system.

Our results are consistent with those experienced by Jung et al. (2009) using a rotating culture system. There was too little development in control embryos to make reliable conclusions about the effects of space flight. In conclusion, our initial attempt to achieve development of bovine embryos in space was limited by inadequate culture conditions and time to optimize these conditions before launch.

## Day 9 Fixation    Day 16 Fixation

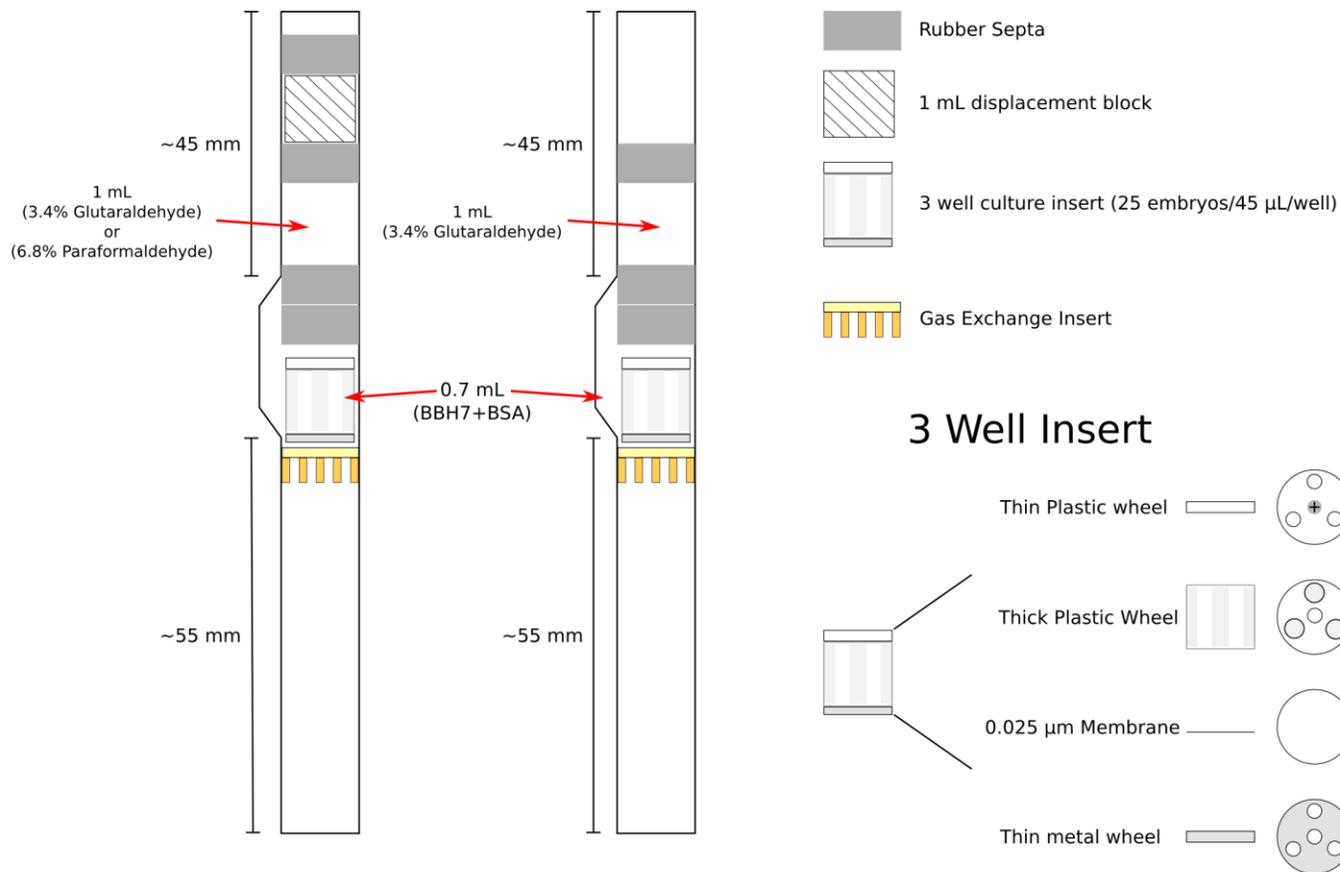


Figure A-1. Schematic drawing the the fluid processing apparatus (FPA). Two different FPA layouts were used to determine the timing of fixation. FPAs to be fixed on Day 9 of culture a 1 mL plastic displacement block was added. FPA assembly consisted of rubber septa (dark gray), 3-well culture inserts, and a gas exchange insert (yellow). 3-well culture inserts consisted of four pieces held together with a small screw. Pieces were assembled as shown, holes for the inserts were aligned for loading, and then offset to prevent embryos from falling out.



Figure A-2. Plunger apparatus. Each plunger contained a single FPA and were gassed with 5% CO<sub>2</sub>, 5% O<sub>2</sub>, ~90% N<sub>2</sub> for 30 sec and sealed. Plungers are loaded into the GAP and on Day 9 or Day 16 of culture plungers were depressed to mix fixative with culture medium.



Figure A-3. Group activation pack (GAP). GAPs consisted of a plastic cylinder with two aluminum lids. Eight FPAs were loaded per GAP. To activate fixative, a crank was inserted on the top of the GAP and a metal plate was lowered depressing the plunger apparatus, mixing fixative and culture medium.



Figure A-4. Flight incubator. The flight incubator contained 9 GAPs. Incubator was maintained at 37°C.

Table A-1. Developmental distribution of embryos recovered from FLIGHT and GROUND.

	Recovered	Cleaved	2-cell	3-cell	4-cell	6-cell	8-cell	16-cell	>16-cell
Flight	547	50	10	14	15	0	9	2	0
Ground	520	63	25	17	14	1	4	2	0

Table A-2. Comparison of embryonic development coincident with orbital flight.

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	Cleaved	≥6-cells	X <sup>2</sup>	P-Value
Flight	50	11	2.4681	0.1162
Ground	63	7		

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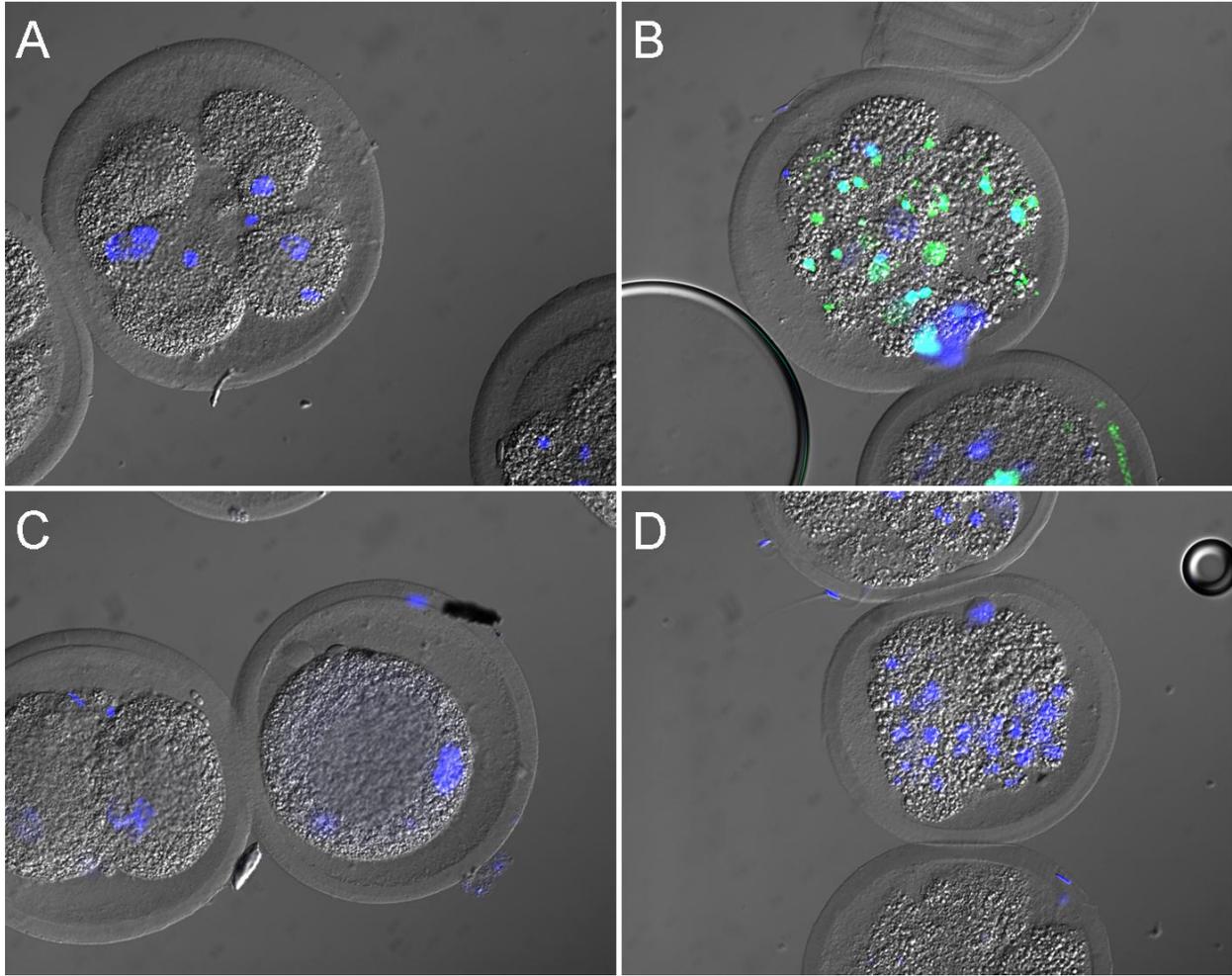


Figure A-5. Representative images of TUNEL analysis. Images are merged and pseudo colored with differential interference contrast (DIC), Hoechst 33342 (blue), and TUNEL (green). Panels (A, C) are experimental embryos and Panels (B, D) are control Embryos. A) 4-cell FLIGHT embryo, C) 2-cell and uncleaved GROUND embryo, B) Positive control, D) Negative control.

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## BIOGRAPHICAL SKETCH

Justin Matthew Fear was born on July 15, 1982, the second of two sons, to Karen M and Jerry J. Fear, in St. Louis Missouri. In 2004, he received his Bachelor of Science degree in animal science from the University of Missouri-Columbia. He then enrolled at The Ohio State University as a non-degree graduate student in Fisheries and Wildlife in 2005. In 2006 he began to work as a laboratory manager for Dr. Joy L. Pate at The Ohio State University. In the summer of 2007 he enrolled at the University of Florida on a research assistantship in the laboratory of Dr. Peter J. Hansen. He is currently a candidate for the Master of Science degree in the Animal Molecular and Cellular Biology Graduate Program while conducting research in the Department of Animal Sciences. On completion of this degree, he will continue his education by pursuing a Doctor of Philosophy in the Genetics and Genomics Program at the University of Florida.