OVERCOMING ENDOMETRIOSIS-ASSOCIATED PREIMPLANTATION
EMBRYO DEVELOPMENTAL ANOMALIES BY CULTURE

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EMBRYO DEVELOPMENTAL ANOMALIES BY CULTURE

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ABBREVIATIONS

$\alpha v\beta 3$	Alpha v Beta 3 Integrin
AQN4	Aquaporin
ATP	Adenosine Triphosphate
Bcl	B-Cell Lymphoma
BDNF	Brain-Derived Neurotrophic Factor
BSA	Bovine Serum Albumin
CAC	Citric Acid Cycle
CaCl ₂	Calcium Chloride
cAMP	Cyclic Adenosine Monophosphate
Cdx2	Caudal Type Homeobox 2
CG	Cortical Granules
c-mos	Moloney Murine Sarcoma Viral Oncogene Homolog
DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxyribonucleic Acid
DUB	Deubiquitinating Enzyme
ECM	Extracellular Matrix
GAD	Glutamic Acid Decarboxylase
GnRH	Gonadotropin Releasing Hormone
HEPES	4-(2-hydroxyethyl)-1-Piperazineethanesulfonic Acid
HMP	Hexose Monophosphate Pathway
ICM	Inner Cell Mass
LHRH	Luteinizing Hormone Releasing Hormone
MATER	Maternal Antigen that Embryos Require
MEA	Mouse Embryo Assay
MEM NEAA	Minimal Essential Medium Non-Essential Amino Acids
MEM EAA	Minimal Essential Medium Essential Amino Acids
miRNA	Micro-Ribonucleic Acid
MgCl ₂	Magnesium Chloride
MMP	Matrix Metalloproteinase
mR1ECM	modified Rat 1-Cell Embryo Medium
mM	Millimolar
mHTF	Modified Human Tubal Fluid
MU	University of Missouri
MZT	Maternal to Zygotic Transition
NaCl	Sodium Chloride
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NANOG	Nanog Homeobox

NFκB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
NGS	Normal Goat Serum
OCT4	Octamer-Binding Transcription Factor 4
PBS	Phosphate Buffered Saline
PC	Peritoneal Cavity
pCREB	cAMP Response Element-Binding Protein
ped	Preimplantation Embryo Development
PF	Peritoneal Fluid
PPP	Pentose Phosphate Pathway
PVA	Polyvinyl Alcohol
PVP	Polyvinyl Pyrrolidone
RNA	Ribonucleic Acid
ROS	Reactive Oxidative Species
SOX2	(Sex Determining Region Y)-Box 2
TCA	Tricarboxylate Acid Cycle
TE	Trophectoderm
Tead4	TEA Domain Family Member 4
TIMP	Tissue Inhibitor of Metalloproteinases
TNFα	Tumor Necrosis Factor α
Triton X	Polyethylene Glycol p-(1,1,3,3-tetramethylbutyl)-phenyl ether
UBAL	Ubiquitin Aldehyde
UCHL1	Ubiquitin C-Terminal Hydrolase L1
UCHL3	Ubiquitin C-Terminal Hydrolase L3
Usp	Ubiquitin Specific Protease 45
UTR	Untranslated Region
VEGF	Vascular Endothelial Growth Factor
VHL	Von Hippel-Lindau Tumor Suppressor
WCH	Women and Children Hospital
Xist	X Inactive Specific Transcript
ZP	Zona Pellucida

CHAPTER 1

A REVIEW OF THE LITERATURE

Endometriosis

Endometriosis is an enigmatic gynecologic disorder that afflicts millions of women worldwide. Present in menstruating mammals (i.e. human and non-human primates), endometriosis is defined by the ectopic localization and establishment of endometrial tissue throughout the body, commonly founded on peritoneal surfaces [Benagiano and Brosens 1991]. Another common location of this tissue's abnormal ectopic presence is on the surface of the ovary, termed endometriomas [Koger et al. 1993]. Histologically, the lesions are defined by the presence of stroma and epithelial glandular cells that respond to the milieu of sex hormones released cyclically throughout the menstrual cycle [Benagiano and Brosens 1991]. Responses include tissue growth and remodeling, localized angiogenesis and vasculogenesis, and a variable release of biochemical factors such as cytokines, matrix metalloproteinase 3 (MMP3) and prostaglandin E2 alpha (PGE2 α) [Karck, et al. 1996; Cox et al. 2001]. Lesions may appear as a variety of phenotypes, ranging from those that are active, maintaining a red, glandular appearance, to those that are inactive, exhibiting a white, fibrotic appearance [Kennedy et al. 2005]. According to the American Fertility Society (AFS), a female can exhibit one or more types of these lesions, regardless of the severity of the disease.

Endometriosis Etiological Hypotheses

There are several hypotheses concerning the etiology of endometriosis. The most cited is that of Sampson's Theory of Retrograde Menstruation [Sampson 1927]. Sampson suggests that, during menstruation, a portion of the sloughed endometrial tissue 'backflows' through the oviducts and into the

peritoneal cavity instead of exiting the lower portion of the uterus. This anomalously displaced endometrium may then transplant itself in an ectopic location and establish endometriotic lesions [Sampson 1927]. Interestingly, retrograde menstruation occurs in about 90% of women worldwide [Halme et al. 1984]. This raises the question as to what specific biological factors cause lesion formation in some, but not all women with retrograde flow.

Numerous studies have observed abnormal gene expression in both the eutopic and ectopic endometrium of endometriosis patients compared to healthy controls [Liu et al. 2011; Stilley et al. 2012]. It has not been yet determined which of these abnormalities is causal or an effect of the disease.

Well-characterized mechanisms supporting Sampson's Theory are known. For example, several members of the matrix metalloproteinase (MMP) family are implicated in the establishment of endometriotic ectopic lesions in the peritoneal cavity [Cox et al. 2001; Becker et al 2010]. Tumor necrosis factor alpha (TNF α) is known to activate MMPs, but suppress tissue inhibitors of metalloproteinases (TIMPs). Increased TNF α in the peritoneal fluid (PF) of women with endometriosis plays a role in the elevated expression of MMP2 and 9 in lesions, leading to their increased ability to degrade the underlying peritoneal basement membrane and establish themselves [Gottschalk et al. 2000]. Also, the reduced functionality of natural killer (NK) cells and phagocytes observed in women with endometriosis has been predicted to lessen the body's ability to remove abnormally located, potentially harmful tissue [Oosterlynck et al. 1991].

Nuclear kappa factor B (NFκB), an important transcription factor that responds to a variety of cellular stressors, is also upregulated in the PF of women with endometriosis [Defrere et al. 2011; Gonzalez-Ramos et al. 2012]. Along with being partially involved in the increased expression of ectopic endometrial MMP3, NFκB also plays a role in mediating apoptosis due to its regulation of a variety of anti-apoptotic genes. These include TNF- Receptor Associated Factor 1 (TRAF1) and TNF-Receptor Associated Factor 2 (TRAF2) [Sheikh and Huang 2003]. Constitutively expressed NFκB will lead to overexpression of these anti-apoptotic molecules, reducing the ability of the endometrial cells to undergo normal programmed cell death in the PC. In turn, the elevated survival rate of retrograde menstruated endometrium may increase its ability to form characteristic endometriotic lesions within the PC.

Another theory implicated in the pathogenesis of endometriosis is that of Robert Meyer [Gruenwald 1927]. This theory describes the metaplastic transformation of coelomic epithelium, a cellular ancestor of endometrial cells, as potentially triggered by an elevated inflammatory response. This is potentially due to an excessive presence of certain cytokines such as interleukin-6 (IL6), interleukin-10 (IL10), and TNFα [Gomez-Torres et al. 2002]. This may lead to the establishment of endometriotic lesions in the peritoneal cavity of specific individuals, dependent upon their immune status. Finally, one recent theory is that endometriosis may develop from stem cells present within the eutopic endometrium [Gargett et al. 2007; Chan et al. 2012]. Candidate endometrial

epithelial and stromal stem/progenitor cells are currently being evaluated [Gargett et al. 2007; Sasson and Taylor 2008].

Symptoms of Endometriosis

The two primary symptoms of endometriosis are pain and infertility, which vary in severity between women. For instance, women with endometriosis may suffer from an excruciating level of abdominal and/or back pain throughout their menstrual cycle while others will experience only a small amount of discomfort [Benagiano and Brosens 1991; Allaire 2006]. Also, while some of these women with endometriosis will become pregnant without assistance, others will undergo multiple cycles of assisted reproduction therapy (ART) without conceiving [Dokras and Olive 1999].

There are many known causes of the abdominal pain exhibited by endometriosis patients. Excessive levels of inflammatory factors and macrophages found in the peritoneal cavity of women with endometriosis act as contributing factors to this pain [Gonzalez-Ramos et al. 2012; Halme 1991]. This response is exacerbated by the cyclic retrograde shedding of the vascular, iron-rich endometrium into the PF. Menstrual fluid-red blood cell (RBC) lysis in the PC leads to an excessive presence of iron-rich hemoglobin, establishing a high prevalence of macrophages laden with hemosiderin, an insoluble form of stored iron [Van Langendonckt et al. 2002; Defrere et al. 2011] . While necessary for a variety of oxidative-reductive reactions, abnormally high levels of iron can lead to oxidative injury and inflammation, amplifying the effects of macrophage-produced cytokines [Van Langendonckt, 2002].

It has been observed that women with endometriosis had significantly lower levels of hemopexin, an iron-binding protein present in blood plasma that typically prevents the toxic effects of free floating iron [Wolfler et al. 2013]. It can be suggested that varying hemopexin concentrations may be a contributing factor to why some women develop the disease, even though it is known that 90% of the female population experiences retrograde menstruation [Halme et al. 1984; Wolfler et al. 2013]. Also, an increase in nerve growth factor (NGF) secretion has been observed in the peritoneal fluid (PF) of women with endometriosis, suggesting that localized peritoneal production of NGF may be a risk factor for endometriosis-induced pain [Anaf et al. 2000; Tokushige et al. 2007]. Nerve growth factor is involved in a number of different pathways, including neuronal survival, development, and function, along with being a mediator of inflammation [Frade and Barde 1998]. Finally, endometriotic lesions may cause adhesions to form between different organs (i.e. bladder and bowels) leading to tissue damage and organ misplacement [Benagiano and Brosens 1991].

Endometriosis causes a second detrimental phenotype in reproductive-aged women: subfertility [Garrido et al. 2002]. It has been reported that 30 to 40% of infertile women are diagnosed with endometriosis [Verkauf, 1987]. A large number of factors contribute to this subfertility. Specifically, the increased presence of reactive oxygen species (ROS), induced by the prevalence of pro-inflammatory cytokines in the PF can have damaging effects on multiple facets of female reproduction [Ngo et al. 2009]. Using an animal model, Stilley et al. have

shown that PF which bathes the ovary is able to traverse throughout the female reproductive tract, moving into the oviducts, past the utero-tubal junction, and distally through the uterus and vagina [Stilley et al. 2009]. This suggests that reproductive tissues are exposed to the PF in women with endometriosis, predictably containing a variety of anomalous endometriotic secretions. This exposure may lead to the numerous difficulties involving fertility.

For example, ovulation is reportedly impaired in animal models of endometriosis. One predicted cause of ovarian dysfunction is the presence of lesion-produced TIMPs in the thecal cells of antral, pre-ovulatory follicles [Stilley et al. 2009]. TIMP1 is a primary contributor, suppressing MMPs 2 and 9, inhibiting their gelatinase activity on the basement membrane of the follicle [Stilley et al. 2009; Stilley et al. 2010]. In turn, TIMP1 decreases the ovulatory potential of a Graafian follicle. These observations about TIMP1 localization positively correlate with the increased number of luteinized unruptured follicles (LUFs) observed in a rat model of endometriosis [Stilley et al. 2009]. In this same rat model [Stilley et al. 2009], MII-stage oocytes collected from endometriosis animals display greater levels of darkened, irregular ooplasm and abnormal nuclei compared to those collected from healthy animals. Abnormal pre-implantation embryonic development is also a common occurrence in women with endometriosis [Brizek et al. 1995]. The level of blastomere fragmentation and the presence of vacuoles, darkened cytoplasm, and uneven cells are elevated in embryos collected from women with endometriosis, for example

[Pellicer et al. 1995; Brizek et al. 1995]. All of these factors may reduce reproductive health.

There is an increased level of nuclear abnormalities, such as DNA/nuclear fragmentation and irregular chromosomal orientation throughout mitosis in these embryos [Stilley et al. 2009; Stilley et al. 2010]. A primary explanation is an elevated exposure to reactive oxygen species (ROS). Reactive oxygen species lead to DNA strand breaks, inducing the transcription of nucleotidyl-damage markers [Laurent et al. 2005]. Dependent upon the severity of the damage, this may activate the apoptotic pathway, leading to embryonic cell death [Laurent et al. 2005].

Tumor necrosis factor alpha, another component secreted by ectopic lesions into the PC, binds to the tumor necrosis factor receptor (TNFR) on the cell membranes of blastomeres [Sheikh and Huang 2003]. This is an alternative upregulator of apoptosis, acting through the intracellular tumor necrosis factor type 1/Fas associated protein with death domain (TRADD/FADD) pathway [Sheikh and Huang 2003]. Both apoptotic triggers lead to the activation of caspase-dependent signaling pathways, involved in the degradation of components necessary for cellular survival (e.g. DNA and nuclear lamins). Blastomeric blebbing, characterized as the decoupling of the cytoskeleton from the plasma membrane, is common in apoptotic cells [Mills et al. 1998; Coleman et al. 2001]. This event is followed by the formation of distinctive apoptotic bodies, characterized as cellular fragmentation in abnormal embryos, a

predominant characteristic found in preimplantation embryos collected from mothers with endometriosis [Brizek et al. 1995].

Tissue inhibitor of metalloproteinase 1 is another potential contributor to the detrimental phenotypes exhibited in embryos collected from women with endometriosis. It was observed that treating healthy rat zygotes with endometriotic concentrations of TIMP1 for 24 hours lead to the development of two cell embryos with an increase in nuclear fragmentation, abnormal nuclear pore complex distribution, and a reduced potential to process sperm tails [Stilley et al. 2010].

There is also a significant reduction in the presence of antioxidants, such as glutathione and superoxide dismutase, observed in a non-human primate model of endometriosis [Hastings et al. 2006]. This factor has been observed in human studies [Szczepańska et al. 2003]. This is another potential contributing factor to the decreased ability of endometriosis patients to mediate tissue and embryonic oxidative damage.

Another cause of subfertility observed in women with endometriosis is a reduction in uterine receptivity to the developing embryo, predominantly due to a dysregulation of integrins [Apparao et al. 2001; Casals et al. 2012]. Integrins are transmembrane receptors that mediate contact between cells. The integrin $\alpha\beta3$, an important contributor to embryonic-uterine receptivity [Lessey et al. 1994], has been observed to be downregulated in the endometrium in endometriosis patients [Kim et al. 2007]. One explanation for this decreased integrin expression involves homeobox protein A10 (HOXA10). Homeobox protein A10

is a homeobox transcription factor involved in the expression of the $\beta 3$ subunit of the $\alpha v\beta 3$ integrin, also known to be downregulated in the eutopic endometrium of women with endometriosis [Lessey et al. 1994; Vitiello et al. 2007]. Under normal circumstances, HOXA10 peaks during the window of implantation, elevating the level of intact, functional $\alpha v\beta 3$, allowing for the recognition and binding of the conceptus to the uterine lining.

Homeobox protein A11 (HOXA11) is another downregulated transcription factor observed in the endometrium of individuals with endometriosis [Jana et al. 2013]. Homeobox protein A11 expression is negatively correlated with MMP2 and MMP9 expression, and positively correlated with the presence of uterine pinopodes [Jana et al. 2013]. Increased MMP2 and MMP9 activity leads to an elevated level of extracellular matrix (ECM) turnover during the window of implantation, while the downregulation of endometrial pinopodes causes a reduction in uterine-embryo recognition [Jana et al. 2013].

Leukemia Inhibiting Factor (LIF), an important regulator of stromal decidualization and embryo implantation, is also downregulated in the eutopic endometrium of patients with endometriosis [Dimitriadis et al. 2006]. These observations strongly suggest a malfunction in uterine receptivity due to abnormal expression of important transcription factors.

Treatments of Endometriosis

The most common treatments for endometriosis involve the down regulation of sex hormones, anti-inflammatory medications and/or surgery [Belaisch 2009; Macer and Taylor 2012]. The hormonal treatments include gonadotropin releasing hormone (GnRH) analogs, female contraception, and

progestin capsules [Belaisch 2009; Macer and Taylor 2012]. Aromatase inhibitors are another potential treatment of this disease that have yet to be approved for human use [Langoi et al. 2013].

In general, hormone treatments are beneficial as they target a reduction in estradiol production, a well-characterized sex steroid known to exacerbate the development of endometriotic lesions [Benagiano and Brosens 1991]. For example, continued administration of progesterone negatively feeds back on the anterior pituitary, reducing the production of follicle stimulating hormone (FSH) and luteinizing hormone (LH), concomitantly reducing ovarian estrogen production. These same treatments typically lead to the cessation of menses, reducing the presence of necrotic menstrual tissue in the peritoneal cavity due to a simultaneous reduction in retrograde menstruation [Belaisch 2009; Macer and Taylor 2012; Langoi 2013].

Commonly prescribed in combination with surgery, these treatments have been moderately effective when it comes to the alleviation of symptoms associated with endometriosis. Yet, even under optimal circumstances, this disease frequently recurs, capable of exhibiting a recurrence rate of up to 50% in in a five year period [Vercellini et al. 2003].

Endometriosis is not curable by any known treatment. Further, treatments come at the cost of negative side-effects, varying from patient to patient. For example, many current treatments lead to sterility due to the disruption of sex steroid production and subsequent deregulation of folliculogenesis, ovulation, and/or pituitary hormone release [Belaisch 2009]. Dependent upon the severity

of the disease, a physician's recommendation may range from lesion excision using a laparoscopic approach to a hysterectomy, followed by combination drug therapy [Belaisch 2009].

Animal Models of Endometriosis

Validated animal models are important when studying human maladies. In the case of endometriosis, the non-human primate is a suitable model [Hastings and Fazleabas 2006; Dehoux et al. 2011]. The Rhesus macaque and baboon are two species most commonly utilized [Dehoux et al. 2011]. Primates, which undergo ~30 day menstrual cycles and exhibit retrograde menstruation, are capable of spontaneously developing endometriosis [Story et al. 2004; Dehoux et al. 2011]. Data extrapolated from primates is more relevant to human disease and medicine than data accumulated from other animal models. There are some disadvantages to working with primates. These include ethical limitations associated with both human and non-human primate research, and the high cost of animal care and housing [Hastings and Faxleabas 2006; Dehoux et al 2011].

Induced endometriosis in the rodent is an important and frequently used model of this disease. A surgical model developed by Vernon and Wilson [1985] involves the autotransplantation of uterine tissue into the vasculature of the small intestine. This location mimics the abdominal localization of displaced endometrial tissue in women with endometriosis, while nourishing the implants with necessities such as oxygen and nutrients. Over a period of four weeks, the implants are allowed to grow and develop in response to the cyclicity of reproductive hormones [Vernon and Wilson 1985].

The primary advantage of the rat model involves their short reproductive cycle, exhibiting 70 to 80 estrous cycles per year and a 20 to 23 day gestation period. These factors allow labs to accumulate a greater amount of data in a year compared to other animal models [Vernon and Wilson 1985; Sharpe-Timms 2002]. Rats are also much less costly to care for and house, along with being easier to handle in comparison to non-human primates.

Another method of imitating endometriosis in rodents includes the injection of homogenized eutopic endometrium into the rodent's abdominal cavity in order to simulate retrograde menstruation [Hirata et al. 2005]. Dependent upon the species and strain of rodent, some of the endometrial tissue will attach to the peritoneum and form lesions, exacerbated by the addition of estradiol [Hirata et al. 2005].

It has been determined that both the surgical and injection models exhibit similar characteristics to those of women with endometriosis, including the phenotype of subfertility [Vernon and Wilson 1985; Sharpe-Timms 2001; Story et al. 2004]. Established endometriotic implants contribute to the presence of inflammatory, anti-apoptotic, and pro-angiogenic/pro-neovascular factors in the rat and mouse PF [Story et al. 2004; Sharpe-Timms 2002; Stilley et al. 2009]. The excess of these factors is predicted, as in humans, to act as a primary cause of the reproductive anomalies observed in these models.

The primary disadvantage of a rodent model is attempting to relate collected data to human health and development [Sharpe-Timms 2002]. For example, rodents display an estrous cycle, exhibiting endometrial proteolytic

breakdown of its extracellular matrix (ECM), followed by subsequent absorption. Therefore, in the wild, rats and mice are incapable of naturally developing the disease, due to their inability to menstruate. Even when one considers this fact, it is important to remember that advantages of rodent models are still plentiful.

Fertilization

Accumulating evidence from a variety of human studies suggests that endometriotic secretions disrupt normal sperm-oocyte interactions, contributing to female subfertility [Sueldo et al. 1987]. Elevated levels of ROS and free-floating iron in the PF of endometriosis women could induce lipid peroxidation, and possible sperm DNA damage in the female reproductive tract [Allaire et al. 2006; Aitken et al. 2012]. Spermatozoa also express TNFR on their surfaces, capable of binding to the high levels of TNF α located in Endo patient PF, potentially leading to high levels of blastomere apoptosis [Mansour et al. 2009].

Cumulatively, all of these factors may lead to the apoptosis of spermatozoa, due to the activation of the enzymatic caspase cascade, preventing their interaction with the oocyte. Alternatively, spermatozoa may be severely damaged, reducing their motility and ability to undergo the acrosomal reaction. It has also been observed that sperm-binding to the endosalpinx, the mucous membrane lining the interior portion of the fallopian tube, may be disrupted in women with endometriosis [Reeve et al. 2005]. Further, attachment to the endosalpinx of the oviductal isthmus has been associated with spermatozoa capacitation and hyperactivation, two events necessary for oocyte penetration.

Oviduct Dynamics

As an embryo continues to develop past the 2 cell (2C) to 4 cell (4C) stage, it moves down the oviduct, from the ampulla toward the isthmus. This movement is modulated by a set of carefully regulated mechanisms including oviductal cilia dynamics, smooth muscle contractions, and luminal fluid composition [Lee and Yeung 2006]. These mechanisms are controlled by hormonal influences, primarily progesterone, estrogen, and prostaglandin E and their corresponding receptors. As can be expected, a balanced proportion of these hormones is required to temporally and spatially control pre-implantation embryo development and migration [Lee and Yeung 2006]. Embryos spend a defined period of time in the oviduct prior to uterine implantation, from the zygote to 8 cell (8C) stage in both bovine and rodents [Hamilton & Laing 1946], and the zygote to 4C or 8C stage in porcine and ovine, respectively [Pomeroy 1955; Holst 1974]. The primary reason behind this extended stay is that the oviduct is a much more conducive environment for embryonic growth than the uterus during the first few days post-fertilization due to the presence of necessary metabolites and amino acids. Table 1.2 shows common uterine and oviductal compositions present in a healthy human female [Gardner 2008].

Considering that estrogen and prostaglandin E₂α are two molecules known to be elevated in the PF of endometriosis women, it is logical to conclude that oviductal dynamics in these same women may be disrupted, subsequently reducing the tightly regulated signaling that occurs between the maternal and embryonic environment. Using a baboon model of endometriosis, an association between progesterone resistance and the abnormal upregulation of both

estrogen receptor 1 (ESR1) and oviductal glycoprotein 1 (OVGP1) (involved in sperm:zona pellucida binding and ESR expression) was observed in the oviducts of the diseased animals during the window of implantation [Wang et al. 2009]. These dysregulated components, if similarly present in human oviductal tissue, may also contribute to endometriosis-induced subfertility.

Early Stage Preimplantation Development (One Cell to Morula)

As early development progresses, there are many events that must appropriately occur in order to establish a healthy embryo. These include the regulated expression of *Pcd*, a regulator of cleavage rate, and the totipotency genes *Oct4*, *Nanog*, and *Sox2* [Wang and Dey 2006]. Another event is that of the Maternal to Zygotic Transition (MZT). After fertilization takes place, maternally stored transcripts and organelles drive embryonic growth and proliferation during the first few cleavage divisions [Wang and Dey 2006]. At a certain point, this being species-specific, a large majority of these transcripts and, in turn, maternal protein products, are degraded, leaving exclusive control of development to the zygotic genome. The MZT in mice occurs at the 2C stage, the 4C stage in pigs, and the 4,000 cell to 8000 cell stage in frogs [Kanka 2003; Wang and Dey 2006]. In mice, up to 90% of maternal mRNA is degraded by the middle of the second cleavage [Hake and Richter 1997]. It is important to mention that transcription of the embryonic genome begins as early as the zygotic stage in many different species, but the levels of transcriptional activity are extremely minimal [Hake and Richter 1997].

There are two important categories of maternal mRNA: 1) that which is replenished upon activation of the zygotic genome and 2) that which is not

subsequently expressed in later stage embryos [Walsch et al. 2011]. In reference to the former, these specific transcripts code for a variety of important cellular components, ranging from cytoskeletal scaffolding proteins (e.g. tubulin and actin) to glucose metabolizing enzyme (e.g. glucose-6-phosphate dehydrogenase). Dysregulation of these important transcripts may lead to an abnormal phenotype in the pre-implantation embryo. A good example involving the second category of maternal mRNA would be the regulated expression of *c-mos*, a protein present in the mouse oocyte that is important in the timed regulation of meiosis and mitosis [Kim et al. 2002]. Delayed degradation of the *c-mos* transcript leads to embryonic growth arrest at the MZT in mice [Kim et al. 2002]. Also, microRNA, involved in epigenetic regulation, is an important contributor to the temporal and orchestrated degradation of maternal transcripts [Walsch et al. 2011].

While there have been very few studies performed on the effects of endometriosis on the MZT, there have been multiple associations made between abnormal pre-implantation embryos and endometriosis. For example, the exposure of 2C stage murine embryos to the PF of women with endometriosis leads to increased levels of nuclear fragmentation and blastomere apoptosis, reduced developmental potential, and an overall increased level of embryo toxicity compared to controls [Tzeng et al. 1994; Gomez-Torre et al. 2002]. Utilizing microarray technology, Birt found that 8C stage embryos collected from rats with surgically induced endometriosis exhibited abnormal expression of multiple genes related to apoptosis, cell cycle progression, RNA processing, and

oxidative stress responsiveness [Birt et al. 2013]. This data leads to the logical conclusion that there may be multiple regulatory gene pathways dysregulated throughout early embryo development, potentially reducing the ability of the endometriosis embryos to respond to external stressors.

Later Stage Preimplantation Development (Morula to Blastocyst)

At approximately the 16 cell (16C) stage in humans, reaching the utero-tubal junction, an embryo begins to develop into a morula. The time point of morula formation is dependent upon the species. Up to this point in development, the blastomeres of the pre-implantation embryo have been loosely connected through microvilli and a low number of gap junctions, allowing intercellular communication and small molecule exchange [Watson et al. 2004; Eckert and Fleming 1997]. Prior to the morula stage of development, cells begin undergoing a calcium-dependent process known as compaction [Watson et al. 2004]. This is the point and time of development when the embryo goes through a physical change that causes its apical surface cells to flatten and form connecting tight junctions, while the interior cells maintain increased connections due to an upregulation of gap junctions [Watson et al. 2004]. It is interesting to note that, even under a high powered microscope, it is impossible to distinguish individual cells of a compacted morula.

This distinctive change in embryonic morphology is associated with important developmental events. First of all, embryos begin to exhibit polarity around the time of compaction [Watson et al. 2004; Motosugi 2005; Eckert and Fleming 2008]. Polarity allows an embryo to establish and distinguish cellular position, leading to the outer, flattened cells to later differentiate into

trophectoderm (TE), and the inner cells to maintain their pluripotency and form the inner cell mass (ICM). The second purpose is to aid in embryonic fluid accumulation and retention, leading to the formation of an embryonic blastocoel. Using a rat model, Stilley made the observation that Endo embryos frequently arrest at the 8C stage, or the time point in which healthy rat embryos begin to undergo compaction [Stilley et al. 2009]. This shows another potential association between endometriosis, abnormal preimplantation development, and the inability to undergo compaction.

The final stage of mammalian pre-implantation embryo development is that of blastocyst formation. Up to this point, all cells of the embryo have been considered totipotent. Blastocysts, on the other hand, are formed from two different cell types, as referenced above: the TE and the ICM. The TE is programmed to differentiate into the extra-embryonic tissue known as the placenta, and the ICM is destined to develop into the embryo proper [Watson et al. 2004]. While the TE is programmed to become placental tissue, induced through the expression of genes such as *Tead4* and *Cdx2*, the ICM maintains a pluripotent state due to its continued, upregulated expression of *Sox2*, *Nanog*, and *Oct4* [Watson et al. 2004].

One of the distinctive features of a blastocyst is that of blastocoel formation. A blastocoel is a fluid-filled cavity that is formed due to the increased TE expression of Na⁺/K⁺ ion transporters, localized to its basolateral membrane. These active transport ion channels form a concentration gradient across the TE that leads to inner-embryo water accumulation, facilitated by the up regulation of

aquaporin channels [Watson et al. 2004]. Along with acting as a liquid medium for a variety of metabolites and molecular building blocks, the blastocoel plays a vital role in the process of blastocyst hatching out of the ZP.

In many mammals, after the ZP-enclosed blastocyst has migrated to the upper portion of the uterus, the embryo becomes much more dynamic in movement, expanding and contracting against the ZP until it opens up to allow for blastocyst hatching [Watson et al. 2004]. Aided by several enzymes, including a trypsin-like protease [Watson et al. 2004], the blastocoel plays a significant role in reducing the integrity of the ZP. If the embryo is unable to hatch, it is incapable of implantation and further growth.

One final important purpose of the TE is to prevent a maternal immune attack against the ICM [Eckert and Fleming 1997]. This tightly associated cellular barrier also reduces the overall level of harmful osmolites that may otherwise diffuse into the blastocyst, preventing further damage to the ICM [Eckert and Fleming 1997]. Table 1.1 compares the basic requirements of mammalian precompaction versus postcompaction embryos.

Retrospective studies, using in vitro fertilization (IVF) clinical records, have shown that blastocysts from women with endometriosis exhibit reduced blastomere number and decreased blastomere mitosis rates [Brizek et al 1995; Pellicer et al. 1995; Tanbo et al. 1995]. Additional research will be needed to elucidate a relationship between endometriosis and blastocyst quality as a means of further determining the association between the disease and subfertility.

Metabolism and Preimplantation Development

Metabolism plays a very important role in all cell types. This process aids in the production of a variety of substrates necessary for nucleotide formation and cellular growth and development. Metabolism provides cells with energy in the form of adenosine triphosphate (ATP), utilized in a variety of cellular processes ranging from cell signaling to enzyme activity [Conaghan et al. 1993; Bavister 1995]. The body has a tightly regulated group of associated metabolic pathways that include glycolysis, pentose phosphate pathway (PPP), and the citric acid cycle (CAC). These pathways are controlled in a way that optimally provides the body with what is required at any specific time. Metabolism is equally important in the context of early embryonic development.

The general dogma suggests that early cleaving embryos from the zygote to the morula stage produce the majority of their ATP through oxidative phosphorylation [Bavister 1995; Houghton and Leese 2004]. This has been observed in mouse, hamster, sheep, cattle, and human [Hillman and Flynn 1980; Bavister 1995; Kito et al. 1997; Kanka 2003]. Moderate to high levels of glucose are toxic to early cleaving embryos in culture medium [Conaghan et al. 1993; Miyoshi et al. 1994]. Elevated glucose may inhibit the transcription of certain genes involved in the progression of in vitro embryonic development. Other in vitro studies performed in mice attempt to disprove this idea, showing that the early detrimental effects of glucose may be alleviated by the addition of amino acids, metal chelators, and/or vitamins [Bavister 1995; Leese 2012].

Early stage preimplantation embryos of numerous species are more inclined to utilize pyruvate and lactate as their primary and immediate energy

sources, as opposed to glucose. Pyruvate is a 3-carbon molecule that can be directly shuttled into the mitochondrial citric acid cycle and further broken down to produce ATP for the quickly developing early embryo [Conaghan et al. 1993; Noordin 2008]. Lactate can be reversibly oxidized to pyruvate, and thereby similarly contribute to immediate embryonic energy production.

Early stage embryos are also considered metabolically quiescent, explaining their noticeably high ATP: ADP ratio compared to later stage embryos [Houghton and Leese 2004]. It is important to note that all stages of preimplantation embryos express membrane glucose transporters, as well [Pantaleone and Kaye 1998]. The low levels of early stage glucose taken up by these transporters have been shown to be primarily shunted into the pentose phosphate pathway (PPP), sustaining stores of reducing molecules necessary for other cellular processes. If the balance of glucose, pyruvate, and lactate is dysregulated, the embryos may undergo atresia. Moderate to high concentrations of phosphate have been observed to be detrimental to early embryos in a large majority of mammalian species [Miyoshi et al. 1994; Lane and Gardner 2007].

Post morula stage, glucose becomes the preferential energy substrate [Houghton and Leese 2004; Leese et al. 2008]. The increase in consumption has been attributed to the many essential processes that occur during this latter developmental stage, including the initiation of cellular differentiation and blastocoel formation. There is also an elevated requirement for ATP in blastocysts due to the increased necessity for macromolecular synthesis (e.g.

DNA, RNA, and proteins). While the ICM has been shown to rely completely on glycolysis for energy, the TE metabolizes glucose principally through oxidation-based metabolism [Robinson and Benos 1991; Houghton et al. 2004].

There is a sharp increase in glucose consumption at the blastocyst stage, yet it only immediately accounts for ~17% of the total ATP produced [Thompson et al. 1996]. The oxidation of pyruvate is still predominant in the fact that it is accountable for ~40% of ATP production at this time point [Thompson et al. 1996]. A logical explanation for this occurrence is that glucose is necessary for other purposes in the blastocyst, potentially contributing to the accumulation of important glycolytic intermediates such as glucose-6-phosphate and fructose 1, 6-*bis*phosphate [Thompson et al. 1996]. Glucose-6-phosphate, for example, may be alternatively shuttled into the PPP, where it is involved in replenishing stores of nicotinamide adenine dinucleotide phosphate (NADPH), ribulose-5-phosphate, and/or nucleotide precursors.

Similar to oocytes, embryos tend to have a general requirement for fatty acid oxidation. While a majority of culture media are not supplemented with lipids, they do include some type of supplemental serum, such as bovine serum albumin (BSA). Bovine serum albumin is a known carrier of fatty acids [Richieri et al. 1993] and as such indirectly adds fatty acids to embryo culture media. As stated, specifically at the blastocyst stage, ~57% of ATP production is accounted for by glucose and pyruvate metabolism [Thompson et al. 1996]. Fatty acid metabolism is predictably the best explanation for the unaccounted 43% of embryonic ATP [Yamada et al. 2012].

It has been observed that octanoate, a medium-chain fatty acid, can act as a fairly efficient energy source throughout the entirety of embryonic development [Yamada et al. 2012]. While early stage embryos tend to express the enzymes necessary to break down small- and medium-chain fatty acids, blastocysts are capable of utilizing small-, medium, and long-chain fatty acids [Hillman and Flynn 1980; Yamada et al. 2012].

It is very easy to disrupt the overall process of embryonic development. In vivo, it takes a distinct spatial and temporal coordination of pH, osmolality, and temperature, along with proper metabolite uptake, to supplement embryonic growth. This is why, when designing a successful embryo culture system, it is important to mimic this normal in vivo environment [Han and Niwa 2003; Jang et al. 2007; Rizos et al. 2008].

In Vitro and Cell Culture Media Conditions

The initial observation that mouse embryonic growth could be improved when calcium chloride was replaced with calcium lactate triggered an interest in culture media optimization in the late 1950s [Whitten 1957]. Around this time, a researcher by the name of Whitten observed that mouse embryos could grow from the 8C to blastocyst stage in a Krebs-Ringer bicarbonate system, supplemented with glucose, bovine plasma albumin, and antibiotics [Whitte 1957]. These initial studies determined that embryos have different developmental requirements from somatic cells, specifically in relation to glucose. Also, Whitten's studies laid the foundation for future researchers to begin optimizing culture media in a variety of different species, attempting to

overcome the developmental block that corresponds to the MZT [Brackett et al. 1982; Miyoshi et al. 1994].

It is important to mimic the oviductal environment when designing a culture media for early embryonic development in vitro. Along with the variety of important proteins and metabolic components produced by the embryo itself, the oviduct provides a wide array of growth factors, cytokines, and other embryotrophic components that contribute to the normal growth of an embryo [Lee and Yeung 2006]. For example, porcine oviducts have been observed to secrete at least 14 different proteins when grown in culture, including TIMPs and oviduct specific glycoprotein [Stokes et al. 2005]. Additionally, these proteins can be differentially expressed dependent upon hormonal influence.

The oviduct also helps to maintain pH, temperature ($\sim 37^{\circ}\text{C}$ to 40°C , depending on the species), and solute concentrations necessary to sustain development [Lee and Yeung 2006]. In this respect, most mammalian culture systems are buffered accordingly, and in an incubator with an atmosphere of 5% CO_2 in air. This percentage may vary, dependent upon the makeup of the medium, in order to sustain a consistent physiological pH. A bicarbonate based buffer system is used to provide a stable pH in the presence of high levels of CO_2 , while a 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer system aids in pH sustainment outside of a CO_2 incubator under normal environmental conditions [Hentemann et al. 2011].

The pH and osmolality of an early embryonic microenvironment must stay consistent in order to stabilize a growing embryo's internal conditions, to prevent

blastomere lysis and/or apoptosis. In the oviduct, a variety of non-essential amino acids, metabolites, and serum albumin play this significant role [Lee and Yeung 2006]. This is especially important in early embryos, due to the fact that they maintain a poorly developed ion transporter system and have difficulty protecting their internal environment from stress in comparison to post-compaction embryos [Watson et al. 2004]. Early stage embryos take up less oxygen compared to later-stage expanding blastocysts in vivo. It has been documented that, while early stage murine embryos take up $\sim 4 \mu\text{l}/\text{mg}$ of oxygen, blastocysts increase consumption by over twice this amount [Thompson et al. 1996]. In reference to embryo culture, there are many animal studies which indicate that high oxygen tension, ranging between 10% and 20%, is detrimental to embryos of certain species, while lower, environmental oxygen tension may be beneficial [Gardner 2008].

For example, cattle and rabbits demonstrate optimal embryonic developmental potential at an O_2 concentration of 5%, while rats and mice thrive at much lower concentrations [Fischer and Bavister 1993; Guerin et al. 2001]. The adverse effects of high oxygen are believed to be due to the production of ROS, or molecules that have the ability to induce DNA damage and blastomere apoptosis [Yang et al. 1998]. This may be avoided by including antioxidants in culture media, such as the tripeptide glutathione; but, this only helps to a certain degree [Gardiner et al. 1995]. Glutathione is a nonessential nutrient that is considered a reducing agent, capable of decreasing the reactivity of harmful, unstable molecules such as free radicals and ROS by acting as a proton donor.

Even with this information, researchers have yet to develop optimal in vitro human culture media. As a precautionary measure, IVF clinics may maintain a low incubator O₂ concentration throughout embryo culture [Burton et al. 2003] .

Amino acids have important functions in the developing embryo, as well. Along with being necessary for protein production, amino acids can act as embryonic chelators, anti-oxidants, osmolytes, and metabolic substrates [Partridge and Leese 1996; Gardner and Lane 1998]. For example, glutamine, a polar, charged amino acid, is beneficial due to the fact that it can be broken down into varying components utilized in the citric acid cycle, along with being a primary nitrogen source for the embryo. Glycine is also an important osmotic regulator in early stage embryos (Partridge and Leese 1996; Gardner and Lane 1998). This is why amino acid supplementation in culture media is extremely important.

Amino acids spontaneously break down in a 37°C culture medium environment, producing embryotoxic by-products such as ammonium. Glutamine, due to its volatile nature, tends to be responsible for the vast majority of the ammonium [Lane et al. 2001]. A stable di-peptide version of glutamine, in the form of either alanyl-L-glutamine or glycyl-L-glutamine, is supplemented as a replacement [Lane et al. 2001]. Embryonic amino acid profiles can be utilized to judge overall quality and developmental potential of embryos cultured in vitro [Booth et al. 2007].

All embryonic stages, from the early cleaving to post-compaction, necessitate non-essential amino acids (NEAA) in culture media [Bavister 1995;

Gardner 2008]. Essential amino acids (EAA) may be detrimental to early cleaving embryos, but required for post-compacted embryo development [Gardner and Lane 1993]. There are a few theories that attempt to explain this phenomenon. One example is that early stage embryos, known to have minimal control over transmembrane transport may be unable to take up a variety of essential amino acids due to a lack of appropriate transporters. This excessive presence of non-metabolized EAAs in the media leads to an increased prevalence of toxic products (e.g. ammonium) that would be harmful to the embryos (due to increased EAA degradation) [Gardner and Lane 1993].

Post-compacted embryos maintain a well-established system of Na⁺/K⁺ pumps, and amino acid transporters capable of controlling their internal environment and taking up both EAAs and NEAAs [Watson et al. 2004]. This well-developed transport system is why morulae and blastocysts are less sensitive to environmental changes compared to early cleaving embryos.

There are a wide variety of non-metabolic factors important to in vitro culture. For instance, in order to avoid osmotic dysregulation, media must maintain a salt composition (e.g. NaCl and KCl) to maintain osmolality in the range of 275 to 290 mOsm [Baltz 2012]. This is especially important in early stage embryos, which, as mentioned previously, have difficulty in maintaining a stable internal environment.

There are a variety of molecular transporters that require the presence of specific ions in order to function, including chloride and sodium [Van Winkle 2001]. These ions are obtained through the dissociation of the salts within the

liquid medium. For example, in mice, glycine transporters, predominant in early stage embryos, have been observed to be Cl^- and Na^+ dependent. Magnesium, frequently present as the stable MgCl in culture media, is another important ion, acting as a cofactor in a variety of enzymatic systems involved in embryonic growth [Van Winkle 2001].

In the past, maternal serum, with a plethora of factors beneficial to both somatic cell and embryonic growth, including cytokines and vitamins, was used in embryo culture media [Pinyopummintr and Bavister 1991; Lim et al. 1994]. This type of supplementation has predominantly gone out of practice due to the fact that there are many uncharacterized components in serum. Rather, serum albumin or synthetic serum substitutes have replaced it. Albumin is one of the most abundant macromolecules located within the oviduct [Lippes et al. 1972; Gardner 2008]. It has benefits ranging from the negation of increased colloidal osmotic pressure in the embryo to inducing an increase in the surface tension of culture media. The latter effect greatly aids in embryo manipulation. Finally, commercially developed albumin is free from specific types of biological contamination, including HIV particles, lending it to be safer to use in human embryo culture [Desai et al. 1996]. The above discoveries came about thanks to well controlled experiments performed over the last 60+ years. Culture media optimization is still of great interest in research laboratories. As of yet, there is no perfect in vitro medium for any species. Along with composition, there is the importance of general culture system design [Bavister 1995; Vutyavanich et al. 2011].

Some researchers place embryos in small microdrops of medium, while others prefer to use a one milliliter volume of medium in double-well culture plates. In either case, it is extremely important to use materials that are non-embryotoxic [Bavister 1995; Rizos et al. 2008]. There have been observed benefits of group culture versus individual culture [Vutyavanich et al. 2011; Menezo et al. 2012]. Studies suggest that group culture may be preferential due to the release of paracrine factors beneficially effecting adjacent embryos.

Group culture maintains elevated levels of embryotoxic metabolites when compared to individual culture increasing the frequency of necessary media culture change. Group vs. single culture tends to be more of a personal preference based on individual researcher observations. It is also always recommended to cover media with light mineral oil to allow for it to equilibrate its pH, osmolality, and temperature overnight in a CO₂ incubator prior to performing experiments [Bavister 1995; Vutyavanich et al. 2011]. Supplementation of media with antibiotics is also important, in order to reduce bacterial contamination of the culture medium.

Media may be of a single or sequential type. Single culture includes a single set of components that successfully nourishes the embryo from the zygote to blastocyst stage [Reed et al. 2009; Paternot et al. 2010]. The rationale behind using a sequential system is that it takes into consideration the changing environment of the oviduct, and uterus, as an in vivo embryo travels down the reproductive tract. The elements addressed above (e.g. amino acids and

carbohydrates) are included in both types of media at variable concentrations [Reed et al. 2009; Paternot et al. 2010].

Rat embryos, for example, are grown optimally using a sequential media protocol [Miyoshi et al. 1997; Miyoshi et al. 1998]. An early stage growth medium is maintained at a higher osmolarity than the later stage medium, as a means of more stably controlling amino acid and metabolite uptake in the pre-compact rat embryos that are more susceptible to external stress [Miyoshi et al. 1998]. The sequential version of the rat culture media reportedly leads to a 75% blastocyst developmental success rate [John Critser, unpublished].

There are a variety of human media, both of the monoculture and sequential culture categories [Gardner and Lane 1998; Sepulveda et al. 2009]. There have been many studies performed, with predominant use of the mouse model, that attempt to determine the differences in these media types. A meta-analysis performed on a variety of murine studies has shown that sequential media is more beneficial at producing a greater quality of blastocyst, maintaining a statistically higher number of ICM and TE cells compared to those grown in a single medium [Lane and Gardner 2007].

These same blastocysts also tend to exhibit higher implantation rates when compared to single culture grown embryos. The G-1™ (*VitroLife AB, Göteborg, Sweden*) and G-2™ (*VitroLife AB, Göteborg, Sweden*) media is an example of an industry-produced human sequential system. The differences in G-1 and G-2 are the concentrations of pyruvate, lactate, and glucose (Table 2.1) [Gardner and Lane 1998].

Preimplantation 1 ® (early stage) and Multiblast® (later stage) (*Irvine Scientific, Santa Ana, California*) is another example of a human sequential system. Multiblast® contains glucose and essential/non-essential amino acids, while P1® does not. Global® medium (*LifeGlobal, Guilford, Connecticut, USA*) is an example of an industry-produced single culture system. In vitro fertilization clinics throughout the United States use varied embryo culture media, including P1®_Multiblast® and Global®. This choice is primarily dependent upon embryo development in each laboratory, taking into consideration available data from research studies.

Murine embryo assays (MEAs) are a common protocol used IVF clinics to validate the quality of human embryo culture media [Fleetham et al. 1993; van den Bergh et al. 1996]. Murine embryo assays are also used to perform quality control studies to ensure the safety of petri dishes, mineral oil, incubators and air systems used in each specific IVF clinic to mitigate embryotoxicity [Fleetham et al. 1993]. Due to ethical restrictions, human embryos cannot be used in quality control protocols.

The MEA involves the process of growing murine embryos from the pre-compaction to blastocyst stage, in culture media developed for human embryos. The one-cell MEA is recorded as being the most appropriate embryotoxicity assay in IVF clinics. The lack of IVF clinical standardization is due to the fact that there is no definitive consensus in the medical community about an optimal process that should be used to test embryotoxicity. Many IVF clinics perform MEAs on a monthly or bi-monthly basis. Production companies also use MEAs

as a means of testing the quality of each lot of their media products prior to distributing them to laboratories.

The percentage of blastocyst formation is the most common endpoint for a MEA [Fleetham et al. 1993; van den Bergh et al. 1996; Gardner et al. 2005].

There has been a recent focus on determining alternative endpoints of MEA analysis. For example, there have been studies performed looking at the effects of toxic volatile compounds, such as formaldehyde, on embryonic amino acid turnover (e.g. leucine) in an MEA and its association with embryo quality [Gada et al. 2012]. The degree of inner cell mass outgrowth on a gelatin-covered plate has also been shown to be correlated with different levels of formaldehyde exposure, potentially relating to blastocyst development in media of variable qualities [Gada et al. 2012]. It is important to mention that these two types of protocols have yet to be utilized in a human IVF environment.

Different types of endpoints, such as amino acid metabolism and ICM outgrowth assays, are of increasing interest because there is a significant amount of existing literature that disagrees with the association between current MEA results and human IVF [van den Bergh et al. 1996]. More sensitive types of quality control are needed as a means of optimizing media. One idea is that a basic MEA should be performed, in adjunct with other quality control protocols.

Ubiquitin-Proteasome System

The 26S proteasome, a multi-protein holoenzyme, is a primary example of how cellular processes are tightly regulated. Proteasomal degradation involves the targeting and degradation of specifically marked proteins and short-chain peptides [Driscoll 1994; Tipler et al. 1997]. A typical mammalian 26S

proteasome is made up of two types of complexes: a 20S proteolytic core, and a 19S regulatory cap. The recognition element in proteasomal proteolysis is an 8.5 kDa protein known as ubiquitin. The 19S complex is capable of recognizing and binding to chains of ubiquitin that have been linked together through covalent bonds, and attached to a targeted protein [Driscoll 1994; Tipler et al. 1997]. Once the recognition event takes place, the ubiquitin chain is removed, and the target substrate is transported into the 20S core where its peptide bonds are proteolytically cleaved.

There are many important temporal and spatial cues that trigger proteasomal degradation due to the variety of pathways that are regulated by this process. These include the following: cell cycle progression and division, DNA transcription and repair, apoptosis, meiotic resumption, and the modulated response to cellular stressors [Driscoll 1994; Tipler et al. 1997; Dawson et al. 1997]. Without proper regulation maintained by the ubiquitin-proteasome system, cellular function and proliferative ability would be completely deregulated, and severe damage to both the designated cell and surrounding cells may occur [Driscoll 1994; Dawson et al. 1997].

The 20S proteolytic core is a barrel-shaped structure made up of two beta rings flanked on each end by two alpha rings, each ring composed of seven subunits [Dawson et al. 1997]. The core beta rings contain proteolytic enzymes that have chymotrypsin-like, trypsin-like, and postglutamyl peptide hydrolyzing activities that function in the degradation of target proteins. The alpha ring is strictly involved in modulating the entrance of proteins/peptides into the beta ring

core [Dawson et al. 1997; Hendil et al. 1998]. The 19S cap is also formed from two distinctive groups of subunits: the 'base' and the 'lid'. The base and lid are further broken down into two different types of subunits, based on whether their activity is either ATP dependent or ATP-independent. The ATP-independent components of the 'base' are involved in the recognition and binding of polyubiquitin-tagged proteins, while the ATP-dependent subunits are involved in protein unfolding and controlled entrance into the 20S portion of the proteasome [Dawson et al. 1997; Hendil et al. 1998]. It is important to note that, prior to entrance into the proteasomal core, the branching ubiquitin chains are cleaved off by a group of proteasomal subunit proteins known as de-ubiquitinating enzymes (DUBs) [Zhu et al. 1996; D'Andrea and Pellman 1998]. It has been hypothesized that these DUBs are targeted to the active proteasome through the non-ATPase subunits of both the 19S base and lid [Hendil et al. 1998]. It is important to note that not all DUBs are involved in substrate-ubiquitin cleavage. Cellular processes regulated by DUBs can range from subcellular localization to protein activation/inactivation [Chung and Baek 1999].

There are three components that are of primary importance in the context of the ubiquitin-proteasome system. These include the proteasome itself, the DUBs, and the targeting protein molecule(s) known as ubiquitin (Ub). Ubiquitin is a 76-amino acid protein that is conjugated to specific proteins in the form of polyubiquitin chains [Dawson et al. 1997]. While targeted protein degradation is an important role of ubiquitin binding, it is not the sole purpose of this type of molecule. Ubiquitin can also bind as monoubiquitin to histones, acting as an

epigenetic transcriptional regulator, or other cytoplasmic and nuclear proteins, aiding in controlled subcellular localization [Wang et al. 2004]. For example, mono-ubiquitin has been observed to interact frequently with the histone variant H2A at Lys119, regulating chromatin availability for transcription [Baarends et al. 1999; Wang et al. 2004].

It is important to understand the mechanisms behind ubiquitin activation and conjugation in order to comprehend just how tightly the ubiquitin proteasome system is regulated. This process involves three different types of enzymes: Ubiquitin-activating enzyme (E1), Ub-conjugating enzyme (E2), and a Ub-ligase (E3) [Ciechanover 2003]. The enzyme E1 is an ATP-dependent enzyme that functions in the activation of ubiquitin through Ub acetylation. This event leads to the formation of a thioester bond between ubiquitin and a cysteine residue located in the E1 active site. This E1:Ub complex interacts with and subsequently transfers the Ub onto an E2 enzyme [Ciechanover 2003]. Next, E2 interacts with a type of ligation enzyme known as E3. This E2:E3:Ub multiprotein complex will, in turn, recognize the product that is to be targeted for ubiquitination, and E3 will ligate the Ub molecule onto a designated lysine residue of the substrate [Ciechanover 2003]. Further rounds of ubiquitination occur, forming a polyubiquitin chain on the targeted peptide/protein, mediated by the 'E3-like protein' E4. This chain is then recognized by the proteasomal 19S complex, triggering polypeptide degradation. These Ub chains can occur in a variety of branching conformations, leading to varying levels of proteasomal recognition [Ciechanover 2003]. The primary structure of ubiquitin is conserved across a

wide array of eukaryotic species, exhibiting 96% sequence homology between humans and yeast [Ciechanover 2003; Zheng et al. 2009].

Deubiquitinating enzymes are the final type of regulatory component involved in the proteasomal-ubiquitin pathway [D'Andrea and Pellman 1998]. Deubiquitinating enzymes are proteases that target the isopeptide bonds that are formed between either two Ub molecules, or a Ub molecule and a targeted substrate. Commonly, this targeted region is the bond formed between a substrate's lysine residue, and the C-terminal carboxylate group of ubiquitin [D'Andrea and Pellman 1998]. Deubiquitinating enzymes primarily aid in the disassembly of polyubiquitin chains prior to insertion into the 20S proteolytic proteasomal core, along with salvaging proteins that had been incorrectly targeted for degradation [D'Andrea and Pellman 1998]. In an indirect manner, these deubiquitinating enzymes are important in preventing the inappropriate regulation of a variety of cellular processes, including cell cycle progression and the response to stressors such as reactive oxidative species (ROS).

In the last 10 years, researchers have begun to speculate on alternative 'proteolytic regulatory mechanisms' of DUBs. The primary model of interest involves the idea that DUBs may bind directly to the E3 ligase enzyme, inhibiting its ability to perform the final step of ubiquitination [Sowa et al. 2009]. Numerous DUB/E3 binding pairs exist. It has been stated that this type of pairing may act in one or two different ways: (1) the DUB deubiquitinates E3 before it is able to transfer the Ub molecule to the targeted substrate or (2) the DUB destabilizes the E2:E3:Ub complex, reducing its functionality [Sowa et al. 2009]. From the

literature, it can be interpreted that the core role of DUBs is to regulate the attachment and presence of ubiquitin, whether it be in a direct or indirect manner. Upon disassembly of ubiquitin chains from the targeted substrates, monoubiquitin molecules are regenerated and capable of being recycled for further protein tagging [D'Andrea and Pellman 1998; Ciechanover 2003].

Similar to the ubiquitinating enzymes, there are multiple classes of DUBs [Chung and Baek 1999]. Two primary categories include cysteine proteases, and metalloproteinases. Metalloproteinase DUBs contain active sites that require zinc binding in order to maintain activity [Chung and Baek 1999]. Alternatively, cysteine protease DUBs, also known as thiol proteases, do not require the binding of metal ions for activity, and have a specific catalytic mechanism that is involved in cleaving protein-protein bonds [Chung and Baek 1999].

There are four known families of cysteine proteases, which are defined primarily by the manner in which they interact with Ub molecules. Two examples include the ubiquitin C-terminal hydrolases (UCHs) and ubiquitin specific proteases (USPs) [Chung and Baek 1999]. Ubiquitin C-terminal hydrolases may either directly bind to and increase the half-life of monoubiquitin, or trim Ub off at the distal ends of polyubiquitin chains. In contrast, USPs bind to non-ubiquitin tagged regions of a Ub-targeted substrate prior to Ub cleavage and removal. In reference to cysteine protease activity, it seems that the majority of these enzymes use a mechanism that involves an important catalytic triad of amino acids made up of cysteine, histidine, and aspartic acid/asparagine. Each specific

amino acid plays a role in the proteolytic cleavage of ubiquitin [Chung and Baek 1999]. Interestingly, while it had been previously thought that ubiquitin pools stay constant, it has now been shown that this protein can either be degraded by the proteasome, or upregulated in expression, dependent upon specific cellular conditions [Proctor et al. 2010].

There is a subset of cysteine protease DUB enzymes which contain a 230 amino acid catalytic UCH domain at their N-Terminus. In humans, there are four UCH DUBs that have been identified, including UCHL1, UCHL3, UCHL5, and BRCA1 associated protein-1 (BAP1). Ubiquitin C-Terminal Hydrolase L1 and UCHL3 are the smaller of the UCHs, made up entirely of the UCH domain [Larsen et al. 1998]. These two enzymes preferentially bind to small proteins that have been ubiquitin-tagged. Interestingly, neither UCHL1 nor UCHL3 is capable of cleaving Ub-Ub bonds. In contrast, BAP1 and UCHL5 have extended strings of amino acids adjacent to their UCH domains (~500 and ~100 residues, respectively) along with preferentially cleaving Ub-Ub bonds [Larsen et al. 1998; Chung and Baek 1999].

Ubiquitin C-Terminal Hydrolase L1 and UCHL3 are extremely interesting molecules in reproductive physiology, primarily due to their varied expression patterns and localization in developing gametes and embryos [Yi et al. 2007; Mtango et al. 2012]. It has been observed that UCHL1 is selectively expressed in neuronal tissue, gametes, and preimplantation embryos [Yi et al. 2007; Mtango et al. 2012]. Ubiquitin C-Terminal Hydrolase L3 is ubiquitously expressed in all known types of tissues. While both of these DUBs maintain 52% homology, their

enzymatic activity differs when tested in vitro [Boudreax et al. 2010]. The activity of UCHL3 is known to be >200x higher than UCHL1. Using *gad* mutant, or UCHL1 deficient, and *Uchl3* knockout mice, the importance of each of these Ubiquitin C-Terminal Hydrolases have also been elucidated in the context of fertility and organismal survival. For example, UCHL1 has been shown to be predominantly present in dividing spermatogonia, but not in meiotically maturing spermatocytes [Kwon et al. 2005]. Ubiquitin C-Terminal Hydrolase L3, on the other hand, is present at all stages of spermatogenesis. This implies that UCHL1 is primarily important in the control of the mitotic proliferation of spermatogonia, and UCHL3 may be involved in meiotic sperm development and differentiation [Kwon et al. 2005].

The DUBs and the ubiquitin-proteasome system are involved in the mediation of cell apoptosis. The *gad* mutant mice deficient in UCHL1 exhibited an elevated expression of the antiapoptotic proteins Bcl-2 and Bcl-xL, along with a reciprocal increase in the prosurvival proteins known as p cAMP response element-binding protein (pCREB) and brain-derived neurotrophic factor (BDNF), when cryporchid injury was induced [Kwon et al. 2004]. Under normal circumstances, this type of injury would have led to an increase in activity of the apoptotic pathway, not a decrease. From this, it is inferred that UCHL1 is involved in balancing levels of pro-apoptotic and anti-apoptotic gene products [Kwon et al. 2004]. Ubiquitin C-Terminal Hydrolase L1 loss, which is normally responsible for binding to and maintaining stable monoubiquitin, may lead to a

reduction in freely available Ub, reducing the overall capability of the cell to sustain the appropriate levels of proteasome-targeting polyubiquitin chains.

In the male reproductive system, it has been observed that UCHL1 and UCHL3 maintain distinctive expression patterns throughout the epididymis [Kwon et al. 2005]. Ubiquitin C-Terminal Hydrolase L1, for instance, is expressed at a higher level in the caput epididymis, while UCHL3 is more highly expressed in the cauda epididymis [Kwon et al. 2005; Baska et al. 2008]. It is interesting to note that ubiquitin is expressed throughout the epididymus similarly to UCHL1, being more highly present in the caput compared to the cauda. This seems logical considering the Ub stabilization function of UCHL1. From this observation, it is tempting to suggest that the high levels of monoubiquitin and UCHL1 at the caput contributes to the increased level of apoptosis present in this epididymal region, aiding in the elimination of abnormal sperm [Baska et al. 2008]. This theory is supported by the fact that levels of p53 and Bax, two pro-apoptotic proteins, are greater in the caput compared to either the epididymal cauda or corpus. Along with being involved in the mediation of male gametogenesis, UCHs have been proven to play an important role in sperm capacitation and acrosomal exocytosis [Zimmerman and Sutovsky 2009].

The UCHs also play a key responsibility in female reproductive function as well, ranging from the progression of oogenesis to the 'block to polyspermy' [Yi et al. 2007; Mtango et al. 2012]. Ubiquitin C-Terminal Hydrolase L1 accumulates in the cortex of the oocyte. The cortex includes the plasma membrane and the portion of cytoplasm directly adjacent to it; the thickness ranging from 0.1-20

micrometers dependent upon species [Sardet et al. 2002]. This section of a cell is considered a scaffold for multiple types of components, including groupings of endoplasmic reticulum, mitochondria, microtubules/microfilaments/intermediate filaments, and a variety of signaling molecules and mRNA. The proportion of what is located in the cortex is dependent upon the species, and the specific stage of embryo development [Sardet et al. 2002]. As UCHL1 is tethered to the cortical cytoskeleton, it may be involved in important processes regulating oocyte maturation, fertilization, and preimplantation development.

It has been observed that UCHL1 contributes to polyspermy block [Yi et al. 2007]. There have been studies performed that show that the block to polyspermy occurs through the regulation of cortical granule (CG) reorganization throughout the cortex. In the mutant *gad* mice deficient in UCHL1, it is noticeable that CG exocytosis is delayed upon sperm-zona pellucida (ZP) penetration [Yi et al. 2007]. This delays the subsequent hardening of the ZP, allowing for the entrance of multiple spermatozoa into the perivitelline space. In a large majority of species, including rodent, bovine, and human, polyspermy is detrimental to the developing zygote [Sun 2003].

Several factors contribute to the abnormal release of cortical granules in fertilized oocytes upon the knockout of UCHL1. An increased accumulation of misfolded proteins has been observed in the endoplasmic reticulum (ER) of *gad* mouse oocytes [Koyanagi et al. 2012]. This is accompanied by the abnormal increase of a few different maternally derived proteins, one example being maternal antigen that embryos require (MATER). Based on this study, it seems

that one primary role of UCHL1 is to regulate the turnover of specific maternally-derived proteins, and misfolded polypeptides [Koyanagi et al. 2012]. The accumulation of MATER in the cortex of the oocyte has been previously associated with oocyte maturation failure. This resultant lack of oocyte maturation, in turn, may lead to delayed ER accumulation in the cortex, reducing the overall level of cortical calcium availability. Calcium release is extremely important in the context of cortical granule exocytosis. The excess of unfolded proteins in the endoplasmic reticulum causes ER stress, as can be observed by the increased expression of heat shock protein A5 (HSPA5), Endoplasmin (ENPL), and Calreticulin (CALR), three chaperone proteins involved in moderating ER stress [Koyanagi et al. 2012]. Similar to the excessive presence of MATER, this elevated level of ER stress can lead to a subsequent decrease in calcium release.

Mtango et al has shown that both *Uchl1* and *Uchl3* are expressed predominantly as maternal transcripts throughout oogenesis, and are steadily reduced in expression upon fertilization and throughout preimplantation development [Mtango and Latham 2007]. These results were consistent in both mice and Rhesus monkeys [Mtango et al. 2007]. The UCHL1 protein is localized to the cortex of both the oocyte and embryonic blastomeres in the mouse [Mtango et al. 2012]. Ubiquitin C-Terminal Hydrolase L3 accumulates in the oocyte spindle and blastomere nuclei during oocyte maturation and embryonic development, respectively. This has been observed in a variety of species, including bovine, porcine, primate, and murine. Further, in order to further

elucidate the functions of UCHL1 and UCHL3, in vitro experiments were performed in which oocytes were injected with either an UCHL1 inhibitor, UCHL3 inhibitor, or an overall UCH inhibitor known as UBAL [Mtango et al. 2012]. Inhibiting UCHL3 led to abnormal spindle microtubule length and configuration, leading them to hypothesize that UCHL3 plays a role in microtubule spindle pole focusing, and chromosomal organization and segregation. Ubiquitin C-Terminal Hydrolase L1 and UBAL inhibitor injections led to the extrusion of an abnormally large first polar body and abnormal distribution of cortical granules [Mtango et al. 2012]. Thus, along with maintaining cortical ER calcium release, UCHL1 may also be involved in controlling the dynamics of microtubules and microfilaments, aiding in the actual allocation of CGs.

One final interesting note concerning oocytes and the localization of UCHL1 and UCHL3, is that UCHL3 may be partially compensatory for UCHL1 [Mtango et al. 2012]. It was recognized that, in the mutant *gad* mice deficient in UCHL1, UCHL3 localized at the cortex of mature oocytes, along with displaying a typical association with spindle microtubules and nuclei. In oocytes from wild type mice, this association does not occur. Considering that mutant *gad* oocytes are prone to polyspermy and embryonic arrest, it is difficult to interpret the importance of this UCHL3 cortical translocation/'replacement' in these mutant *gad* mice deficient in UCHL1.

It has also been observed in UCHL1-deficient *gad* mice that the majority of collected embryos were incapable of developing past the morula stage [Mtango et al. 2012]. These morulas were suspended in growth around the time of

compaction. Mtango et al also reported that *gad* mice have 3.6 pups per litter, as opposed to the 7.3 pups per litter present from wild type mice [Mtango et al 2012]. These observations demonstrate that even though UCHL1 deficient oocytes are capable of being fertilized, they tend to have reduced developmental potential. More research needs to be done in order to determine why UCHL1 and/or UCHL3 are required for later stage preimplantation development.

Project Goals

Endometriosis is a common disorder associated with female infertility. The goal of this project was to determine if zygotes from rats with surgically induced endometriosis may be salvaged by removing them from the inflammatory environment of the Endo reproductive tract, placing them into an established culture system and growing them to either Day 2 or Day 4. This in vitro culture regimen was designed to reduce embryo exposure to harmful endometriotic secretions present in vivo. The outcomes of our research may relate to human in vitro fertilization and IVF protocols, specifically referring to patients who have endometriosis.

A secondary goal of my research was to compare the developmental potential of healthy murine zygotes in a sequential media system versus a single medium system. Based on previous observations, our expected outcome was that one medium will be superior over the other in supplementing murine embryonic growth, which can predictably be associated with the culture of human embryos. It is important to determine which embryo culture protocol most successfully supplements human embryo growth and development in vitro, in

order to help increase the pregnancy and birth rates associated with subsequent embryo transfer performed at IVF clinics.

Table 1.1: Precompaction Versus Postcompaction Mammalian Embryo Requirements¹

<u>Precompaction Stage</u>	<u>Postcompaction Stage</u>
Low biosynthetic activity	High biosynthetic activity
Low QO_2 (Metabolic Quotient)	High QO_2 (Metabolic Quotient)
Pyruvate-based metabolism	Glucose-based metabolism
Maternal genome	Embryonic genome
Low ability to maintain cellular homeostasis	Transport epithelium to maintain cellular homeostasis

¹ Modified from [Lane and Gardner 2007]

Table 1.2: Media Component Comparison in Humans¹

	Pyruvate (mM)	Lactate (mM)	Glucose (mM)
Oviduct	0.32	10.5	0.5
G-1 (cleavage stage)	0.32	10.5	0.5
Uterus	0.1	5.87	3.15
G-2 (blastocyst stage)	0.1	5.87	3.15

¹ Modified from [Gardner 2008]

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Figure 1.1: Ubiquitin-Proteasome System. A depiction of proteasomal protein degradation by the 26S Proteasome, involving the Ub-activating/conjugating enzymes E1, E2, and E3 as a means of conjugating ubiquitin onto a specific proteinaceous substrate [Ciechanover 2003].

CHAPTER 2

PREIMPLANTATION EMBRYONIC DEVELOPMENT OF RAT ZYGOTES TRANSFERRED FROM AN IN VIVO ENDOMETRIOTIC ENVIRONMENT TO AN IN VITRO CULTURE SYSTEM

INTRODUCTION

Subfertility is a primary symptom associated with endometriosis, a gynecological disorder that afflicts millions of women worldwide [Garrido et al. 2002]. Known to traverse the lumen of the entire reproductive tract while present in peritoneal fluid (PF), endometriotic lesion secretions (e.g. pro-inflammatory factors) localize in the thecal cells of the ovary [Stilley et al. 2009]. Furthermore, developing embryos are exposed to these same adverse secretions. Embryos obtained from women diagnosed with endometriosis exhibit elevated levels of blastomeric fragmentation and developmental arrest [Brizek et al. 1995; Pellicer et al. 1995; Yanushpolsky et al. 1998]. Early stage murine embryos are also negatively affected when exposed to the PF of endometriosis women [Gomez-Torres et al. 2002]. While a variety of genes and proteins have been shown to be dysregulated in preimplantation embryos collected from rats with surgically-induced endometriosis, a definitive cause of the aforementioned embryo anomalies has yet to be determined [Stilley et al. 2012; Birt et al. 2013].

It would be beneficial to know at what stage of embryo development endometriosis has the most predominant effect. This would subsequently aid researchers and clinicians in narrowing their focus when studying the origin of endometriosis-associated subfertility. Based on this idea, a hypothesis was formulated: utilizing a rat model for endometriosis, Endo embryos, or embryos collected from these same rats, may be salvaged by removing them from their mothers at the zygote stage and placing them into a defined in vitro environment. Numerous women who experience endometriosis-associated subfertility seek the

aid of IVF clinics in an attempt to become pregnant. The implications behind our study thus relate to human in vitro fertilization and IVF clinical protocols. Our experiments will help further characterize advantages and/or disadvantages of removing embryos from women with endometriosis and placing them into culture medium.

MATERIALS AND METHODS

Rat Husbandry

Sexually mature female Sprague Dawley rats (*Harlan, Indianapolis, Indiana, USA*) of ~225 to 250 grams and ~70 days of age were placed into cages, two rats per cage, in a dedicated room in the University of Missouri (MU) Medical School vivarium. The room was maintained at a consistent humidity and temperature, following a light:dark cycle of 14 hours:10 hours. One hundred watt incandescent bulbs were used in the room. The primary purpose behind this light/dark cycle was so that the Sprague Dawley rats maintained a regular estrous cycle. The rats were housed and experiments were conducted in accordance with The University of Missouri Institutional Animal Care and Use Committee (ACUC) Rules and Regulations, in accordance with the National Research Council's guidelines.

A two week acclimation period was allocated prior to initiating experiments to reduce post-travel anxiety and familiarize the rats to their new living environment. During the first week, human contact was minimized to reduce the external stressor of human manipulation. During the second week, the rats were carefully handled at the same time each day, by the same person, to allow recognition by the animals.

Toward the end of the second week, daily vaginal lavage was performed between 9 and 10 a.m. and the cytology evaluated microscopically as an indirect indicator of reproductive cyclicity. The lavage was performed using a sterile glass pipette. Approximately 200 μ l volume of sterile phosphate buffered saline

(PBS) containing 1 µg/ml penicillin and 1 µg/ml streptomycin was aspirated into the pipet. The blunted, flat, open end of the pipette was then placed 1-1.5 cm into the vagina, where the PBS was gently pipetted two or three times to remove cells from the vagina. The PBS, containing vaginal cells, was expelled onto a microscope slide and the cells were observed under a Leica DMLB light microscope (200X, Leica, Buffalo Grove, IL, USA) to determine the predominant cell type, which in turn, was used to predict the specific stage of each rat's estrous cycle. The cycle stages were distinguished as described in Table 2.1.

Endometriosis and Sham Surgeries

The endometriosis surgical model used in this research was developed by Vernon and Wilson (1985). Rats were anesthetized using isofluorane (*VetOne, Meridian, Idaho, USA*) and a unilateral ovariectomy was performed via a ventral midline incision. Silk sutures (4-0, *Ethicon, Somerville, New Jersey, USA*) were used to ligate above the ovary and approximately 2/3 of the uterine horn. The uterine horn was sectioned down its longitudinal axis with small surgical scissors, cut into six equal size 2 x 2 mm pieces. Using non-absorbable 4-0 monofilament nylon suture (*Ethicon, San Lorenzo, Puerto Rico*), the pieces were sutured onto the vasculature of the rat's small intestine. Immediately after the uterine implants had been sutured in place, 1 mL of the sterile PBS with penicillin and streptomycin was instilled into the abdominal cavity to hydrate and help prevent adhesive disease. The abdominal incision was closed using continuous, interlocking absorbable suture (4-0, *CPMedical, Portland, Oregon, USA*). The skin was closed using four to six AutoClip® Wound Clips (9 mm, *Becton Dickinson, Sparks, Maryland, USA*). There were two control groups for this

project, including a sham surgery where one ovary and uterine horn were removed but no tissue was sutured into the peritoneal mesentery (Sham rats) as well as no-surgery controls (Control rats) (Table 2.2).

The analgesic Flunixin meglumine (2.5 mg/mL; *Prevail, Dallas, Texas, USA*) was injected subcutaneously at 0.1 mL per gram of body weight to relieve any potential pain. The rats were subsequently monitored post-operatively at approximately 30 minute intervals for four hours to assure that they were sternal and healthy. The following morning, the health status was evaluated and the rats were injected with a second identical dose of Flunixin meglumine. For the next four weeks, the rats were permitted time to recover from surgery and, in the Endo group, let the endometrial implants grow and develop in response to the cycling rats' hormonal milieu. Beginning one week after the surgeries, daily evaluation by vaginal cytology was reinstituted.

Timed Breeding Experiments

To facilitate breeding experiments, proven breeder males were used (*Harlan, Indianapolis, Indiana, USA*). Males were housed individually. Females were reproductively synchronized through the use of a synthetic luteinizing hormone releasing hormone (LHRH) (*Sigma Aldrich, St. Louis, Missouri, USA*). Females received 40 µg intraperitoneal injections of LHRH suspended in PBS supplemented with 1% bovine serum albumin (BSA) between 9 am and 11 am. The LHRH induced a luteinizing hormone (LH) surge, stimulating ovulation the following day. Bovine serum albumin acted as a carrier protein for LHRH. Rats were placed into a male's cage five days later, on the evening of proestrus, two females per male. The injection and breeding schedule is displayed in Table 2.3.

The next day, during the morning of estrus, mating was validated through the use of two different observations: (1) presence of a vaginal plug and/or (2) the presence of sperm in the vaginal cytology. All females that were determined to be pregnant on this Day 1 (D1) of pregnancy were then placed into one of four different groups, based on whether their embryos were to be cultured, or allowed to gestate to a specific day of development. Figure 2.1 illustrates these groups and the number of rats included in each.

The night before embryo collections were to take place, the in vitro embryo culture system was prepared. Three types of culture media were utilized: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) –modified Rat Embryo Culture Medium 1 (mR1ECM) ([Miyoshi et al. 1995] and modified by the Transgenic Core, University of Missouri, Columbia, Missouri): This medium was used in manipulating reproductive tissues outside of the CO₂ incubator (e.g. embryo collection and embryo isolation). The HEPES in the mR1ECM medium was necessary to maintain physiological pH outside of a CO₂-rich environment.

- 1) 1C to 2C mR1ECM ([Miyoshi et al. 1995] and modified by the Transgenic Core, University of Missouri, Columbia, Missouri): This high salt medium promoted the proper development of rat zygotes into two-cell stage embryos. This bicarbonate-based buffer required CO₂ in order to upkeep physiological pH. The addition of BSA functioned in supplementing the growing embryos and increasing the surface tension of the medium.

2) 2C to Blastocyst mR1ECM ([Miyoshi 1995] and modified by the Transgenic

Core, University of Missouri, Columbia, Missouri): This low salt medium

supported development of rat two-cell stage embryos into blastocysts.

This bicarbonate-based buffer required CO₂ in order to maintain

physiological pH.

The Heracell 150i incubator atmosphere was set at 5% CO₂, 95% air, 37°C, and 98% humidity (*ThermoFisher Scientific, Palm Beach, Florida, USA*). See Table 2.4 for a list of all media components and concentrations, osmolality, pH, and temperatures.

Initially, the HEPES-mR1ECM, placed into a tightly capped container, was transferred into a CO₂ incubator (5% CO₂, 95% air, 37°C, 98% Humidity). This acted to prevent the disruption of the medium's pH, while maintaining appropriate temperature prior to experiments. The 1C to 2C mR1ECM medium was mixed to a final concentration of 0.1% BSA and subsequently sterile filtered through a Millex® 0.22 µm filter (*Millipore, Billerica, Massachusetts, USA*) attached to a 5 mL pipette (*BD Syringe, Franklin Lakes, New Jersey, USA*). There is twofold reasoning for adding the BSA: (1) to supplement the growth of the embryos as a protein source and (2) to prevent the collapse of the mineral oil-covered microdrops and aid in embryo manipulation.

Three microdrops of 1C to 2C mR1ECM were pipetted into a flat, non-embryotoxic culture plate (Figure 2.2). The two smaller drops were considered washes for the embryos prior to being cultured, while the larger drop was the embryo 'resting home' throughout development inside the incubator. After one

milliliter of sterile-filtered, non-embryotoxic light mineral oil (*LifeGlobal, Guilford, Connecticut, USA*) was pipetted over the microdrops, the culture plates were placed into the Heracell 150i to equilibrate overnight after being loosely covered with their corresponding lids. All of these steps were performed on the evening prior to Developmental D1.

The 2C to Blastocyst mR1ECM medium is prepared similarly to the 1C to 2C mR1ECM medium (Figure 2.3). This specific solution was prepared on the evening prior to *in vitro* Developmental Day 2. This later embryonic stage medium does not require additional components. This is due, in part, to the presence of polyvinyl alcohol (PVA) (*Irvine Scientific, Santa Ana, California*) in the medium itself, which plays a similar role to that of BSA in the context of medium viscosity and embryo manipulation.

Embryo Collections (In Vitro Groups)

After rats were verified to have successfully bred on the morning following co-caging with a male, they were euthanized using a CO₂ overdose, followed by an aortic transection. The abdominal cavity of each female was then transected, and appropriate tissues were collected, including the entire intact reproductive tract. Removal and measurement of the endometriotic implants in the Endo groups took place at this time, to validate that they had appropriately developed to 5 mm x 5 mm in size. The Endo and Sham groups contained a single uterine horn, oviduct, and ovary, due to the surgery that they had undergone four weeks prior. The no-surgery controls had two horns, two oviducts, and two ovaries. The tracts were carefully stripped of the attached fat with a pair of small surgical

scissors and then placed into warmed HEPES-mR1ECM medium, set at 37°C, and taken to a sterile cell culture room.

Under sterile conditions, the reproductive tracts and the HEPES medium were poured out into a non-embryotoxic plastic culture plate sitting on a 37°C heated stage of a Leica s8 AP0 light stereoscope (*Leica Microsystems, Buffalo Grove, Illinois, USA*). All remaining embryo manipulations were performed under this microscope. Removing the remainder of the fat, the oviducts and uterine horns were separated. A pair of small dissecting scissors and 26G needles (26G x 3/8, *BD Syringe, Franklin Lakes, New Jersey, USA*) attached to 1 mL syringes (*BD Syringe, Franklin Lakes, New Jersey, USA*) were used during this protocol.

In order to collect the zygotes, the protruding area at the oviductal ampulla was first identified. The ampulla was then torn and punctured using the two 26G needles. After this puncture, the cumulus oophorus complexes were flushed out of the oviduct. These were immediately collected using an Origio Stripper Pipette® (*MidAtlantic, Mt Laurel, New Jersey, USA*) with an attached 300 µl pipette tip, and placed into a 1% Bovine Hyaluronidase solution, diluted in HEPES-mR1ECM medium. By gently pipetting the zygotes up and down in the Stripper Pipette®, the cumulus cells were removed over a period of three to five minutes. The zygotes were then immediately placed into the 300 µl drop of 1C to 2C mR1ECM that had been prepared the night before and equilibrated in the CO₂ incubator (Figure 2.4). This first drop was one of two washes, necessary to dilute and remove any of the toxic hyaluronidase solution from the zygotes. The zygotes were then transferred into the second wash of mR1ECM.

Zygotes were placed into the final 500 μ l drop of mR1ECM medium (Figure 2.3). After placing a lid on top of the culture plate, this culture system was transferred back into the CO₂ incubator, set to an atmosphere of 5% CO₂, 95% air, 37°C, and 98% humidity. At this point, the embryos were cultured to either developmental Day 2 or developmental Day 4. For those that were cultured to D2, images were taken on a Nikon N8008 camera (*100X, 200X, and 400X, Nikon, Tallahassee, Florida, USA*) under a light microscope equipped with Hoffman optics on the afternoon of Developmental D2. After these images were taken, the embryos were fixed in 2% formaldehyde, and prepared to be processed. For those that were chosen to grow to D4, the embryos were switched from the 1C to 2C mR1ECM medium, to the '2C to Blastocyst' mR1ECM medium on the afternoon of D2. They were then allowed to grow out to the afternoon of D4 in the CO₂ incubator without further disturbance. At this point, the embryos were imaged using the Nikon light scope, and fixed, similarly to the D2 embryos. A diagram of the embryo culture timeline can be found in Figure 2.3.

Embryo Collections (In Vivo Groups)

The in vivo derived embryos were used as a means of determining a 'baseline' for embryo quality in the context of our experiments. The rats gestated to either Pregnancy Day 2, or Pregnancy Day 4, prior to being euthanized with a CO₂ overdose, followed by an aortic transection. Reproductive tracts were then removed, identically to the in vitro rat groups, placed into the HEPES-mR1ECM medium heated to 37°C, and transferred to the sterile cell culture room.

For the in vivo D2 group, the oviducts were carefully teased away from the ovary and uterine horns with a small pair of surgical scissors. Each Fallopian Tube was subsequently flushed with HEPES-mR1ECM medium using a 26G needle attached to a 1 ml syringe, two to three times per oviduct. Afterwards, the embryos were quickly located under the Leica s8 APO light stereoscope, and fixed in formaldehyde. For the in vivo D4 group, the oviduct and uterine horn were allowed to remain attached to one another, while the ovary was removed with small surgical scissors. Using a 30g needle (30G, *BD Syringe, Franklin Lakes, New Jersey, USA*) attached to a one mL syringe, the components of the uterine horn were flushed up through the attached oviduct with HEPES-mR1ECM medium at least twice. Next, the oviductal contents were flushed down through the uterine horn with this same solution using a 26c needle attached to a one mL syringe. This flushing was performed at least two individual times, as well. This dual flushing protocol was utilized because the 8 cell to 16 cell embryos were observed to be located at both the distal end of the oviduct, and uterotubal junction, on embryonic D4. These multicell embryos were then located in a culture plate under the Leica s8 APO light stereoscope, equipped with a 37°C heated stage, and immediately placed into formaldehyde for fixation.

Embryo Fixation

As referred to above, all embryos were fixed in a formaldehyde solution post-collection. Nine-well glass plates were placed onto a heated tray, and allowed to warm up to 37°C. Following this preparation, per each group of embryos, 400 µl of warmed, pure PBS was placed into two individual wells located side-by-side. Four hundred µl of PBS, maintaining a 1.0% concentration

of polyvinyl pyrrolidone (PVP) (*Irvine Scientific, Santa Ana, California*), was then added to the third, adjacent well. The PVP was used to help prevent the embryos from attaching/sticking to the bottom of the glass well during fixation. A single cohort of embryos was pipetted into the well containing the 1.0% PVP solution. Next, 100 μ l of 10% formaldehyde was slowly added around the edges of the 'PVP well' in order to create a 2% formaldehyde/0.8% PVP solution. The embryos then incubated for 40 minutes at room temperature. This was done per every in vivo and in vitro derived rat embryo cohort.

After the incubation period, embryos were removed and briefly washed in the two adjacent PBS wells to remove any remaining formaldehyde. The embryos were subsequently transferred to a well in another 9 well glass plate that was labeled with the rat number.

The nine well plates containing fixed embryos were covered with a second nine-well glass plate, and tightly wrapped in Saran Wrap® (*Johnson and Sons™, Racine, Wisconsin, USA*) in order to prevent evaporation and potential loss of the embryos upon transportation. Embryos were immunolabeled and imaged no later than five to seven days after fixation.

Embryo Processing and Imaging

A protocol from the laboratory of Peter Sutovsky was used to immunolabel the embryos [Schatten 2004]. The secondary antibodies conjugated to fluorescein isothiocyanate (FITC) and tetramethyl rhodamine isothiocyanate (TRITC), along with the nuclear stain 4',6-diamidino-2-phenylindole (DAPI) were utilized. Per each embryo cohort, this protocol took place in a single 9-well glass plate, utilizing a Stripper® Pipette with attached 300 μ l pipette tips. Embryos

were placed into a 400 µl volume of 0.1%% Triton X-100 in 0.1 M phosphate buffered saline (PBS) for 40 minutes in order to permeabilize the zonae and oolemmas. The permeabilized embryos were transferred to 400 µl of a 0.1% Triton X-100/5% normal goat serum (NGS) solution diluted in 0.1 M PBS for 30 minutes as a means of suppressing non-specific antibody binding. Finally, embryos were placed into 100 µl of a 1:400 diluted solution of primary antibodies targeting the two de-ubiquitinating enzymes (DUBs), Ubiquitin C-Terminal Hydrolase L1 (UCHL1) and Ubiquitin C-Terminal Hydrolase L3 (UCHL3) overnight. Primary antibodies were diluted in 0.1% TritonX/1% NGS in 0.1 M PBS.

After an overnight incubation at +4°C in the primary antibody solution, embryos were briefly placed through two 400 µl washes of 0.1% TritonX/1% NGS in 0.1 M PBS. Embryos were then transferred into 400 µl of a 1:200 diluted secondary antibody solution for 40 minutes. As with the previous solutions, these secondary antibodies were diluted using 0.1% Triton X-100/1%NGS in 0.1 M PBS. Due to the light sensitive nature of these fluorescent secondary antibodies, this solution was covered with tin foil throughout the incubation period. The FITC-conjugated antibody tagged the UCHL1-primary antibody, the TRITC-conjugated antibody tagged the UCHL3-primary antibody, and the DAPI targeted nucleic acids. Finally, the embryos were mounted onto a glass slide using a small drop of VectaShield® mounting medium (*Vector Labs, Burlingame, California, USA*), covered with a glass cover slip, and the cover slip sealed with Insta-Dri® translucent nail polish. Once labeled with the fluorescent antibodies,

the embryos were minimally exposed to light. All steps of the fixation protocol were performed under the Nikon SMZ645 microscope (*Nikon, Tallahassee, Florida, USA*).

Images were taken on a Nikon Eclipse 800 epifluorescence microscope (*Nikon, Tallahassee, Florida, USA*) equipped with a Cool Snap camera (*Photometrics, Tuscon, Arizona, USA*). These images, along with those taken under the dissecting microscope, were analyzed, evaluated, and compared between the three groups: Sham, No-Surgery Control, and Endo. A list of specific comparisons and criteria is referenced in Figure 2.4.

General Categories of Analysis

All in vivo and in vitro derived embryos were evaluated through the utilization of two primary categories: (1) morphology and (2) UCHL1 and UCHL3 localization. The morphological comparisons were broken down into categories based on levels of blastomere quality, nuclear quality, and total cell number.

Comparison of Embryo Fragmentation

Embryo fragmentation was evaluated by utilizing a scale that contained two categories of blastomere fragmentation: 0%-<50% fragmentation versus 50%-100% fragmentation. This category was evaluated based on what percentage of the entire embryo volume appeared to be fragmented. See Figure 2.5 for a breakdown of each fragmentation category. Each embryo cohort per rat was evaluated by referencing images taken under a light microscope. Blastomere fragmentation levels were then compared among all of the groups on either Day 2 or Day 4 of development. This was done by separating out

embryos that showed <50% fragmentation, from those that showed >50% fragmentation, and performing a logistic analysis to compare between all groups.

Developmental Stage

Each embryo per each rat cohort was evaluated on the basis of appropriate embryonic stage on either developmental D2 or D4. The average number of nuclei per embryo per rat was determined and compared between Control, Sham, and Endo groups on either Day 2 or Day 4 of development. Light microscope images were used as a means of performing this data analysis. Only embryos in which individual nuclei could be distinguished were included. See Figures 2.6 and 2.7, respectively, for an example of proper D2 and D4 developmental stages.

Nuclear Quality

Each embryo nucleus per each embryo cohort was evaluated on the basis of nuclear quality. Any embryo that exhibited nuclear fragmentation, nuclear pyknosis, and/or chromosomal misalignment was considered abnormal. The average number of abnormal nuclei per embryo per rat was calculated, and compared between all groups on either D2 or D4 of development. To accurately represent the data, only embryos with distinguishable blastomeres were included (e.g. those without severe fragmentation). Images of DAPI-labeled embryos were referenced as a means of evaluating this specific category of morphological analysis. See Figure 2.8 for an example of normal versus abnormal nuclear quality in a 2C stage embryo.

Other Embryonic Abnormalities

Presence of vacuoles within the cytoplasm, uneven blastomere size, and granular cytoplasm are other proven signs of reduced embryo quality. These characteristics were identified by reviewing light microscope images taken on a Nikon N8008 camera per each rat embryo. No statistical analysis was performed using these specific categories of data, due to the fact that it was difficult to consistently define these characteristics between two different observers. See Figure 2.9 for an example of each type of embryonic anomaly.

UCHL1 Localization

Each embryo cohort per rat was evaluated immunolocalization of UCHL1. Ubiquitin C-Terminal Hydrolase L1 was immunolabeled green by using a FITC-conjugated fluorescent secondary antibody. Localization was characterized by referencing images taken on a Nikon Eclipse 800 microscope. The two categories used in this analysis included 'Diffuse UCHL1' and 'Cortically-Located UCHL1'. Localization was compared between the different groups on either developmental D2 or D4.

UCHL3 Localization

Each embryo cohort per rat was evaluated by determining the localization of UCHL3. Ubiquitin C-Terminal Hydrolase L3 was immunolabeled red by using a TRITC-conjugated fluorescent secondary antibody. The two categories used in this analysis included 'Diffuse' and 'Nuclear' localization of UCHL3. Localization was compared between the different groups on either developmental D2 or D4.

Statistical Analysis

Logistic regression analysis using SAS GLIMMIX software was chosen to compare differences in levels of embryo fragmentation, and UCHL1 or UCHL3

localization between the different groups. This form of analysis was utilized due to the variability in sample size per group, the multilevel data (Trial Number, In Vitro/In Vivo, and Experimental Group), and the binomial nature of the data. The statistical model for this analysis is as follows: $\log(P(Y = 1)/P(Y = 0)) = XB + ZU + e$, where 'B' and 'U' are considered vectors of the specific parameters that were estimated from the data. The 'B' denotes the fixed effects, 'U' the random effects, and 'e' the error term. The 'Z' refers to a set of indicator variables denoting the 'batch effect', referring to the three separate experimental trials included in the analysis. The 'X' is denoted as Control, Sham, or Endo, and 'Y' the specific category of analysis. The left side of the equation, expressed as $\log(P(Y=1)/P(Y=0))$, is known as the log odds, and takes the binomial nature of the data into consideration. Any $p < 0.05$ was considered statistically significant. Statistical comparisons between Control, Sham, and Endo groups are displayed as Odds Ratios for each of the aforementioned categories. An Odds Ratio is the ratio of the probability of an event 'occurring', to the probability of that same event 'not occurring'.

A hierarchical regression model of analysis was also utilized to determine the differences in the mean total nuclei number per embryo per rat, along with the differences in the mean total abnormal nuclei number per embryo per rat, for similar reasons (difference being that this type of data is not binomial). The statistical model of analysis is slightly different, being expressed as follows: $Y = XB + ZU + e$. All basic terms in the equation are defined similarly as in the previous paragraph. A $p < 0.05$ was considered statistically significant.

Table 2.1: Stages of Estrous

STAGE	CELL TYPES IN VAGINAL CYTOLOGIES
Proestrus	Rounded epithelium
Estrus	Cornified epithelium
Diestrus I	Few cornified epithelial cells and white blood cells
Diestrus II	Predominantly white blood cells and a few rounded epithelial cells

Figure 2.1: Experimental Groups. A breakdown of the multiple groups that were utilized throughout the embryo culture experiments. Each of the in vivo and in vitro groups were formaldehyde-fixed on either developmental D2, or developmental D4. Each of these included three different subgroups of rats: No-Surgery Control, Sham, or Endo. 'n' = total number of rats per each group.

Table 2.2: Surgical Groups

GROUP TYPES	DESCRIPTION
Sham	Unilateral ovariectomy
Endo	Unilateral ovariectomy+ Endometrial Implants
No-Surgery Control	No surgery performed

Table 2.3: Rat Estrous Synchronization Schedule

D0	D1	D2	D3	D4	D5
	Estrus	Diestrus	Diestrus	Proestrus	Estrus
LHRH Injection				Breed	Pregnant

Table 2.4: Comparison of Rat Embryo Culture Media¹

	<u>mR1ECM +HEPES</u>	<u>mR1ECM (High NaCl)</u>	<u>mR1ECM1 (Low NaCl)</u>
Sodium Bicarbonate	5 mM	25 mM	25 mM
Sodium Pyruvate	0.5 mM	0.5 mM	0.5 mM
MEM NEAA 100x	0.5x	0.5x	0.5x
MEM EAA 50X	0.5x	0.5x	0.5x
GlutaMAX 1*	0.1 mM	0.1 mM	0.1 mM
CaCl ₂ -2H ₂ O	2 mM	2 mM	X
MgCl ₂ -6H ₂ O	0.5 mM	0.5 mM	X
NaCl	110 mM	110 mM	80 mM
KCl	3.2 mM	3.2 mM	3.2 mM
D-Glucose	7.5 mM	7.5 mM	7.5 mM
Penicillin G K Salt	100 µg/ml	100 µg/ml	100 µg/ml
Streptomycin Sulfate	50 µg/ml	50 µg/ml	50 µg/ml
Sodium Lactate (60% syrup)	13.53 mM	13.53 mM	13.53 mM
HEPES	22 mM	0	0
PVA	0	0	1 mg/mL
BSA	0	0.1%	0
<u>OSMOLALITY:</u>	290-320 mOsm	280-300 mOsm	235-255 mOsm
<u>pH:</u>	7.4	7.4	7.4
<u>TEMPERATURE:</u>	37°C	37°C	37°C

¹ Critser [Unpublished] as modified from [Miyoshi et al. 1995]

Table 2.5: Comparison of Embryo Development in Chemically Defined Media

<u>Culture Media</u>	<u>Category</u>	<u>% Embryo Development</u>	<u>Collection Stage</u>	<u>Investigator/ Researcher</u>
mR1ECM	single stage	90% Blastocyst	Day 5	Miyoshi [Miyoshi et al. 1995]
mR1ECM	sequential stage	75% Blastocyst	Day 5	Critser [Unpublished]
mR1ECM	sequential stage	60% Morula	Day 4	Hennings ¹

¹ Dr. John Critser's media protocol was utilized for Hennings' experiments. These are the media that are displayed and compared in Table 2.4.

Figure 2.2: Embryo Culture Diagram. A diagram of the embryo culture setup used for the rat in vitro experiments. Both the 1C to 2C mR1ECM and 2C to Blastocyst mR1ECM are set up in an identical manner.

Figure 2.3: Timeline of Rat Embryo Culture. A diagram of rat embryo collection, culture, and fixation in a sequential media system (mR1ECM). 'D1-D4' = days of embryonic development.

Figure 2.4: Experimental Design. Experimental comparisons between Control, Sham, and Endo groups, in vivo vs. in vivo (red line) and in vitro vs. in vitro (black line).

Figure 2.5: Comparison of Embryo Fragmentation. Original magnification 200X. Examples of varying degrees of blastomere fragmentation, including 0% to <50%; 50% to 100%.

Figure 2.6: Stages of D2 Embryo Development. Original magnification 200X. Representative cohort of embryos on D2 from a no-surgery control rat, developed in vitro. In rats, D2 embryos should be at the 2C stage or greater.

Figure 2.7: Stage of D4 Embryo Development. Original magnification 40X. Examples of characteristically normal embryos on D4 of development. In rats, D4 embryos should be at the 8C stage or greater.

Figure 2.8: Nuclear quality. Original magnification 600X. Normal vs. abnormal nuclear morphology. (A) A 10 cell to 12 cell stage rat embryo. Nuclei are blue (DAPI stain) Red arrows indicate abnormal nuclei (B) A DIC image(A). The black circle indicates a cluster of blastomere fragments.

(A)

(B)

(C)

(D)

Figure 2.9: Other Embryonic Abnormalities. Original magnification 200X.
(A) Zygotes with darkened, granular cytoplasm (B) D2 embryo with uneven, different sized blastomeres (C) Fragmented embryos with abnormally-shaped blastomeres and vacuoles (D) Atretic D4 embryo. Blue arrows indicate abnormal embryos.

RESULTS

Day 2 of Embryonic Development

In Vitro and In Vivo Comparison

There were no significant differences observed between the in vivo and in vitro derived embryos on D2, when comparing within each of the three experimental groups (Control, Sham, and Endo), in the categories of blastomere fragmentation, total nuclei number per embryo, abnormal nuclei number per embryo, or UCHL1/UCHL3 localization. Thus, all Control, Sham, and Endo values in each of the in vitro and in vivo groups were pooled for subsequent analyses. In vivo and in vitro group data is separated out and compared in Figures 2.10, 2.11, and 2.12, and Tables 2.6b, 2.7, and 2.8.

Embryo Fragmentation

The number of embryos exhibiting greater than 50% blastomere fragmentation did not statistically differ between Control, Sham, and Endo (Figure 2.10 and Table 2.6b). The majority of embryos per each group fell into the less than 50% fragmentation category. Table 2.6a shows each experimental group to another by using Odds Ratios, or the relationship between the proportion of >50% fragmented embryos to <50% fragmented embryos between Control, Sham, and Endo.

Appropriate Stage of Development

On D2 of development, rat embryos should be at the two-cell stage or greater. The average nuclei count per embryo per rat did not statistically differ

when comparing between Control, Sham, and Endo (Figure 2.11 and Table 2.7). On average, the majority of embryos in each of the three groups contained a mean total of two nuclei.

Abnormal Nuclei

The average number of abnormal nuclei per embryo per rat did not differ between Control, Sham, and Endo (Figure 2.12 and Table 2.8). The majority of embryos in each group, on average, exhibited less than one abnormal nucleus per embryo per rat. It is important to note that the in vitro Endo group exhibited a high level of variance.

UCHL1 and UCHL3 Localization

The localization of UCHL1 and UCHL3 did not differ between Control, Sham, and Endo groups. Ubiquitin C-Terminal Hydrolase L1 was predominantly localized around the periphery of individual cells in the two and four cell stage embryos in all groups. Ubiquitin C-Terminal Hydrolase L3 showed predominant localization around intact, healthy nuclei, while remaining diffuse in blastomeres that had fragmented nuclei, in all three experimental groups.

Figure 2.10: Percentage of D2 Embryos with >50% Fragmentation^{1,2}

¹ Values within each bar denote the total number of embryos that were analyzed per each group (corresponding to Table 2.6b)

² a: Same letter above all bars indicates no significant differences between groups ($p > 0.05$)

Table 2.6a: D2 Embryo Fragmentation Analysis¹

	p-value	Odds Ratios
Control vs. Endo	0.4	0.6
Control vs. Sham	0.2	0.9
Endo vs. Sham	0.1	0.8

¹ No statistical differences were observed ($p > 0.05$)

Table 2.6b: D2 Embryo Fragmentation Group Numbers

	IN VITRO			IN VIVO		
	Control	Sham	Endo	Control	Sham	Endo
N=rats(embryos)	9 (55)	6 (47)	11 (76)	1 (11)	6 (22)	5(25)

Figure 2.11: Average Number of Nuclei Per D2 Embryo^{1,2}

¹ Values within each bar denote the total number of embryos that were analyzed per each group (corresponding to Table 2.7)

² a: Same letter above bars indicates no significant differences between groups ($p > 0.05$)

Table 2.7: D2 Embryo Mean Total Nuclei Counts¹

	IN VITRO			IN VIVO		
	Control	Sham	Endo	Control	Sham	Endo
Mean # Nuclei/Embryo/ Rat ($\bar{x} \pm \text{SEM}$)	2.5 $\pm$ 0.1 ^a	2.1 $\pm$ 0.1 ^a	2.3 $\pm$ 0.1 ^a	2.01 $\pm$ 0.1 ^a	2.2 $\pm$ 0.1 ^a	2.3 $\pm$ 0.1 ^a
N=rats(embryos)	4 (38)	5 (54)	12 (79)	4 (28)	4 (15)	2 (12)

¹ a: Same letter across the same row indicates no significant differences between groups ($p > 0.05$)

Figure 2.12: Average Number of Abnormal Nuclei Per D2 Embryo ^{1,2}

¹ Values within each bar denote the total number of embryos that were analyzed per each group (corresponding to Table 2.8).

² a: Same letter above bars indicates no significant differences between groups (p > 0.05)

Table 2.8: D2 Embryo Mean Abnormal Nuclei Counts¹

	IN VITRO GROUPS			IN VIVO GROUPS		
	Control	Sham	Endo	Control	Sham	Endo
Mean # Abnormal Nuclei/Embryo/Rat ($\bar{x} \pm \text{SEM}$)	0.4 $\pm$ 0.1 ^a	0.6 $\pm$ 0.02 ^a	0.5 $\pm$ 0.1 ^a	0.2 $\pm$ 0.03 ^a	0.2 $\pm$ 0.01 ^a	0.1 $\pm$ 0.1 ^a
N=rats(embryos)	4 (38)	5 (54)	12 (79)	4 (28)	4 (15)	2 (12)

¹ a: Same letter across the same row indicates no significant differences between groups ($p > 0.05$).

Day 4 of Embryonic Development

In Vitro and In Vivo Comparison

There were no significant differences observed between the in vivo and in vitro groups on D4, when comparing within each of the three experimental groups (Control, Sham, and Endo), in the categories of blastomere fragmentation, total nuclei number per embryo, abnormal nuclei number per embryo, and UCHL1/UCHL3 localization. Thus, all Control, Sham, and Endo values in each of the in vitro and in vivo derived groups were pooled for subsequent analyses. In vivo and in vitro derived group data is separated out and compared in Figures 2.13, 2.14, and 2.15, and Tables 2.9b, 2.10, and 2.11.

Embryo Fragmentation

There was a difference in the number of embryos that develop greater than 50% fragmentation when comparing Control and Endo groups ($p < 0.05$). There was no statistical difference observed in this category of analysis when comparing Control and Sham, and, Sham and Endo, groups (Figure 2.13 and Table 2.8b). Table 2.4a statistically compares each experimental group to another by using Odds Ratios, or the relationship between the proportion of >50% fragmented embryos to <50% fragmented embryos between Control, Sham, and Endo. P-value of <0.05 denotes a significant difference.

Stage of Development

Rat embryos should be at the 8C stage or greater on D4 of embryonic development. Endo Day 4 embryos had fewer cells than Sham or No Surgery

Control groups ($p < 0.05$) (Figure 2.14 and Table 2.10). There was no difference observed between the No Surgery Control and Sham rat groups.

Abnormal Nuclei

The Endo embryos, on average, showed approximately two more abnormal nuclei per embryo per rat compared to the No-Surgery Controls, and Shams (Figure 2.15 and Table 2.11). These differences were considered to be significant, exhibiting $p < 0.05$. The average number of abnormal nuclei per embryo per rat was not different between Sham and Control groups.

UCHL1 and UCHL3 Localization

There was no difference in the localization of UCHL1 (diffuse vs. cortical) and UCHL3 (diffuse vs. nuclear) when comparing Control, Sham, and Endo. While there was a tendency for Endo embryos to show diffuse UCHL1 localization when compared to Control and Sham embryos, this did not reach statistical significance. Also: fragmented embryos had a greater tendency to exhibit diffuse UCHL1, and diffuse UCHL3, when compared to intact embryos, in all groups. Typically, UCHL3 would be expected to co-localize with the nuclei and mitotic spindles, and UCHL1 with the cortex. See Figure 2.16 for a representative panel per each of the D4 Control, Sham, and Endo embryos. Figures 2.17a and 2.17b display the percentages of embryos with diffuse UCHL1 and UCHL3, respectively.

Figure 2.13: Percentage of D4 Embryos with >50% Fragmentation^{1,2}

¹ Values within each bar denote the total number of embryos that were analyzed per each group (corresponding to Table 2.9c)

² a,b: Different letters above bars indicate significant differences ($p < 0.05$)

Table 2.9a: D4 Embryo Fragmentation Analysis

	p-value	Odds Ratio
Control vs. Endo*	0.04	0.2
Control vs. Sham	0.4	0.5
Endo vs. Sham	0.2	3.0

* Indicates a significant difference ($p < 0.05$)

Table 2.9b: D4 Embryo Fragmentation Analysis (vivo vs. vitro)¹

	p-value
Control (vitro) vs. Control (vivo)	0.9
Sham (vitro) vs. Sham (vivo)	0.3
Endo (vitro) vs. Endo (vivo)	0.4

¹ No significant differences were observed ($p > 0.05$)

Table 2.9c: D4 Embryo Fragmentation Group Numbers

	<i>IN VITRO</i>			<i>IN VIVO</i>		
	Control	Sham	Endo	Control	Sham	Endo
N=rats(embryos)	10 (67)	17 (104)	14 (124)	2 (11)	4 (38)	4 (37)

Figure 2.14: Average Number of Nuclei Per D4 Embryo^{1,2}

¹ Values within each bar denote the total number of embryos that were analyzed per each group (corresponding to Table 2.10)

² a,b: Different letters above bars indicate significant differences ($p < 0.05$)

Table 2.10: D4 Embryo Mean Total Nuclei Counts¹

	IN VITRO			IN VIVO		
	Control	Sham	Endo	Control	Sham	Endo
Mean # of Nuclei/Embryo/Rat ($\bar{x} \pm \text{SEM}$)	10.9 $\pm$ 1.1 ^a	9.1 $\pm$ 1.3 ^a	6.4 $\pm$ 1.8 ^b	9.3 $\pm$ 0.5 ^a	8.0 $\pm$ 0.01 ^a	7.4 $\pm$ 0.1 ^b
N=rats(embryos)	8 (55)	13 (89)	11 (100)	2 (11)	4 (27)	3 (29)

¹ a,b: Different letters across the same row indicate significant differences (p < 0.05)

Figure 2.15: Average Number of Abnormal Nuclei Per D4 Embryo^{1,2}

¹ Values within each bar correspond to the total number of embryos that were analyzed per each group (corresponding to Table 2.11)

² a,b: Different letters above bars indicate significant differences ($p < 0.05$)

Table 2.11: D4 Mean Abnormal Nuclei Counts¹

	IN VITRO			IN VIVO		
	Control	Sham	Endo	Control	Sham	Endo
Mean # of Abnormal Nuclei/Embryo/Rat ($\bar{x} \pm \text{SEM}$)	2.4 $\pm$ 0.2 ^a	2.1 $\pm$ 0.5 ^a	3.9 $\pm$ 1.1 ^b	1.0 $\pm$ 0.1 ^a	1.9 $\pm$ 0.3 ^a	1.7 $\pm$ 0.2 ^b
N=rats(embryos)	8 (55)	13 (89)	11 (102)	2 (11)	4 (27)	3 (29)

¹ a,b: Different letters across the same row indicate significant differences (p < 0.05)

Figure 2.16: D4 Embryo UCHL3 and UCHL1 Immunolocalization. (A,B,C) DAPI-labeled images of Control, Sham, and Endo embryos. Nuclei counts were performed on these images. Normal (green arrows) and abnormal nuclei (red arrow) are denoted appropriately. (D,E,F) UCHL1 immunolabeling of Control, Sham, and Endo embryos. Cortical UCHL1 is denoted with a blue arrow, and diffuse UCHL1 with a red arrow. (G,H,I) UCHL3 staining of Control, Sham, and Endo embryos. Nuclear UCHL3 is denoted with a blue arrow, and diffuse UCHL3 with a red arrow. (J,K,L) Merged images of Control, Sham, and Endo embryos.

Figure 2.17a: Percentage of Embryos with Diffuse UCHL1¹

¹ Values within each bar correspond to the total number of embryos that were analyzed per each group (corresponding to Table 2.11)

² a: similar letter above all bars indicates non-significance (p > 0.05)

Figure 2.17b: Percentage of Embryos with Diffuse UCHL3¹

¹ Values within each bar correspond to the total number of embryos that were analyzed per each group (corresponding to Table 2.11)

² a: similar letter above all bars indicates non-significance (p > 0.05)

Table 2.11: D4 Embryo UCHL1 and UCHL3 Odds Ratio Analysis

	UCHL1		UCHL3	
	p-value	Odds Ratio	p-value	Odds Ratio
Control vs. Sham	0.4	0.9	0.2	1.1
Control vs. Endo	0.1	0.4	0.2	0.7
Sham vs. Endo	0.1	0.6	0.3	0.9

DISCUSSION

On D2 and on D4, no differences were found between in vitro and in vivo derived embryo development within Control, Sham or Endo rats for any of the experimental outcomes. Hence, in vivo and in vitro derived data were pooled and analysis was conducted between surgical groups.

On D2, the majority of Control and Sham embryos exhibited a fairly high quality phenotype and had similar patterns of UCHL1 and UCHL3 protein localization. Endo embryos had a similar total cell count and UCHL1/UCHL3 immunolocalization pattern when compared to Sham and Control groups. While no difference was observed in the number of abnormal nuclei between all groups, the in vitro Endo group displayed a high level of variance. This is potentially indicative of either a treatment effect, or an adverse effect of the culture system. The data shows that the effects of endometriosis on developmental D2 did not readily impact embryo development, did not become apparent at this stage or could not be discerned by morphology or UCHL1/L3 localization.

On D4, differences in embryonic development between Control, Sham and Endo embryos became apparent. Control and Sham D4 embryos did not differ in developmental morphology, nuclear numbers, fragmentation or the number of abnormal nuclei per embryo. Endo D4 embryos, on the other hand, were of a poorer quality, displaying significantly fewer total nuclei, greater fragmentation and more abnormal nuclei per embryo. Compared to the D2 data, the in vitro D4

embryos exhibited a high level of variance in the total number of abnormal nuclei when compared to all other group types. The data shows that in vitro culture may exacerbate the adverse effects of endometriosis on embryo development. Another potential conclusion is that the effects of endometriosis begin to predominantly manifest at some time point between D2 and D4 of preimplantation development. This range of time corresponds to the maternal to zygotic transition (MZT) in rats and the point at which the multicellular embryos begin to undergo compaction and cellular differentiation. A final conclusion from the D4 embryo data involves the null hypothesis. Endo embryos are unable to be rescued from the effects of endometriosis when removed from the mother at the zygote stage and transferred into an established culture system.

Temporal and spatial gene expression and epigenetic control is important for many embryonic physiological processes, ranging from the degradation of maternal mRNA throughout the MZT to the timed expression of proteins involved in cellular cleavage and DNA replication [Kanka 2003; Harvey et al. 2007]. If these processes are disrupted, embryos may not properly develop. In the context of our in vitro endometriosis rats, it seems that early exposure to elevated levels of ROS and negative proinflammatory factors such as tumor necrosis factor alpha (TNF α) prior to cleavage may disrupt later stage processes. It has been reported that external stressors (e.g ROS and TNF α) can disrupt the transcriptome of the oocyte and early stage embryos, in which one effect may be a reduction in functional microRNA (miRNA) [Sirard 2012]. This, in turn, would lead to the delayed removal of maternal mRNA, in which an accumulation can be

detrimental to the later stage embryo. This may be the cause of the increased abnormalities observed in our D4 Endo embryos. Also, previous studies documented that Endo embryos tended to arrest at the 8C stage of development in vivo [Stilley et al. 2009], which is well correlated with observations made in the present study.

In conclusion, early exposure to Endo lesion secretions may lead to delayed or disrupted embryonic development, such as the MZT, which could very well be causative of delayed embryo development and an increased number of abnormal nuclei per embryo. This in turn may explain why Endo embryos cannot be salvaged by growing them in an established culture system, and why culture seems to exacerbate the abnormal effects of surgically induced endometriosis.

The UCHL1 and UCHL3 localization was determined per each of the D2 and D4 embryos. It has been previously observed in our lab that multiple apoptotic genes were dysregulated in the 8C embryos of Endo rats, including Diablo, Casp3, Parp1, and Dnaja3 [Birt et al. 2013]. An accumulation of cytoplasmic proteasomes was also observed within the early-stage preimplantation embryos from this same Endo rat model, indicative of elevated cellular stress [Stilley et al. 2010]. As a way to further characterize the effects that endometriosis has on programmed cell death, the de-ubiquitinating enzymes UCHL1 and UCHL3 were analyzed [Driscoll 1994; Larsen et al. 1998]. While there was no observed statistical difference between any of the groups, there was a tendency for the D4 embryos in Control and Sham rats to display cortically located (normal) UCHL1 when compared to Endo rats, in which UCHL1

localization was predominantly diffuse. It is important to mention that a large proportion of the diffuse UCHL1 embryos were severely fragmented.

It is impossible to determine if UCHL1 destabilization from the cortex is a cause or an effect of the embryonic detriment. While little is known about the involvement of UCHL1 in preimplantation development, it was reported that mutant *gad* mice lacking UCHL1 have a tendency to arrest at the morula stage, exhibiting an inability to compact [Mtango et al. 2012]. It is possible that UCHL1 is involved in the tightly regulated interaction between the outer cells of the compacting morula.

Ubiquitin c-terminal hydrolase L1 is also important in binding to and increasing the half-life of ubiquitin [Yi et al. 2007; Koyanagi et al. 2012]. This DUB may be necessary to maintain stable pools of ubiquitin at the cortex, significant in regulating the internalization of certain transmembrane proteins, and degradation of misfolded peptides present in the endoplasmic reticulum [Bonifacino and Weissman 1998]. This may be an explanation behind the elevated number of abnormal embryos observed in the Endo group and a proposal that UCHL1 deregulation may contribute to embryonic abnormalities.

In conclusion, our research data indicates that the in vitro culture of preimplantation embryos from women with endometriosis in IVF clinics may not be a solution for improved preimplantation development. This is predominantly due to the early-stage effects of endometriotic lesion secretions before embryos are taken out of the endometriotic environment, potentially exacerbated by culture medium. These adverse effects become obvious on D4 of development,

as observed in our experiments. In order to further characterize our data, gene analysis should be performed on Control, Sham, and Endo embryos to determine the expression levels of UCHL1, UCHL3, and MZT-associated transcripts on D2 and D4 of development.

CHAPTER 3

COMPARING THE QUALITY OF BLASTOCYST DEVELOPMENT IN A SEQUENTIAL CULTURE SYSTEM VERSUS A SINGLE CULTURE SYSTEM

INTRODUCTION

Culture media for embryonic growth and development include amino acids, carbohydrates, a variety of salts and other components. Many of these components play roles in the regulation of embryonic processes, ranging from metabolism to the maintenance of intracellular osmolality and redox potential [Naaktgeboren 1987; Oh et al. 1998; Van Winkle 2001; Han and Niwa 2003]. They are also known to interact with each other, leading to an elevated complexity in the development of different types of culture media.

For example, the effect of glucose toxicity on early cleaving mammalian embryos can be alleviated by including non-essential amino acids and glutamine in the media. Lactate, at a low concentration, is also known to be beneficial to early embryo development in conjunction with amino acids and pyruvate, but toxic when present by itself and at elevated concentrations [Bavister 1995, Lane and Gardner 1998]. Early cleaving embryos, of all studied mammalian species, have a limited control over their internal environment, compared to later-stage, compacted embryos [Eckert et al. 1997; Rizos et al. 2003; Yamada et al. 2012]. This concept has been taken into consideration during optimization of culture media systems.

According to the Center for Disease Control, an estimated 85,000 women undergo in vitro fertilization (IVF) procedures in the United States each year; on average spending \$12,400 per cycle. Further, the number of assisted reproductive technology (ART) cycles per year is steadily on the rise, the current average reported as 135,000 cycles per year.

Numerous studies conducted over decades have utilized a variety of animal models to create an optimal culture system for human embryos [Whitten 1957; Miyoshi et al. 1995; Rizados et al. 2003]. Human embryos cannot be used for experimentation due to ethical restrictions. There are numerous brands of culture media on the market, both of the single and sequential types. An example of a single medium is Global® (*LifeGlobal, Guilford, Connecticut, USA*), while an example of a sequential media is EmbryoAssist™/BlastAssist™ (*Origio, Mt. Laurel, New Jersey, USA*). The issue of using single culture versus two-step culture remains controversial. The argument is that while single culture may be more beneficial due to a consistent culture environment and reduced embryonic manipulation, a sequential system more successfully mimics the changing environment of the entire human reproductive tract [Artini et al. 2004; Lane and Gardner 2007].

Human ART began in the late 1970s with single medium and then moved to sequential media as our knowledge of embryo requirements advanced [Lane and Gardner 2007]. Early IVF protocols would grow embryos for two days in culture and transfer them back to the recipient at the 2 cell (2C) to 4 cell (4C) stage of development. Approximately 20 years later, embryo culture systems had improved to permit culture to the blastocyst stage (developmental D5) [Lane and Gardner 2007]. Zygotes that are capable of developing to the blastocyst stage on developmental D5 are regarded as being of a higher quality in comparison to slower developing embryos. Predictably, this protocol is capable

of selecting the best quality embryo(s) for transfer. Further, fewer blastocysts can be transferred, reducing the risk of multiple pregnancies.

In 2000, Biggers et al. found no differences in the hatching rates, or number of trophectoderm (TE) cells or inner cell mass (ICM) cells of murine blastocysts that were grown in a one-step culture KSOM^{AA} (*Sigma, St. Louis, Missouri, USA*) versus a sequential system G1.2/G2.2 (*Scandinavian IVF Science, Gothenburg, Sweden*) [Biggers et al. 2000]. Conversely, a 2004 retrospective meta-analysis concluded that sequential media lead to a greater number of developed human blastocyst stage embryos and a higher number of live births compared to single medium [Blake et al. 2004]. During this same time, other studies observed that while a specific single-stage culture lead to poorer quality human blastocysts compared to sequential, culture birth rates did not differ [Ben-Yosef et al. 2004]. This indicated a need for more sophisticated methods of embryo selection for transfer beyond embryo morphology to help in predicting pregnancy and live birth outcomes.

Then, coming full circle, in 2009 and 2010, Paternot et al. and Reed et al. reported that a single media system led to the development of a greater number of human blastocysts when compared to a sequential system [Reed et al. 2009; Paternot et al. 2010]. The different outcomes observed among the aforementioned research studies may be due to the variability in media compositions, and specific embryo growth conditions used per each research laboratory.

The purpose of the present study was to determine the developmental potential, quality and possible mechanisms by which murine embryos differentially develop in sequential versus single media systems over a period of five days. Nuclei numbers and nuclear quality were two primary categories evaluated and compared between embryos grown in either media type.

MATERIALS AND METHODS

Murine Embryo Collection and Experimental Plan

All murine embryos that were used in these experiments were obtained from EmbryoTech™, a company chosen due to its reputation for a high level product quality control. At EmbryoTech™, superovulated female B6C3F-1 and male B6D2F-1 mice were bred, and their zygotes subsequently collected. These zygotes were pooled and randomly distributed into ThawAlert™ (*EmbryoTech™, Haverhill, Massachusetts, USA*) straws, 20 to 23 embryos per straw. They were then cryopreserved and shipped to consumers.

All in vitro culture experiments were performed at the Missouri Center for Reproductive Medicine and Fertility in vitro fertilization (IVF) laboratories at the Women's and Children's Hospital (WCH) of Columbia, MO. Embryos were fixed in 2% formaldehyde on developmental day 5, followed by processing and imaging. All experiments were performed in accordance with the Animal Care and Use Committee regulations.

The purpose of this section of my Thesis was to determine the developmental potential of murine embryos in each of two different media types. One of these includes a single culture medium known as Global® medium (*LifeGlobal, Guilford, Connecticut, USA*), and the other is a sequential media system known as the P1® (Preimplantation Stage 1®)_Multiblast® media (*Irvine Scientific, Santa Ana, California*). Global® medium is the current media type being used at the Women's and Children's Hospital of Columbia, MO. The P1®_Multiblast® system is commonly used in human IVF laboratories throughout

the United States, but has been replaced at Columbia's WCH due to the observation that Global® tends to perform better than the former. This chapter tackles the comparison between these two media, looking at quality and quantity of mouse blastocysts developed in these established in vitro environments.

Murine Embryo Culture

On the morning of D1, two straws of murine embryos were thawed in accordance with EmbryoTech™'s protocol (*EmbryoTech™, Haverhill, Massachusetts, USA*). Briefly: each ThawAlert™ straw was removed from the storage tank, placed at room temperature for two minutes, and then set into a 37°C water bath for one minute. Afterwards, both straws were cut open, and the embryos expelled into a drop of HEPES-buffered, modified Human Tubal Fluid (mHTF) (*Irvine Scientific, Santa Ana, California, USA*) using the provided stylet. Subsequently, the embryos were briefly washed in two more drops of the same medium and allowed to rehydrate in the second drop for a total of 10 seconds. Afterwards, the embryos were split between one of the two different culture media that had been equilibrated overnight in a Heracell 150i CO₂ incubator (*ThermoFisher Scientific, Palm Beach, Florida, USA*), using an Origio Stripper® Pipette (*MidAtlantic, Mt Laurel, New Jersey, USA*) and 300 µl pipette tips. One milliliter volumes of media were set into double-well, non-embryotoxic culture plates. The plates were then placed into the Heracell 150i incubator, set to an atmosphere of 6% CO₂, 94% air, 37°C, and >98% humidity. Observations on cell number and embryo quality were made on the mornings of developmental D2, D3, D4, and on the afternoon of D5 using an Olympus IX-71 Inverted Microscope (*Olympus America, Center Valley, Pennsylvania, USA*). Light microscope

images were taken on D1, and D5, of development using an Olympus DP70 digital camera (*Olympus America, Center Valley, Pennsylvania, USA*).

In both culture medium systems, the volume was changed on the afternoon of developmental D3. See Table 3.1 for a comparison of each medium's components, pH, osmolality, and temperatures. In the context of the Global® group, embryos were transferred into fresh Global® medium using a Stripper® Pipette on D3. In reference to the P1®_Multiblast® system, embryos were removed from the P1® medium supplemented with protein (D1 to D2), and switched to the Multiblast® medium (D3 to D5), using a Stripper® Pipette, on the afternoon of developmental D3. All media were equilibrated the night before in a CO₂ incubator set to an atmosphere of 6% CO₂, 94% air, 37°C, and >98% humidity, covered in 1 mL of light mineral oil, before coming in contact with the embryos. See Figure 3.3 for a basic timeline of the murine embryo culture experiments.

Many IVF clinics routinely use mouse embryo culture as a means of human media quality control. This protocol is known as a mouse embryo assay (*EmbryoTech™, Haverhill, Massachusetts, USA*). The developmental potential of the P1®_Multiblast® culture system was compared to that of the Global® system during four of these scheduled MEAs.

Embryo Fixation

As referred to above, all in vivo and in vitro derived embryos were fixed in a formaldehyde solution post-collection. Nine-well glass plates were placed onto a heated tray, and allowed to warm up to 37°C. Following this preparation, per each group of embryos, 400 µl of warmed, pure PBS was placed into two

individual wells located side-by-side. Four hundred μ l of PBS, maintaining a 1.0% concentration of polyvinyl pyrrolidone (PVP) (*Irvine Scientific, Santa Ana, California*), was then added to the third, adjacent well. The PVP was used to help prevent the embryos from attaching/sticking to the bottom of the glass well during fixation. A single cohort of embryos was pipetted into the well containing the 1.0% PVP solution. Next, 100 μ l of 10% formaldehyde was slowly added around the edges of the 'PVP well' in order to create a 2% formaldehyde/0.8% PVP solution. The embryos then incubated for 40 minutes at room temperature. This was done per every in vivo and in vitro rat embryo cohort.

After this incubation period, the embryos were removed and briefly washed in the two adjacent PBS wells to remove any remaining formaldehyde. The embryos were subsequently transferred to a well in another 9 well glass plate that was labeled with the rat number. This was considered the 'final resting well' for the embryos prior to processing.

The nine well plates containing the fixed embryos were then covered with a second nine-well glass plate, and tightly wrapped in Saran Wrap® (*Johnson and Sons™, Racine, Wisconsin, USA*) in order to prevent evaporation and potential loss of the embryos upon transportation. Embryos were immunolabeled and imaged no later than five to seven days after fixation.

Embryo Processing and Imaging

A protocol from the laboratory of Peter Sutovsky was used to fluorescently immunolabel the embryos [Schatten 2004]. The secondary antibodies conjugated to Fluorescein isothiocyanate (FITC) and tetramethyl rhodamine isothiocyanate (TRITC), along with the nuclear stain 4',6-diamidino-2-

phenylindole (DAPI) were utilized. Per each embryo cohort, this protocol took place in a single nine well glass plate, utilizing a Stripper® Pipette with attached 300 µl pipette tips. Embryos were placed into a 400 µl volume of 0.1%% Triton X-100 in 0.1 M phosphate buffered saline (PBS) for 40 minutes in order to permeabilize the zonae and oolemmas. These permeabilized embryos were transferred to 400 µl of a 0.1% Triton X-100/5% Normal Goat Serum (NGS) solution diluted in 0.1 M PBS for 30 minutes as a means of suppressing non-specific antibody binding. Finally, embryos were placed into 100 µl of a 1:400 diluted solution of primary antibodies targeting the two de-ubiquitinating enzymes (DUBs), Ubiquitin C-Terminal Hydrolase L1 (UCHL1) and Ubiquitin C-Terminal Hydrolase L3 (UCHL3) overnight. Primary antibodies were diluted in 0.1% TritonX/1% NGS in 0.1 M PBS.

After an overnight incubation at +4°C in the primary antibody solution, embryos were briefly placed into two 400 µl washes of 0.1% TritonX/1% NGS in 0.1 M PBS. Embryos were then transferred into 400 µl of a 1:200 diluted secondary antibody solution for 40 minutes. As with the previous solutions, these secondary antibodies were diluted using 0.1% Triton X-100/1%NGS in 0.1 M PBS. Due to the light sensitive nature of these fluorescent secondary antibodies, this solution was covered with tin foil throughout the incubation period. The FITC-conjugated antibody tagged the UCHL1-primary antibody, the TRITC-conjugated antibody tagged the UCHL3-primary antibody, and the DAPI stain targeted nucleic acids. Finally, the embryos were mounted onto a glass slide using a small drop of VectaShield® mounting medium (*Vector Labs*,

Burlingame, California, USA), covered with a glass cover slip, and the cover slip sealed with Insta-Dri® translucent nail polish. Once labeled with the fluorescent antibodies, the embryos were minimally exposed to light. All steps of the fixation protocol were performed under the Nikon SMZ645 microscope (*Nikon, Tallahassee, Florida, USA*).

Epifluorescence images were taken on a Nikon Eclipse 800 epifluorescence microscope (*Nikon, Tallahassee, Florida, USA*) equipped with a Cool Snap camera (*Photometrics, Tuscon, Arizona, USA*). These images were analyzed, evaluated, and compared between the two groups: P1®_Multiblast® and Global®.

General Categories of Analysis

On D5 of embryogenesis, the embryos are expected to reach the blastocyst stage of development. Blastocysts were imaged in each group, and simultaneously analyzed. The following five categories of data were collected, analyzed, and compared between the Global® and P1®_Multiblast® grown embryos: total cell number, presence of an inner cell mass (ICM), ICM cell count, trophoblast cell count and nuclear quality. Immunofluorescent localization of UCHL1 and UCHL3 was used as a reference point for nuclear counts and embryo morphology. Numbers of each embryo stage were also analyzed and compared on both D3 and D5 of development.

Total Nuclei Number

Total embryo nuclei numbers per blastocyst were determined by counting nuclei under a Nikon Eclipse 800 fluorescent microscope (600X), using a DAPI filter, per blastocyst. The nuclei of each blastocyst were counted three

individual times, and averaged, by analyzing multiple planes of focus. Any of the three nuclei counts that was considered to be an outlier (cell count mean $\pm 3 \times$ standard deviation) was removed from the data set, and all counts were re-done.

Presence of an Inner Cell Mass

Using the UCHL1 and UCHL3 immunolocalization as a reference point, a presumptive ICM was distinguished under the Nikon Eclipse 800 microscope (400X, 600X) by analyzing multiple planes of focus per blastocyst. The UCHL1 had a tendency to weakly immunolocalize, and UCHL3 strongly, at the ICM. Intense DAPI labeling aided in the identification of the ICM.

Inner Cell Mass Cell Count

Once the presumptive ICM was identified, a presumptive ICM cell number was determined. The predicted ICM cells were counted three individual times at 600X (using the DAPI filter) under the Nikon Eclipse 800 microscope, with a five minute time gap between each count, compared, and then averaged. Any of the three cell counts that was considered an outlier (cell count mean $\pm 3 \times$ standard deviation) was removed, and all counts re-done in that specific blastocyst. If an ICM could not be definitively located in a specific blastocyst, there was no ICM cell count performed.

In embryos in which a presumptive ICM was clearly identified under the Nikon fluorescent microscope, presumptive ICM nuclear counts were re-done on DAPI-labeled images of blastocysts opened up in ImageJ [Abramoff et al, 2004]. twice, averaged, and then compared to the averaged microscope counts by using a One-Way ANOVA analysis. One other researcher also counted and recorded presumptive inner cell mass numbers, twice, in an identical manner to

validate this counting protocol by using images opened up in ImageJ. After the ImageJ count method was validated by statistically comparing the counts determined by both researchers, using a One-Way ANOVA, presumptive ICM counts were compared between the P1®_Multiblast® and Global® embryo groups.

Trophoblast Cell Count

The following calculation was performed to determine the total number of presumptive trophoblast cells per embryo:

$$\text{Total Cell Count} - \text{Presumptive ICM Cell Count}$$

These values were then compared between the P1®_Multiblast® and Global® groups.

D3 and D5 of Embryo Development

The D3 embryos should ideally be at the 8C+ stage, while D5 embryos, should be at the blastocyst stage. The total number of appropriately-staged embryos was compared on either developmental D3 or D5, between each of the two groups. These numbers were obtained by examining images recorded on the Olympus IX-71 Inverted Microscope (400X). These values were then compared between P1®_Multiblast® and Global® embryos on D3, and D5.

Abnormal Nuclei

Each blastocyst in each mouse cohort was evaluated on the basis of nuclear quality. Any embryo that exhibited nuclear fragmentation, nuclear pyknosis, and/or chromosomal misalignment was considered abnormal. Abnormal nuclei were identified and counted under a Nikon Eclipse 800 microscope equipped with a Cool Snap camera (200X, 400X, and 600X), using

UCHL1 and UCHL3-immunolabels as a reference point for individual cells. Multiple planes of focus were used in order to allow all nuclei to be taken into consideration. These counts were performed as described above. Averaged values were then statistically compared between the P1®_Multiblast® and Global® embryos. See Figure 3.1 for an example of normal versus abnormal nuclear quality in a blastocyst.

Hatching vs. Unhatched Blastocysts

The number of hatching/hatched blastocysts and non-hatching blastocysts was determined and compared between the P1®_Multiblast® and Global® groups. All completely hatched and partially hatched blastocysts were combined into one group. These characteristic were determined by referencing both fluorescent images taken on the Nikon Eclipse 800 microscope, and light microscope images taken on the Olympus IX-71 Inverted Microscope (400X) at WCH . These numbers were then statistically compared between P1®_Multiblast® and Global® embryos. See Figure 3.2 for an example of a hatching blastocyst vs. an unhatched blastocyst.

Statistical Analysis

A general linear mixed model (GLMM) was used to analyze differences in total nuclear number, presumptive ICM nuclear number, and presumptive TE nuclear number between the blastocysts grown in P1®_Multiblast® and Global® media. Considering that four replicates of embryo growth and fixation were performed, a random effect was taken into consideration. This allowed for data, collected at multiple different times, to be combined and analyzed together.

Once the least square means were calculated, an approximate t-test was used to compare differences in total nuclei number through the software known as SAS 9.22 PROC MIXED. The statistical model for this analysis is expressed as follows: $Y = XB + ZU + e$, where 'B' and 'U' are considered vectors of the specific parameters that were estimated from the data. 'B' denotes the fixed effects, 'U' the random effects, and 'e' the error term. The Z refers to a set of indicator variables denoting the 'batch effect', referring to the four separate experimental trials included in the analysis. The X is denoted as either Global® or P1®_Multiblast®, and 'Y' the specific category of analysis. Any $p < 0.05$ was considered statistically significant.

A logistic regression analysis, using SAS 9.22 PROC LOGISTIC software, was utilized to statistically compare embryonic development of D3 and D5 of embryos between the P1®_Multiblast® and Global® medium systems. The ratios of hatching/hatched:unhatched blastocysts, TE:ICM counts, and abnormal nuclei:normal nuclei counts were also compared through this same analytical approach. The statistical model is set up as follows: $\log(P(Y = 1)/P(Y = 0)) = XB + ZU + e$. The left side of the equation, displayed as $\log(P(Y = 1)/P(Y = 0))$, is known as the log odds, and takes the binomial nature of the data into consideration. All basic terms in the equation are defined similarly as in the previous paragraph. A $p < 0.05$ was considered significant. Statistical comparisons between the P1®_Multiblast® and Global® embryos are displayed as Odds Ratios for each of the aforementioned categories. An Odds Ratio is the

ratio of the probability of an event occurring, to the probability of that same event not occurring.

<u>COMPONENTS</u>	<u>P-1® (Step 1)</u>	<u>Multiblast® (Step 2)</u>	<u>Global®</u>
Sodium Bicarbonate	X	X	X
Phenol Red	X	X	X
Sodium Pyruvate	X	X	X
Taurine	X	X	
Gentamycin	X		X
Gentamicin Sulfate		X	
Sodium Chloride	X	X	X
Calcium Chloride	X	X	X
Potassium Chloride	X	X	X
Sodium Citrate	X	X	
Sodium Lactate	X	X	X
Magnesium Sulfate	X	X	X
Potassium Phosphate		X	X
Glucose		X	X
EDTA			X
Alanyl-glutamine		X	X
Alanine		X	X
Asparagine		X	X
Aspartic Acid		X	X
Glutamic Acid		X	X
Glycine		X	X
Proline		X	X
Serine		X	X
Arginine		X	X
Cysteine		X	X
Histidine		X	X
Isoleucine		X	X
Leucine		X	X
Lysine		X	X
Methionine		X	X
Phenylalanine		X	X
Threonine		X	X
Tryptophan		X	X
Tyrosine		X	X
Valine		X	X
— Osmolality (mOsm)/pH/Temperature (°C)	290/7.3/37	290/7.3/37	270/7.25/37

¹ Concentration of components are proprietary

² Media are from Irvine Scientific (P-1® and Multiblast®) and LifeGlobal (Global®)

Figure 3.1: Nuclear Morphology. Original magnification 600X. Pyknosis (shrinking/condensation) and karyorrhexis (disintegration/granulation) are forms of nuclear damage, and are identified in this blastocyst with red arrows.

Figure 3.2: Hatching vs. Unhatched Blastocysts. Original magnification 600X. (A) A hatching blastocyst. A breach in the ZP can be observed (red circle). (B) An unhatched blastocyst. A fully intact ZP is present (red arrow).

Figure 3.3: Timeline of Murine Embryo Culture. Timeline of murine embryo collection, culture, and fixation.

RESULTS

Total Blastocyst Nuclei Number

Total blastocyst nuclei number was significantly higher in embryos that were grown in single medium as compared to those grown in sequential media. Single media blastocysts had on average had 35 more total nuclei than the sequential media blastocysts (Figure 3.4 and Table 3.2).

ICM and TE Nuclei Number

While there was no significant difference in the average number of presumptive ICM nuclei per blastocyst between the single medium and the sequential media groups, the single media group had a significantly higher average number of presumptive trophoblast nuclei per blastocyst (Figure 3.5 and Table 3.3a). There was also a significant difference in the ratio of presumptive TE nuclei: presumptive ICM nuclei per blastocyst between the two groups, with single media being 1.7X more likely to maintain a higher TE:ICM ratio compared to sequential media. This is expressed as an Odds Ratio in Table 3.3b.

Abnormal versus Normal Nuclei Number

There was no significant difference in the average number of abnormal nuclei per blastocyst between the single medium and the sequential media groups (Figure 3.6 and Table 3.4a). Further, there was no significant difference observed in the ratio of abnormal nuclei:normal nuclei per blastocyst between either of the two groups. This is expressed as an Odds Ratio in Table 3.4b.

Unhatched vs. Partially/Fully Hatched Blastocysts

There was a statistically higher number of total hatched/hatching blastocysts found in the single medium when compared to the sequential media (Figure 3.7 and Table 3.5a) (p-value not shown). Further, there was a significant difference in the ratio of hatched/hatching blastocysts:unhatched blastocysts between the two groups. The single medium was 8X more likely to develop a hatching/hatched blastocyst when compared to the sequential system. This is expressed as an Odds Ratio in Table 3.5b.

Development on D3 or D5

Murine embryos should be at the 8 cell stage or greater on developmental D3. Zygotes that were grown in the single medium did not show a statistical difference in the number of embryos $\geq$ 8C stage vs. those $<$ 8C stage compared to those grown in the sequential media on developmental D3 (Figure 3.8 and Table 3.6a). The ratio of $\geq$ 8C stage : $<$ 8C stage was also non-significant. This is expressed as an Odds Ratio in Table 3.6b. Murine embryos should be at the blastocyst stage on D5 in vitro. The zygotes that were grown in the single medium showed a statistical difference in the ratio of blastocyst to morula stage embryos when compared to the sequential medium. This is expressed as an Odds Ratio in Table 3.6b. The odds of observing a D5 blastocyst-stage embryo in the single medium are 3.65X the odds of observing a blastocyst-stage embryo in the sequential media. Also, single medium exhibited a statistically higher total number of blastocysts when compared to the sequential media on D5 (Figure 3.8 and Table 3.6a).

Figure 3.4: Mean Nuclei Count Per Blastocyst^{1,2}

¹ Values within each bar denote the total number of blastocysts that were analyzed per each group (corresponding to Table 3.2)

² $p < 0.05$ is significant

Table 3.2: Mean Nuclei Count Per Blastocyst

	Sequential	Single
Mean Number of Nuclei ($\bar{x} \pm \text{SEM}$)	56 ± 7.0	$91 \pm 6.8^*$
N = straws (blastocysts)	4 (34)	4 (46)

* Statistically different between sequential and single ($p < 0.05$)

Figure 3.5: Mean Number of TE and ICM Nuclei Per Blastocyst^{1,2}

¹ Values within each bar denote the total number of blastocysts that were included per each group (corresponding to Table 3.3a)

² p-value is comparing the TE:ICM ratios between groups (p < 0.05 is significant)

Table 3.3a: Mean Number of TE and ICM Nuclei Per Blastocyst¹

	Sequential	Single
Mean Number of TE Nuclei ($\bar{x} \pm \text{SEM}$)	41 ± 5.5^a	73 ± 5.7^b
Mean Number of ICM Nuclei ($\bar{x} \pm \text{SEM}$)	17.8 ± 1.2^a	19.1 ± 1.1^a
N= straws (blastocysts)	4 (29)	4 (37)

¹ a,b: Different letters across the same row indicate significant differences ($p < 0.05$)

Table 3.3b: Mean Number of TE and ICM Nuclei Count Odds Ratio Analysis¹

	p-value	Odds Ratio
Single vs. Sequential	<0.0001	1.7

¹ $p < 0.05$ is significant

Figure 3.6: Mean Number of Abnormal and Normal Nuclei Per Blastocyst^{1,2}

¹ Values within each bar denote total number of blastocysts that were analyzed per each group (corresponding to Table 3.4a)

² p-value refers to the Odds Ratio Analysis (p > 0.05 is nonsignificant)

Table 3.4a: Mean Number of Abnormal and Normal Nuclei Per Blastocyst

	Sequential	Single
Mean Number Abnormal Nuclei ($\bar{x} \pm \text{SEM}$)	6 ± 0.3	4 ± 0.3
Mean Number Normal Nuclei ($\bar{x} \pm \text{SEM}$)	52 ± 3.6	84 ± 3.8
N= straws (blastocysts)	4 (32)	4 (46)

Table 3.4b: Abnormal versus Normal Nuclei Per Blastocyst Odds Ratio Analysis¹

	p-value	Odds Ratio
Sequential vs. Single	0.4	0.9

¹ p > 0.05 is nonsignificant

Figure 3.7: Percentage of Hatched/Hatching Blastocysts^{1,2}

¹ Values within each bar denote the total number of blastocysts that were included per each group (corresponding to Table 3.5a)

² p-value refers to the Odds Ratio Analysis ($p < 0.05$ indicates significance)

Table 3.5a: Total Number of Hatched/Hatching and Unhatched Blastocysts

	Sequential	Single
Total Number of Hatched/Hatching Blastocysts	10.0	37.0
Total Number of Unhatched Blastocysts	23	9
N= straws (blastocysts)	4 (33)	4 (46)

Table 3.5b: Odds Ratio Analysis of Hatched/Hatching versus**Unhatched Blastocysts¹**

	p-value	Odds Ratio
Single vs. Sequential	<0.0001	8.9

¹ p < 0.05 is significant

Figure 3.8: Percentage of Embryos at $\geq 8C$ (D3) or Blastocyst (D5) Stage^{1,2}

¹ Values within each bar denote the total number of blastocysts that were included per each group (corresponding to Table 3.6a)

² p-values refer to the Odds Ratio Analyses ($p < 0.05$ is significant). Comparisons were made between the Single and Sequential groups on either D3 or D5

Table 3.6a: Total Number of Embryos at $\geq$ 8C (D3) or Blastocyst (D5) Stage

	D3		D5	
	Sequential	Single	Sequential	Single
Total Number $\geq$ 8C Stage Embryos (D3)	65	73	NA	NA
Total Number <8C Stage Embryos (D3)	21	10	NA	NA
Total Number Blastocysts (D5)	NA	NA	53	71
Total Number <Blastocysts (D5)	NA	NA	33	12
N=straws (embryos)	4 (86)	4 (83)	4 (86)	4 (83)

Table 3.6b: Odds Ratio Analysis of Developmental D3 and D5 Embryos¹

	p-value	Odds Ratio
Single vs. Sequential (D3)	0.058	2.4
Single vs. Sequential (D5)	0.0007	3.7

DISCUSSION

Culture in single medium resulted in blastocysts with a greater total number of nuclei, more blastocysts, more hatched blastocysts and a higher proportion of trophoblast nuclei to ICM nuclei compared to those grown in sequential media. This data suggests one of two potential conclusions: (1) the sequential system contains a mixture of components that restricts murine embryo development or (2) the single culture environment is more conducive to embryo growth.

Others have observed that murine, bovine, and porcine embryos that rapidly divide in vitro (developing a higher nuclei number) tend to be of a higher quality compared to those that do not [McLaren and Bowman 1973; Grisart et al. 1994; Lonergan et al. 1999]. These same embryos displayed significantly higher birth and pregnancy rates when transferred, as well. Also, a 2008 study by Luna et al. reported that faster cleaving D3 human embryos tended to develop to a higher quality D5 blastocyst than those dividing slowly, showing a greater degree of blastocoel expansion and higher quality ICM and TE morphological scores [Luna et al. 2008]. These same embryos also trended toward displaying a higher birth rate after being transferred. Relating these previous findings with our own, it seems logical to conclude that growth in the tested single medium leads to a higher quality blastocyst than development in the sequential media. As reported in our results, the trophoblast nuclei contributed to the significant difference observed in the average total blastocyst nuclei number between single and sequential media groups.

It has been reported that an increased number of trophoblast nuclei leads to a more complete and cohesive TE layer, predictably improving the ability of the developing embryo to undergo cavitation [Aplin 2000; Ahlstrom et al. 2011]. The increased number of TE nuclei corresponds to an elevated level of Na⁺/K⁺ ATPase activity, and aquaporin channel accumulation, potentially contributing to the elevated rate of fluid accumulation and blastocoel formation [Ahlstrom et al. 2011]. The significantly higher number of TE nuclei in the single medium group blastocysts potentially leads to a higher rate of blastocoel expansion. Further, this may explain the increased number of hatching/hatched blastocysts observed in the Global® over the P1®_Multi® group.

Trophectoderm that is made up of a cohesive layer of many small cells is associated with a higher quality blastocyst, as compared to TE which has a loose layer of larger cells [Ahlstrom et al. 2011]. This information further suggests that the tested single culture medium is more successful at nourishing preimplantation development when compared to the sequential media, based on the significantly higher number of trophoblast nuclei.

One explanation behind the differences observed in embryo quality between the single and sequential media may be their composition. The early stage media of the sequential system lacks amino acids, glucose, and EDTA. Along with being involved in protein production, amino acids have been known to serve as osmolytes, regulators of intracellular pH and ROS protectants [Miyoshi et al. 1995; Biggers et al. 2000; Van Winkle 2001; Gardner 2008; Baltz 2012].

These functions are predominantly associated with glycine and glutamine [Baltz 2012].

There are mouse studies that have observed that exposing early stage embryos to a pool of amino acids for just a brief period of time improves developmental rates by nearly 20% [Biggers et al. 2000]. Early stage amino acid treatment has been associated with increased blastocyst cell number and a decreased incidence of apoptotic cellular death, as well [Hardarson et al. 2003]. Glutamine, which is a known substrate of the glycine transporter GLY1 (expressed predominantly in pre-compacted embryos), is metabolized to glutamate, a precursor of nucleic acids, and alpha-ketoglutarate, a primary citric acid cycle intermediate [Gardner and Lane 1996; Hashimoto et al. 2008]. A lack of early stage amino acid supplementation may lead to the sequential media embryos to be more susceptible to external stressors, and exhibit a suboptimal metabolism. This may either lead to increased embryotoxicity, or a delay in blastomere cleavage rates.

Early stage exposure of mouse embryos to glucose has been associated with an upregulation of GLUT1, an important glucose transporter present throughout all stages of development, along with monocarboxylate transporter 1 (MCT1), a pyruvate/lactate transporter [Purcell and Moley 2009]. Glucose is also known to be taken up by pre-compaction embryos at a fairly low rate in many species in vivo, ranging from bovine to rodent, and contributing to the accumulation of biomass necessary for proper growth and development (e.g. glucose-6-phosphate, which is shuttled into the pentose phosphate pathway). It

is predictive that the early exposure of embryos to glucose in the single medium contributed to their increased developmental potential. This could, in turn, be due to the improved ability of these same embryos to take up glucose at all embryonic stages.

Interestingly, based on light microscopic image analysis, single and sequential media were not statistically different in the number of embryos reaching ≥ 8 -cell stage on developmental D3. Rather, differences in development were first observed on D4 and D5. This may indicate that in these studies, embryonic phenotypic anomalies do not manifest themselves until after D3 of in vitro embryonic development. This information further points to the idea that the sequential system somehow disrupts proper blastocyst formation, potentially involving abnormal metabolite uptake throughout all stages of development. This may be influenced by a lack of important components in the early stage medium of the sequential system. Alternatively, it is possible that the first medium in the sequential pair was insufficient to provide developmental cues for the embryo to develop past the 8-cell stage.

The percentage of presumptive ICM nuclei per blastocyst was not significantly different between the two groups. This suggests that cellular differentiation and migration may not be impacted in one culture system versus the other. Caution should be taken in interpreting this last finding due to the fact that TE and ICM nuclei were not differentially stained and could not be completely distinguished from one another during the counting protocol.

It is possible that the greater number of trophoblast nuclei observed in the blastocysts developing in the single media may lead to improved implantation due to the increased surface area of the TE that is able to associate with the endometrium. Another benefit of a blastocyst having a higher TE nuclear number is the subsequent development of a more robust placenta, leading to the increased nourishment of the fetus in utero.

In conclusion, the sequential media may not be the best choice in the context of ART, in comparison to single media, when choosing a culture system for human preimplantation embryo development. Single culture media may lead to higher quality blastocysts and is easier to prepare. More side-by-side comparisons must be performed, using mouse embryo assays, in order to further conclude which media type and manufacturer is most optimal. It is also possible that certain media types may be more conducive to human embryo development when compared to murine embryo development, and vice versa. This must be taken into consideration when interpreting the results of a murine embryo assay.

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VITA

Justin Hennings was born in Mattoon, IL on August 26th, 1987. He was raised in the small farming community of Shelbyville, IL, in which his grandparents and two uncles each owned individual, productive farms. His father worked as a part-time agricultural consultant, and full time semi-truck driver for Rural King, while his mother was employed as a Medical Transcriptionist. Justin's interest in the Animal Sciences peaked throughout his teenage years, in which he spent large periods of time helping his family care for their bovine, porcine, feline, and canine companions. This interest continued to grow as he took nearly every biology and agriculture course that was available to the ~400 students present at Shelbyville High School (less Advanced Genetics). Equipped with a 4.0 GPA, he was chosen as the co-Valedictorian of his high school, and voted as one of the two individuals that was 'Most Likely to Succeed'. After high school, Justin headed to Southern Illinois University (SIU) in Carbondale, IL to pursue an educational path that would potentially lead him to becoming a large animal veterinarian, equipped with a scholarship that would pay for the entirety of his first two years. He also received a multitude of other academic awards that helped him to fund the majority of his remaining time at SIU. Throughout his undergraduate career, Justin was frequently involved in a variety of organizations including the Pre-Vet Club, Microbiology Club, Zoology Club, and SIU Academic Honor Society. Justin worked in two different Microbiology laboratories as 'general help' throughout his Sophomore and Junior

years, performing duties that ranged from washing dishes, to preparing a variety of bacterial culture media. He also worked in the biochemistry laboratory of Dr. Perez-Alvarado throughout his Senior year, learning the concepts and techniques involved in protein purification and nuclear magnetic resonance (NMR) analysis. Justin graduated from SIU with a bachelor of science in zoology, and a minor in biochemistry. Due to the great experience that he had gained in the research laboratories in which he was employed as an undergraduate, Justin decided to switch paths and apply to Graduate Schools instead of Veterinary Programs post-graduation.

In August 2011, Justin began his Master's program in Animal Science with emphasis in Reproductive Physiology/Molecular Biology at the University of Missouri in the laboratory of Dr. Kathy Sharpe-Timms in Obstetrics and Gynecology. His primary research focused on endometriosis-associated infertility linked to abnormal preimplantation embryo quality, and how placing early stage zygotes in an established culture system may salvage them.