MOLECULAR AND CLINICAL INVESTIGATIONS USING ANIMAL AND HUMAN SYSTEMS TO IMPROVE ASSISTED REPRODUCTIVE TECHNOLOGIES

By

JOSEPH MICHAEL KRAMER

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To my wife, Raeanna, who provided constant encouragement and support while I continued my studies, and to my children, Nadia and Colt, who may have slowed my writing, but through it have helped me become more tolerant and develop patience

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ART	Assisted Reproductive Technology
ADO	Allelic Dropout
AMCB	Animal Molecular and Cellular Biology
AMH	Anti-Mullarian Hormone
AREG	Amphiregulin
BMP2	Bone Morphogenic Protein 2
BSA	Bovine Serum Albumin
cDNA	Complimentary DNA
CF	Cystic Fibrosis
CGH	Comparative Genome Hybridization
CL	Corpus Luteum
CLIA 88	Clinical Laboratory Improvement Act of 1988
COC	Cumulus Oocyte Complex
COH	Controlled Ovarian Hyperstimulation
C_T	Critical Threshold
DAPI	4'6-Diamidino-2-Phenylindole Dihydrochloride Hydrate
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
E_2	Estradiol
EGF	Epidermal Growth Factor
FBS	Fetal Bovine Serum
FET	Frozen Embryo Transfer
FF	Follicular Fluid

FISH	Fluorescence In Situ Hybridization
FSH	Follicle Stimulating Hormone
GnRH	Gonadotropin-Releasing Hormone
GnRHa	GnRH agonist
GnRHant	GnRH antagonist
GNRH-R	Gonadotropin-Releasing Hormone Receptor
hCG	Human Chorionic Gonadotropin
hGC	Human Granulosa Cells
HSA	Human Serum Albumin
ICM	Inner Cell Mass
ICSI	Intracytoplasmic Sperm Injection
IGF-I	Insulin-Like Growth Factor-1
IhGC	Immortalized Human Granulosa Cell Line
IVF	In Vitro Fertilization
IVP	In Vitro Produced
KO	Knockout
LH	Luteinizing Hormone
LHRH	Luteinizing Hormone-Releasing Hormone
mRNA	Messenger Ribonucleic Acid
miRNA	MircroRNA
OHSS	Ovarian Hyperstimulation Syndrome
P4	Progesterone
PBS	Phosphate Buffered Saline

PCC	Premature Chromosome Condensation
PCCs	Prematurely Condensed Chromosomes
PCR	Polymerase Chain Reaction
PGD	Preimplantation Genetic Diagnosis
PGS	Preimplantation Genetic Screening
PP	Protein Phosphatase
PAPPA	Pregnancy Associated Plasma Protein A
pre-miRNA	Precursor-miRNA
PVA	Polyvinylalcohol
RISC	RNA-Induced Silencing Complex
RNA	Ribonucleic Acid
SEM	Standard Error of Mean
siRNA	Small-Interfering RNAs
SNP	Single Nucleotide Polymorphism
SSS	Synthetic Serum Substitute
T	Testosterone
TGF	Transforming Growth Factor
UTR	Untranslated Region
VEGF	Vascular Endothelial Growth Factor
WGA	Whole Genome Amplification

Abstract of Dissertation Presented to the Graduate School
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MOLECULAR AND CLINICAL INVESTIGATIONS USING ANIMAL AND HUMAN SYSTEMS TO IMPROVE ASSISTED REPRODUCTIVE TECHNOLOGIES

By

Joseph Michael Kramer

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More than 30 years have passed since the birth of the first in vitro fertilization (IVF) baby. Since that time, advances in reproductive technologies have been remarkable. Yet with all that has been achieved so far, there is still much to be learned. To further our understanding, this dissertation investigated several aspects of IVF important to assisted reproductive medicine.

The first area of investigation focused on the effects of gonadotropin-releasing hormone (GnRH) analogs on granulosa cells. Important components of stimulation protocols are the suppression of endogenous gonadotropins with GnRH analogs and the timely administration of exogenous gonadotropins to promote follicular development and final maturation of oocytes for use in IVF. Central to this process is the response of granulosa cells to gonadotropins by regulating the production of steroids and growth factors during folliculogenesis. In this study, GnRH agonist and antagonist protocols were shown to differentially affect the expression of microRNAs (miRNAs) in granulosa cells. This could have important consequences for the function of granulosa cells as these small non-coding RNAs have been shown to post-transcriptionally regulate the

expression of numerous genes essential for folliculogenesis, which may affect stimulation response and reproductive success.

The ensuing investigation focused on the effects of globulin proteins in solutions used for embryo cryopreservation. Successful cryopreservation is dependent upon the osmoregulation of water during freeze/thaw to avoid the formation of potentially detrimental ice crystals. Globulin-rich proteins, which have previously been shown to promote embryo development, have been suggested to alter the embryo microenvironment through interactions with water molecules and therefore were tested in cryopreservation solutions to improve post-thaw embryo survival. Results showed more blastocysts survived, as measured by re-expansion following freezing and thawing, in solutions supplemented with globulin-rich proteins compared to human serum albumin. These results indicate inclusion of globulins promotes blastocyst thaw-survival, which may prove beneficial for clinical cryopreservation of embryos.

The final investigation studied the effects of a protein phosphatase inhibitor, calyculin-A, used to induce premature chromosome condensation (PCC) within single embryonic cells to identify a complete chromosome complement for genetic analysis. Traditional methods for analyzing chromosomal abnormalities in embryos utilize interphase fluorescence in situ hybridization (FISH), which is limited by the number of chromosomes that can be analyzed at one time. In an effort to improve these methods, this study exposed single blastomeres from bovine and murine embryos to calyculin-A to induced PCC. Results demonstrated blastomeres underwent rapid chromatin condensation; however, the quality of condensed chromosomes was inconsistent so that application of this technology was unreliable for genetic screening of embryos.

CHAPTER 1
INTRODUCTION

Long before the birth of the world's first in vitro fertilization (IVF) baby, scientific experiments using animal and later human systems, were conducted to lay the foundation for the field of assisted reproductive technology (ART). The early pioneers of this field were not just clinicians, but included reproductive biologists and geneticists whose scientific training was instrumental to overcoming the many problems of achieving fertilization and embryo development in the laboratory. Science continues to be a part of the field of ART today, but the approach is much slower because of the presence of ever increasing regulation. Since the passing of the Clinical Laboratory Improvement Act of 1988 (CLIA 88), directors of embryology laboratories within the United States are required to hold doctorate degrees in related sciences or medicine; however, there are few academic programs of study with emphasis in embryology to educate and train persons for such positions. This dissertation is part of the requirements of a newly developed doctorate program in the Animal Molecular and Cellular Biology (AMCB) graduate program with emphasis in clinical embryology. The multidisciplinary approach of this dissertation is somewhat unconventional, covering various topics of ART, including ovarian stimulation, embryo cryopreservation and preimplantation genetic diagnosis (PGD), but as a whole the approach provides the basis of quality control and scientific methods essential for persons seeking careers in clinical IVF.

One of the most important advances in IVF has been the development of ovarian stimulation. From the beginning, the founders of clinical IVF, Patrick Steptoe and Robert Edwards, believed ovarian stimulation of multiple follicles would increase the likelihood

of achieving a fertilized oocyte that develops to an embryo stage suitable for transfer to the uterus. This approach was briefly abandoned in favor of natural IVF cycles, as none of Steptoe and Edwards' patients became pregnant. Despite the success with the birth of Louise Brown in 1978 with the use of a natural IVF cycle, a few groups began to reexamine ovarian stimulation, namely Georgeanna and Howard Jones of the Norfolk IVF program and John Leeton and Alan Trounson of the Melbourne program. Amid controversy, the persistence of these groups paid off and was eventually proven to be more effective than natural cycles. Today, ovarian stimulation is a hallmark of IVF and has spawned the fertility pharmaceutical industry.

Among the current approaches to ovarian stimulation are the utilization of gonadotropin-releasing hormone (GnRH) analogs, GnRH agonists and GnRH antagonists, which control endogenous production of gonadotropins and prevent premature surge of luteinizing hormone (LH). Despite overwhelming improvements in controlled ovarian hyperstimulation (COH), the use of GnRH analogs is not without controversy as patient response and outcomes differ between agonist and antagonist protocols. Currently, an explanation for these differences is unknown. In an attempt to help elucidate these differences, chapter 3 of this dissertation investigates the expression of messenger ribonucleic acid (mRNA) and microRNA (miRNA) in granulosa cells from IVF patients and cell lines treated with GnRH agonists and antagonists. Comparisons of patterns of mRNA and miRNA expression are warranted as results may provide insight into a potential molecular mechanism functioning within the ovary in response to GnRH analog therapy which may have profound effects on future treatment of patients along with the development of new drug therapies.

Another area of importance for IVF is cryopreservation of embryos. Improvements in ovarian stimulation and laboratory culture conditions often result in the production of multiple embryos of transferable quality. While in the past it was common practice for patients to receive numerous embryos, current efforts to reduce the risk of multiple pregnancy restrict the number of embryos that can be transferred. As such, following the transfer of restricted numbers of embryos there are usually at least some remaining in culture. Among the options for disposition of remaining embryos, cryopreservation for future use offers perhaps the greatest benefit and is the least controversial choice for the remaining embryos. Unfortunately, early attempts at cryopreservation often resulted in poor thaw-survival rates of frozen embryos, in particular at the blastocyst stage. In fact, many groups abandoned blastocyst cryopreservation, only to revisit the technique after a few groups reported frozen embryo transfer (FET) pregnancy rates greater than 40% [1-4]. Attempts to duplicate this success have not been uniformly successful possibly due to differences in freezing and thawing protocols. Included in one of the more successful cryopreservation protocols are protein sources rich in α- and β-globulins [2]. While the function of these macromolecules is unknown, they have been suggested, to alter the physiochemical environment surrounding the embryo through interactions of hydrogen bonds with water molecules [5]. As osmoregulation of water molecules is an essential component of embryo cryopreservation, chapter 4 of this dissertation investigates the use of globulin-rich protein sources in freezing and thawing protocols using a murine system in an attempt to improve post-thaw embryo survival.

The last area of IVF investigated in this dissertation is preimplantation genetic diagnosis (PGD). This powerful tool allows testing of embryos for monogenic disorders

or chromosomal abnormalities prior to transfer to the uterus to thereby avoid potential miscarriages or decisions to terminate affected pregnancies. Traditional approaches to screening embryos for chromosomal abnormalities, such as structural rearrangements or numerical abnormalities, utilized fluorescence in situ hybridization (FISH) on fixed interphase nuclei from blastomeres removed from cleavage stage embryos. In recent years, this procedure has been an area of heated debate as pregnancy and delivery rates of healthy children have not improved, which in theory should have increased with detection and transfer of normal embryos. A number of reasons for this discrepancy have been proposed with most focus on the utilization of FISH to diagnose a single interphase nucleus. This technique is not only limited by the number of chromosomes that can be analyzed at one time, but also has notoriously high error rates ranging up to 50% [6-11]. These inefficiencies are unfortunate as the frequency of numerical chromosome abnormalities in preimplantation embryos has been reported to range from 50-70% [6, 12-19]. Accordingly, there is a compelling need for a new technology to replace interphase FISH. A number of promising technologies have been proposed, but each has drawbacks that must be overcome before their implementation. Chapter 5 of this dissertation discusses the investigation of premature chromosome condensation in blastomeres from bovine embryos using the protein phosphatase (PP) inhibitor, calyculin-A, in an attempt to produce condensed chromosomes suitable for full chromosome analysis. [20]. Cytogenetic protocols for utilization of PP inhibitors in cell lines are well established and with little modification may be able to replace interphase FISH for preimplantation genetic screening of embryos.

CHAPTER 2
LITERATURE REVIEW

Use of GnRH Analogs for Controlled Ovarian Hyperstimulation

Gonadotropin-releasing hormone (GnRH) is the initiator reproductive hormone responsible for maintaining the physiology and function of mammalian reproduction. As such, a vast number of hormone dependent diseases, including prostate cancer, breast cancer, endometriosis, uterine fibroids, polycystic ovarian syndrome, and precocious puberty, as well as infertility are treated with synthetic GnRH analogs to regulate reproductive hormone effects. This review will focus on the use of GnRH analogs for preventing a premature surge of luteinizing hormone (LH) during controlled ovarian hyperstimulation (COH) of infertile couples seeking in vitro fertilization.

Gonadotropin-releasing hormone, also referred to as luteinizing hormone-releasing hormone (LHRH) or gonadorelin, is a decapeptide (pyroGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH2)[21] synthesized in specialized neurons within the arcuate nucleus of the medial basal hypothalamus (MBH) and preoptic anterior hypothalamus [22]. Following synthesis, GnRH is transported down axons to the median eminence where it is released into the hypophysial blood portal system. The release of GnRH appears to be under the control of a GnRH “pulse generator” within the MBH. In humans, the pulsatile release occurs every 60 to 100 minutes, but varies across the normal female reproductive cycle [23]. Upon release, GnRH binds to a seven-transmembrane G-protein-coupled receptor (GnRH-R) localized on the plasma membrane of the gonadotroph cells within the anterior pituitary stimulating synthesis and release of the gonadotropins, LH and follicle stimulating hormone (FSH)[24].

In mammals, several isoforms have been identified for GnRH and its receptors. The type-I form of GnRH is responsible for regulating gonadotropins, while the type-II form is a neuromodulator and stimulates sexual behavior [25]. Likewise, two isoforms of GnRH receptors have been identified; however, the GnRH type-II receptor has been silenced in most mammals including humans [26]. The GnRH type-I receptor is characterized as a typical G-protein coupled receptor with the exception of the absence of a carboxy terminal tail which is believed to serve an important role in the functioning of the receptor [25]. Both GnRH type-I and -II can bind and activate the functional GnRH type-I receptor [26].

Activation of the GnRH receptor stimulates the phospholipase C (PhL-C) enzyme cascade, which promotes the rapid formation of inositol-triphosphate (IP3) and diacylglycerol (DAG) [27]. The formation of IP3 subsequently mobilizes calcium which activates protein kinase C [27]. This cascade culminates in the synthesis and release of gonadotropins, LH and FSH, and is controlled by the frequency of GnRH release as well as feedback by estrogen and androgens [28]. Generally, low frequency GnRH leads to FSH release, and high-frequency GnRH stimulates LH release. In addition, the frequency of GnRH release can have profound effects on availability of GnRH-Rs. While pulsatile GnRH release patterns stimulate the up-regulation of GnRH receptors through rapid dimerization, internalization, and recycling back to the cell surface, continuous exposure to GnRH results in down-regulation of GnRH-Rs and suppression of gonadotropins. This suppressive effect by down-regulation of GnRH-Rs is the basis for the fundamental principal for controlling ovarian stimulation with GnRH agonists.

The use of GnRH agonists to control ovarian stimulation by suppressing pituitary release of LH and FSH while stimulating follicular development with exogenous gonadotropins was first demonstrated in 1984 [29]. Today, this procedure, although slightly modified, is the primary protocol for preventing premature LH surge in patients undergoing COH for IVF treatment. Unlike endogenous GnRH, which has a half life of 2 to 4 minutes [30], GnRH agonists are less susceptible to enzymatic breakdown and have increased binding efficiency to GnRH-R. This increase in biologically potency is made possible through substitutions of glycine at position six with D-amino acids such as D-Leu or D-Ser found in Lupron and Buserelin, respectively [31]. In addition, the replacement of the C-terminal glycinamide residue by an ethylamide group can lead to a 10-fold increase in GnRH-R affinity [31]. Together, these changes improve the effectiveness of synthetic GnRH agonists up to 200-fold more than endogenous GnRH making them highly efficient for desensitizing the gonadotroph cells and causing suppression of pituitary gonadotropins.

GnRH antagonists offer an alternative, opposing mechanism, for preventing premature LH surge during COH. Contrary to agonists, GnRH antagonists bind to the GnRH-R without inducing gonadotropin synthesis by competitively inhibiting endogenous GnRH from activating the GnRH receptor [25]. Competitive inhibition is advantageous in that its effects are immediate and reversible. The properties of GnRH antagonists arise mainly from modifications of the N-terminal region which are thought to be necessary for GnRH actions, but modifications at the pyroglutamic region (positions 1) or deletion and substitution of hydrophobic D-amino acids at position 2 and 3 have also been shown to be effective in generating antagonistic effects [31].

The protocols for GnRH agonist and antagonist are fairly standard for preventing premature LH surge in patients undergoing ovarian stimulation for IVF. The major differences between protocols are with the timing and duration of the GnRH analog treatment. Generally, with the GnRH agonist “long protocol”, patients receive a daily dose of a GnRH agonist following menses up to the time of trigger with human chorionic gonadotropin (hCG), which induces final oocyte maturation. Stimulation with FSH is initiated when the patient is suppressed as evident by low LH, progesterone (P4), estradiol (E_2) (approximately 10 days after the first dose of GnRH agonist). Protocols using GnRH antagonists differ in that the patients are not suppressed when they begin stimulation with FSH. Instead, approximately 6 days after stimulation with FSH (or when the leading follicle reaches 14 mm in diameter) patients receive a daily dose of GnRH antagonists (or a large single dose) to inhibit premature LH surge up to the time the oocytes are triggered for final maturation.

Since the introduction of GnRH antagonist protocols for COH [32], a number of studies have compared patient response and outcome to the GnRH agonist long protocol. Most studies have found patients treated with GnRH antagonist protocols were associated with lower gonadotropin dose requirements [33-35], shorter durations of stimulation [33-38], absence of flare-induced effects (hot flashes, headaches, sleep deprivation) and decreased risk of ovarian hyperstimulation syndrome (OHSS)[34, 39-42]. Studies were not in agreement in patient outcomes, however. While a number of prospective randomized trials have found no differences in pregnancy rates in patients receiving GnRH antagonists compared to agonists [33, 38, 43, 44], a few studies reported a small advantage in favor of GnRH agonists, although not statistically

significant [34, 45, 46]. In an effort to clarify these discrepancies, at least four meta-analyses have been undertaken but findings are equally conflicting. Two meta-analyses by Al-Inany and Aboulghar [47] and Al-Inany [48] reported significantly higher pregnancy rates after treatment with GnRH agonists. In contrast, Ludwig and coworkers [41] and Daya [49] found no differences in pregnancy rates between protocols. Given inconsistencies between studies and the potential for reduced outcomes, widespread acceptance of GnRH antagonist protocols has been slow and met with resistance despite the apparent benefits with patient response.

GnRH agonist and antagonist protocols have also been associated with differences in concentrations of serum hormones and steroids as well as follicular steroid concentrations. Several reports have found lower E_2 [50-52], T [50], and P4 [50] in follicular fluid from patients receiving GnRH antagonist compared to agonist protocols. Ferrari and coworkers also found higher concentration of insulin-like growth factor-1 (IGF-1) [52] and vascular endothelial growth factor (VEGF) [52, 53] in follicular fluid obtained from patients receiving the GnRH antagonist protocol. In addition, they found IGF-1 and VEGF to be inversely correlated with serum LH. Based on their observations, they suggest the reduced LH release during late follicular maturation in GnRH antagonist protocols may cause a decrease in steroidogenesis in thecal and granulosa cells. While their suggestion could explain the reduced concentrations of E_2, T and P4, it does not explain the observed increases in IGF-1 and VEGF in GnRH antagonists. Alternatively, many researchers have suggested the differences between GnRH agonist and antagonist may arise because of direct ovarian affects as receptors

for GnRH have been localized in granulosa cells [54, 55]. However, the function of these ovarian receptors remains unknown.

Direct ovarian action by GnRH analogs has been reported in a number of in vitro animal and human studies. Hsueh and Erickson [56] were among the first to demonstrate reduced estrogen production in cultured rat granulosa cells after treatment with a GnRH agonist. In humans, the direct effects of GnRH analogs on steroidogenesis of granulosa cells are controversial as no effect [57, 58], inhibitory [59] and stimulatory [60] effects have been reported. Interestingly, Bussenot and coworkers [61] tested five different GnRH agonists on steroidogenesis of human granulosa cells and found that GnRH analogs were able to differentially modulate steroid production, despite all the analogs having common pituitary effects. Based on their results, they suggested the activation and function of GnRH-R differs in pituitary and ovarian cells. Recently, another study investigated the effects of GnRH analogs on the expression of CYP19A1, a key enzyme in the synthesis of E_2 [62]. GnRH antagonists directly suppressed CYP19A1 in cultured human granulosa cells while GnRH agonists promoted CYP19A1. Together, these studies lend support to direct and differential actions of GnRH agonists and antagonists on ovarian cells and suggest their actions may affect expression of genes essential for ovarian function. A molecular explanation for differential regulation of gene expression by GnRH analogs remains unknown. Such findings would be of major concern as any direct ovarian effects of GnRH analogs might affect the quality of gamete maturation and subsequent embryo development. Further research is warranted.

Ovarian Expression of miRNAs

MicroRNAs (miRNAs) are members of a newly identified class of small non-coding ribonucleic acid (RNA) molecules that function post-transcriptionally to regulate gene expression. Since the identification of the first miRNA in *Caenorhabditis elegans* in 1993 [63], several hundred more miRNAs have been identified in various species. As of June 1st, 2010, 579, 615, and 721 miRNAs have been recorded in murine, bovine, and human databases, respectively (www.mirbase.org), and computational algorithms predict the existence of several thousands more that may regulate the expression of up to 30% of genes in humans [64, 65].

The biogenesis and functionality of miRNAs is a complex process (Figure 2-1). The gene sequences encoding for miRNAs are found throughout the genome and reside mainly in the intergenic regions and in a few cases in exon regions [66]. In addition, promoters and regulatory units are included in many of the miRNA genes [67]. MicroRNA biogenesis initially results in the synthesis of primary miRNA molecules (pri-miRNA) that can range up to a two kilobases long and can also contain sequences of several different miRNAs as a result of clustering [68]. The pri-miRNAs are cleaved by type III RNA endonuclease Drosha and its mammalian partner DiGeorge Syndrome Critical Region 8 (DGCR8) into 70- to 90-nucleotide stem-loop precursor miRNA (pre-miRNA) in the nucleus [69]. The pre-miRNAs are transported to the cytoplasm by the nucleocytoplasmic shuttle exportin 5, where they undergo a second cleavage by another RNA endonuclease, Dicer, generating a 19-24 base-pair-long double stranded miRNA duplex [68, 70]. The double stranded miRNA duplex unwinds with usually, but not always, one strand selected to function as a mature miRNA [71]. The mature miRNA is then incorporated into the RNA-induced silencing complex (RISC), also known as

micro-ribonucleoprotein (miRISC) complexes, which contain a number of proteins, most notably Argonaute proteins [72]. The Argonautes contain RNA binding domains, PAZ and PIWI, that bind the single stranded 3’ end of the mature miRNA and the 5’ end of the guide strand and orient the mature miRNA for interaction with the target messenger RNA (mRNA) [72]. The interaction between a miRNA and its target usually occurs in the 3’ untranslated region (3’UTR) of the mRNA [73]. The complementary region of the miRNA is referred to as the “seed” region and contains 7-8 nucleotides. This small “seed” sequence may be complimentary to hundreds of potential mRNA targets giving rise to the diversity of gene regulation through only a few miRNA. In plants, the complementary region has nearly perfect base pairing with the target RNA [74], while in animals interaction with 2-8 nucleotides is sufficient to regulate gene expression [75]. In general, miRNA binding to the complimentary target induces translational suppression by blocking assembly of 80S ribosome [76], or through inhibition of translation after initiation [77]. However, other reports have also suggested miRNAs can cause an increase the in degradation of the mRNA target by endonucleolytic cleavage [77] similar to small-interfering RNAs (siRNAs). This complexity in miRNA function may also be target gene and tissue specific. As such, full understanding of miRNA functionality will likely take many more years of research.

Currently, little is known about the role of miRNAs in reproductive tissues. To date, a few reports have identified the expression of miRNAs in the pituitary [78], ovary [79-81], testis [82], uterus [83, 84], several reproductive tissue cancers [85-87], and recently the embryo [88, 89]. With regards to the ovary, several reports have identified hundreds of miRNAs in fetal and adult mouse ovaries, including several novel miRNAs

preferentially expressed in the ovary [80]. Similar studies in the cow have also identified more than 50 known and 24 novel miRNAs predominantly expressed in fetal ovarian tissue [81]. Many of these miRNA are homologous to known miRNAs in other species, supporting the notion that miRNAs are evolutionary conserved [90, 91]. In addition, functional studies in other tissues have shown these miRNAs to play a central role in various cellular activities, including developmental processes, cell growth, differentiation, apoptosis, cell-cell communication, and inflammatory and immune responses [92-96] and therefore are likely to be involved in similar processes and pathways within the ovary.

The mammalian ovary is a dynamic tissue undergoing cyclic recruitment of growing follicles, atresia, ovulation, and luteal formation and regression. These intricate events involve highly coordinated endocrine and paracrine processes to modulate cell growth, angiogenesis, steroidogenesis, and apoptosis. Coordination is most evident in the development of oocytes and their supporting granulosa cells. Any disruption in this paracrine communication between germ cells and their supporting somatic cells could have profound effects on oocyte competence. As such, the processes of folliculogenesis are tightly regulated by the expression of numerous genes. For instance, more than 100 genes have been shown to be essential for normal folliculogenesis in the mouse [97, 98]. Given that miRNAs have previously been identified in the ovary, and that they function in the regulation of gene expression, miRNAs may play a critical role in controlling the expression of genes essential for maintaining ovarian function and avoiding disruption of folliculogenesis, ovulation and CL formation and regression.

The biological importance of miRNAs has been well documented over the past decade through a number of studies utilizing animal models and in vitro cell culture that lack expression of the key miRNA processing protein, Dicer [99, 100]. Studies that generated homozygous mutant Dicer knockout (KO) embryos found they exhibited retarded growth and pregnancy declined as early as embryonic day 7.5 (E7.5) [99, 100]. Furthermore, yolk sacs from E11.5 Dicer KO were thin and contained disorganized blood vessels suggesting developmental arrest was likely due to impaired angiogenesis [100].

Several studies have since investigated the function of Dicer in growth and development of oocytes and embryos [101, 102]. During normal folliculogenesis, germ cells maintain steady transcription of dicer mRNA [103]; however, after fertilization, dicer mRNA transcript decreases and remains low throughout the blastocyst stage [102, 104]. During this same time, the pool of maternal mRNAs in the mature oocyte decreases throughout early cleavage, likely in part to Dicer function. Reports using conditional oocyte specific KO of Dicer suggest folliculogenesis appears normal, as evidenced by normal ovulation rates. However, oocytes are less likely to extrude polar bodies after mating, and immunostaining indicated defects in spindle formation and chromatin condensation [101, 102]. Furthermore, transplanting germinal vesicles from wild-type mice into Dicer deficient oocytes resulted in similar spindle defects [101]. These results suggest that meiotic defects arise from ooplasm of the oocyte and not the germinal vesicle.

The expression of Dicer has also been selectively KO in mullarian duct derivatives, including oviduct, uterus, cervix and ovary in mice [79, 105, 106]. In one

study [106], Dicer KO females demonstrated normal estrous cycles and successfully mated as evidenced by fertilized oocytes and blastocysts obtained from day 1 and day 3 pregnancies; however, embryos morphologically contained more fragmentation and degeneration compared to wild-type. Furthermore, Dicer KO females failed to produce live offspring. Interestingly, P4 levels were comparable for day 1-3 of pregnancy, but decreased thereafter. While the authors attribute the loss of pregnancy to poorly developed oviduct and uteri, reduced luteal function could not be excluded.

In a similar report, ovaries from female mice containing a hypomorph KO of Dicer (~75% loss of Dicer mRNAs) were transplanted into wild-type females and vice versa [79]. In that study, dicer KO females receiving wild-type ovaries became pregnant, while wild-type females receiving the Dicer KO ovaries failed to establish pregnancy despite apparent normal ovulation, fertilization of oocytes, and cleavage of resulting embryos. In contrast, serum progesterone concentrations were decreased in females with dicer KO ovaries along with reduced expression of genes essential for CL formation, including LH receptor, cytochrome p450 11a1 and prolactin receptor. Further assessment of Dicer KO ovaries revealed decreased formation of blood vessels in the CL correlated with an up-regulation of angiogenic factors, TIMP1 and platelet factor 4. Computational analysis predicted that miRNAs, mir-17-5p and let-7b, may target these genes. Functional studies that knocked down each miRNA in wild-type mice showed impaired CL angiogenesis and decreased P4, similar to dicer KO females. Furthermore, the injection of mir-17-5p and let-7b into the ovarian bursa of dicer KO females restored the vasculature and increased P4; although pregnant females failed to deliver offspring suggesting other miRNAs might be involved and necessary for continuing pregnancy.

While the previous studies suggest miRNAs may function in post-ovulatory events leading to infertility, another study explored the role of dicer during folliculogenesis. Using conditional KO mice lacking dicer in granulosa cells, histological analysis found inactivation of dicer led to an increase in primordial follicle endowment, accelerated early development of secondary and tertiary follicles, more degenerate follicles, and more ovarian cysts in KO animals [105]. In addition, the CL in KO ovaries had abnormal vascularization including the presence of more red blood cells. Analysis of ovarian gene expression in dicer KO revealed several genes were significantly reduced by day 4 and day 8, including genes involved in activin/inhibin (inhiba) assembly, hormone synthesis (cyp11a1, cyp17a1 and cyp19a1), granulosa cell development (Bcl2, Bax, Casp3, Ccnd2, Cdkn1b) and oocyte specific genes (Zp2, Zp3, Gdf9) as well as expression of mir-503. Functional analysis through transfection of pre-mir-503 into primary cultured granulosa cells resulted in significant down-regulation of both target and non-target genes involved in granulosa cell proliferation and luteinization. These results suggest the Dicer-regulated signal pathway plays an important role in follicular cell development through differential regulation of gene expression.

Although the aforementioned studies clearly demonstrate the importance of the miRNA processing protein Dicer in reproductive tissues, it remains unclear how miRNAs are themselves regulated and how their function can regulate expression of genes essential for maintaining ovarian physiology and preserving fertility. Recently, a few reports have investigated the regulatory effects of hormones on the expression of ovarian miRNAs [107, 108]**.** In one study, the effects of LH surge were investigated by

collecting murine granulosa cells from periovulatory follicles before and 4 hours after an ovulatory dose of hCG (mimic LH) [107]. Microarray analysis showed differential expression of 13 miRNAs, including mir-132 and mir-212, despite no changes in the expression of Dicer. In addition, functional assessment of mir-132 and mir-212 demonstrated granulosa cells transfected with specific anti-mir probes and treated with cAMP to simulate the second messenger system downstream of LH/hCG differentially expressed several potential gene targets, including CTBP1, which was recently shown to interact with steroidogenic factor-1[109]. These results suggest hormonal stimulation (LH/hCG) of ovarian tissue can regulate the expression of miRNAs, such as mir-132 and mir-212, which in turn may affect the expression of several genes important for ovulatory events, including the final maturation of oocytes, luteal formation, and steroid synthesis.

A similar study recently investigated the hormonal effects of FSH on ovarian expression of miRNAs [108]. In situ hybridization of murine ovarian tissue revealed select miRNAs (mir-142, let-7a, mir-125b, let-7b, let-7c, and miRr-21) were less detectable in primordial follicles, but were readily detectable in granulosa cells from primary, secondary and tertiary follicles suggesting expression may be under hormonal regulation. Further experimentation using the murine ovarian cell line KK-1 demonstrated treatment with FSH reduced the expression of mir-143, let-7a and mir-125b overtime. Based upon these results, the authors suggest FSH may regulate folliculogenesis through a network of miRNAs.

Given that hormones drive follicular development and steroid production and that the expression of miRNAs has recently been shown to be affected by FSH and LH/hCG,

and that defects in ovarian miRNA are associated with follicular and ovulatory dysfunction, it would be expected that ovarian miRNAs play a role in ovarian steroidogenesis. In support, a recent study using human granulosa cells transfected with a miRNA library containing synthetic precursor miRNAs demonstrated differential release of P4, T, and E_2 [110]. Their results found transfection with 36 out of 80 tested precursor miRNAs significantly inhibited P4 release, including mir-15a and mir-188, while 10 miRNAs enhanced release of P4. Transfected miRNAs had similar effects with T (57/80 miRNAs inhibited T release, while 1 activated T release) and E_2 (51/80 miRNAs suppressed E_2 release, while none activated E2 release). Functional studies showed transfection with specific anti-mir probes successfully knocked down mir-15a and mir-188 and significantly stimulated P4 release. This study suggests that among the ovarian functions of miRNAs, they may regulate the processes of steroidogenesis.

Several studies have shown miRNAs to have an important role in ovarian physiology. Furthermore, other studies have shown the expression of ovarian miRNAs to be influenced by the same hormones responsible for regulating ovarian function. In addition, disruption of the miRNA pathway has been found to result in ovarian dysfunction, including abnormal follicular development, meiotic maturation, luteal regression and formation, and abnormal steroid release. How these findings are related to infertile disease remains unknown. However, the cumulative evidence suggests miRNAs play a crucial part in regulating ovarian function. As such, fertility treatments using different hormonal stimulation regimens may have differential effects on the expression of ovarian miRNAs that may affect ovarian response differently. Therefore, future studies are needed to determine if differences observed in patient response using

different stimulation regimes may be explained through altered regulation of ovarian miRNAs.

Protein Supplementation of In Vitro Systems

Over the last few decades, advances in ART, including culture media, stimulation procedures, and intracytoplasmic sperm injection (ICSI), have brought forth an age where pregnancy rates of 60% can now be routinely achieved. While advances in each of these areas has contributed to the overall improvement of ART, improvements in culture media have perhaps had the greatest impact within the laboratory on sustaining the viability of developing embryos. This review will focus on inclusion of macromolecules in human embryo culture media with emphasis on the development and utilization of Synthetic Serum Substitute (SSS).

In the early days of IVF, practitioners relied on culture media developed for somatic cells (reviewed in detail by Summers and Biggers [111]). Accordingly, the supplementation of embryo culture medium with macromolecules, such as serum, was also borrowed from somatic cell culture. Serum or proteins from serum were initially believed to provide some nutritional necessity for proper embryo development. Caro and Trounson [112] however, were able to demonstrate IVF and pregnancy could be achieved with embryos cultured in protein-free conditions. This result suggested serum or other protein supplementation is not a requirement but rather acts to improve the performance of embryos within a culture medium [113]. Additionally, early studies comparing different sources of serum as well as different lots of the same source of serum demonstrated inconsistencies within patients and batch to batch variation. It is now well recognized that serum is a complex mixture containing a number of undefined components that can vary from lot-to-lot resulting in unpredictable embryo development

and pregnancy. Despite this drawback, serum, including fetal calf, maternal, and fetal cord, continued to be a major component of embryo culture media as it consistently produces greater pregnancy rates than protein-free systems.

Slowly, with improvements in embryo culture conditions, a movement grew towards the use of more defined culture components, including sources of protein, in an effort to overcome the inconsistencies in embryo culture media performance and to avoid the possibility of introducing serum contaminants. Produced from ethanol precipitation of whole serum, serum albumin, initially from bovine (BSA) and later from human (HSA), was a more defined protein component shown to produce equivalent rates of fertilization, cleavage and pregnancy when compared to whole serum [114, 115]. At the time, commercial sources of HSA were limited to plasma volume expanders licensed for intravenous infusion. Two such expanders were Plasmanate and Plasmatein. Like other plasma volume expanders, these expanders are composed predominantly of HSA, but they also contain a significant proportion of α- and β-globulins.

Two groups set out to evaluate the use of Plasmanate and Plasmatein as embryo culture medium supplements. Plasmanate, composed of 88% normal human albumin, 12% α- and β-globulins, and not more than 1% γ-globulins, was studied by the Cornell IVF program [116]. Meanwhile Pool and Martin [5] studied Plasmatein, which contained up to 5% more α- and β-globulins than Plasmanate. Although not directly compared, both Plasmanate and Plasmatein resulted in similar pregnancy rates. Both groups attributed the success of these plasma extenders to the presence of the α- and β-globulins, which might be mediating their effects by binding with available water

molecules and changing the microenvironment to one more conducive for embryonic growth. Interestingly, despite successful fertilization and pregnancy when used as a protein supplement, neither Plasmanate nor Plasmatein were developed for use as a supplement for culture of embryos. In fact, characteristics such as the ionic strength, pH and osmolarity, were optimized for vascular physiology and not embryonic development. Weathersbee and coworkers [117] set out to overcome the drawbacks of these plasma extenders by formulating a protein supplement containing high concentrations of α- and β-globulins prepared in a solution more suitable for inclusion within embryo culture media. Their efforts led to the development of Synthetic Serum Substitute (SSS; Irvine Scientific, Santa Ana, CA, USA), composed of 6% total human protein containing 84% HSA, 16% α- and β-globulins and with less than 1% γ-globulins in a saline solution with a pH of 7.2-7.4 and an osmolarity of 275-285 mOsm/kg.

Since its initial development, SSS has undergone numerous animal and human studies examining its ability to support embryonic development and pregnancy. When tested with the culture of frozen-thawed mouse embryos, SSS supplementation was comparable with Plasmatein and produced high rates of embryo development with 100% blastocyst formation [117]. Another study using mouse embryos compared culture media performance when supplemented with either SSS or serum from patients [118]. Interestingly, media supplemented with SSS produced more total blastocysts and hatched blastocysts compared to media supplemented with serum from a proven fertile donor (83.7% vs. 39.4% and 43.2% vs. 9.1%, respectively) suggesting SSS may be clinically beneficial for IVF.

With regards to culture of human embryos, several studies have compared media performance with SSS and other protein supplements [119-121]. One study compared supplementation with SSS to Plasmanate and maternal serum for sperm preparation and oocyte and embryo culture [119]. Results from that study revealed a trend towards a higher pregnancy rate and a significant increase in implantation rates with SSS compared to maternal serum or Plasmanate. Surprisingly, the rate of fertilization was lower when SSS was used for sperm preparation and insemination compared to Plasmanate. A different study compared supplemented culture media with SSS or fetal cord serum [120]. In this study, there were no significant differences in rates of fertilization, pregnancy, or implantation for embryos produced in media supplemented with SSS or fetal cord serum. Yet another study compared SSS and HSA in the early culture of human oocytes and embryos [121]. In that study, sperm, oocytes, and fertilized embryos were processed and cultured until day 2 with SSS or HSA. Fertilization rates were similar for both treatments and resulting embryos were scored with similar high quality grades on day 2. Pregnancy rate and implantation rate were reported only for the SSS group, but were comparable to the clinics previous experience with HSA. Taken together, these studies demonstrate SSS to be comparable and in some cases better than other protein supplements for the fertilization and development of embryos while sustaining high pregnancy and implantation rates.

In addition to improving development of cleavage stage embryos, globulin-rich supplements have also been tested for improving extended culture of blastocysts. The importance of blastocyst development cannot be understated, especially in today's laboratory where selection of the best one or two embryos from a cohort is necessary to

reduce the risks of multiple pregnancy. Schneider and Hayslip [122] cultured mouse embryos to the blastocyst stage in medium supplemented with either SSS, HSA, or a mixture of globulins and HSA. Their results indicated SSS or HSA+globulins to be superior for the development of embryos to hatched blastocysts compared to HSA alone. They also demonstrated that SSS could "rescue" hatching by transferring embryos from HSA to a globulin-containing medium. Additionally, they showed that hatching can be inhibited by removing embryos from globulin-containing medium. Other groups have made similar observations which have led some to suggest that the transition from morula to the early blastocyst stage is marked by a critical transition phase influencing hatching [123]. Whether or not this holds true, it is clear that protein supplementation during extended culture, and in particular globulin-rich supplements, can promote blastocyst development and hatching.

The mechanism by which macromolecules, such as globulins, enhance in vitro development of embryos is currently unknown. The original developers of SSS suggested the growth promoting effects of globulins were predominantly physical rather than chemical [5]. Central to their hypothesis is the number and orientation of hydroxyl (-OH) groups in glycoprotein macromolecules, such as globulins, which may allow them to interact through hydrogen bonds with water molecules to alter the microenvironment [124]. Interestingly, naturally occurring glycoprotein macromolecules found within the oviduct have also been proposed to modulate the microenvironment by affecting the interaction of cations, sugars and other essential substrates with the developing embryo [125]. Among the macromolecules indentified in the reproductive tract include Mucus glycoproteins [mucins [126-130]] and hyaluronan [131, 132].

Gardner and coworkers [133] have previously examined the effects of supplementing embryo culture medium with hyaluronan. In their study, they compared embryo development and implantation using culture medium supplemented with macromolecules hyaluronan, BSA, polyvinylalcohol (PVA), or dextran. The highest rates of implantation came from embryos exposed to hyaluronan. Murine [134], bovine [135] and human [136] embryos are known to have receptors for hyaluronan so the functions of this macromolecule in culture or in the oviduct are probably not limited to physical interactions with the microenvironment.

Nonproteinaceous macromolecules, such as PVA and dextran, are known to interact strongly with water and may provide support to a physical role of macromolecules such as globulins during in vitro development. Bavister [137] previously demonstrated PVA was able to replace BSA for the development of hamster and bovine embryos. In addition, a protein supplement containing a mixture of dextran and HSA [Dextran Serum Supplement (DSS);Irvine Scientific] was recently made commercially available for inclusion of embryo culture medium. Preliminary reports from the manufacturer demonstrated DSS performed equally to SSS in promoting blastocyst development. The growth promoting effects of dextran are likely similar to globulins, as both are included in intravenous volume expanders used for improving osmotic blood pressure. As such, it is conceivable to think that these macromolecules interact with minute amounts of water in the embryo microenvironment much like the way they help to maintain water volume in the vasculature. In doing so, these macromolecules could be altering the physical interactions of the preimplantation embryo with its microenvironment which may enhance biological effects.

Alternatively, one could argue that there may be some unknown chemical interaction responsible for the enhanced performance of embryo culture media when supplemented with protein macromolecules. The processes of protein purification are not absolute and consequently, trace amounts of embryotrophic peptides, such as growth factors, could be present within the processed macromolecules and be responsible for the improved embryonic potential.

Since their first introduction, macromolecules have played an integral role in the improvement of embryo culture media. Numerous reports have shown enhanced potential of embryos when cultured in the presence of macromolecules. Most notably are globulins, which are now commercially available as supplements for embryo culture media as SSS or Serum Protein Supplement (SPS; Sage In Vitro Fertilization, CT). While the mechanism remains unknown, the enhanced embryonic potential derived from the inclusion of these macromolecules has no doubt helped improve the overall success rates of ART. With the advent of culture medium rich in hyaluronan (EmbryoGlue,Vitrolife, Englewood, CO) and dextran, as well as the availability of recombinant HSA, it will be interesting to see if greater understanding of the interaction between macromolecules and embryos in culture media will explain enhanced viability. Perhaps one day, protein-free culture media will be developed that support embryo growth as effectively as or even better than current available media.

Cryopreservation of Blastocysts

The Successful cryopreservation of mammalian embryos was first demonstrated in 1972, when Whittingham and coworkers [138] obtained live offspring following the transfer of frozen-thawed mouse morula. A year later, these techniques were applied to domestic animals, most notably cattle [139]. In humans, the first pregnancy was

reported by Trounson and Morh [140] in 1983, following transfer of a frozen-thawed eight cell embryo. Shortly thereafter, Cohen and coworkers reported successful cryopreservation of human blastocysts [141].

Today, the importance of blastocyst cryopreservation is greater than ever before as improvements in extended embryo culture often result in the production of more blastocysts than can be transferred to the patient. Consequently patients must make a decision on the disposition of remaining embryos, such as cryopreservation for future use, donation to an infertile couple, donation to research, or discard. Among the choices, donating for research or discarding remaining embryos are controversial and many patients and laboratory personnel have ethical concerns. Alternatively, cryopreservation for future use and donation to an infertile couple, offer perhaps the greatest benefit and are the least unethical solutions; however, both require the laboratory to be proficient at freezing and thawing embryos. This review will focus on current concepts of blastocysts cryopreservation.

The principals of cryopreservation have been well documented [142, 143], and understanding their fundamentals is essential for gaining insight into the cellular and molecular changes occurring in embryos during the freezing and thawing process. During freezing, decreasing temperatures eventually cause extracellular water molecules to transition from a liquid into a solid, generally in the form of ice crystals. The osmolarity of the remaining solution surrounding the embryo then increases as the ratio of free water to salts decreases. This increase in salt concentration in the extracellular environment causes osmoregulatory mechanisms, including diffusion and active transport, to drive water out of cells leading to dehydration. If this process is controlled

by a slow rate of freezing, then the formation of extracellular ice is harmless. Conversely, if the temperature cools too quickly, then cellular water will transition into intracellular ice crystals before the cell is dehydrated. The formation of intracellular ice, in contrast, can damage cytoskeletal structures thereby affecting the integrity of cell membranes. Fortunately, problems associated with intracellular ice formation can be reduced with the aid of cryoprotectants. Theses chemical compounds increase the total concentration of all solutes in the system, thereby reducing the amount of ice formed at any given temperature. In addition, some cryoprotectants, such as glycerol, propandiol, ethylene glycol and dimethyl sulfoxide (DMSO), increase cell membrane permeability thereby allowing water to diffuse out of the cell more rapidly. As such, the inclusion of cryoprotectants, along with controlling the rate of cooling, are important steps in all freezing protocols.

During the thawing process, the transition from a frozen to an aqueous state must occur faster than the rate at which the embryos were originally frozen. The reason for this difference is that slow warming puts cells at a greater risk for ice crystal re-formation or re-crystallization which can lead to membrane damage [142]. In contrast, during rapid warming there is insufficient time for re-crystallization, and any ice crystals melt away [142].

Upon thaw, permeating cryoprotectants need to be promptly removed to again avoid toxic injury. Exposure to lower concentrations of cryoprotectants causes the osmotic uptake of water at a faster rate than the diffusion of cryoprotectants out of the cell [142]. This causes cells to swell above their initial volume. As cells are generally

more sensitive to swelling than shrinking, removal of cryoprotectants tends to be more hazardous than their addition.

With an understanding of the general principles of freezing, embryologists have been able to attain good success from cryopreservation of zygotes and early cleavage stage embryos. The cryopreservation of blastocysts, however, has been more challenging as early results were marred with poor rates of post-thaw survival. It was almost 20 years between the first reported pregnancy from a frozen blastocyst [141] to when Veeck and coworkers [2] reported high clinical pregnancy rates from frozen blastocysts. In the interval, many groups had abandoned blastocyst cryopreservation. Eventually though, with improved extended culture conditions, several groups began to revisit blastocyst cryopreservation and reported pregnancy rates greater than 40% following frozen embryo transfer (FET) [1-4]. Unfortunately, many other programs have not been able to attain similar successes [144-149]. The differences in thaw survival of early cleavage stage embryos and blastocysts suggest blastocysts have unique properties which may require further optimization of freezing and thawing procedures. Those groups that reported high rates of success may have, unbeknownst to them, managed to highlight some differences that are necessary for attaining good post-thaw survival.

There are at least two differences between blastocysts and cleavage stage embryos that may factor into cryopreservation success rates. For one, the cells of the trophoblast and inner cell mass (ICM) are much smaller compared to cells from an early cleavage stage embryo. This size difference would mean the cell volumes from blastocysts should contain less water, which would otherwise be thought to be an

advantage, as cells from blastocysts should be at reduced risk for intracellular ice crystal formation. However, it is plausible that the permeability of cells from the trophoblast and/or ICM differ compared to cells from early cleavage stage embryos because of their reduced size or differentiation, which may affect the rate of exchange of cryoprotectants and water. Another difference that may contribute to the lower thaw survival of blastocysts is the presence of the blastocoele. This fluid filled cavity, if not dehydrated, may lead to the formation of ice crystals within the cavity. While these crystals would not be intracellular, the shear forces that they exert within the blastocyst may damage extracellular structures and interconnections between cells, as observed in the cryopreservation of multicellular organs and tissues [150-152]. Another possibility is that the blastocoele cavity itself may affect the permeation of the cryoprotectants, especially with cells of the ICM, and this may lead to highly damaging intracellular ice crystal formation within those cells.

Overtime, a number of different approaches have attempted to further optimize blastocyst cryopreservation protocols. The most notable studies include investigations into changing the start temperature and cooling rate [153], artificially shrinking the blastocoele cavity [154, 155], incorporating macromolecules into cryo solutions [156, 157], and more recently vitrification [3, 4, 158].

Gardner and coworkers [153] set out to determine the effects of start temperature and cooling rate of slow freezing protocols on blastocyst viability. Using human blastocysts donated for research, they tested freezing with a start temperature of -6˚C and a cooling rate of 0.5˚C/minute compared to a more traditional control rate that starts at 20˚C and cools to -6˚C for seeding and then continues cooling at 0.3˚C/minute. After

thawing, blastocysts frozen with the lower start temperature and slightly faster cooling rate had a thaw survival rate of 75% compared to 15% for the more traditional protocol. Based on these results, they applied the lower start temperature and faster cooling rate clinically, and they were able to attain a 69% thaw-survival, 60% on-going pregnancy rate and 30% implantation rate. The authors concluded that the shorter protocol increased thaw-survival likely by reducing the duration that the blastocysts were exposed to toxic cryoprotectants. Interestingly though, other groups using the traditional longer protocol have reported high thaw-survival and pregnancy rates [2, 3] suggesting other factors may also improve blastocyst cryopreservation.

Recently, several groups have investigated vitrification of blastocysts as a means to improve thaw-survival. Vitrification differs from traditional slow control-rate freezing in that blastocysts are directly plunged into liquid nitrogen causing a rapid decrease in temperature producing a glass-like state without ice-crystal formation [159]. The theory is that if done properly, vitrified embryos are not at risk for injury from ice crystals [160], and therefore should have higher survival rates. Additional advantages of vitrification technology are that expensive control-rate freezers are not needed, the duration of the freezing and thawing procedures are much shorter, and that the same protocol can be applied to all stages of embryonic development, including oocytes [161]. Currently, several groups have reported high rates of survival and pregnancy following vitrification suggesting the technology holds tremendous promise [3, 4]; however, differences in vitrification protocols, including solutions and storage containers, have made the technology difficult to reproduce by inexperienced groups. Complicating the matter is that concentrations of cryoprotectants are much higher than in traditional freezing

protocols requiring laboratory personnel to work within time constraints to avoid toxicity. Eventually, when the protocols become more uniform and laboratory personnel become more experienced, this technology will replace traditional freezing methods. In the meantime, as some of the freezing principals, such as the osmoregulation of water and cryoprotectants, apply to both vitrification and traditional control-rate freezing, it will be interesting to see if other factors that improve control-rate thaw-survival also help improve survival following vitrification.

Several groups have investigated the effects of artificially shrinking the blastocoele cavity prior to freezing, which might reduce the risk for ice crystal formation and potential injury. Three different approaches to artificially shrink the blastocoele cavity have all resulted in improved blastocyst thaw-survival, including puncture with a needle [154, 155, 162], micropipetting [163], and laser [162], These reports, however, were using vitrification technology, and their effects may be limited to that technique. While no study has reported on artificially shrinking of blastocysts frozen by control-rate methods, a study comparing the natural osmotic shrinkage of blastocysts in cryo solutions using control-rate freezing reported a 73% thaw-survival rate from blastocysts that underwent blastocoele collapse compared to a 33% thaw-survival from those that did not collapse [164]. Together these studies suggest that the blastocoele cavity or fluid within negatively effects thaw-survival, but also those blastocysts that are able to undergo osmotic shrinkage are more likely to survive.

Macromolecules are routinely incorporated into freezing and thawing solutions. Proteinaceous macromolecules, such as albumin [165], have been shown to improve thaw-survival of blastocysts. Today, almost all embryo cryopreservation solutions

contain twice the amount of albumin than found in standard culture medium, as doubling the concentration of albumin increases thaw-survival [165, 166]. Another macromolecule, hyaluronan, which is known for its promoting effects on development and implantation of fresh embryos [133, 167], has also been shown to increase blastocyst thaw-survival. Several studies have demonstrated enhanced thaw-survival and development of blastocysts for embryos cultured with hyaluronan [167, 168]. In addition to embryo culture, Palasz and coworkers [157] found that replacement of newborn calf serum with hyaluronan in freezing medium resulted in similar high rates of thaw-survival of bovine blastocysts. While studies such as these demonstrate benefits of macromolecules in cryopreservation, the mechanism by which these compounds affect thaw-survival is unknown. Interestingly, non-proteinaceous macromolecules, such as dextran and ficol, have also been shown to improve thaw-survival [156] suggesting the mechanism may be physical rather than biological.

Although not directly tested on thaw-survival, protein supplements rich in globulin macromolecules have been incorporated into many of the most successful cryopreservation protocols, including slow control-rate [2] and vitrification [4] of blastocysts. Pool and Martin [5] suggested that globulins mediate their effects by interacting with water molecules thereby changing the microenvironment surrounding the embryo. As such, it is conceivable that macromolecules alter the physiochemical environment during freezing and thawing and by doing so improve the embryo's ability to handle osmoregulatory stress. This action could reduce the risk for ice-crystal formation during freezing and also facilitate rehydration and removal of cryoprotectants during thawing. As reduced risk to ice crystal formation could improve thaw-survival,

future studies are needed to determine the effects of globulin-rich sources on thaw-survival.

Preimplantation Genetic Diagnosis

Preimplantation genetic diagnosis (PGD) is a powerful tool used to test individual embryos for genetic abnormalities. This technology is more favorable than prenatal testing as embryos are screened prior to transfer to the uterus avoiding potential miscarriages and termination of affected pregnancies. The first demonstration of preimplantation genetic testing occurred in 1968 with the sexing of rabbit embryos [169]. Many years later, after the development of clinical IVF and polymerase chain reaction (PCR), Handyside and coworkers [170] applied sex selection to human embryos from couples at risk for transmitting an X-linked disorder. Two years later, the same group applied the technique to test for cystic fibrosis (CF)[171]. Since then, preimplantation embryos have been tested for hundreds of monogenic disorders, structural and numerical chromosomal defects, mitochondrial diseases [172], and human leukocyte antigen (HLA) matching [173]. This review will cover historical and current concepts of PGD with emphasis on testing embryos for structural and chromosomal defects.

Preimplantation testing begins in the IVF laboratory with the biopsy. There are three potential sources of genetic material for testing and each has its own set of advantages and disadvantages. The earliest source of genetic material can be obtained by biopsy of the preconception first and post-conception second polar bodies. As meiotic products of oogenesis, the contents of the polar bodies can be used to indirectly make inferences about the genetic composition of the oocyte [174]. The advantage of this method is that the risk to the mature oocyte or the fertilized zygote is low because genetic material is not removed from either source. The downside is that only the

maternal genetic contribution to the embryo can be analyzed. As such, this method of biopsy is rarely used.

Another source for biopsy is blastomeres from day 3 cleavage stage embryos. Embryos at this stage are generally composed of 6-8 blastomeres. In theory, each blastomere should be representative of the genetic composition of the whole; although in practice this is not always true do to the inherit mosaic nature of embryos (discussed in more detail later) [175]. The procedures for removal of a single blastomere are simple, and the potential risk of injury is small relative to the genetic benefit. As such, removal of a single cell from a cleavage stage embryo is currently the preferred method of biopsy by most programs.

The last potential source of genetic material comes from the trophoblast. Removal of 5-10 cells from the outer layer of the blastocyst, if done properly, is considered to pose little risk to the inner cell mass that will eventually become the embryo proper. The main advantage is that genetic analysis of multiple cells reduces the risk for misdiagnosis. The problems with the procedure are that the biopsy is technically challenging, time constraints for genetic analysis usually require the biopsied blastocysts to be cryopreserved, and there is concern that embryos that autocorrect by allocating abnormal cells to the trophoblast may be misdiagnosed [176]. For most laboratories, these risks currently outweigh the potential benefits. Perhaps in the future, with the development of rapid genetic tests and/or better cryopreservation techniques, this will become the preferred biopsy method.

For traditional testing of monogenic disorders, such as CF, Duchenne muscular dystrophy and Huntington’s disease, the biopsied samples are simply loaded into micro

tubes and shipped overnight to a PGD laboratory for analysis. The single most technically challenging aspect for the PGD laboratory is making a genetic diagnosis from as little as one copy of the genome. PCR has traditionally been used to amplify the genes of interest to detectable levels. Initially, amplification inefficiencies limited the practical use of PCR. One of the most common occurrences was failure of one of the two target alleles to amplify (referred to as allelic dropout; ADO) which may lead to misdiagnosis, especially in dominant disorders. Fortunately, numerous strategies have since been developed to overcome ADO [177]. Today, PCR for monogenic disorders has an accuracy of greater than 99% [178] making this technology invaluable in avoiding the transfer of embryos with potentially life-altering genetic disorders.

Testing of preimplantation embryos for structural and numerical chromosomal abnormalities is traditionally done with fluorescence in situ hybridization (FISH). Prior to FISH, the biopsied cell must be fixed to a glass microscope slide. The fixation process is challenging and has been an area of concern for many years [179]. Complicating the procedure is that blastomeres are most frequently found in the interphase stage of the cell cycle. As such, nuclear chromatin must be carefully fixed to allow hybridization without overlapping or distortion of probe signals. Once fixed, the nuclear chromatin is hybridized with chromosome specific DNA probes labeled with different colored fluorochromes. Currently, only 5-6 colors are distinguishable at any given time, limiting the number of chromosomes that can be analyzed with a single hybridization. This, combined with the analysis of interphase nuclei, complicates preimplantation screening of structural and numerical chromosomal defects.

Couples in which one of the partners carry a balanced translocation are at high risk for the resulting embryos to have structural abnormalities; reportedly 72% for Robertsonian translocations and 82% for reciprocal translocations [180]. Since the majority of these structural abnormalities result in miscarriage, there is a strong need to screen individual embryos prior to transfer to the uterus. Fortunately, FISH with as few as three probes can be used to screen against structural abnormalities associated with most reciprocal and Robertsonian translocations [181], regardless of whether the biopsied cell is in interphase. The downside to interphase FISH for structural abnormalities is that the method cannot distinguish between a normal and balanced chromosome complement [182]. In addition, hybridization failure of a single probe may result in misdiagnosis and the transfer of a potentially lethal embryo. The only way to overcome such limitations with FISH would require analysis of condensed chromosomes enabling detection of the breakpoints.

The frequency of numerical chromosome abnormalities in preimplantation embryos has been reported by multiple studies to range from 50-70% [6, 12-19]. However, the frequency of aneuploidy determined by prenatal analysis is not nearly as high. One possible explanation for this discrepancy is that the majority of preimplantation embryos are aneuploid and developmentally arrest before reaching the prenatal stage. Preimplantation genetic screening (PGS) is a procedure of PGD capable of detecting chromosome copy numbers. Theoretically, the culling of embryos containing numerical chromosomal abnormalities, such as monosomies or trisomies, and the transfer of embryos identified as euploid should improve implantation rates and decrease the frequency of miscarriage. As such, there is a tremendous need for PGS.

Unfortunately, traditional methods of PGS using interphase FISH have a number of drawbacks making its use for improving IVF outcomes debatable.

Among the problems with interphase FISH are the limitations of the number of chromosomes that can be screened. PGS protocols typically screen a maximum of 9 to 12 chromosomes, but require multiple rounds of hybridizations to do so. This is a concern as previous reports have associated multiple rounds of FISH with increased error rates [183]. To limit the risk of error, only the most common chromosomes affected by aneuploidy (22, 16, 21 and 15) [184] as well as those in which trisomies are capable of reaching live birth (13, 18, 21, X and Y) are typically screened with PGS. However, this equates to fewer than half of all chromosomes being screened for potential numerical abnormalities.

An additional problem with PGS is the reportedly high error rate estimated to range from 4 to 50% [6-11]. Most often, the contributing factor affecting error rates is the quality of the fixed chromatin. Problems associated with poorly fixed nuclei include: damaged/lost chromatin, probe hybridization failures and misinterpretation of probe signals [179]. Ultimately, these errors can result in the labeling of a normal embryo as abnormal or labeling of an abnormal embryo as normal. While both outcomes are unacceptable, the later could have profound effects if the embryo is trisomy for 13, 18, or 21 and is transferred back to the uterus. As such, there is an urgent need for the development of alternative approaches for determining numerical abnormalities in preimplantation embryos.

Yet another problem with PGS is mosaicism. Mosaic embryos are those where chromosome number differs between cells within the same embryo. For example, some

blastomeres could be diploid and others aneuploid. This phenomenon is believed to be caused by mitotic errors in comparison to traditional aneuploidy that is thought to be caused by meiotic errors [185]. The frequency of mosaicism is estimated to range from ~25-60% [14, 175, 186, 187], however, these estimates are likely confounded by the error rate of interphase FISH. On the other hand, the error rate of FISH may be overestimated because of mosaicism [188].

Several alternative approaches to traditional PGS have been proposed, including comparative genome hybridization (CGH) [189], induced metaphase FISH [190, 191], and more recently microarray [192]. The application of CGH for analysis of blastomeres was first demonstrated by Wells and coworkers in 1999 [189]. This procedure works by comparison of the test DNA with known reference DNA. Both test and reference DNA samples are labeled with different color fluorochromes and then hybridized to a previously fixed set of metaphase chromosomes on a microscope slide. Computerized imaging systems examine fluorescent intensities of the test DNA and the reference DNA to determine chromosome copy number. The main advantage of CGH over interphase FISH is that the copy number of all chromosomes can be determined. In addition, CGH provides coverage of the entire length of each chromosome, enabling the detection of potential breakpoints in translocations. However, CGH cannot distinguish euploid from ploidy errors, such as haploid, triploid or tetraploid. In addition, traditional CGH is labor intensive and generally takes 3-5 days to yield results [193], requiring the embryos to be frozen which may compromise the overall success. Another limitation is that CGH is not sensitive enough to work with a single cell, requiring whole genome amplification (WGA). Traditional WGA methods, including degenerate oligonucleotide primed PCR

(DOP-PCR) and primer extension preamplification (PEP), were often found to unevenly amplify the genome complicating CGH analysis. Only, recently with a newly developed and more efficient methods for WGA is there renewed interest in CGH [194, 195]; however, even with this improvement the overall length of the procedure remains too long for clinical application of PGD.

Another alternative approach to PGS with interphase FISH is the application of DNA microarray technologies. Recently, CGH-arrays and single nucleotide polymorphism-arrays (SNP-arrays) have been proposed for full chromosome analysis of preimplantation embryos. CGH-array is somewhat similar to traditional CGH, requiring WGA and competitive hybridization of labeled test DNA and reference DNA to thousands of chromosome specific probes spotted on the surface of a slide [196]. Hellani and coworkers [197] recently reported clinical use of CGH-array for PGS-aneuploidy screening. In that study they were able to detect chromosomal abnormalities in 60% of the embryos. Selection and transfer of euploid embryos in 5 out of 6 patients resulted in pregnancy. Despite their success, the WGA was not robust and required the removal of two blastomeres to attain a reproducible result with CGH-array. This is a major concern as the removal of more than one blastomere has previously been suggested to impact embryonic potential [198]. An additional concern is that no study has validated the accuracy single cell CGH-array for aneuploidy testing calling into question its clinical application.

SNP-array methodology is similar to CGH-array, but the test DNA following WGA is hybridized to thousands of SNPs instead of chromosome specific probes. Recently, Treff and coworkers [199] validated single cell 24 chromosome aneuploidy screening

using cell lines with known karyotypes demonstrating a 96% success rate of obtaining a reliable result. In addition, they demonstrated the technique could be applied to single blastomeres from cleavage stage embryos with high rates of concurrence. While that study did not analyze any transferrable embryos, a similar report by the same group used SNP-array to successfully DNA fingerprint and molecular karyotype blastomeres from sibling embryos prior to transfer [200]. Based on the studies to date, SNP-array has perhaps the most potential for not only improving assessment of chromosomal abnormalities, but also for improving IVF laboratory performance through sibling embryo investigations of laboratory culture components. The current downside to SNP-array is that the chips are very expensive. Eventually though, as chips become more competitively priced, the potential benefits will outweigh the costs and make SNP-array a very attractive technology for improving ART.

As the previously mentioned technologies are currently limited for PGS, an older but slightly modified approach to induced metaphase FISH may be beneficial. Previous studies by Verlinsky and Evsikov [190, 201] demonstrated the fusion of a mouse zygote with a blastomere from a cleavage stage human embryo could drive the chromatin in the blastomere into mitosis making it possible to do 24 chromosome analysis. Similarly, Willadsen and coworkers [191] demonstrated a bovine metaphase II oocyte could be fused to a blastomere from cleavage stage human embryo to induce condensation of chromosomes suitable for karyotyping in 74% of blastomeres. This conversion rate is much greater than the 30% reported after overnight culture with cytoskeletal inhibitors, such as colcemid [202]. However, the improved rate of obtaining metaphase-like chromosomes is not high enough to warrant clinical application for PGS. One possible

way to potentially increase this rate may be with the incubation of potent protein phosphatase (PP) inhibitors, such as calyculin-A or okadaic acid, which have been shown to rapidly induce premature chromatin condensation (PCC) in cell lines suitable for cytogenetic analysis [20]. Protocols for PP inhibitors are well established for cell lines and should require little modification for application to blastomeres. If PP inhibitors can be used to obtain high conversion rates of metaphase-like chromosomes suitable for karyotyping, then this technology could replace interphase FISH for PGS or at least until other approaches come to fruition.

Premature Chromosome Condensation

Visualization of chromosomes only occurs during the brief mitotic or meiotic stage of the cell cycle. Through the intermiotic (or interphase) period, which can range up to 40 hrs for cultured animal cells, chromosomes remain diffuse and indistinguishable from the nucleus. This long period makes studying chromosomes difficult as few cells at any given time will contain visible condensed chromatin. Overtime, researchers have developed methods to trap or induce chromatin condensation within cells opening the way for more detailed studies of chromosomal rearrangements and aneuploidies. This review will focus on the use of premature chromosome condensation (PCC) for assessment of chromosomal abnormalities.

Premature condensation of chromatin was first described in the 1960's by several groups that fused cells at different stages of the cell cycle [203-205]. Based on similar observations, Rao and Johnson [206] suggested that advanced entry of a non-mitotic cell into mitosis (M-phase) might have been caused by fusion with a mitotic cell containing some factors that induce chromatin condensation. These observations led them to perform additional experiments in which mitotic cells blocked with colcemid

were fused with cells synchronized to G_1, S, or G_2 phase of the cell cycle [207]. Fusion of M-phase and interphase cells resulted in rapid chromosome condensation within 30 minutes. This induction of condensed chromatin from an interphase cell was termed premature chromosome condensation [207]. From their experiments with fused synchronized cells, they were able to determine that cells from the G_1, S, or G_2 phase, when induced into PCC, resulted in distinct patterns of condensed chromatin.

Prematurely condensed chromosomes (PCCs) from G_1 cells consist of single chromatids indicating the unreplicated state of the genome. Several studies have further analyzed changes in the morphology of PCCs at different points in the G_1 phase [208, 209]. These studies revealed a progressive decondensation of chromatin as cells progressed toward the DNA synthetic period. The PCCs of a cell at the beginning of the G_1 phase are relatively short and well condensed. In comparison, the PCCs from later in G_1 are much longer and more attenuated.

Induction of PCC in an S phase cell results in uneven condensation of chromatin. As chromosomes replicate at different rates, the PCCs of an S phase cell can contain a mixture of single chromatids, double chromatids, or finely “pulverized” chromatin, depending on replication. Whereas single chromatids represent chromosomes still within G_1 that have not begun replication, double chromatids represent chromosomes that have completed replication. PCC of chromosomes in the process of replication appear as pulverized pieces of chromatin. These observations can be explained by the molecular model for DNA synthesis that indicates bi-directional replication occurring at multiple sites across the chromosome. As such, each piece of pulverized chromatin represents a region that was undergoing replication.

PCCs from cells in G_2 phase resemble mitotic chromosomes during prophase. The G_2 PCCs consists of two chromatids that are usually attached to each other, but are slightly longer than prophase chromatids. The extended nature of G_2 PCCs likely represents the conformational state of chromatin at that point in the cell cycle.

Although chromosomes induced by PCC have some resemblance to M-phase chromosomes, there are differences. The major difference between PCCs and M-phase chromosomes is the degree of condensation. PCCs from G_1 and G_2 nuclei are not as condensed as M-phase chromosomes. As such, G banding or C banding G_1 and G_2 PCCs are less intensely stained than metaphase chromosomes. In addition, some G_2 PCCs appear to have extra bands, which only become visually separated in the elongated G_2 PCCs [210].

Over the last couple of decades, the mechanism responsible for chromosome condensation has become clearer with the understanding of factors regulating the onset of mitosis. Central to the initiation of both mitosis and meiosis is maturation-promoting factor (MPF; also referred to as mitosis-promoting factor or M-Phase-promoting factor). MPF is a heterodimeric protein composed of cyclin-dependent kinase $p34^{cdc2}$ and cyclins A and B [211]. Kinase $p34^{cdc2}$ is present in growing cells throughout the cell cycle, but is only activated when bound by cyclin B at G_2/M-phase [212]. In addition, the activation of kinase $p34^{cdc2}$ requires phosphorylation of threonine 161. The kinase activity is directed towards serine and threonine residues in histones [213]. Conversely, in interphase, kinase $p34^{cdc2}$ is phosphorylated on threonine and tyrosine residues resulting in inhibition of kinase activity. At the G_2/M transition, these residues are

dephosphorylated allowing kinase activity to appear, resulting in entry into M-phase [211].

Insight into the mechanism regulating the onset of mitosis has been applied to developing new methods for inducing PCC. Investigations of several chemical compounds, including the protein phosphatase (PP) inhibitors okadaic acid, fostriecin, and calyculin-A, have shown their ability to rapidly induce PCC [214, 215]. While the exact mechanism is unknown, these chemical compounds are believed to facilitate PCC through activation of kinase $p34^{cdc2}$ and inhibition of histone PP [216]. Interestingly, the inhibitory effects are not the same for all PP inhibitors. Whereas okadaic acid, fostriecin, and calyculin-A are all strong inhibitors of PP type 2A (PP2A), okadaic acid and fostriecin are weak inhibitors of PP type 1 (PP1). In contrast, calyculin-A is a strong inhibitor of both PP1 and PP2 and therefore, has been suggested to be a more potent inducer of PCC.

Chemical approaches with PP inhibitors offer several advantages over traditional cytogenetic methods of colcemid block or PCC by cell fusion. Compared to cell fusion methods which are difficult and laborious, chemical methods can be applied directly to cultured cells and induce PCC within a few hours. In addition, PP inhibitors result in much higher rates of PCCs (>20%) compared to traditional M-phase from colcemid block treatments (usually 1-2%) [217]. A number of reports have successfully shown these chemical compounds to rapidly induce PCC suitable for studying chromosomal aberrations [214, 218-221].

One area in particular where PP inhibitors may prove beneficial is for inducing condensed chromosomes suitable for preimplantation genetic screening for numerical

or structural chromosomal abnormalities. The traditional method for PGS uses interphase FISH, which is limited to analysis of 5-6 chromosomes per hybridization and a maximum of 9-12 total chromosomes, therefore making it impossible to perform 24 chromosome analysis. In addition, detailed analysis of chromosome breakpoints is not possible with nuclear chromatin found in interphase cells prohibiting distinction of normal and balanced translocations. In an effort to overcome these limitations of interphase FISH, Evsikov and Verlinsky [201] treated blastomeres from human cleavage stage embryos with okadaic acid; however, blastomere incubation in the presence of okadaic acid resulted in only a 22% conversion rate of condensed chromosomes. In most cases, okadaic treatment had no effect on the interphase nucleus. These results were too low for use in PGS. More potent PP inhibitors, such as calyculin-A, may prove to be more effective and therefore more research is needed to determine the efficacy of calyculin-A for inducing PCC of blastomeres because this treatment may become a suitable alternative to the current methods of PGS using interphase FISH.

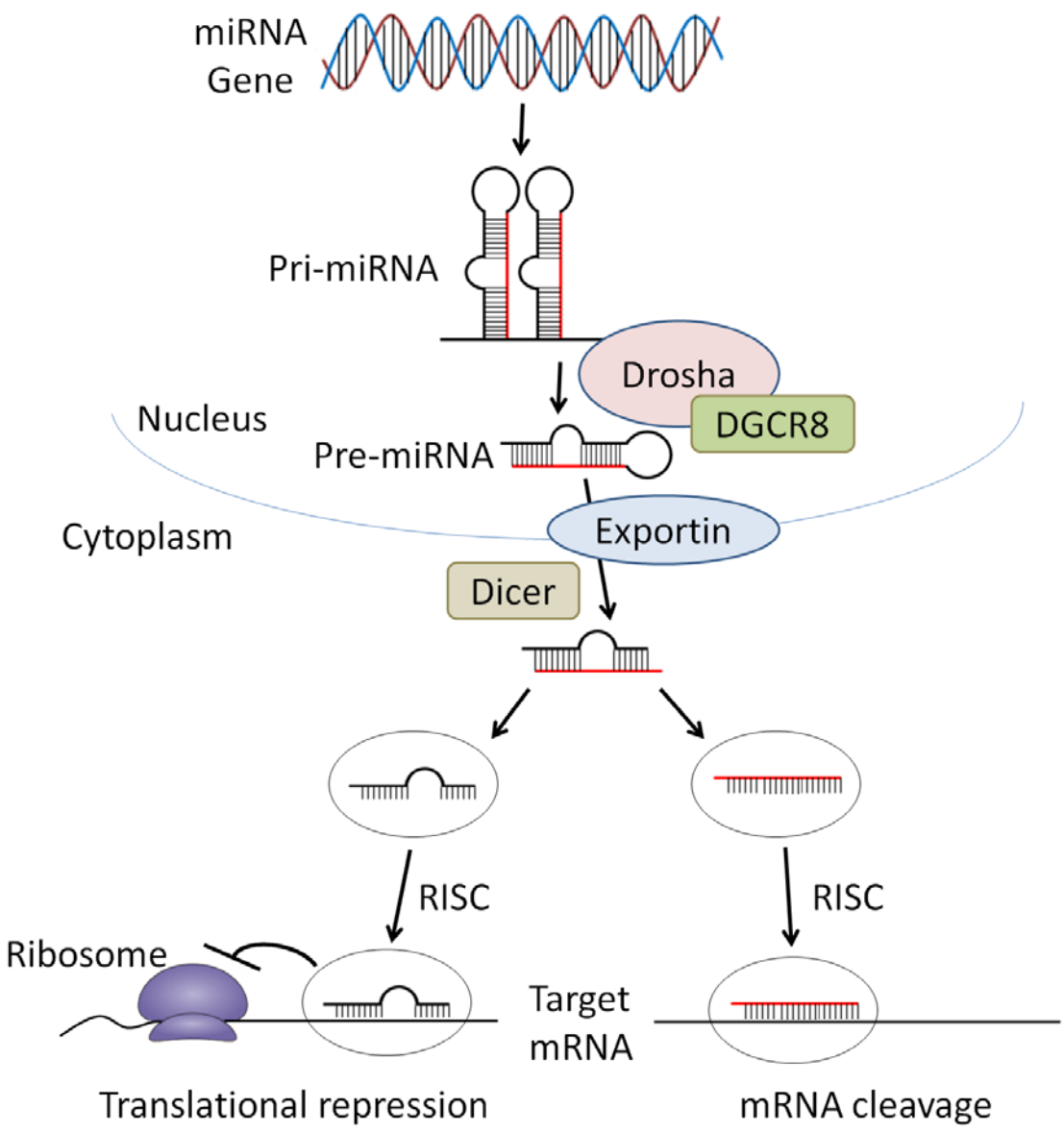

Figure 2-1. Biogenesis and functionality of miRNAs.

CHAPTER 3
COMPARATIVE EFFECTS OF GNRH AGONIST AND ANTAGONIST ON MIRNA AND GENE EXPRESSION PATTERN OF HUMAN GRANULOSA CELLS

Introduction

Gonadotropin-releasing hormone (GnRH) agonists (a) and antagonists (ant) are used in controlled ovarian hyperstimulation (COH) to suppress premature luteinizing hormone surge in patients undergoing in vitro fertilization (IVF). GnRHa acting through GnRH receptor (GnRHR) induces a brief flare of gonadotropins from the pituitary while prolonged administration of a high dose of GnRHa results in suppression of gonadotropins through internalization of available GnRHRs [222]. In contrast, binding of GnRHant to GnRHRs induces an immediate competitive inhibition of GnRHR resulting in a rapid suppression of gonadotropins [223]. Although controversies exist with regard to differences in IVF outcomes, GnRHant use provides some advantages over GnRHa such as lower gonadotropin dose requirements, shorter duration of stimulation [37], absence of flare-induced effects and decreased risk of ovarian hyperstimulation syndrome (OHSS) [40, 41, 47, 224].

Several studies have demonstrated changes in follicular fluid (FF) concentrations of steroids [51, 52] vascular endothelial growth factor (VEGF)[53] and insulin-like growth factor-1 (IGF-I)[52] to account for differences between the action of GnRH analogs. Although a molecular mechanism to explain the differences in the production of these factors is unknown, GnRH analogs have been considered to directly act and differentially regulate their expression through ovarian receptor mediated mechanisms. As such, the expression of GnRH and GnRHRs have been documented in several extrapituitary tissues, including the ovary [54], supporting GnRH local ovarian function. Experiments using cultured granulosa cells have shown GnRH analogs to modulate

steroidogenesis [61] with GnRHant suppression of aromatase expression, the key enzyme responsible for E_2 biosynthesis [62]. Taken together, these observations imply that GnRH analogs can directly act on ovarian cells; however, the extent to which these analogs affect ovarian function remains to be elucidated.

MicroRNAs (miRNAs) are members of a family of small non-protein coding RNAs that function as key regulators of gene expression. Several thousand miRNAs have been identified and/or predicted and are estimated to target the expression of 30% of genes in humans at post-transcriptional/translational levels [225, 226]. Functionally, miRNAs play a central role in various cellular activities, including developmental processes, cell growth, differentiation, apoptosis, cell-cell communication, and inflammatory and immune responses [92-96]. Aberrant expression of specific miRNAs has been associated with developmental abnormalities, disorders of inflammatory and immune response, infection and tumorigenesis. Furthermore, conditional inactivation of Dicer, a poll II enzyme necessary for miRNA processing, has been shown to impact ovarian, oviductal and uterine development through altered expression of many miRNAs causing female sterility [106, 227, 228]. Additional studies have also implicated the importance of Dicer and regulatory function of miRNAs in overall gene expression necessary for early embryonic development [99].

Recently, the expression profile of many miRNAs have been reported in the pituitary [78], ovary [79, 80], testis [229], uterus [83], and embryo [230] suggesting miRNAs may play a role in reproductive function. With regards to ovarian miRNAs, a few reports have demonstrated granulosa cells differentially express miRNAs in response to hormone treatment, including hCG/LH [107] and FSH [108]. In addition,

another report found the expression of miRNAs to differentially affect the release of ovarian steroids P4, T, and E_2 [110]. Studies like these imply hormonal stimulation may regulate the expression of miRNAs which in turn may affect the expression of several genes important for follicular development, including the final maturation of oocytes, luteal formation, and steroid synthesis.

To further our understanding of hormonal regulation of miRNAs, the present study assessed the expression of a select number of miRNAs in granulosa cells from IVF patients receiving GnRHa and GnRHant protocols. In addition, using an immortalized human granulosa cell line we examined the direct action of GnRHa and GnRHant on the expression of these miRNAs and a number of their predicted target genes. We hypothesize that GnRHa and GnRHant differentially regulate the expression of ovarian miRNAs which may affect the expression of their predicted target genes to influence ovarian function.

Materials and Methods

This study utilized human granulosa cells (hGC) from clinical IVF patients as well as an immortalized human granulosa cell line (IhGC). The collection and use of hGC was approved by the University of Florida institutional review board. All patients involved in the study signed informed consent for collection and use of hGC to study gene regulation.

Experiments

Six women, aged between 26 and 35, underwent COH with either a GnRHa (standard ‘long-leuprolide acetate’ suppression; n=3) or GnRHant (ganirelix acetate flexible protocol; n=3) protocol for IVF cycles with indications of male factor, tubal factor or oocyte donation. All patients were pre-treated with combined birth control pills. None

of the patients were poor responders and all IVF patients and all anonymous oocyte recipients from these cycles ended up with ongoing pregnancies. Hormonal profiles were comparable in patients treated with GnRHa or GnRHant protocols. Follicles were aspirated 35 hours after hCG administration for final oocyte maturation. After recovery of oocytes in the IVF laboratory, the remaining FF from each patient was collected and processed through repeated centrifugation at 400g and washing with phosphate buffered saline (PBS) to isolate hGC. The cell pellets containing granulosa cells were kept at -80°C until utilization for RNA extraction.

Cell culture experiments were performed using a well characterized human granulosa cell line previously immortalized with SV40 large T antigen and was reported to express functional GnRHR [231]. These cells were kindly provided by Dr. Leung from the University of British Columbia. Cells were cultured in DMEM:F12 (1:1, Invitrogen, Carlsbad, CA), 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) antibiotic at 37˚C in a humidified atmosphere of 5% CO_2 in air. Culture medium was changed every 48 hours. Once cells reached 70% confluence, they were washed once with medium and then serum starved for 24 hours before initiating GnRH analog treatment. Cells were treated with either 0.1 µM GnRH agonist (leuprolide acetate, Sigma-Aldrich Corp., St. Louis, MO), 0.1 µM GnRH antagonist (cetrorelix acetate, Cetrotide; Serono, Geneva, Switzerland), or vehicle control for 24 hours. Each treatment was performed in triplicate.

RNA Extraction, cDNA Synthesis and Real-Time PCR

Total RNA was isolated from granulosa cells using TRIzol reagent (Invitrogen) and then quantified using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE). All the reagents for cDNA synthesis and real-time PCR, including primers and probes were purchased from Applied Biosystems (Foster City, CA).

Complimentary DNA was synthesized by reverse transcription from 2 µg of total RNA using random primers for mRNA and specific stem-loop primers for miRNA (Table 3-1). Real-time PCR was carried out as previously described [232] at 95°C for 10 minutes, 95°C for 15 seconds, and 60 °C for one minute for 40 cycles using an Applied Biosystems 7300 Sequence Detection System. MicroRNA and mRNA expression values were analyzed using the comparative C_T (critical threshold) method following transformation and normalization to RUB6 and18S rRNA expression, respectively, with threshold cycle set within the exponential phase of the PCR. Final results were reported as relative expression by setting the expression values of miRNAs and mRNAs at 1 in one of the samples and the expression value of other samples were calculated relative to this control.

Statistical Analysis

All data were analyzed using software SPSS statistics 17.0 (SPSS Inc., Chicago, IL). Means for hGC treatments were compared using two-tailed student *t* test. Means for IhGC were analyzed using analysis of variance with post hoc Tukey's test for multiple comparisons. Results for all comparisons are presented as means ± standard error of the mean (SEM). In addition, correlations between each mRNA and miRNA were analyzed using Pearson's correlations coefficient. Data were considered significantly different if $P < .05$.

Results

The Expression of miRNAs and Their Predicted Target Genes in Granulosa Cells

The regulation by gonadotropins and local actions of aromatase (CYP19A1), amphiregulin (AREG), GATA4, pregnancy associated plasma protein-A (PAPPA) and transforming growth factor (TGF)-β system, including TGFβ1, TGFβ-RII, bone

morphogenetic protein 2 (BMP2) and Smads, have been associated with various ovarian cellular activities, including oogenesis, granulosa and thecal cell growth and differentiation and steroid biosynthesis. Using real-time PCR, we assessed the expression of these genes and miRNAs that are predicted to target their expression, including miR-16, miR-17, miR-23a, miR-23b, miR-26, miR-34a, miR-98, miR-133b, miR-138, miR-145, miR-195, miR-424, miR-496, miR-497, and miR-512-3p (Table 3-1), in hGC from patients undergoing COH with either GnRHa and GnRHant protocol. In addition, the direct action of GnRHa and GnRHant on the expression of these genes and miRNAs was determined in vitro using IhGC. Both hGC and IhGC expressed higher levels of miR-16 as compared to the other miRNAs assayed and undetectable levels of miR-512-3p and low levels of miR-138 (Figure 3-1). Similar analysis indicated higher overall expression of PAPPA and TGFβ1 and lower overall expression of CYP19A1, PAPPA2 and BMP2 in both cell types (data not shown). The level of expression of the other genes varied with the exception of AREG, which was the highest expressed gene in hGC and only moderately expressed in IhGC.

GnRHa and GnRHant Protocols Instigate Differential Effects on hGC miRNA Expression

Comparative analysis revealed that hGC obtained from patients that underwent COH with GnRHant express higher levels of miR-26a, miR-34a, miR-98, mir-133b, miR-195, and miR-497 than in cells obtained from women treated with the GnRHa protocol ($P < .05$) (Figure 3-2). In addition, there was a trend toward higher expression of miR-17-5p, miR-23a, miR-23b, and miR-497 in hGC from patients treated with GnRHant protocol compared to those treated with GnRHa protocol. No significant differences were observed in relative expressions of mRNAs predicted as targets of these miRNAs

in hGC from both GnRHa and GnRHant treated cohorts, although AREG, BMP2, SMAD3, TGFβ1, and TGF-βRII were expressed at lower levels in hGC obtained from patients receiving GnRHant protocol. In addition, there were no significant inverse correlations between the relative expressions of miRNA and predicted target genes. A positive correlation was determined between the expression of miR-195 and miR-497, and PAPPA in hGC ($P < .05$).

Direct Effects of GnRH Analogs on miRNA and Gene Expression

Using a well established immortalized human granulosa cell line (IhGC) we assessed the direct action of GnRH analogs on the expression of miRNAs and predicted target genes. Treatment of IhGC with GnRHant moderately increased the relative level of expression of a number of miRNAs as compared with GnRHa and untreated controls although the comparisons were not statistically significant (Figure 3-3). Similar analysis of predicted target mRNAs indicated significantly lower expression of AREG and CYP19A1 and higher expression of GATA4 in GnRHa treated cells as compared with cells treated with GnRHant or vehicle control ($P < .05$). Meanwhile, the expression patterns of BMP2, SMAD3, TGFβ1, TGFβ-RII, PAPPA and PAPPA2 were not significantly different between treatments or controls. No significant correlations were determined from miRNA and predicted target genes in IhGC.

Discussion

In the present study, we selected and analyzed the expression and regulation of a number of miRNAs and their predicted target genes by GnRHa and GnRHant in hGC obtained from IVF patients as well as in IhGC. We found that both hGC and IhGC express miR-16, miR-17, miR-23a, miR-23b, miR-26, miR-34a, miR-98, mir-133b, miR-145, miR-195, miR-424, miR-496, and miR-497 at varying levels. These miRNAs are

predicted to target many genes, including CYP19A1, AREG, GATA4, PAPPAs, TGFβ1 and TGFβ-RII, BMP-2 and Smads. Gonadotropins, through a receptor-mediated action, regulate the expression of these and many other locally expressed genes. The products of these genes modulate various ovarian cellular activities, including oogenesis, granulosa and thecal cell growth and differentiation and steroidogenesis, and alteration in their expression has been associated with disruption in follicular development negatively impacting oocyte maturation and subsequent embryo development.

Accumulated evidence supports a central role for miRNAs in gene expression stability by acting through mainly translational repression or mRNA degradation [90, 233, 234]. Among the 10 miRNAs analyzed, miR-16 was the most abundant, while miR-512-3p and miR-138 were either not expressed or expressed at very low levels in both hGC and IhGC, without any difference in their expression as a result of GnRH analog treatments. In contrast, miR-26a, miR-34a, miR-98, miR-133b, miR-195, and miR-497 were expressed at significantly higher levels in hGC from patients receiving GnRHant compared to GnRHa. These results suggest that granulosa cells express varying levels of miRNAs, and GnRH analogs may differentially affect their expression, although in IhGC these effects were limited and not significantly different from untreated controls. Whereas it remains unclear whether the observed differences in miRNAs and mRNAs expression in hGC is due to direct action of GnRH analogs, a recent in vitro study has demonstrated that GnRH analogs have differential direct effects on AMH, aromatase, SF-1 and LRH-1 expression in human primary granulosa cells in culture and in HGL5 granulosa cell line [62].

We also selected and confirmed the expression of CYP19A1, AREG, BMP2, SMAD3, TGFβ1, TGFβ2R, GATA4, PAPPA and PAPPA2 which are among the predicted targets of the tested miRNAs expressed in hGC and IhGC. Although, co-expression of these miRNAs and their target genes imply a possible regulatory interaction, we found no inverse correlations between their relative expressions; however, there was a positive correlation between the expression of miR-195 and miR-497, and PAPPA in hGC. Since miRNAs regulatory action on gene expression may occur mainly at translational rather than transcriptional level, further studies are required to confirm these observations. In addition, lack of correlations between the expression of miRNAs and their target genes was not surprising since computational algorithms rather than experimental validation was used for selecting these predicted target genes. As such, the seed sequence of these miRNAs can potentially target many other genes not tested in the current study.

Of the selected genes analyzed in this study, the expression of amphiregulin (AREG) mRNA was differential affected in response to GnRH analog treatment in cultured cells. AREG is a member of the epidermal-like growth factor (EGF-like) family, which has been shown using both in vitro as well as animal systems to play a central role in steroidogenesis, cumulus cell expansion and oocyte maturation, probably as mediators of LH action in the ovulatory follicle [235-237]. EGF-like ligands have been shown to accumulate in FF obtained from IVF patients, with concentrations of amphiregulin up to 3,000 times greater than that in the serum [238]. The source of FF AREG is most likely from granulosa cells in which AREG mRNA expression increases in response to LH surge [236]. In the present study, we observed differential expression of

AREG in IhGC with GnRH analog treatment showing suppressed expression in GnRHa treated cells. This finding suggests that GnRH can likely modulate AREG expression in granulosa cells as well. Whether regulation of AREG expression by GnRH is mediated through LH/hCG receptor cross-talk is unknown; however, GnRH has also been shown to induce cyclooxygenase-2 expression in rat granulosa cells similar to LH [239] and granulosa cell AREG mRNA expression increases in response to PGE2 [240] indicating multiple mechanisms may regulate AREG. The molecular pathways of granulosa cell expression of AREG are beyond the scope of the current study, however, our observations warrant future investigations into possible role of GnRH and its analogs in expression of AREG.

In the immortalized granulosa cell line used in this study, we could not replicate some of the findings reported by Winkler and coworkers [62] using cultured human granulosa-lutein cells and a HGL5 granulosa-lutein cell line. We observed a significant suppression of CYP19A1 mRNA expression by GnRHa treatment while GnRHant treatment did not show any significant effects. On the contrary, in HGL5 cell line, Winkler and coworkers reported suppression of both basal and cAMP stimulated mRNA expression of CYP19A1, the gonadal promoter of aromatase [62]. We used the same GnRH antagonist, cetrorelix, at an intermediate dose of 0.1 µM rather than the doses of 1 nM or 1 µM. We consider this finding as another supporting evidence that the effects of GnRH agonists and antagonists can differ in different cell lines, in different cell types and in different culture conditions [241]. Furthermore, we noted that GnRHa increased expression of GATA4 mRNA expression, a transcription factor interacting with the promoter regions of many genes including AMH, steroidogenic factor (SF)-1, CYP19A1

and many other enzymes and factors involved in steroidogenesis and reproductive system development and function [242, 243]. Hence, while GnRHa suppressed mRNA expression of CYP19A1, it induced GATA-4 mRNA expression, which is a known positive regulator of CYP19A1 expression. The observed diversity in GnRH analog effects in different cell types have been thought to be related to treatment conditions (dose and duration), different Gα subtypes or other G protein subunit components, GnRHR expression level, ligand specificity, GnRHR splice variants, intrinsic cellular properties and lastly cross-talk between different receptor systems [243, 244]. As such our findings with different action of GnRHa and GnRHant on miRNAs and mRNAs expression in granulosa cells may involve one or all the above pathways whose identity requires detailed investigation [245].

The results of our study in human granulosa cells suggest that GnRH analogs differentially regulate the expression of miRNAs and some of their target genes involved with steroidogenesis and follicle development. Because miRNAs are estimated to regulate the expression of over 30% of genes [246], any change in ovarian cellular expression of a given miRNA could have a profound effect on the expression of many genes, including specific genes whose products are known to influence granulosa cell function. However, detailed investigation is required to identify the specific genes targeted by these miRNAs allowing association of miRNAs biological function in human granulosa cells. In addition, further studies using different GnRH antagonist preparations is warranted in order to identify their specific and common miRNA and genes targets and mechanism that regulate their expression.

Table 3-1. Predicted miRNA gene targets.

MiRNA	Mature sequence	Predicted gene targets
hsa-miR-17	caaagugcuuacagugcagguag	TGFBR2, BMPR2, MMP2, ADAM9, MMP24, IGF2BP1, EREG, SMAD6, FGF4, BMP2, VEGFA
hsa-miR-23a	aucacauugccagggauuucc	TGFBR2, FGF2, CASP7, TGFA, PGR,
hsa-miR-23b	aucacauugccagggauuacc	PTGFR, SMAD5
hsa-miR-26a	uucaaguaauccaggauaggcu	PTEN, SMAD1, INHBB, IGF1, GATA4
hsa-miR-34a	uggcagugucuuagcugguugu	BMP3, FGF23, AREG, CASP2, PDCD4, SMAD4, INHBB,
hsa-miR-98	ugagguaguaaguuguauuguu	IGF2BP1, TGFBR1, IGF1R, GDF6, IGF2BP3, CASP3, IGF2BP2, FGF11, GHR, INSR, IGF1, SMAD2, MMP11, CYP19A1
hsa-miR-133b	uuuggucccuucaaccagcua	FGF1, EGFR, MMP14, MMP15, INSR, TGFBR1, IGF1R, TGFB1
hsa-miR-138	agcuggugugugaaucaggccg	CASP3, PAPPA, IGF2BP2, AMH
hsa-miR-145	guccaguuuucccaggaaucccu	PTGFR, SMAD3, SMAD5, FGF5, ACTB, TGFBR2, SMAD4, BMP3, ESR1, IGF1R, INHBB, SP1, CASP10
hsa-miR-496	ugaguauuacaugccaaucuc	PAPPA, MMP10, TGFBR2
hsa-miR-512-3p	aagugcugucauagcugaggc	SMAD2, FGF7, ESR1, PAPPA2
hsa-miR-16	uagcagcacguaaauauuggcg	PAPPA, FGF7, FGFR1, SMAD3, SMAD5, SMAD7, VEGFA, GHR, TGFbR3, IGF2R, IGF1R, GATA4
hsa-miR-424	cagcagcaauucauguuuugaa	
hsa-miR-195	uagcagcacagaaauauuggc	
hsa-miR-497	cagcagcacacugugguuugu	

Notes: Prediction of miRNA targets based on TargetScanHuman version 5.1. High-lighted bases in mature sequence indicate seed region. Not all predicted targets shown for each miRNA.

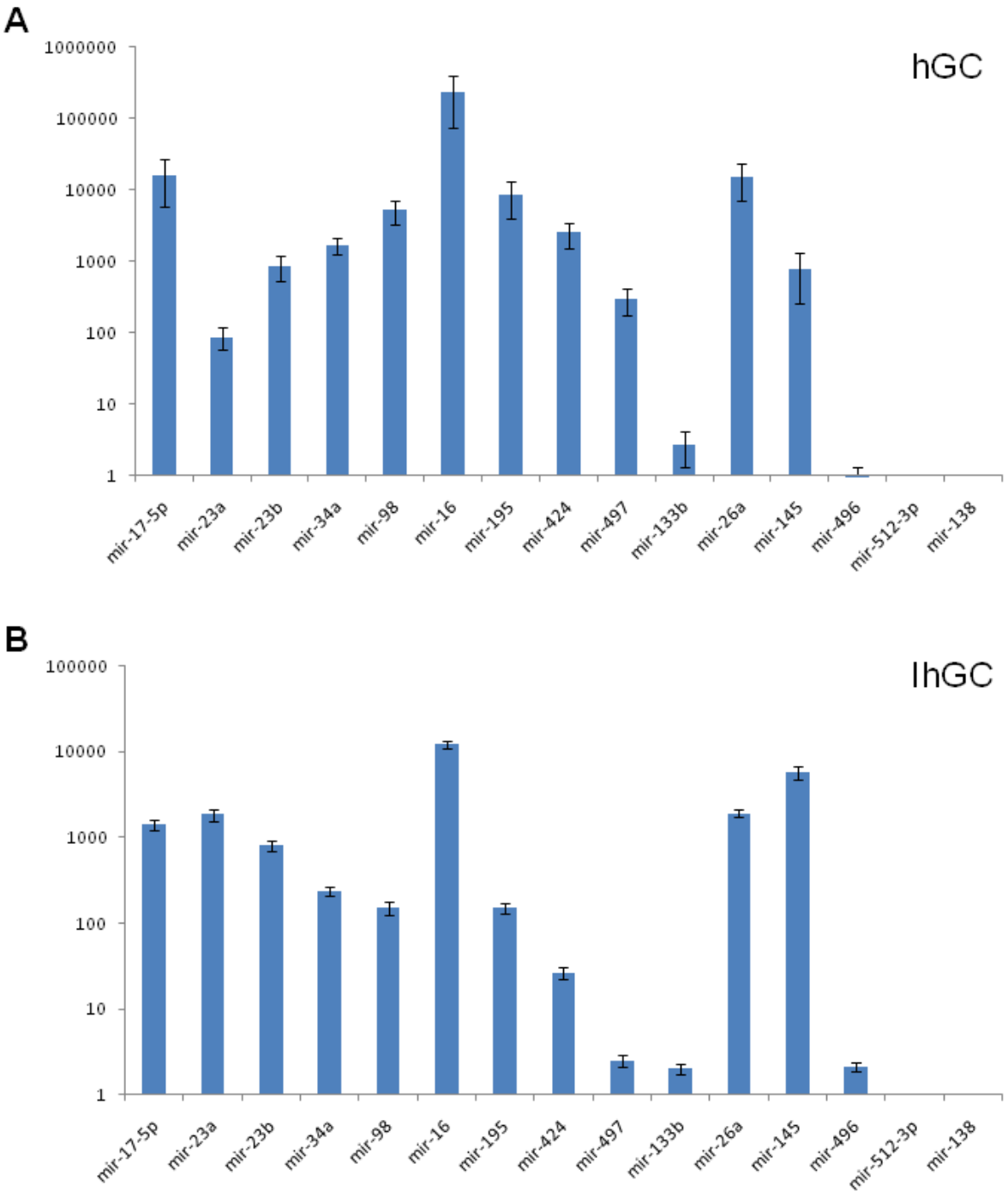

Figure 3-1. Overall expression patterns of miRNA in (A) hGC and (B) IhGC. The y-axis in each graph represents the average expression for each miRNA relative to a calibrator. Mir-133b was chosen as the calibrator for hGC and IhGC following normalization with RU6B.

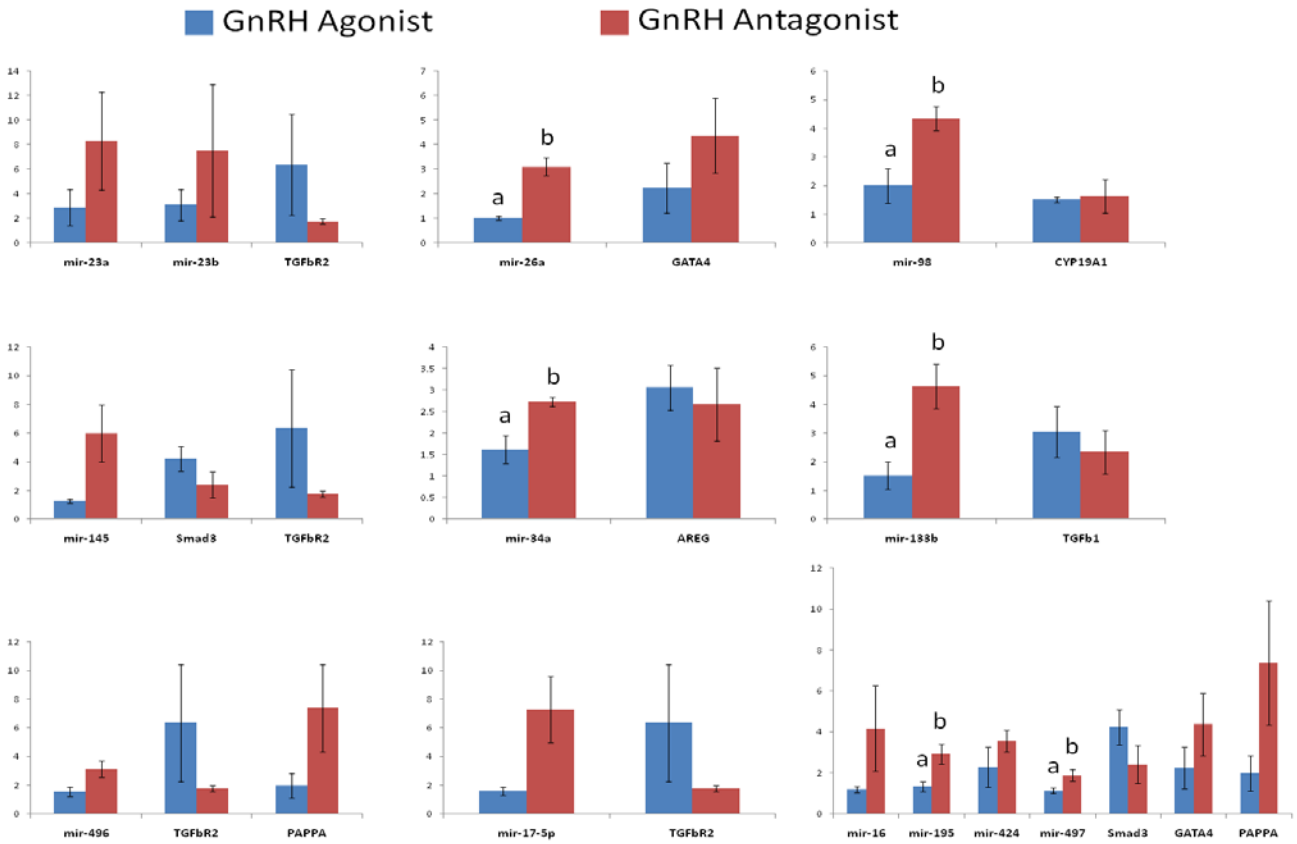

Figure 3-2. Comparative analysis of the relative expression of miRNA and predicted mRNA in hGC. Granulosa cells were recovered from women undergoing COH with either GnRH agonist or GnRH antagonist protocols. The y-axis of each graph represents the expression of miRNAs and predicted targets relative to a selected calibrator. The calibrator for each miRNA and mRNA was chosen based on the sample with the highest ΔC_T following normalization to RU6B and 18S, respectively. Differing letters within miRNA or mRNA indicate significance ($P < .05$).

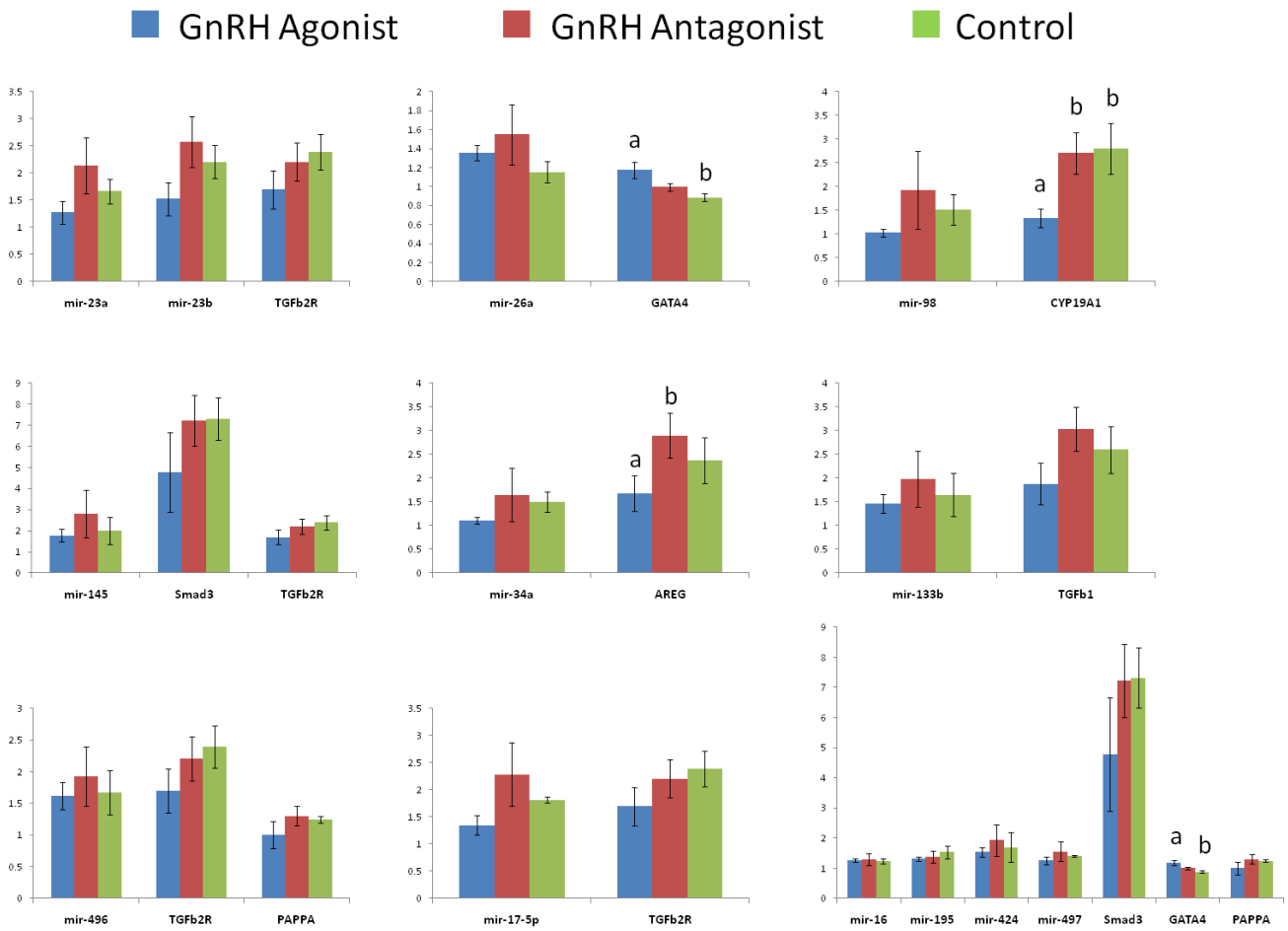

Figure 3-3. Comparative analysis of the relative expression of miRNA and mRNA targets in IhGC. Granulosa cell line was treated with either 0.1 µM GnRH agonist, 0.1 µM GnRH antagonist, or vehicle control. The y-axis of each graph represents the expression of miRNAs and predicted targets relative to a selected calibrator. The calibrator for each miRNA and mRNA was chosen based on the sample with the highest ΔC_T following normalization to RU6B and 18S, respectively. Differing letters within miRNA or mRNA indicate significance ($P < .05$).

CHAPTER 4
SUPPLEMENTATION OF FREEZE AND THAW SOLUTIONS WITH A GLOBULIN-RICH PROTEIN SOURCE IMPROVES POST-THAW SURVIVAL AND IMPLANTATION OF CONTROL-RATE CRYOPRESERVED BLASTOCYSTS

Introduction

For more than two decades, the inclusion of globulin-rich protein sources in culture medium has been suggested to benefit in-vitro development of embryos. Initial studies investigating plasma volume extenders containing a mixture of serum albumin and globulins intended for intravenous use, demonstrated high continuing pregnancy rates if included in embryo culture medium [5, 116]. Furthermore, these studies attributed the success largely to the high fractions of α- and β-globulins. Results from those early studies led to the development of commercial preparations of globulin-rich protein supplements, such as Synthetic Serum Substitute (SSS; now manufactured under name Serum Substitute Supplement, Irvine Scientific)[117] and Serum Protein Substitute (SPS; SAGE In Vitro Fertilization). These serum substitutes are now formulated to provide a more suitable pH range as well as appropriate osmolarity for inclusion in embryo culture media. Numerous human and animal studies have demonstrated that these commercially available protein supplements promote blastocyst hatching [118, 122, 247] and implantation [119, 120, 248]. While a mechanism for the interactions of globulins with embryos remains to be established, Pool and Martin [5] suggested that the polyhydroxy nature of globulins may influence their physiochemical interactions with water molecules within the culture environment. These interactions may enhance developing embryo viability through physical mechanisms involving altered water dynamics. As such, these interactions may also

benefit embryo cryopreservation, where osmoregulation of the embryo is essential for post-thaw re-expansion and survival.

Modern trends seeking to limit the number of freshly transferred embryos along with general improvements in culture conditions often result in embryos remaining untransferred so as to place emphasis on successful blastocyst cryopreservation. Previous attempts to improve blastocyst cryopreservation have focused on various components, such as type of cryoprotectant [249, 250], altering start temperatures and rate of slow-freezing [153], utilization of straws verse cryo-vials [251], inclusion of various macromolecules [156] and more recently vitrification [3]. One of the earlier reports demonstrating high pregnancy rates from frozen-thawed blastocysts was accomplished with freeze and thaw solutions supplemented with a globulin-rich protein source [2], suggesting globulins may promote post-thaw blastocyst survival. Surprisingly, no studies have evaluated the effects of globulins on blastocyst cryopreservation.

In the present study, supplementation of freeze and/or thaw solutions with SSS compared to HSA alone was assessed for control-rate cryopreservation of murine blastocysts in an effort to determine if globulin-rich protein sources offer advantages during cryopreservation. We hypothesize that freeze and thaw solutions supplemented with a globulin-rich protein, such as SSS, will improve thaw-survival, as measured by blastocyst re-expansion and total cell number. In addition, we retrospectively examined human blastocysts thawed and cultured in solutions supplemented with SSS compared to HSA alone to determine if globulin-rich protein supplementation benefited clinical frozen embryo transfer (FET) outcomes.

Materials and Methods

Freezing and Thawing of Blastocysts

Freeze and thaw procedures followed a modified version of Menezo's protocols [143, 252] using kits of commercially available solutions (G-FreezeKit Blast and G-ThawKit Blast; Vitrolife, Englewood, CO). Each freeze and thaw solution was supplemented with protein in the form of HSA (10 mg/ml albumin; Vitrolife) or SSS (10 mg/ml albumin + 2 mg/ml globulins; Irvine Scientific,Santa Ana, CA) according to the experimental design. Unless noted, all procedures were carried out at 37°C. For freezing, blastocysts were exposed for 5 minutes to a MOPS buffered incubation medium containing no cryoprotectant or sucrose. They were then transferred into a solution of 5% glycerol + 0.1 M sucrose for 10 minutes, and then to a solution of 10% glycerol + 0.2 M sucrose for 7 minutes. Blastocysts were loaded into CBS high security straws (Cryo Bio Systems, IMV corp, New York, NY), sealed and transferred into a programmable rate controlled freezer (Cryologic, Victoria, AU). The freezing program initially cooled from 18°C to -7°C at a rate of -2°C/minute. Ice crystallization seeding was performed manually by touching the straw with pre-cooled forceps. The temperature was held for 15 minutes and then further cooled at a rate of -0.3°C/minute to -38°C. Straws were then plunged directly into LN_2 (-196°C) for storage. For thawing, straws were removed from LN_2, held in air at room temperature for 1 minute, and immersed into a water bath for 30 seconds at 37°C. Straws were wiped to remove excess water and then cut to expel the contents into a petri dish containing 10% glycerol + 0.2 M sucrose. Once located, the blastocysts were transferred into a solution of 5% glycerol + 0.1 M sucrose for 5 minutes, then into a solution of 0.1 M sucrose for 5 minutes, and then held in MOPS buffered incubation medium for 5 minutes.

Animal Experimental Design

Frozen two-cell murine embryos (Embryotech, Haverhill, MA) were thawed and cultured in G1v5 PLUS (Vitrolife) containing 5 mg/ml HSA for 72 hours. Only resulting expanded blastocysts, containing a discernable inner cell mass and a well defined layer of trophoblast cells were cryopreserved. Blastocysts remained in liquid nitrogen storage for at least 24 hours but less than 1 week. Following thaw, blastocysts were cultured for 24 hours and then assessed for thaw-survival. Blastocysts exhibiting greater than 50% blastocoele re-expansion were considered to survive.

Experiment 1: Effects of SSS Supplementation of Freeze and Thaw Solutions on Post-Thaw Murine Blastocyst Re-Expansion

Murine blastocysts were randomly distributed to one of four treatments: frozen and thawed in cryopreservation solutions supplemented with HSA (group I), frozen in solutions supplemented with HSA and thawed in solutions supplemented with SSS (group II), frozen in solutions supplemented with SSS and thawed in solutions supplemented with HSA (group III), and frozen and thawed in solutions supplemented with SSS (group IV). Following thaw, each treatment was cultured for 24 hours in G2v5 PLUS (Vitrolife) containing HSA (5 mg/ml).

Experiment 2: Effects of post-thaw culture medium with SSS on re-expansion of murine blastocyst frozen-thawed with different protein supplements

Murine blastocysts were randomly distributed to one of four treatments as described in experiment 1. Following thaw, each treatment was cultured for 24 hours in G2v5 supplemented with SSS (5 mg/ml albumin + 1 mg/ml globulins).

Experiment 3: Effects of protein supplementation in freeze-thaw and post-thaw culture solutions on murine blastocyst re-expansion and total cell number

Murine blastocysts were randomly distributed to freeze-thaw solutions supplemented with HSA followed by 24 hours culture in G2v5 PLUS containing HSA (5 mg/ml) (group I) or freeze and thaw in solutions supplemented with SSS followed by 24 hours culture in G2v5 supplemented with SSS (5 mg/ml albumin + 1 mg/ml globulins) (group II). Total cell numbers were calculated from all blastocysts that re-expanded using a simple fixation and staining procedure. Briefly, individual blastocysts were exposed for 2 minutes in hypotonic solution [1.0% sodium citrate (Sigma-Aldrich, St. Louis, MO) + 3 mg/ml BSA (Sigma-Aldrich)], then transferred into a softening solution [0.01 N HCl (Sigma-Aldrich) + 1% Tween 20 (Sigma-Aldrich)] for 10 seconds and loaded onto a slide containing 10 µl softening solution. As soon as the softening solution evaporated, one drop of fixative solution (methanol:acetic acid; 3:1) was added to fixate nuclei to the slide. The central location on the slide containing the nuclei was encircled with a carbide pen. Coverslips were mounted with 10 µl of vectashield mounting medium with 4',6-diamidino-2-phenylindole (DAPI)(Vector Laboratories, Burlingame, CA) applied to the slide area containing nuclei. The total number of nuclei corresponding with each blastocyst were counted using a Nikon Eclipse E600 (Nikon, Melville, NY) epifluorescent microscope equipped with a 60X dry objective.

Human Retrospective Analysis

A retrospective study was conducted that included 28 patient couples that initiated FET cycles from January 2006 to March 2009 at Shands hospital at the University of Florida, Gainesville, Florida. Only patients who had frozen blastocysts thawed for the purpose of FET were included. No other exclusion criteria were used. All

women were pre-treated by combined oral contraceptive pills followed by down-regulation with daily leuprolide acetate. Following hormonal suppression, confirmed by estradiol concentrations <40 pg/ml and transvaginal ultrasound assessment of ovaries and the endometrial stripe, patients were placed on transdermal estradiol with increasing incremental doses to develop endometrium. Once the endometrial thickness reached 7 mm, progesterone in oil was injected IM to synchronize endometrium for embryo transfer. On day 5 of progesterone administration, blastocysts, originally frozen on developmental day 5 or 6 with at least a grade of 3BB or better using Gardner's scoring system [253], were thawed. One to two blastocysts were transferred to each patient on day 6 of initiation of progesterone treatment regardless of the developmental stage of the blastocyst. Pregnancy was first determined 9 days post transfer on the basis of a serum hCG greater than 25 mIU/ml. Ongoing clinical pregnancy was determined 4 weeks post transfer using transvaginal ultrasound to assess the presence of fetal heartbeat.

We compared two groups of patients. Group I (n=19) included patients who had frozen blastocysts thawed and cultured in solutions supplemented with HSA. Group II (n=9) included patients who had frozen blastocysts thawed and cultured in solutions supplemented with SSS. Thaw-survival was characterized as blastocysts exhibiting fewer than 20% degenerative cells assessed at two hours post-thaw. Blastocyst expansion was assessed at 24 hours post-thaw with only blastocysts exhibiting greater than 50% blastocoele expansion considered to have re-expanded. The institutional review board and ethics committee of the University of Florida approved this study.

Statistical Analysis

All data were analyzed using software SPSS statistics 17.0 (SPSS Inc., Chicago, IL). Chi-Square testing was used to analyze blastocyst re-expansion for animal experimental studies 1, 2, and 3, as well as the clinical retrospective study. Multiple comparisons with Bonferroni adjustments were used to compare individual treatments in animal experimental studies 1 and 2. Student's t-test was used to compare mean total cell numbers for animal experiment 3. Fisher's exact test was used to analyze clinical pregnancy and implantation rates for the retrospective study. Significance was deemed $P<.05$.

Results

Experiment 1: Effects of Supplementing Freeze and/or Thaw Solutions with SSS on Post-Thaw Murine Blastocyst Re-Expansion

Initially 306 murine blastocysts were frozen using freeze solutions supplemented with either HSA (n=152) or SSS (n=154). Table 4-1 shows the distribution of 300 recovered murine blastocysts following thaw. Overall, there was no significant difference in re-expansion from blastocysts frozen in HSA compared to SSS, whereas re-expansion was greater if blastocysts were thawed in solutions supplemented with SSS (85.3%) compared to HSA (64.0%) ($P<.05$). Individually, 89.5% of recovered blastocysts re-expanded from group IV compared to 70.7% and 57.3% in groups I ($P<.05$) and III ($P<0.001$), respectively.

Experiment 2: Effects of post-thaw culture medium with SSS on re-expansion of murine blastocyst frozen-thawed with different protein supplements

In experiment 2, 158 of 160 frozen murine blastocysts were recovered following thaw and were grouped similarly to experiment 1 (Table 4-2). Following 24 hours of

culture in post-thaw medium supplemented with SSS, blastocyst re-expansion was excellent in all groups with no significant differences between treatments.

Experiment 3: Effects of protein supplementation of freeze-thaw and post-thaw culture solutions on murine blastocyst re-expansion and total cell number

Experiment 3 included 111 murine blastocysts frozen, thawed and cultured for 24 hours using cryosolutions and post-thaw culture medium supplemented with HSA or SSS (Figure 4-1 and Table 4-3). A greater percentage of blastocysts re-expanded if frozen, thawed and cultured in solutions supplemented with SSS (85.5%) compared to HSA alone (66.1%; $P<.05$). In addition, the mean total cell number of re-expanded blastocysts, as determined by staining of fixed nuclei with DAPI (Figure 4-2), was moderately greater for SSS group compared to HSA group (80.0 vs 70.5; $P=.057$).

Retrospective Analysis of Frozen-Thawed Human Blastocyst Re-Expansion Using Thaw and Post-Thaw Solutions Supplemented with SSS

The retrospective analysis included 82 blastocysts originally frozen in freeze solutions supplemented with HSA (Table 4-4). Cycles were grouped based on the thaw protocol in use at the time. Between January 2006 and June 2008, 58 embryos were thawed using thaw and post-thaw culture medium supplemented with HSA (group I). Beginning in July 2008 through March 2009, 24 additional blastocysts were thawed, but in these cases using thaw and post-thaw culture medium supplemented with SSS (group II). Blastocysts thawed and cultured in solutions supplemented with SSS had significantly greater survival at 2 hours post-thaw and greater blastocyst re-expansion at 24 hours than blastocysts thawed and cultured in HSA alone (83.3% and 66.7% vs 43.9% and 15.8%, respectively; $P<.01$). In addition, there was a higher clinical pregnancy rate per thaw and higher implantation rate from frozen blastocysts thawed

and cultured in solutions containing SSS compared to HSA (55.6% and 53.8% vs 10.5% and 13.3%; $P<.05$).

Discussion

The aim of this work was to improve current freezing and thawing protocols in order to enhance post-thaw survival and implantation of blastocysts. The present study was designed to test whether inclusion of the commercially available globulin-rich protein supplement SSS in freeze, thaw and post-thaw solutions would increase thaw-survival as compared to HSA. The results indicate that the type of protein supplement provided impacted thaw-survival, as measured by post-thaw re-expansion and total cell number. As several studies have demonstrated post-thaw re-expansion and total cell number are predictors of implantation, the selection of protein type used in freeze, thaw and post-thaw solutions could affect frozen embryo transfer outcomes.

Accumulated evidence supports a role for globular protein supplementation in embryo culture medium. Results from the current study suggest globulin-rich protein sources, such as SSS, may also promote thaw-survival of cryopreserved blastocysts. The results show that blastocysts that were cryopreserved and thawed in freeze-thaw solutions using SSS had significantly higher rates of re-expansion compared to HSA alone. If we consider that cellular mechanisms involved during blastocyst expansion are likely to also be involved in re-expansion of collapsed blastocysts following thaw, then these results are consistent with previous studies demonstrating the positive effects of globulin-rich protein supplements on blastocyst hatching [118, 122, 247]. Interestingly, transitioning from SSS used during freeze to HSA used during thaw negatively affected post-thaw re-expansion. Schneider and Hayslip [122] observed a similar phenomena during extended embryo culture where compact embryos cultured with a mix of HSA

and globulin proteins resulted in poor production of hatched blastocysts when moved to a sequential medium devoid of globulins, suggesting an active role for globulins in stimulation of blastocyst hatching. In addition, the present study found that the culture of thawed blastocysts overnight in SSS promotes excellent blastocyst hatching, regardless of protein type in freeze-thaw solutions. These results imply supplementation of post-thaw culture medium with a globulin-rich protein source, like SSS, may restore the potential for blastocyst re-expansion even if previously frozen and/or thawed with HSA.

Total cell numbers were also analyzed to determine if re-expanded blastocysts from SSS treatments were generally healthier compared to HSA alone. As total blastocyst cell number has been correlated with implantation potential [254], our results suggest that blastocysts cryopreserved and thawed in solutions containing SSS are more robust than blastocysts cryopreserved and thawed in HSA. Although murine blastocysts were not transferred to recipients, similar studies assessing protein supplementation in routine embryo culture found globulins enhanced total cell numbers [255] and implantation [119, 120, 248], further supporting the impression that blastocysts cryopreserved, thawed and cultured in solutions containing SSS are more likely to implant.

Based on the research results using murine embryos, our clinical ART laboratory which previously solely used HSA, incorporated SSS into freeze-thaw protocols as well as post-thaw culture of human blastocysts. Since this change, our program has shown a remarkable improvement in thaw-survival. In addition, as our standard procedure is to culture thawed embryos overnight, we have observed significant improvements in blastocyst re-expansion prior to transfer. These changes have led to significant

increases in pregnancy per thaw and implantation rates. However, as these numbers are small, additional studies are needed to determine whether these trends are clinically significant.

Given the osmotic dynamics imposed on embryos undergoing exposure to high concentrations of cryoprotectants, minor alterations in the physiochemical environment may dramatically enhance an embryo's ability to restore osmotic function. Pool and Martin [5] propose that the high number and orientation of hydroxyl groups (-OH) in polyhydroxy compounds may interact through hydrogen bonding with water molecules affecting physical properties such as water exchange dynamics. The finding presented here that thawed blastocysts are more likely to re-expand if cultured with a protein source containing globulins lends support to the idea that polyhydroxy compounds improve osmotic function. Intriguingly, other polyhydroxy compounds, some of which are currently used in IVF medium, may function better than globulins for cryopreservation. Hyaluronan, which is naturally found in the reproductive tract [131] and has been reported to stimulate in vitro embryo development and improve implantation [133], was previously shown to produce equally high thaw-survival rates in mouse and cow embryos when substituted for newborn calf serum in freezing solutions [157]. While no study (to the author's knowledge) has evaluated the affects of hyaluronan in freeze-thaw solutions of human blastocysts, a recent study comparing blastocyst FET using hyaluronan-rich transfer medium, embryoglue (VitroLife), demonstrated no improvement in implantation as observed with fresh blastocyst transfer [256]. While it remains to be elucidated, Korosec [256] suggested that the freeze-thaw process itself may affect receptor-mediated actions of hyaluronan thereby disrupting

signaling between the endometrium and the embryo. As this could have major implications affecting implantation, further research is needed.

Alternative mechanisms for the thaw-survival promoting effects of globulins are plausible and warrant further investigation. A previous in vitro culture study of murine embryos cultured in medium supplemented with SSS showed reduced presence of reactive oxygen species (ROS) accompanied by lower incidences of apoptosis when compared to embryos cultured in medium supplemented solely with HSA [247]. Our study was limited to observational data, including blastocyst re-expansion and total cell number. We did not analyze post-thaw culture medium for ROS or re-expanded blastocysts for apoptosis and therefore cannot exclude this as a potential mechanism promoting thaw-survival. In addition, globulin proteins within SSS have also been suggested to act as energy substrates or serve as carriers of growth promoting factors. While this is plausible, it is more reasonable that the –OH groups within polyhydroxy compounds, like SSS, influence osmotic tolerance of embryos. In support, a recently made available supplement containing a mixture of HSA and dextran (DSS, Irvine Scientific) is being promoted to be as effective as SSS for in vitro mouse embryo development and more relevantly produce comparable thaw-survival to SSS when used as the protein component in vitrification solutions for all stages of development. As dextran is a nonproteinaceous polymer known to interact strongly with water, future studies incorporating DSS may provide additional support for a mechanism in which polyhydroxyl compounds interact in a physiochemical manner with the embryo and its environment to enhance osmoregulation rather than function as growth promoting factors.

The results of this study suggest that supplementation of freeze, thaw and post-thaw solutions with a protein source containing high fractions of globulins, such as SSS, may benefit thaw-survival. As this study was limited to slow-cool cryopreservation, it would be interesting to see if similar results can be obtained through vitrification. Given that most referenced vitrification solutions are formulated using SSS, it is likely that the success of those solutions may at least in part be due to the presence of globins.

Table 4-1. Effects of protein supplement in freeze-thaw solutions on murine post-thaw re-expansion.

Treatment	Thawed-Recovered	Re-Expanded (%)
Group I: HSA-->HSA	75	53 (70.7)[ab]
Group II: HSA-->SSS	74	60 (81.1)[ac]
Group III: SSS-->HSA	75	43 (57.3)[b]
Group IV: SSS-->SSS	76	68 (89.5)[c]

Results with different superscripts indicate significance. ab vs c $P<0.05$, b vs. ac $P<0.01$, b vs. c $P<0.001$.

Table 4-2. Effects of post-thaw culture medium with SSS on re-expansion of murine blastocyst frozen-thawed with different protein supplements.

Treatment	Thawed-Recovered	Re-Expanded
Group I: HSA-->HSA	38	36 (94.7)
Group II: HSA-->SSS	40	37 (92.5)
Group III: SSS-->HSA	41	37 (90.2)
Group IV: SSS-->SSS	39	37 (94.9)

No significance observed between treatments.

Table 4-3. Effects of protein supplementation of freeze-thaw and post-thaw culture solutions on murine blastocyst re-expansion and total cell number.

Treatment	Thawed	Expanded (%)	Mean Total Cell Count	SEM
Group I: HSA	56	37 (66.1)[a]	70.5[c]	4.03
Group II: SSS	55	47 (85.5)[b]	80.0[c]	2.96

Treatments within columns with different superscripts are significantly different. a vs. b $P<0.05$, c vs. c $P = 0.057$.

Table 4-4. Retrospective analysis of frozen-thawed human blastocyst re-expansion using thaw and post-thaw solutions supplemented with SSS

	Group I	Group II	Statistical Significance
Number of Cycles	19	9	
Number of Embryos Thawed	58	24	
Average Number of Embryos Thawed	3.1	2.7	NS
Number of Embryos Recovered (%)	57/58 (98.3)	24/24 (100.0)	NS
Number of Embryos Survived (%)	25/57 (43.9)	20/24 (83.3)	P<0.01
Number of Embryos Expanded (%)	9/57 (15.8)	16/24 (66.7)	P<0.01
Number of Embryo Transfers (%)	11/19 (57.9)	8/9 (88.9)	NS
Average Number of Embryos Transferred	1.4	1.6	NS
Number of Biochemical Pregnancy	4	5	
Per Thaw (%)	4/19 (21.1)	5/9 (55.6)	NS
Per Transfer (%)	4/11 (36.4)	5/8 (62.5)	NS
Number of Clinical Pregnancy	2	5	
Per Thaw (%)	2/19 (10.5)	5/9 (55.6)	P<0.05
Per Transfer (%)	2/11 (18.2)	5/8 (62.5)	NS
Number of Implants	2	7	
Per Embryo Transferred (%)	2/15 (13.3)	7/13 (53.8)	P<0.05

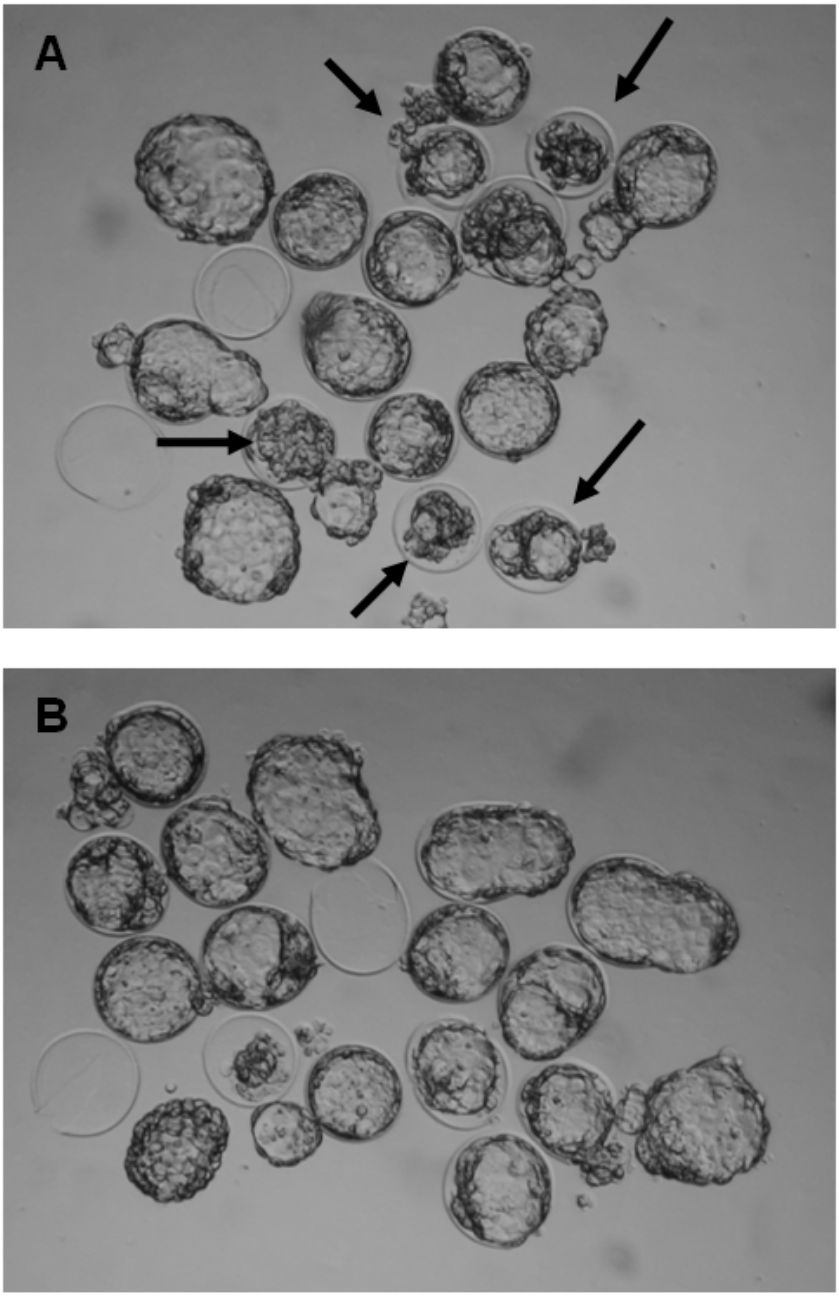

Figure 4-1. Thaw-survival of murine blastocysts following cryopreservation in solutions supplemented with (A) HSA or (B) SSS. Arrows indicate blastocysts that did not meet re-expansion criteria for survival.

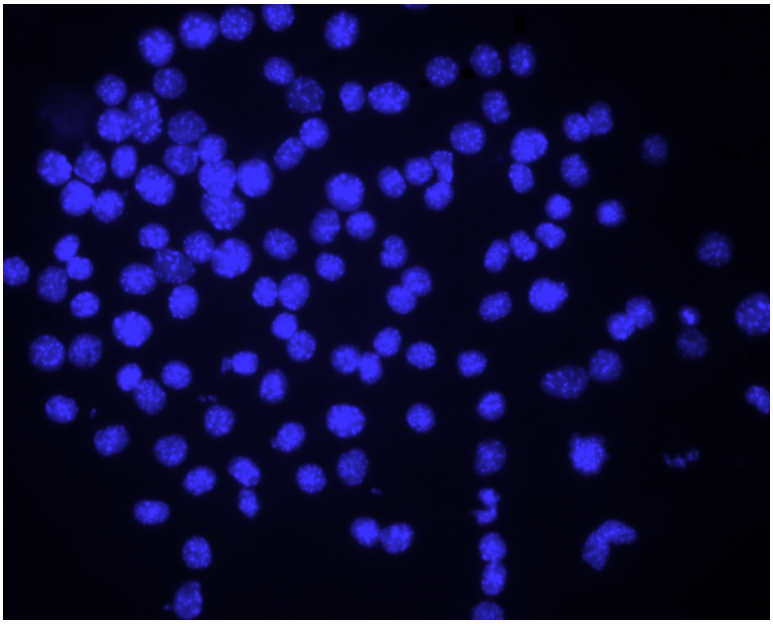

Figure 4-2. Epifluorescence imaging of total cell number from a frozen-thawed murine blastocyst fixed and stained with DAPI. The total number of nuclei corresponding with each blastocyst were counted at 600X total magnification.

CHAPTER 5
CALYCULIN-A IMPROVES CHROMOSOME CONDENSATION FOR CYTOGENETIC ANALYSIS OF BLASTOMERES FROM BOVINE AND MURINE EIGHT-CELL STAGE EMBRYOS

Introduction

Cytogenetic analysis of preimplantation embryos has traditionally used fluorescent in situ hybridization (FISH) to examine individual chromosome copy number, chromosome rearrangements, or sex determination. Comprehensive and simultaneous assessment of all chromosomes however, cannot be achieved with interphase FISH. Metaphase FISH is an attractive alternative, but is biologically limited since the majority of cells, including blastomeres, spend only a short duration in metaphase. Consequently, this technique requires large numbers of cells to increase the likelihood of obtaining sufficient metaphase spreads for analysis, making this inappropriate for preimplantation embryos. An improved method for obtaining condensed chromosomes therefore, would improve the efficiency of cytogenetic analysis of preimplantation embryos.

Premature chromosome condensation (PCC) has been well documented and in many respects mimics the chromatin changes seen during mitosis, enabling preparation of chromosome spreads from interphase cells in G_1, G_2 and M phases of the cell cycle [207, 209]. Interestingly, the final structure of prematurely condensed chromosomes is influenced by the cell cycle stage, with three basic morphologies corresponding with G_1, S and G_2/M cell cycle phases. Cells in G_1 phase generally exhibit longer chromosomes than traditional metaphase chromosomes, especially when in late G_1 [209]. In contrast, cells in G_2/M appear as doublets, reflecting replicated chromosomes. Cells in S phase

are often described as “pulverized”, as the chromatin appears smeared or fragmented [209]. Therefore, PCC of S phase cells is not suitable for cytogenetic analysis.

Chromatin condensation of blastomeres has been achieved by fusing blastomeres with metaphase stage oocytes, enabling analysis of chromosome aberrations in interphase nuclei [190, 191]. However, the methods used for cell fusion are technically difficult and laborious, and fusion results are often inconsistent. Furthermore, rates of sufficient chromatin condensation remain low. Therefore, an alternative approach is needed to overcome these inadequacies in order to evaluate the early embryo prior to embryo transfer.

Chemical approaches have traditionally used colcemid to depolymerize microtubules and prevent spindle formation, thereby trapping cells at metaphase [257]. This method, however, is often time consuming, as it requires the cell to enter mitosis. Alternatively, protein phosphatase (PP) inhibitors, such as okadaic acid, fostriecin and calyculin-A, have been used successfully to obtain condensed chromatin in lymphocytes sufficient for karyotyping [214, 215]. Protein phosphatases play an essential role in cell cycle regulation including mitosis [see review [258]]. Whereas okadaic acid, fostriecin and calyculin-A are strong inhibitors of PP2A, okadaic acid and fostriecin are weak inhibitors of PP1. In contrast, calyculin-A is a strong inhibitor of both PP1 and PP2 and therefore, has been suggested to be a more potent inducer of PCC.

The aim of the present study was to demonstrate PCC in blastomeres using calyculin-A as an improved method for obtaining condensed chromosomes for cytogenetic analysis of embryos. We tested dose and duration to determine the optimal conditions for using calyculin-A on eight-cell stage bovine and murine embryos, and

also examined whether chromosome spreads induced by calyculin-A could be used in combination with whole chromosome painting for assessment of individual chromosomes and blastomere ploidy.

Materials and Methods

Production of Bovine Embryos

Blastomeres were obtained from in vitro produced bovine embryos that were produced according to a modified procedure of that previously described [259]. Briefly, ovaries were collected from an abattoir and cumulus oocyte complexes (COCs) were recovered by slashing 2–8 mm follicles with a scalpel blade. Cumulus oocyte complexes with at least one layer of intact compact cumulus and evenly granular ooplasm were matured in maturation medium incubated in 5% CO_2 in humidified air at 39°C. After 22 hours maturation (Day 0), COCs were washed in Hepes-Tyrodes Albumin Lactate Pyruvate (TALP) medium and transferred in groups of 30 to wells with 425 µl of pre-equilibrated Synthetic Oviduct Fluid-FERT [SOFF; [260]] overlaid with mineral oil. Frozen–thawed Angus spermatozoa (Southeastern Semen Services, Wellborn, FL, USA) were prepared by Percoll gradient centrifugation, and COCs were fertilized at a concentration of 1×10^6 spermatozoa/ml. Following 18 hours incubation, putative zygotes were stripped of cumulus by vortexing in hyaluronidase (0.3 mg/ml; Sigma, St. Louis, MO, USA), washed in Hepes-TALP, transferred to pre-equilibrated 20 µl drops of KSOM + amino acids (Specialty Media; Phillipsburg, NJ, USA) with BSA (EFAF; 3 mg/ml; Sigma) overlaid with mineral oil (Sigma), and cultured in a humidified environment of 5% O_2, 5% CO_2, and 90% N_2 at 39°C.

Harvesting Bovine Blastomeres

Three days after fertilization (Day 3), developmental stage-appropriate eight-cell embryos were selected and incubated in 0.5% pronase (Sigma) for 2 minutes to dissolve the zona pellucida. Embryos were then transferred to calcium- and magnesium-free phosphate buffered saline (Invitrogen, Gaithersburg, MD, USA) with BSA (fraction V; 3 mg/ml; Sigma) and were pipetted to assist disaggregation into individual blastomeres.

Harvesting Murine Blastomeres

Frozen two-cell mouse embryos (Day 1; Embryotech, Wilmington, MA, USA) were thawed according to manufacturer's protocol and cultured overnight in G-1v3 PLUS medium (Vitrolife, Englewood, CO, USA) in a humidified environment of 5% CO_2 at 37°C. Approximately 16 hours post-thaw, zona pellucida were removed from embryos with acidified Tyrode's solution (Irvine Scientific, Santa Ana, CA, USA). Embryos were then transferred to calcium- and magnesium-free G-PGD biopsy medium (Vitrolife) + human serum albumin (HSA; 5 mg/ml; Vitrolife) and pipetted to mechanically facilitate disaggregation into individual blastomeres.

Induction of Premature Chromosome Condensation

Calyculin-A stock solution (5 µM, Sigma) was prepared in DMSO (Sigma) and stored at −20°C. On the day of blastomere harvest, working solutions of calyculin-A were prepared by diluting the stock solution in KSOM medium for bovine blastomeres or G-1v3 PLUS medium for murine blastomeres. Blastomeres that were intact after disaggregation were randomly allotted to treatments in 500 µl wells (4-well plates, Nalge Nunc International, Rochester, NY, USA) and incubated in a humidified environment of

5% CO_2. Blastomeres were observed every 30 minutes and evaluated for morphological changes, such as cell lysis or blebbing of the plasma membrane.

Experiment 1: effects of calyculin-A duration on bovine blastomeres

The effects of calyculin-A duration on bovine blastomeres were examined by incubating blastomeres in 50 nM calyculin-A for 0 (control), 60, 120, or 180 minutes at 39°C. Calyculin-A concentration of 50 nM was chosen based upon PCC experiments by Kanda et al. [261] using human fibroblast cell lines. Blastomeres from eight-cell stage, Day 3 embryos were randomly distributed across the four treatments.

Experiment 2: effects of calyculin-A concentration on bovine blastomeres

The effect of calyculin-A concentration on bovine blastomeres was examined by incubating blastomeres in 10, 50, or 100 nM calyculin-A or vehicle control (1% DMSO in KSOM) for 120 minutes or in colcemid control (0.1 µg/ml) for 16 hours at 39°C. Blastomeres from eight-cell stage, Day 3 embryos were disaggregated and randomly distributed among the treatments.

Experiment 3: effects of calyculin-A on murine blastomeres

The effects of calyculin-A duration on murine blastomeres were examined by incubating blastomeres in 50 nM calyculin-A for 0 (control), 60, 90, or 120 minutes at 37°C. Blastomeres from eight-cell stage, Day 2 embryos were randomly distributed between the four treatments.

Experiment 4: calyculin-A use for cytogenetic analysis of Day 3, eight-cell stage bovine embryos

The usefulness of calyculin-A for inducing chromosome condensation suitable for cytogenetic analysis of bovine embryos was examined using FISH. Intact Day 3, eight-cell stage bovine embryos were incubated individually in 50 µl drops of 50 nM calyculin-

A overlaid with mineral oil for 120 minutes at 39°C, disaggregated with gentle pipeting, and blastomeres were fixed individually on a slide, while maintaining embryo of origin identity. Fluorescent in situ hybridization was used to determine ploidy and gender of each embryo. Chromosome spreads obtained from adult male bovine fibroblasts treated with either calyculin-A (50 nM, 30 minutes) or colcemid control (0.1 µg/ml for 30 minutes) were used as FISH controls.

An additional group of calyculin-A treated embryos were cultured to Day 7 to determine if the effects of calyculin-A treatment were reversible in embryos. Embryos were treated with calyculin-A (50 nM) for 0 (vehicle control), 30, 60, or 120 minutes at 39°C. Embryos were then washed in KSOM and placed into culture drops as described above. Treatments were evaluated for blastocyst development rates on Day 7.

Fixation of Blastomeres

Fixation of blastomeres was performed as previously described [262]. Briefly, 1 hour prior to fixation, room humidity was recorded using a digital hygrometer (Fisher Scientific), and if necessary a ReliOn ultrasonic humidifier (Wal-mart, Bentonville, AR, USA) was used to increase the level of humidity to 40%. Blastomeres were incubated in hypotonic solution [1% (w/v) sodium citrate and BSA (2 mg/ml; fraction V); Sigma] for 1–3 minutes, washed briefly in softening solution [0.01 N HCl (Sigma) + 0.1% Tween 20; (Sigma)] and individually loaded with minimal volume (~1 µl) onto a clean glass slide. Just prior to completely drying, one drop of fixative solution [3:1 methanol:acetic acid; (Sigma)] was applied adjacent to the blastomere to induce chromosome spreading. If necessary, additional drops of fixative were applied prior to evaporation to remove residual cytoplasm. Throughout the fixation process, the specimen was monitored using an inverted microscope (Nikon Instruments Inc., Melville, NY, USA; model TMS-F) at

100X. The chromatin was encircled with a diamond tip scribe to help mark the location for analysis.

Analysis of Premature Chromosome Condensation

Data were collected on morphological appearance of the blastomeres, PCC rate, and the degree and quality of chromosome condensation for each treatment. The level of condensation and quality of chromosome spreads were determined by 4′,6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI) staining. Slides containing fixed chromatin were mounted under coverslips with Vectashield mounting medium containing DAPI (Vector Laboratories, Burlingame, CA, USA) and visualized at 1000X under oil using a Zeiss Axioplan 2 epifluorescence microscope equipped with an Axiocam MRM digital camera and Axiovision 3.1 software (Carl Zeiss MicroImaging Inc., Thornwood, NY, USA). Fixed blastomeres containing an interphase nucleus were considered not having undergone PCC. Blastomeres that did not contain interphase nuclei, but contained condensed chromatin were considered to have undergone PCC. This group was further subdivided based on the quality of the chromosome condensation. Condensed chromatin containing defined chromosomes (blastomeres in G_1 or G_2/M phase), in which individual chromosomes could be counted or used for chromosome painting were considered suitable for cytogenetic analysis, whereas chromatin that was loosely condensed, or chromatin which was pulverized (blastomeres in S phase) and individual chromosomes could not be well defined were deemed unsuitable for cytogenetic analysis.

Fluorescence In Situ Hybridization Analysis

Chromosome spreads obtained from bovine blastomeres treated with calyculin-A and deemed suitable for cytogenetic analysis were analyzed using FISH with a two-

color X–Y chromosome paint set following a modified version of the manufacturer's protocol (Cambio, United Kingdom). Briefly, slides containing chromosome spreads were washed in 2× SSC (Sigma) for 5 minutes at room temperature and then dehydrated by washing through a series of 70, 85, and 100% ethanol solutions for 1 minute each. After air drying at room temperature, a 10 μl premix of biotin-labeled X and Cy3-labeled Y was applied to the area of the slide containing the chromosomes, coverslipped, and sealed with parafilm. The chromosomes and probes were co-denatured at 75°C for 2 minutes on a dry bath incubator (Fisher Scientific, Pittsburgh, PA, USA), and then the slide was transferred to a prewarmed humidified chamber and incubated in the dark at 37°C overnight. Following hybridization, coverslips were removed and slides were washed at 45°C for 5 minutes each in Coplin jars (Fisher Scientific) containing stringency wash solution [formamide:1X SSC (1:1); (Sigma)], 1X SSC (Sigma), and detergent wash solution [4X SSC (Sigma) + manufacturer supplied detergent DT (Cambio)]. Blocking protein and biotin detection agent (100 μl) were applied to each slide, covered with parafilm, and incubated in a humidified box for 20 minutes at 37°C. Slides were then washed for 10 minutes in detergent wash solution at room temperature and allowed to air dry before applying 20 μl of mountant containing DAPI (Cambio) and a coverslip. Chromosome spreads were visualized at 1000X under oil using a Zeiss Axioplan2 epifluorescence microscope as described above.

Chromosome counting and ploidy assessment were determined by cytogenetic criteria previously described by McCauley and coworkers [263]. Briefly, embryos in which all blastomeres counted contained 2 sets of chromosomes (2*N* = 60 chromosomes) were scored as diploid. Likewise, embryos in which all blastomeres

counted contained one set of chromosomes (1*N* = 30 chromosomes) were scored as haploid, and embryos in which all blastomeres counted contained three or more sets of chromosomes were scored as polyploid. Embryos containing a mixture of haploid, diploid, or polyploid blastomeres were considered mixoploid.

Statistical Analysis

Data for PCC rate and degree and quality of condensation were analyzed by chi-square. Multiple comparisons were made using PROC MULTTEST procedures of SAS with Bonferroni *P*-value adjustments. Significance was deemed $P < 0.05$.

Results

Experiment 1: Effects of Calyculin-A Duration on Bovine Blastomeres

Blastomeres from 16 bovine IVP eight-cell stage embryos were evaluated for calyculin-A duration during 4 repetitions of Experiment 1 (Table 5-1). No morphological differences were observed between blastomeres prior to fixation for each treatment. In addition, no blastomeres lysed during hypotonic treatment. Duration of calyculin-A treatment affected the frequency of blastomeres undergoing PCC, as fewer blastomeres treated for 0 and 60 minutes underwent PCC when compared to 120 and 180 minutes ($P < 0.005$). Blastomeres not responding to calyculin-A treatment contained intact interphase nuclei (Figure 5-1A). The frequency of blastomeres with defined chromosomes varied by treatment, with 120 minutes giving the best results (57%). Non-treated control blastomeres had 6% mitotic cells.

Despite >90% of blastomeres undergoing PCC after 120 minutes, not all blastomeres were suitable for cytogenetic analysis. The effects of calyculin-A were dependent upon cell cycle stage of the blastomeres. Chromatin of blastomeres was sometimes incompletely condensed (Figure 5-1B), whereas blastomeres in S phase

appeared to be chaotically condensed (Figure 5-1D), or pulverized, in which distinct chromosomes could not be differentiated (Figure 5-1E). Chromosomes deemed suitable for analysis were either from blastomeres treated in G_1 or G_2/M phase of the cell cycle (Figure 5-1C and 5-1F).

Experiment 2: Effects of Calyculin-A Concentration on Bovine Blastomeres

Individual blastomeres (*n* = 283) from 35 bovine IVP embryos at the eight-cell stage were used during six repetitions of Experiment 2 to determine optimal concentration (Table 5-2). No morphological differences were detected in blastomeres between each treatment, and no blastomeres lysed during hypotonic treatment. Preliminary experiments testing doses up to 750 nM calyculin-A (data not shown) indicated that when concentrations exceeded 150 nM, blastomeres also underwent cellular blebbing and subsequently ruptured when transferred to hypotonic solution. Concentration of calyculin-A affected the frequency of blastomeres undergoing PCC, resulting in nearly a 3-fold increase over colcemid-treated control blastomeres. Blastomeres treated with 50 or 100 nM calyculin-A had a higher frequency of PCC compared to blastomeres treated with 10 nM calyculin-A ($P < 0.005$). In addition, blastomeres treated with 50 nM calyculin-A had a higher frequency of defined chromosomes deemed suitable for cytogenetic analysis, as compared to blastomeres treated with 10 nM calyculin-A ($P < 0.005$). Blastomeres that underwent PCC, but were deemed unsuitable for cytogenetic analysis had chromatin that appeared chaotically condensed (Figure 5-1D) or pulverized (Figure 5-1E). In comparison, vehicle treated controls had only 3.5% mitotic cells after 2 hours, whereas colcemid treatment captured 32.7% in metaphase after 16 hours.

Experiment 3: Effects of Calyculin-A on Murine Blastomeres

Experiment 3 included 207 blastomeres recovered from 62 Day 2 murine embryos during two repetitions to evaluate optimal dose (Table 5-3). No morphological differences were observed between treatments; however, during the fixation process 17 blastomeres (29%) from the 120 minutes calyculin-A treatment lysed in hypotonic solution and could not be used for analysis. Blastomeres remained intact in all other treatments. Following fixation, blastomeres treated with calyculin-A for 90 and 120 minutes had higher frequencies of PCC (90 and 100%, respectively) than 0 or 60 minutes ($P < 0.05$). This was 13-fold greater than mitotic cells observed in the control group. There were no differences in the degree of chromosome condensation between calyculin-A treatments and averaged 24.7%. Blastomeres undergoing PCC appeared to be similar to bovine blastomeres, exhibiting similar cell cycle effects, with no preference between treatments (Figure 5-2).

Experiment 4: Calyculin-A Use for Preimplantation Cytogenetic Analysis of Day 3, Eight-Cell Stage Bovine Embryos

Chromosome spreads from blastomeres of 11 bovine IVP embryos were assessed by FISH, to determine the usefulness of calyculin-A treatment for cytogenetic analysis and sex determination of embryos (Table 5-4). Seven blastomeres were lost during fixation and those remaining were used for analysis. Overall, 100% of the blastomeres analyzed underwent PCC, and 85% of blastomeres contained defined chromosomes.

Normal, diploid blastomeres were observed in 93% of all blastomeres with defined chromosomes. Four embryos (36%) were considered mixoploid, containing combinations of diploid (Figure 5-3A and B), haploid (Figure 5-3C), and tetraploid

(Figure 5-3D) blastomeres, however, numbers of chromosomally abnormal blastomeres were low in mixoploid embryos (14–22%). Overall, no sex chromosome specific aneuploidies were observed among blastomeres and the ratio of female to male embryos was 6–5 (Table 5-4; Fig. 5-3B and A, respectively).

Male fibroblast control slides were used to validate the FISH assay. Twenty metaphase spreads from each FISH fibroblast control slide were analyzed with greater than 80% detection of both X and Y chromosomes. There were no differences in hybridization efficiencies between blastomeres and control fibroblasts.

Finally, intact bovine embryos treated with calyculin-A at the optimal dose (50 nM) for 30, 60 or 120 minutes failed to resume development when returned to culture in KSOM to Day 7, as compared to vehicle treated control embryos that developed to the blastocyst stage (40%).

Discussion

In this study we tested calyculin-A, an inhibitor of serine/threonine phosphatases, as an improved chemical approach for inducing premature chromosome condensation suitable for cytogenetic analysis of preimplantation bovine and murine embryos. The degree and quality of condensed chromosomes was affected by blastomere cell cycle phase, as well as dose and duration of calyculin-A treatment. In addition, using a whole chromosome paint set along with DAPI-counterstaining, we demonstrated that condensed chromosomes from calyculin-A treated bovine blastomeres were suitable for cytogenetic analysis by FISH.

Multiple studies of IVP embryos have reported chromosomal abnormalities varying between 15 and 80% in domestic species [263-267] and in humans [15, 268-271]; however, the incidence of live born mammals with chromosomal abnormalities

remains lower, suggesting that chromosomal abnormalities contribute to the high incidence of embryonic and fetal losses following embryo transfer. This has led to considerable interest in trying to study chromosomal abnormalities in IVP embryos. To date, most cytogenetic studies of embryos have relied on interphase FISH and therefore, have not provided insight into full karyotypes. Although alternative approaches to trap or induce metaphase chromosomes for more detailed cytogenetic studies of embryos have proven to be more informative than interphase FISH, the techniques involved are laborious, inefficient and time consuming.

Our approach, using calyculin-A to achieve chromosome condensation in blastomeres, was more efficient and required a shorter duration than colcemid, which is traditionally used for cytogenetic studies. The potency of calyculin-A can be attributed to its ability to induce chromosome condensation of cells in G_1, S, and G_2/M of the cell cycle, whereas colcemid is limited to cells that have entered mitosis. This versatility clearly makes calyculin-A advantageous for attaining condensed chromosomes in the shortest duration.

Since the selection or induction of cells in G_1 or G_2/M prior to PCC is not possible with blastomeres, this study evaluated all blastomeres regardless of cell cycle stage. Retrospective analysis of chromosomes showed PCC in blastomeres treated in G_1 and G_2/M were representative of those spreads deemed suitable for cytogenetic analysis. Chromatin from blastomeres in S phase appeared either pulverized or presented interspersed blocks of condensed and non-condensed chromatin, as described by El Achkar and coworkers [272], and were not suitable for cytogenetic analysis.

The fourth cell cycle post-fertilization in the bovine embryo is the longest and is associated with embryonic genome activation. It takes 38–49 h to complete cell divisions in order to develop from the eight-cell stage to the 16-cell stage [273-275], which allows ample time to complete embryo biopsies and blastomere treatments. We demonstrate here that calyculin-A, under optimal conditions, can induce PCC in 100% of bovine blastomeres treated at the eight-cell stage. However, between 15 and 47% are unsuitable for analysis, probably due to cells being treated in the S phase of the cell cycle. This suggests that 53–85% of blastomeres were in G_1/G_2/M phases of the cell cycle.

Extended exposure of blastomeres to calyculin-A had an adverse effect on chromosome condensation. Although calyculin-A treatment of bovine blastomeres for 3 hours had the greatest frequency of PCC, chromosomes were often over-condensed and could not be utilized for further analysis. Interestingly, murine blastomeres showed a similar adverse effect with prolonged exposure to calyculin-A; however, the cutoff time for optimal condensation was shorter than for bovine. This over-condensed chromatin morphology was also reported by Swain and coworkers [276], when murine oocytes were cultured with calyculin-A for an extended period of time (7 hours) and no distinct resolution of chromosomes was achieved. They attributed this effect to hyperphosphorylation of histone H3 on Ser 10 and Ser 28, that may be detrimental to proper chromatin remodeling required for the formation of chromosomes.

Calyculin-A also had adverse effects when blastomeres were exposed to doses of 100 nM or more. Whereas blastomeres treated with 10 nM frequently had incomplete chromatin condensation, resembling early prophase chromosomes, treatment with

100 nM calyculin-A resulted in over-condensed chromatin similar to blastomeres exposed to durations longer than 2 hours. Taken together, we inferred that duration and concentration of calyculin-A must be carefully monitored to achieve optimal chromosome condensation and to avoid deleterious effects, such as over-condensation, which will negatively affect cytogenetic analysis.

Employing optimal conditions in Experiment 4, we demonstrated that blastomeres treated with calyculin-A can be used to screen for chromosome abnormalities. Out of 11 embryos analyzed, 4 (36%) were mixoploid containing haploid or polyploid blastomeres in combination with normal diploid blastomeres; however, only a small percentage of cells within each of these embryos were chromosomally abnormal. Mixoploidy can arise either from the fusion of blastomeres or the failure of cell division following chromosome replication [277, 278]. Although live offspring may be produced from mixoploid embryos if polyploid blastomeres are sequestered within the extraembryonic tissues, Viuff and coworkers [279] suggest that embryos with a large contribution of polyploid cells to the trophoblast lineage could contribute to the incidence of large offspring syndrome. It is also important to note that given the limitations of determining stage of cell cycle of bovine blastomeres, along with the resolution of bovine chromosomes, we cannot ignore the possibility that some of the tetraploid cells observed in embryos 7 and 11 could have been G_2/M cells that underwent premature centromere division (PCD)[218]. Notwithstanding, our results were similar to previous studies of bovine IVP embryos that reported a range of mixoploidy from 12 to 72% [279, 280].

Interestingly, the only sex chromosomal abnormalities we observed were limited to the mixoploid embryos. In addition, we did not observe any sex chromosome aneuploidies, which was consistent with a detailed preimplantation genetic study of human embryos that reported only 1.2% of analyzed embryos had aneuploidies associated with sex chromosomes [184]. Although sex chromosome aneuploidies are low, we chose this as our model, since we have good positive control male cell lines and X–Y paint probes are commercially available. Future efforts can focus on specific chromosomes of interest, but will require developing new chromosome paint probes.

Fixation of chromosomes from bovine blastomeres proved to be challenging. Unlike human and murine blastomeres, bovine blastomeres from in vitro produced embryos have a high lipid content, which can readily be seen upon addition of fixative solution. The lipid droplets tend to associate with chromatin, making it challenging to remove excess lipid without losing chromatin. Future studies therefore, will need to develop fixation methods or improve culture conditions [281] that can overcome the problems associated with this high lipid content in bovine IVP embryos. In addition, future cytogenetic studies of bovine IVP embryos will need to address methods to perform detailed karyotypes. A normal bovine cell contains 60 chromosomes, of which all the autosomes are acrocentric making traditional staining methods, such a G-banding, more difficult. Spectral karyotyping using whole chromosome paint probes could overcome the shortcomings of G-banding, however chromosome painting probes are commercially available only for chromosomes 1, 29, X, and Y.

Murine embryos were also evaluated in this study in an attempt to demonstrate the versatility of calyculin-A for inducing PCC in embryos other than bovine. Although

murine embryos had been cryopreserved at the two-cell stage, they were all thawed and cultured overnight, and all embryos developed to the eight-cell stage. Therefore, we concluded that embryo cryopreservation did not alter results. Interestingly, murine blastomeres were more sensitive to calyculin-A, so dose titrations tested differed from the bovine experiments taking this into account. Although we did achieve high frequencies of blastomeres undergoing PCC similar to bovine, the degree and quality of condensation was often poor and insufficient for further cytogenetic analysis. Likewise, using human blastomeres, Shkumatov and coworkers [282] found calyculin-A produced low rates of well-spread metaphase chromosomes. We inferred that calyculin-A may have species-specific activity.

An alternative possibility is that PCC induced by calyculin-A may be affected by Ca^{2+}-free medium. Interestingly, we obtained higher frequencies of chromosomes suitable for cytogenetic analysis in Experiment 4, in which embryos were not predisposed to Ca^{2+}-free medium prior to calyculin-A treatment. We speculated that these differences could be due to a disruption in intracellular calcium release. To support this hypothesis, studies using cell lines of human fibroblasts and lymphocytes demonstrated that inclusion of a calcium ionophore with calyculin-A improved PCC and quality of chromosome spreads [261, 283].

Another unexpected finding was that treating whole embryos with calyculin-A (Experiment 4) improved PCC over treating individual blastomeres (Experiments 1–3). Methods for preimplantation genetic diagnosis (PGD) routinely involve the isolation of individual blastomeres, whereas the biopsied embryos are returned to culture during the analysis. Eriksson and coworkers [284] previously reported that the effects of calyculin-

A were reversible in BHK-21 fibroblasts. Therefore, as the next step for improving this technique for possible use in PGD, we tested whether calyculin-A effects were reversible when treating whole bovine embryos. Unfortunately, exposure of embryos to calyculin-A for as little as 30 minutes was sufficient to induce irreversible cellular arrest, and treated embryos failed to resume development. Although the highest frequency of PCC is obtained with whole embryo incubation, it is not feasible for use in blastomere biopsy schemes due to its irreversible toxicity in embryos at this dose.

In conclusion, we inferred that calyculin-A can rapidly induce PCC in blastomeres for preimplantation cytogenetic studies of bovine and murine embryos, but the degree of chromosome condensation may not always be suitable for detailed cytogenetic analysis from a single blastomere, depending upon its cell cycle phase.

Table 5-1. Effects of calyculin-A duration on inducing PCC in bovine blastomeres

Duration (minutes)	No. of blastomeres analyzed	No. of blastomeres undergoing PCC (%)	No. of blastomeres suitable for cytogenetic analysis (%)
60	26	8 (31)[a]	3 (12)[a]
120	35	32 (91)[b]	20 (57)[b]
180	34	33 (97)[b]	17 (50)[b]
Non-treated	33	2 (6)[c]	2 (6)[a]

Treatments within columns with different superscript letters differed ($P < 0.01$).

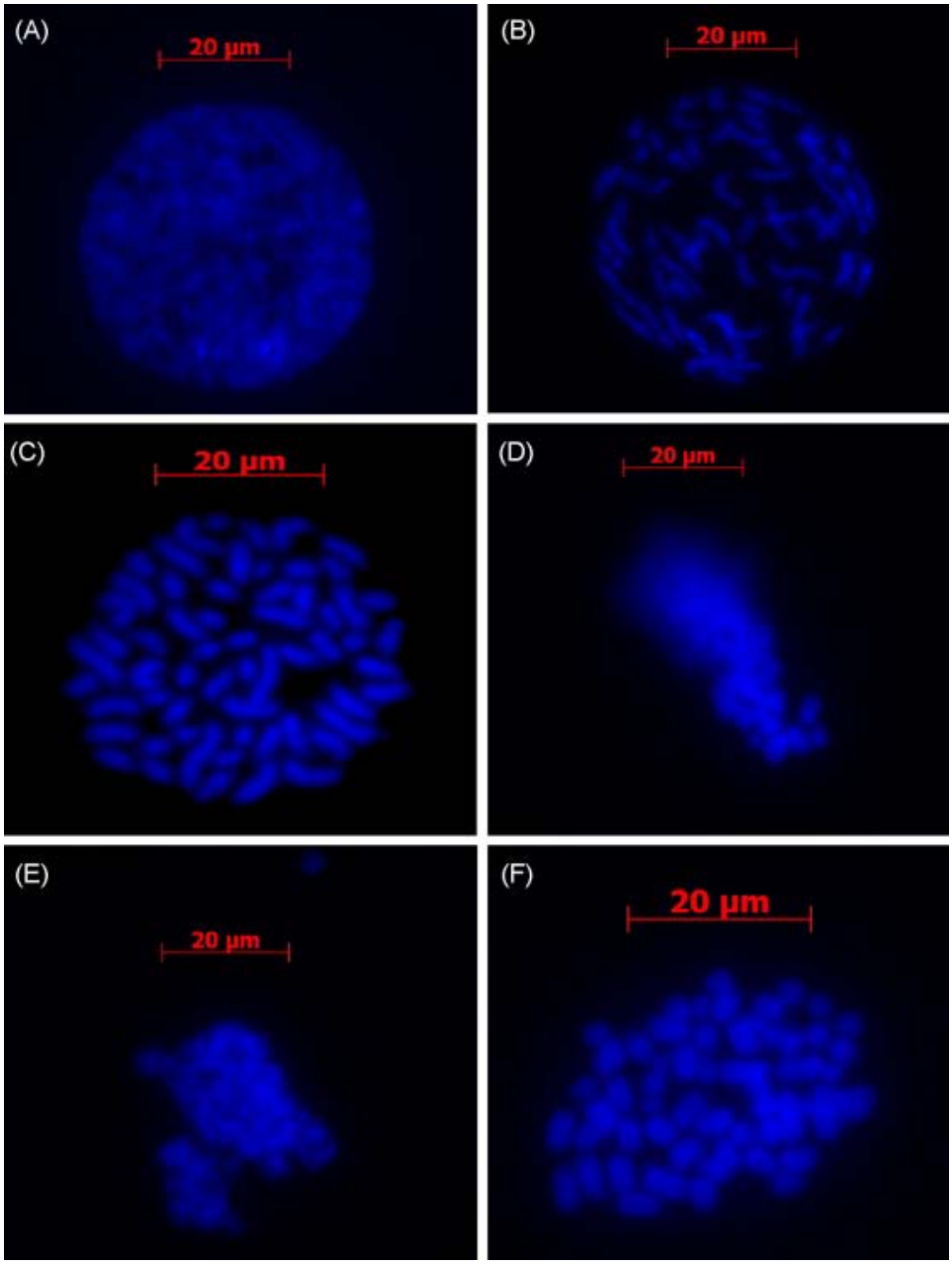

Figure 5-1. Calyculin-A induces premature chromosome condensation (PCC) in blastomeres from Day 3, eight-cell stage bovine IVP embryos (Day 0 = fertilization). The degree of chromatin condensation was determined by DAPI counter-staining and epifluorescence microscopy at 1000×. (A) Interphase nucleus, considered to not have undergone PCC. (B) Loosely condensed bovine chromatin, as is seen in late G_1 phase of the cell cycle. (C) Defined bovine chromosomes, normal G_1. (D) Chaotically condensed bovine chromatin, indicative of a cell treated in S phase of the cell cycle. (E) Pulverized bovine chromatin, indicative of a blastomere treated in S phase of the cell cycle. (F) Defined bovine chromosomes, 60 chromosomes, normal G_2/M.

Table 5-2. Effects of calyculin-A concentration on inducing PCC in bovine blastomeres.

Concentration (nM)	No. of blastomeres analyzed	No. of blastomeres undergoing PCC (%)	No. blastomeres suitable for cytogenetic analysis (%)
10	53	19 (35.8)[a]	12 (22.6)[ab]
50	59	56 (94.9)[b]	31 (52.5)[c]
100	59	58 (98.3)[b]	22 (37.3)[bc]
Control (DMSO)	57	2 (3.5)[c]	2 (3.5)[a]
Control (colcemid)	55	18 (32.7)[a]	18 (32.7)[bc]

Treatments within columns with different superscript letters differed ($P < 0.005$).

Table 5-3. Effects of calyculin-A duration on inducing PCC in murine blastomeres.

Duration (minutes)	Reps	No. of blastomeres analyzed	No. of blastomeres undergoing PCC (%)	No. of blastomeres suitable for cytogenetic analysis (%)
60	1	35	15 (42.9)[a]	7 (20.0)[ab]
90	2	59	53 (89.8)[b]	20 (33.9)[b]
120	1	41	41 (100.0)[b]	8 (19.5)[ab]
Control (non-treated)	2	55	4 (7.3)[c]	4 (7.3)[a]

Treatments within columns with different superscript letters differed ($P<0.05$).

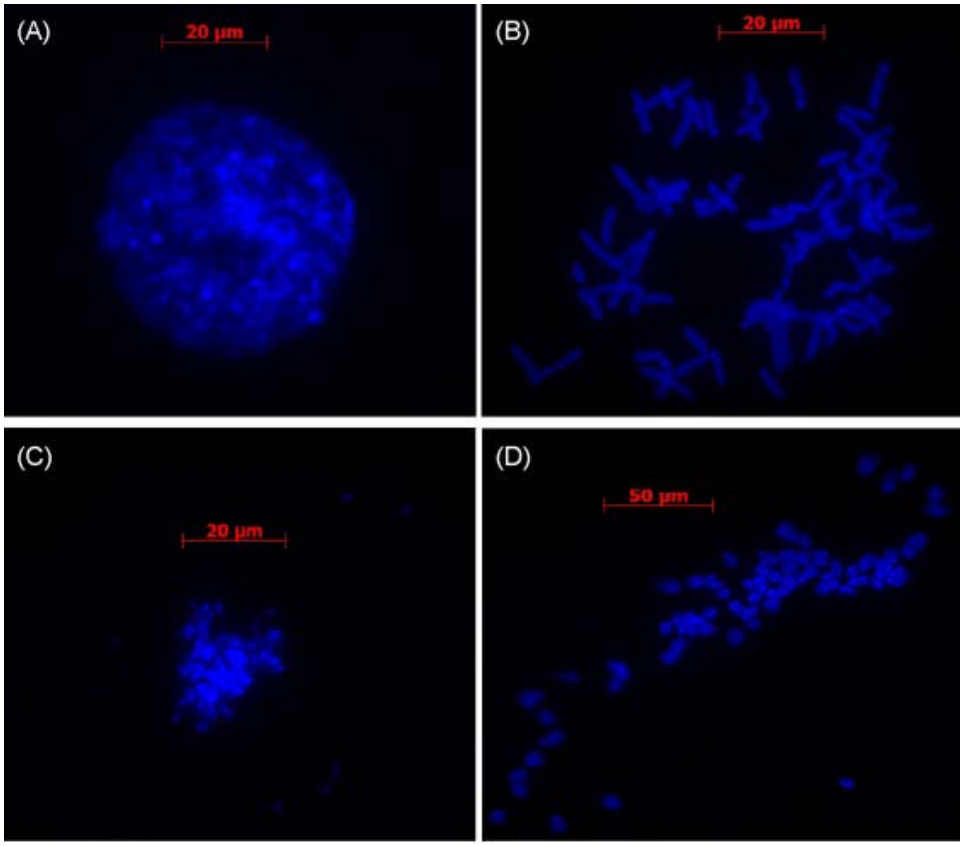

Figure 5-2. Calyculin-A induces PCC in blastomeres from Day 2, eight-cell stage murine embryos (Day 0 = fertilization). The degree of chromatin condensation was determined by DAPI counter-staining and epifluorescence microscopy at 1000×. (A) Interphase nucleus, considered to not have undergone PCC. (B) Defined murine chromosomes, abnormal 80 chromosomes, tetraploid, G_1. (C) Chaotically condensed murine chromatin, as seen in a blastomere in S phase. (D) Defined murine chromosomes, 80 chromosomes, tetraploid, G_2/M.

Table 5-4. Cytogenetic analysis of blastomeres from bovine embryos treated with calyculin-A

Embryo	No. of blastomeres per embryo	No. of blastomeres analyzed	No. of analyzed blastomeres undergoing PCC (%)	No. of analyzed blastomeres suitable for cytogenetic analysis (%)	Ploidy	Gender
1	8	8	8 (100.0)	8 (100.0)	Diploid	Male
2	12	10	10 (100.0)	9 (90.0)	Mixiploid (haploidx2, diploidx7)	Male
3	8	8	8 (100.0)	6 (75.0)	Diploid	Female
4	8	8	8 (100.0)	7 (87.5)	Diploid	Female
5	7	5	5 (100.0)	5 (100.0)	Diploid	Female
6	7	7	7 (100.0)	7 (100.0)	Diploid	Male
7	7	6	6 (100.0)	5 (83.3)	Mixaploid (diploidx4, tetraploidx1)	Female
8	9	8	8 (100.0)	7 (87.5)	Diploid	Male
9	10	10	10 (100.0)	4 (40.0)	Diploid	Female
10	8	8	8 (100.0)	7 (87.5)	Mixiploid (haploidx1, diploidx6)	Male
11	8	7	7 (100.0)	7 (100.0)	Mixiploid (diploidx6, tetraploidx1)	Female
Total	92	85	85 (100.0)	72 (84.7)	7 Diploid (63.6%)	6 Female (54.5%), 5 Male (45.5%)

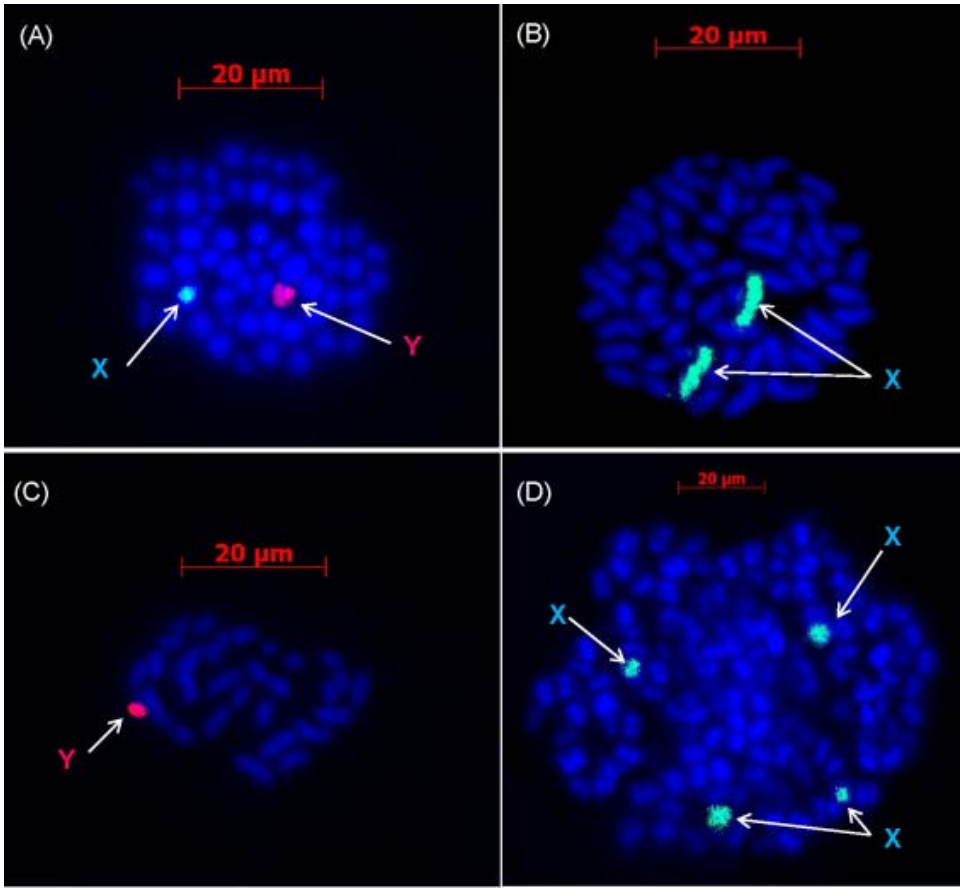

Figure 5-3. Cytogenetic analysis of bovine blastomeres treated with calyculin-A. Blastomeres with defined chromosomes were hybridized with an X–Y specific, whole chromosome paint probe set [biotin-labeled X (green) and Cy3-labeled Y (red); Cambio] and counter-stained with DAPI. Images were captured at 1000× using epifluorescence microscopy. (A) Diploid, 60,XY, normal male. (B) Diploid, 60,XX, normal female. (C) Haploid, 30, 0Y, abnormal male. (D) Tetraploid, 120, XXXX, abnormal female.

CHAPTER 6
SUMMARY, CONCLUSIONS AND FINAL REMARKS

The field of assisted reproduction has undergone many changes since the birth of the first IVF baby more than 30 years ago. Compared to the early days of IVF, the success and range of treatment options has seen tremendous improvement. Initially, IVF was limited to treatment of female infertility, covering only 1/3 of all infertility cases. For the laboratory's part, the early procedures were fairly simple and involved mixing of sperm with oocytes within a petri dish and following fertilization selecting resulting embryos for transfer back to the uterus. Today though, with the development of complex procedures such as ICSI, assisted hatching, oocyte and embryo cryopreservation and PGD, treatment options are no longer limited to female infertility. For instance, most male factor infertility can now be routinely treated with ICSI. In addition, patients diagnosed with cancer have a number of oncofertility options to help preserve their fertility following treatment, including cryopreservation of oocytes, sperm and embryos. Furthermore, patients who carry genetic abnormalities can undergo PGD to avoid the transfer of affected embryos and in some cases even select embryos that match an older sibling's immune components, providing a source of stem cells that can treat and hopefully save a sibling's life. As these IVF procedures have increased in complexity, so have government regulations enacted to ensure that laboratories and persons within the laboratories performing such complex procedures are qualified.

This dissertation is part of the requirements of a newly developed doctorate program with an emphasis in clinical embryology. Among the goals of this program are to expose students to the fundamental principles of embryology, general laboratory knowledge, quality control and quality assurance, and the development of clinical and

experimental research programs. As such, this dissertation incorporates many of these aspects through investigations of the effects of GnRH analogs on ovarian expression of miRNAs, effects of macromolecules during embryo cryopreservation, and the effects of a specific protein phosphatase inhibitor, calyculin-A, on the induction of chromosome condensation for the purpose of chromosomal analysis of in vitro produced embryos.

Effects of GnRH Analogs on Ovarian Expression of MiRNAs

Common practice for patients seeking IVF treatment is to undergo controlled ovarian stimulation utilizing GnRH analogs in order to prevent a premature surge of LH. Since the introduction of GnRH agonist and antagonist protocols, there have been observed differences in follicular and serum steroid concentrations and pregnancy outcomes between analog protocols that have led to the suggestion that GnRH agonists and antagonists may directly and differentially affect ovarian function. While a molecular mechanism remains unknown, hormone treatment, including hCG/LH [107] and FSH [108] have recently been shown to differentially affect the expression of miRNAs in granulosa cells. In addition, differential expression of miRNAs were shown in another report to affect the release of ovarian steroids P4, T, and E_2 [110].

Based partially on these studies, a hypothesis was formulated for the current study stating that GnRH agonists and GnRH antagonists directly and differentially regulate the expression of ovarian miRNAs which affects the expression of their predicted target genes and influences ovarian function. To test this hypothesis, granulosa cells, collected from patients undergoing stimulation with GnRH agonist and antagonist protocols, as well as a granulosa cell line treated with GnRH agonist and antagonist, were analyzed using realtime quantitative PCR to compare differences in expression of select miRNAs and mRNAs.

Results from these experiments indicate greater expression of mir-26a, mir-34a, mir-98, mir-133b, mir-195 and mir-497 in granulosa cells from patients treated with GnRH antagonists compared to GnRH agonist, lending support to the hypothesis that GnRH analogs differentially affect miRNA expression in ovarian tissue. However, the expression patterns of the same miRNAs were not significantly different in the granulosa cell line treated with GnRH agonist and antagonists, suggesting the observed differences in miRNAs in granulosa cells from patients were not a direct affect of GnRH analogs. An alternative explanation is that the granulosa cell line is not a representative model of in vivo function. However, overall expression patterns of miRNAs in granulosa cells from patients were similar to miRNA expression patterns in the granulosa cell line. In addition, GnRH analogs differentially affected the expression of AREG, GATA4, and CYP19A1 mRNA in the granulosa cell line demonstrating that these cells were capable of directly responding to GnRH agonist and antagonist treatments. Based on these results, the observed miRNA differences in granulosa cells from patients are most likely explained by GnRH analogs indirectly affecting the expression of ovarian miRNAs, possibly through modulation by endogenous hormones.

A limitation of this study is that a traditional non-treated control was not included among the patients. The inclusion of a group that does not receive either GnRH analog would have helped elucidate the indirect effects of GnRH agonist and antagonists on ovarian expression of miRNAs. However, to not administer either GnRH analog is not practical as the patient would be at an increased risk for premature LH surge. This could lead to loss of oocytes and cancelation of the IVF cycle.

Another limitation was the sample size. With so few patients included in the study, the differences in miRNA expression may not necessarily be clinically significant. Perhaps, with a larger sample size and more detailed patient information, it may be possible to determine the relevance of the differential expression of miRNAs.

Conclusions drawn from this study are that GnRH analog stimulation protocols differentially regulate ovarian expression of miRNAs. Since miRNAs may regulate the expression of 30% of all genes (246), any changes in ovarian expression of a given miRNA could have profound effects on the expression of many other genes affecting processes essential to ovarian function. This is a major concern that warrants further investigations, as folliculogenesis and steroidogenesis may be affected by differential ovarian miRNA expression in response to GnRH analog stimulation protocols and ultimately affects patient response and reproductive success.

Effects of Macromolecules on Embryo Cryopreservation

This area of investigation focused on the utilization of a globulin-rich protein source in cryopreservation protocols for improving thaw-survival of blastocysts. Previous studies have shown that the inclusion of macromolecules, such as α- and β-globulins in embryo culture medium, promotes blastocyst hatching [118, 122, 247] and implantation [119, 120, 248]. Several mechanisms have been proposed to explain the promoting effects, including the alteration of the physiochemical environment surrounding the embryo through interactions of macromolecules with water molecules [5]. Since osmoregulation of water molecules is a vital component of embryo cryopreservation, the current investigation tested the hypothesis that freeze and thaw solutions supplemented with globulin-rich proteins would improve embryo thaw-survival. To test this hypothesis, animal experimental studies compared thaw-survival rates of

murine blastocysts cryopreserved using freeze and/or thaw solutions supplemented with HSA or the globulin-rich protein source, SSS.

Following the thaw of murine blastocysts, higher rates of re-expansion and moderately higher total cell numbers were found in those frozen and thawed in solutions containing SSS compared to HSA, supporting the hypothesis that globulin-rich protein supplementation improves embryo thaw-survival. While the current study did not investigate a potential mechanism, the findings are consistent with the proposed mechanism by Pool and Martin that high numbers and orientation of hydroxyl groups (-OH) in polyhydroxy compounds, such as α- and β-globulins, interact with water molecules affecting physical properties such as water exchange dynamics.

While the overall results are promising, there are several limitations with the current study that need to be addressed. The true potential of the thawed blastocysts is unknown, as murine blastocysts were not transferred to recipients. In defense, total cell numbers, which have been positively correlated with implantation potential [254], were higher for the SSS treatment, and therefore implantation potential would be expected to be greater for blastocysts frozen and thawed in solutions containing SSS compared to HSA. The present study utilized a commercially available protein source containing a fixed mixture of 84% HSA, 16% α- and β-globulins and less than 1% γ-globulins. While this mixture produced good overall thaw-survival rates, other concentrations of globulins may be more beneficial. The experimental design used a murine system and the results may not translate to human embryo cryopreservation. Murine embryos, however, are an accepted system for quality control testing of clinical conditions. In addition, results from the retrospective analysis of human clinical data, although limited by sample size, are

consistent with the results obtained from the murine experiments. The experiments within the current study focused on control-rate cryopreservation, which is slowly being phased out in favor of vitrification. Whether globulins would improve survival of vitrified embryos remains unknown; however, many of the successful published vitrification protocols incorporate globulin-rich proteins sources, such as SSS, suggesting the observed results are probably applicable to vitrification technology.

The conclusion from this investigation is that supplementation of freeze and thaw solutions with a protein source containing high fractions of globulins, such as SSS, promotes thaw-survival. With increasing restrictions placed on the number of embryos that can be transferred to the uterus, improvements in embryo cryopreservation, as discussed in this study, will undoubtedly allow for better utilization of remaining embryos.

Effects of Calyculin-A on the Induction of Chromosome Condensation for Genetic Analysis of Embryos

Chromosomal abnormalities are estimated to occur in 50-70% of preimplantation embryos [6, 12-19]. As these abnormalities are thought to be a major cause of implantation failure, there is a tremendous need for technologies capable of screening embryos prior to transfer to the uterus. Unfortunately, traditional cytogenetic approaches for assessment of chromosomal abnormalities utilize FISH technology on interphase nuclei, which limits the number of chromosomes that can be analyzed at one time and more importantly is associated with high error rates [6-11]. In an attempt to overcome the limitations of interphase FISH, the present study investigated the induction of PCC in bovine and murine blastomeres using a specific protein phosphatase inhibitor, calyculin-A, to obtain condensed chromosomes suitable for full cytogenetic analysis.

Results from this study demonstrate calyculin-A induced chromosome condensation of bovine and to a lesser extent murine blastomeres; however, the quality of condensation was dose, time, and cell cycle stage dependent. Overall, the most effective conditions for inducing PCC in bovine blastomeres were 120 minute exposure to 50 nM calyculin-A.

Though optimized conditions resulted in high rates of PCC in blastomeres, there are several limitations that may hinder the application of this technology. 1) A large fraction of blastomeres yielded condensed chromatin unsuitable for cytogenetic analysis, presumably because blastomeres were in S phase. PCC of cells in S phase is associated with chaotic chromatin which cannot be further studied. As the phase of the cell cycle is difficult to ascertain prior to embryo biopsy, there is a risk that the selected blastomere may be in S phase and therefore a diagnosis of the biopsied embryo will not be able to be determined. 2) Calyculin-A may be limited to bovine blastomeres. In the present study, calyculin-A was less effective for inducing PCC in murine blastomeres. Additionally, another study reported fewer than 60% of human blastomeres underwent PCC with calyculin-A, and of those only 17% were suitable for analysis [282]. Together these results suggest induction of PCC in blastomeres with calyculin-A is species limited, such as bovine. 3) High lipid content in bovine embryos complicates fixation and impedes analysis. Lipid droplets, which can be readily seen upon addition of fixative solution, tend to associate with chromatin making it difficult to remove excess lipid without losing chromatin. This is a major concern as any lost chromatin can lead to misdiagnosis.

Conclusions to be drawn from this study are that calyculin-A can rapidly induce PCC in blastomeres, but the quality of chromosome condensation may not be suitable for cytogenetic analysis. As such, the application of PCC is not an acceptable method for chromosomal screening of embryos.

Final Remarks

For more than 30 years, the field of assisted reproduction has been pioneered by clinicians and scientists whose achievements in clinical and basic research have led to improvements in technical applications advancing reproductive medicine. Unfortunately, many of these the early pioneers are preparing to retire, leaving a void of scientific knowledge and clinical experience. As such, the field of assisted reproduction is at a crossroads and will require a new generation of uniquely qualified personnel to continue scientific and clinical efforts. Within the field of assisted reproduction, the area most likely to be affected is the laboratory, as few academic programs exist to prepare future leaders in embryology laboratory sciences. Unlike technical laboratory experience, which can be gained with repetition, the acquisition of the fundamental principles embryology requires careful study of scientific and academic literature. In addition, general understanding of quality control and clinical and basic research are necessities, as embryology is a clinical science requiring careful monitoring of clinical parameters, as well as knowledge of scientific methodology to ensure clinical advances.

The investigations within this dissertation were designed to incorporate knowledge of embryology with quality control, clinical research, and basic scientific research to further our understanding of several areas of assisted reproduction, including the effects of ovarian stimulation on granulosa cells, the effects of globulin proteins on embryo cryopreservation, and the usefulness of chemical induction of

premature chromosome condensation for chromosomal screening of preimplantation embryos. The majority of the results from these individual investigations are typical of many studies, in that some outcomes, although significant, have little or no clinical relevance; however, a few observations, such as the potential for improved post-thaw survival with the utilization of globulin-rich proteins in cryopreservation solutions, may have clinical application. In any case, these investigations together provide an understanding of clinical and scientific methods that are fundamental for the knowledge base of future embryologists who will be able to bring translational research from the laboratory to the reproductive bedside.

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BIOGRAPHICAL SKETCH

Joseph Michael Kramer was born in 1977 in Easy Patchogue, New York, to Doris and John Kramer. He was raised with his older sister, Jennifer, and younger brother, David, in Dunnellon, Florida. Joseph married Raeanna Fox on August 15, 1998. They have two children, Nadia, born June 2004, and Colt, born February 2010.

Joseph graduated from Dunnellon high school in May, 1995, and then he enrolled at the University of Florida, in Gainesville. Joseph received his bachelor's degree in animal sciences in August 2000. The following year, he began a Master of Science program in animal sciences at the University of Florida, Gainesville, under the direction of Dr. Karen Moore, and he completed the program in April 2005. From August 2003 to June 2006, Joseph began his career in the field of human reproductive medicine as an embryologist and later as a cytogeneticist for Reproductive Medicine Associates of New Jersey. Joseph returned to the University of Florida in August 2006 to begin a Doctor of Philosophy in animal molecular and cellular biology, as part of a unique joint venture between the Department of Animal Sciences and the human in vitro fertilization laboratory at Shands hospital, Gainesville, under the direction of Dr. Kenneth Drury. After graduation, Joseph will apply for certification as a High-Complexity Clinical Laboratory Director through the American Board of Bioanalysis. In the future, Joseph plans on directing a human clinical in vitro fertilization laboratory and pursuing his research interests in ovarian tissue and oocyte vitrification and embryo metabolomics and proteomics.