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The Impact Of Mitochondria On Preimplantation Development In The Rhesus Macaque

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THE IMPACT OF MITOCHONDRIA ON PREIMPLANTATION DEVELOPMENT IN THE RHESUS MACAQUE

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DEDICATION

To my family and friends for all their love and support.

To the memory of my pal, Morgan. We had countless adventures together and for fourteen years she never left my side. She will always be missed.

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I would first like to acknowledge my parents: Mom, Dad, Garlyn, Jeannie, Mikki and Kit. Thank you all for your unwavering love, confidence and support throughout my life and especially this process, for never letting me forget where I come from or what is important and for accepting that, while you may not agree, I have to make my own path through life. Thank you to my brothers, Scott and Josh. I am very fortunate to have unique relationships with each of my two brothers who are both extraordinary in their own ways. To my major professor, Carol Brenner. It has been a long and twisted road to the finish, but the trip has changed my life; thank you for the ride. I will always be grateful for your support and confidence in me. To Edmundo Kraiselburd, Janis Gonzales, Carol Sariol and the staff at the University of Puerto Rico and the Caribbean Primate Research Center; thank you for making me feel so welcome at CPRC and for the generous support you have given me. I look forward to an exciting and rewarding future working together. Thank you to Barry Bavister. I am grateful for your wealth of knowledge and your willingness to teach and help guide me through this process. Thanks to my PhD committee members; Randall Armant, Michael Diamond, James Rillema, Derek Wildman and Maik Huttemann for all the advice and assistance. A special thanks to Christine Cupps for making the transition from UNO to WSU as easy as possible and for everything you have done throughout this process. Your work is truly appreciated. Thank you to Leann and Michael Spake for your endless generosity, giving me a home away from home and for bringing a little bit of Texas to Michigan. To all of my family and friends. Each of you has contributed to my life and to this journey and I cannot thank you all enough. Finally, a thank you to Dustin. You have understood the magnitude of what I have been trying to achieve and have been endlessly patient and supportive. Thank you for enriching my life.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ADP-adenosine diphosphate

ART-Assisted Reproduction Technologies

ATP- adenosine triphosphate

ATSC - adipose stromal cell

B-hCG-biological human chorionic gonadotrophin

BMSC-bone marrow stromal cell

bp - base pare

CO_2 - carbon dioxide

COX - cytochrome c oxidase

CPRC - Caribbean Primate Research Center

D-Loop - mitochondrial displacement loop

dbcAMP - dibutyryl cyclic adenosine 3', 5'-monophosphate

DNA - deoxyribonucleic acid

dNTP - Deoxyribonucleotide triphosphate

Drp1 - dynamin-related protein

ESC - embryonic stem cell

ETC - electron transport complexes

Fis1 - mitochondrial fission protein 1

Fzo - Drosophila mitofusin gene

GAPDH-Glyceraldehyde 3-phosphate dehydrogenase

GV - germinal vesicle

GVBD - germinal vesicle breakdown

H_2O_2 - hydrogen peroxide

hCG - human chorionic gonadotrophin

HECM-6 or 9 - hamster embryo culture media 6 or 9

HEPES - (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HSP - heavy strand promotor

ICM - inner cell mass

IU - international unit

IVC - *in vitro* culture

IVF - *in vitro* fertilization

IVP - *in vitro* produced

KSS - Kearns Sayer syndrome

LN_2 - liquid nitrogen

LSP - light strand promotor

Mfn1 - mammalian mitofusin gene 1

Mfn2 - mammalian mitfusin gene 2

MI - metaphase I oocyte

MII - metaphase II oocyte

Miro1/2 - mitochondrial Rho 1/2

MOMP - mitochondrial outer membrane permeabilization

mRNA - messenger RNA

mtDNA - mitochondrial DNA

MTP18 - mitochondrial protein 1

mtPTP - mitochondrial permeability transition pore

N_2 - nitrogen

NADH - nicotinamide adenine dinucleotide

NHP - non-human primate

O_2 - oxygen

O_2^- - superoxide anion radical

OH - hydroxyl radical

O_H - origin of heavy strand replication

O_L - origin of light strand replication

Opa1 - optic atrophy 1

ORMES - Oregon monkey embryonic stem cell line

ORPRC - Oregon Regional Primate Research Center

OXPHOS - oxidative phosphorylation

PCR - polymerase chain reaction

PEO - progressive external opthalmoplegia

PGC - primordial germ cell

PGD - Preimplantation Genetic Diagnosis

Pink1 - serine/threonine protein kinase 1

PolG - mitochondrial DNA polymerase gamma

PolRmt - mitochondrial RNA polymerase

PVA - polyvinyl alcohol

R-hCG - recombinant human chorionic gonadotrophin

rFSH - recombinant follicle stimulating hormone

rLH - recombinant lutenizing hormone

RNA - ribonucleic acid

ROS - reactive oxygen species

rRNA - ribosomal RNA

RT-PCR - reverse transcription - polymerase chain reaction

SOD - superoxide dismutase

SSBP1 - mitochondrial single stranded binding protein 1

TALP - Tyrode's Albumin Lactate Pyruvate

TFAM - mitochondrial transcription factor A

TFB1M - mitochondrial transcription factor B1

TFB2M - mitochondrial transcription factor B2

tRNA - transfer RNA

UPR - University of Puerto Rico

PREFACE

INTRODUCTION

Assisted Reproduction Technologies (ART) have been developed to treat infertility since 1790, when the first case of artificial insemination was performed. Since then, multiple and dramatic advances have been made in the field of infertility research through the employment of a variety of species; enabling people, whom without the use of these techniques, would not be capable of reproducing. While the development of techniques to overcome infertility has been at the forefront of reproductive research, investigation into the causes of infertility have also been, and continue to be, equally as important as the remedies. It is well established that one major contributor to female infertility is maternal age. According to the Society for Assisted Reproductive Technology (SART) statistics for 2007, oocyte retrievals in women <35yrs had a 43.1% live birth rate, while women 41-42yrs of age had a 14.3% live birth rate. Furthermore, the average number of embryos per transfer was 2.2 in the <35yr age group and increased to 3.1 in the 41-42yr age group, indicating that there is a decline in either oocyte or embryo quality with age as well. While maternal age is known and can be a fairly reliable indicator of fertility; oocyte and embryo quality contribute significantly to fertility as well, but are much more difficult to determine. Oocyte quality is generally assessed by appearance and meiotic stage of the oocyte at the time of collection and insemination. There are few guidelines that aid in discriminating a competent from a non-competent oocyte and the process is subjective and heavily dependent upon the embryologists' skill and expertise. Embryo quality is more defined in that there is an expected, timely progression of cell division and development through preimplantation. It is reasonably simple to identify poor or bad embryos based upon morphology and embryonic arrest during the cleavage stages; however, it remains difficult to identify a high quality embryo based upon anything besides development rate and morphological quality score.

Because of poor predictive value of embryo morphology, virtually all human ART clinics practice multiple embryo transfers, which while increasing pregnancy and live birth rates, also result in multiple births which can lead to pre and perinatal complications. Techniques such as preimplantation genetic diagnosis (PGD) have been developed in attempts to remedy this problem, however the results have been unreliable and the procedure is detrimental to the embryo. Due to the fact that there are currently no reliable, objective ways to determine oocyte, and even embryo, quality, it is imperative to ascertain methods to measure oocyte quality that will improve selection of oocytes and embryos in ART clinics and increase pregnancy success rates. These methods are only possible with a more complete understanding of the cellular and molecular properties of oocytes and preimplantation embryos. A key component of all cells, including oocytes and embryos, is the mitochondrion, which produces energy and maintains its own DNA for encoding proteins necessary for energy production. Defects in either the proteins responsible for energy production, the mitochondrial DNA (mtDNA) genome, or other contributing factors can lead to loss of oocyte competence (failed fertilization) and embryo quality (embryonic arrest, incompetent placentas, pregnancy loss). Unquestioningly, human oocytes and the embryos derived from them would be the optimal material to use for performing studies on oocyte quality and embryo development. However, practical and ethical constraints prevent the use of human material for intensive, potentially terminal studies; thus, compelling researchers to find and use suitable animal models instead. It is important that studies are conducted with animals closely related to humans due to the wide diversity in cellular, molecular and endocrine mechanisms used by different mammals in their reproductive strategies. The non-human primate (NHP) is the most befitting animal model for human reproduction due to evolutionary closeness and the dramatic similarities in reproductive function and preimplantation development. Furthermore, because studies in human preimplantation development are

restricted to the use of discarded oocytes from infertility clinics, the use of the NHP has established the rhesus macaque as an excellent research model for human reproduction due to the availability of normal, viable oocytes and embryos from fertile animals. Therefore, the overall goals of this study were to evaluate mitochondria in NHP oocytes and embryos and to determine how mtDNA mutations and mitochondrial function affect oocyte and embryo quality.

Specific Aim 1.1: To determine if the rhesus macaque can be utilized as a clinically translational model for mitochondrial mutations identified in human IVF.

Specific Aim 1.2: To determine if the rate of mitochondrial mutations in rhesus macaque oocytes and IVF produced embryos is increased due to gonadotrophin stimulation. A high proportion of presumably normal oocytes give rise to developmentally incompetent embryos in human IVF programs. Pre-implantation embryos generated by IVF show high frequencies of abnormal development and early demise. A direct relationship between mitochondrial mutations in oocytes and embryos and reproductive success has not been demonstrated; however, it is generally assumed that there must be a sufficient number of functional mitochondrial genomes in the embryo in order for it to develop and implant successfully. A mitochondrial mutation that has been described in human oocytes and embryos is termed the “common deletion" or ΔmtDNA4977. The reason for this mutation is currently unknown, however, an important question is whether or not this deletion is caused by gonadotrophin stimulation. In order to determine this, employment of the rhesus macaque as a clinically translational model is vital.

Specific Aim 2: Due to the frequency of the rhesus mtDNA common mutation in gonadotrophin stimulated, rhesus macaque MII oocytes and IVP embryos, we hypothesize that the common mutation will also be present in embryonic stem (ES) cells derived from IVP produced embryos. We propose that the presence of numerous mtDNA deletions and point mutations may actually reflect the quality of affected oocytes or primate ES cells lines.

Primate ES cell lines may harbor mutant mtDNA that may further accumulate during cell culture, and thus have consequences for long-term viability.

Specific Aim 3.1: To determine if the presence of mutations in the mtDNA control region, as well as the mitochondrial polymerase, PolG, are associated with aging in the rhesus macaque.

Specific Aim 3.2: To determine if in the presence of mutations in the mtDNA control region are associated with meiotic failure in oocytes. In order to understand the significance of mutations within the mtDNA genome, it is imperative to examine the region of the genome that controls OXPHOS, replication and transcription. In addition to investigating the occurrence of mutations in the mtDNA control region, we can determine if aging is a cause of mtDNA damage as well identifying mutations that may be associated with failure of oocytes to mature to metaphase II, enabling them to be fertilized.

Specific Aim 4: To determine if gonadotrophin stimulation efficiency and oocyte and embryo quality can be improved through optimization of gonadotrophin stimulation protocols.

Our laboratory has been working with the Caribbean Primate Research Center (CPRC) for approximately 4 years in an effort to establish a reputable rhesus macaque IVF program using the CPRC as a resource to provide high quality rhesus macaque oocytes, embryos and sperm. In order to obtain rhesus macaque reproductive material of the highest quality, we tested three modifications to the previously established gonadotrophin stimulation protocols.

Specific Aim 5.1: To determine if expression levels of genes controlling mitochondrial dynamics are correlated to the failure of oocytes to resume meiosis.

Specific Aim 5.2: To determine if expression levels of genes controlling mitochondrial dynamics are correlated to important preimplantation development time points.

Interest in the mitochondrion and its role in the preimplantation mammalian embryo has had a recent resurgence and it is understood that mitochondria undergo a variety of species-specific reorganizations during early preimplantation development. It is possible that the localization of mitochondria in rhesus zygotes is a result of mitochondria undergoing fusion and fission processes during preimplantation resulting in the microtubule network guiding the mitochondria to their pronuclear location. Our lab has recently found that monkey oocytes have an abundance of gene involved in the control of mitochondrial fusion, fission, movement and replication. Therefore we speculate that mitochondrial trafficking is important for fusion and fission mechanisms and may impact oocyte and embryo quality.

CHAPTER 1

BACKGROUND AND SIGNIFICANCE

Oogenesis and Preimplantation Development

Oogenesis begins early in fetal development with the formation of primordial germ cells (PGCs) which populate the fetal ovary. PGCs migrate along the hind gut during gestation to the region adjacent to the mesonephros that becomes the genital ridge. PGCs are the sole source of adult germ cells and once established in the developing ovary are referred to as oogonia. Oogonia undergo several rounds of mitotic division over a period of several months until shortly before birth, when meiosis is initiated. The primary oocytes progress through prophase I of the first meiotic division before arresting at the diplotene stage. Diplotene oocytes are characterized by diffused chromosomes surrounded by an intact nuclear membrane known as the germinal vesicle (GV). With the resumption of meiosis, which occurs shortly after puberty, the interphase chromatin condenses, the germinal vesicle membrane breaks down (GVBD), the spindles form and chromosomes segregate. Extrusion of the first polar body marks the completion of the first meiotic division. Oocytes then arrest again at metaphase II (MII). Completion of the second meiotic division only occurs after fertilization and the extrusion of the second polar body. The interaction of maternal and paternal pronuclei at syngamy is characterized by tight association of both male and female pronuclear envelopes with close alignment of the nucleoli. In humans and non-human primates, pronuclear membrane breakdown and commencement of the first mitotic division ensues without actual fusion of the pronuclear envelopes, as is seen in a multitude of other species (reviewed by (Levron et al., 1995).

Mitochondria and Reproduction

Mitochondria are considered entirely maternally inherited. Although sperm carries paternal mitochondria into the oocyte at fertilization, they are normally eliminated in early

embryonic development by ubiquitin-tagging of the sperm surface. During the first half of fetal development, mitochondria number around 10 in each premigratory PGC and increase to approximately 200 in each original oogonium. Mitochondria then increase exponentially until the diplotene stage of meiosis where they arrest. At birth, there are approximately two million oocytes and each immature primordial oocyte contains about 6000 mitochondria (Jansen and Burton, 2004; Shoubridge and Wai, 2007). Once puberty is reached, primordial follicles contain oocytes arrested at the GV stage of development. Upon fertilization, during mitosis, mtDNA synthesis and mitochondrial replication are inhibited as cleavage divides the mitochondria from the fertilized oocyte among the daughter cells of the developing embryo. This process progressively reduces the mtDNA copy number per cell, or blastomere in the embryo, from the 0.5–5 million found in the oocyte at ovulation to approximately 10 per cell by the time gastrulation begins. This process establishes a genetic bottleneck of mtDNA. The mitochondrial bottleneck theory states that a limited number of mtDNAs (1-200) are the only mtDNAs selected to undergo replication. These genetic founder mtDNAs replicate to give rise to almost all the 100,000 mtDNAs in mature MII oocytes (Jenuth et al., 1996). Typically, prior to mtDNA replication, if oocytes contain a small percentage of mtDNAs with rearrangements, then random selection of a limited number of founder mtDNAs would likely select normal mtDNAs for replication. At this point, the literature varies significantly about both the number of mitochondria in each GV oocyte and the number of mtDNA copies per mitochondria. Upon resumption of meiosis and during maturation, cytoplasmic growth of oocytes recruited for ovulation indicates that mitochondrial numbers have increased, and in some species, i.e. NHP and porcine (Gibson et al., 2005; Spikings et al., 2007) mtDNA replication occurs, increasing the mtDNA copy number from GV to MII. This replication event does not appear to occur in the mouse.

Mitochondrial Function

Mitochondria are traditionally known as the powerhouses of the cell; the primary source of ATP production. While this is true, the mitochondrion is an infinitely more complicated and interesting organelle. The mitochondrion, which evolved from an oxidative α-proteo-bacterium, approximately 2 billion years, ago joined forces with a glycolytic motile cell evolving into what we know currently as a eukaryotic cell. Over approximately 1.2 billion years a symbiotic relationship evolved in which the mitochondrion became specialized in energy production and the nucleus-cytosol became the driving force behind structure. As this relationship evolved further, essentially all genetic function from the mitochondrion (~1500 genes) became part of the chromosomal nuclear DNA. However, the mitochondrial genome did not disappear completely and continues to retain the core subunits of the enzyme complexes of mitochondrial oxidative phosphorylation (OXPHOS) (Wallace, 2008). Thirteen polypeptide-encoding genes, 2 rRNA genes and 22 tRNA genes, all essential to OXPHOS, remain encoded by the mitochondrial genome, rendering the mitochondrial DNA (mtDNA) genome essential to energy production and cell function. Mitochondria generate energy through the electron transport complexes (ETC) by oxidizing hydrogen with oxygen to generate heat and ATP. Two electrons are passed sequentially, either from NADH+H+ to complex I (NADH dehydrogenase) or from succinate to complex II (succinate dehydrogenase), to ubiquinone (coenzyme Q) to give ubisemiquinone (CoQH) and then ubiquinol (CoQH2). Ubiquinol transfers its electrons to complex III (cytochrome c oxidoreductase), which transfers them to cytochrome c. From cytochrome c, the electrons flow to complex IV (cytochrome c oxidase, COX) and then to 1/2 O_2 to give H_2O. The energy released from the ETC is used to pump protons out of the mitochondrial inner membrane through complexes I, III, and IV, creating an electrochemical gradient. The potential energy stored in the inner membrane is coupled to ATP synthesis by complex V (ATP synthase). As

protons flow back into the matrix through a proton channel in complex V, ADP and Pi are bound, condensed, and released as ATP. Matrix ATP is then exchanged for cytosolic ADP by the adenine nucleotide translocator (ANT). As a by-product of OXPHOS, mitochondria generate reactive oxygen species (ROS), in the forms of O_2^- (superoxide anion radical), H_2O_2 (hydrogen peroxide) and OH (hydroxyl radical). Superoxide anion can be converted to hydrogen peroxide which can then be converted to highly reactive hydroxyl radicals, rendering mitochondria key targets for oxidative damage (reviewed by (Wallace, 2005). H_2O_2, in conjunction with superoxide anion, can damage cells by allowing the most reactive metabolite, OH, to form via superoxide dismutase (SOD) and the Haber-Weiss reaction (Halliwell, 1989).

Because gametes and embryos require energy, which is produced by the conversion of ADP to ATP via glycolysis and OXPHOS; a process by which O_2 is intrinsic through its role as an electron acceptor, O_2^-, H_2O_2 and OH have been well established as factors of oxidative stress in *in vitro* culture systems. The production of excessive amounts of intracellular ROS during *in vitro* embryo culture is thought to disrupt metabolic activity and is therefore detrimental to embryo development (Guerin et al., 2001). The concentration of oxygen at which embryos are cultured has shown to be a key factor in regulating OXPHOS and ROS production in several species including bovine (Nagao et al., 1994), mouse (Goto et al., 1993) and human (Catt and Henman, 2000; Dumoulin et al., 1999). Furthermore, reduced O_2 concentration that more closely resembles physiological O_2 results in higher blastocyst rates in mice (Dumoulin et al., 1999), sheep (Bernardi et al., 1996) and bovine (Lonergan et al., 1999). In mammals, ovulation, fertilization, and early embryo development occur in the oviduct. Embryos then migrate to the uterus, where implantation occurs. Oviductal O_2 concentration is dramatically lower than atmospheric concentration (Mastroianni and Jones, 1965) with the uterus maintaining an even lower O_2 concentration than that of the oviduct (Fischer and Bavister, 1993). Thus there is a

decreasing O_2 concentration gradient that embryos encounter as they progress through the reproductive tract. Traditionally, *in vitro* culture systems employed 5% CO_2 in atmospheric air (approximately 20% O_2). Studies in the last two decades have shown that culture of embryos under an O_2 concentration more closely resembling that of the oviduct (5% O_2) result in increased embryonic viability (Dumoulin et al., 1999), decreased H_2O_2 production and DNA fragmentation (Kitagawa et al., 2004) and significantly increased pregnancy and birth rates as well as blastocyst rate and quality (Waldenstrom et al., 2009).

In addition to energy production, mitochondria are key regulators of apoptosis. Apoptosis can be initiated through the activation of the mitochondrial permeability transition pore (mtPTP) in response to energy deficiency, increased oxidative stress, excessive calcium, and other factors. A complete understanding of the mtPTP remains to be elucidated, however it is thought to be composed of the inner membrane ANT, the outer membrane voltage-dependent anion channel (VDAC), Bax, Bcl2, and cyclophilin D. When the mtPTP opens, membrane potential decreases and ions equilibrate between the matrix and cytosol, causing a phenomenon called mitochondrial outer membrane permeabilization (MOMP) and subsequent swelling of the mitochondria. This results in the release of the contents of the mitochondrial inner membrane space (cell death promoting factors such as cytochrome c) into the cytosol. Upon release, cytochrome c activates factors which downstream activate caspases that propagate and execute apoptosis.

The mitochondrial theory of aging (or the oxidative stress theory) holds that oxidative damage to the mtDNA genome can lead to DNA strand breaks, deletions and mutations and subsequent dysfunction of the respiratory chain. The most current version of this theory by (Sohal and Weindruch, 1996) states that

"a chronic state of oxidative stress exists in cells of aerobic organisms even under

> normal physiological conditions because of an imbalance of pro-oxidants and antioxidants. This imbalance results in a steady-state accumulation of oxidative damage in a variety of macromolecules. Oxidative damage increases during aging, which results in a progressive loss in the functional efficiency of various cellular processes."

Mitochondrial DNA is much more susceptible to ROS damage than nuclear DNA due to its proximity to the site of ROS production, as well as the lack of histones and the limited repair function of mtDNA. It is well established that as the proportion of mutated mtDNA increases, the energetic output of the mitochondria subsequently declines, ROS production increases, and the propensity for apoptosis increases, leading to loss of tissue function (Wallace, 2005).

Despite decades of research in support of the mitochondrial theory of aging, the exact connection between aging and mitochondria continues to be a hot topic of debate and study. One question of interest is whether mtDNA mutations are a cause or consequence of aging. The mitochondrial theory of aging supports the idea that mtDNA mutations are a result of the aging process (Sohal and Weindruch, 1996). The alternative theory is the concept of clonal expansion of mtDNA mutations that accumulate over the lifetime of an individual, leading to dysfunction of the respiratory complex, followed by the observed aging phenotypes and age-related diseases (Elson et al., 2001). This is of particular interest in post-mitotic cells and tissues, such as skeletal muscle, myocardium, brain and oocytes. As previously stated, the mitochondrial bottleneck theory states that limited number of mtDNAs are selected for replication. If the oocyte selected for ovulation contains a small percentage of founder mtDNA with rearrangements that escaped the bottleneck, then the resulting oocyte would contain an abnormally high percentage of rearranged mtDNAs and thus a high percentage on non-functional mitochondria. It is well established that mtDNA mutations must meet a critical threshold in order to result in age-related phenotypes. However, it is currently unknown exactly how long this process takes, i.e., do mtDNA mutations occur early in life and through years of replication clonally expand as the

individual ages? Thus, it would follow that the mtDNA mutations are a cause of aging, instead of a result. Most likely, some combination of both of these processes is the culprit of aging and age related diseases.

Genome and Replication

Mammalian mtDNA is a double-stranded, closed-circular molecule of approximately 16.6 kb in the human and non-human primate (Figure P1).

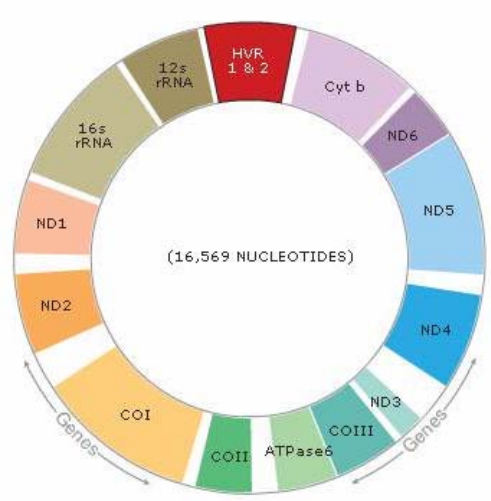

Figure P1. Human mtDNA Genome

The two strands of mtDNA are classified as either the heavy (H) or light (L) strand. The H strand is thusly named because it is guanine rich and it encodes most of the regulatory information: 2 rRNAs, 14 tRNAs and mRNAs for 12 of 13 polypeptides encoded by mtDNA. The L strand only encodes 8 tRNAs and 1 mRNA. There are only two non-coding regions of mtDNA. The primary region is a triple stranded structure called the displacement loop (D-Loop), approximately 1kb long, within which is the site of heavy strand replication (O_H). The D-Loop is triple stranded due to the frequent termination of the nascent H strand approximately 700bp downstream of O_H, which remains annealed to the parental L strand, thus leaving three strands present in the D-Loop. Also located within the D-Loop are the sites of the promoters for both H and L strand transcription, as well as multiple binding sites for mitochondrial

transcription factor A (Tfam). The second non-coding region is approximately two thirds of the mtDNA length from the O_H, is only ~30bp in length, and is the origin of replication for the light strand (O_L).

Mitochondria are unique organelles in that they require the contribution of both the nuclear and mitochondrial genome. The bulk of mitochondrial proteins are encoded in the nucleus, synthesized in the cytoplasm, then imported and processed inside the mitochondrion. The mitochondrial genome contributes a small subset of proteins necessary for the OXPHOS pathway and is quite different from the nuclear genome for several reasons: 1) Cells contain hundreds of mitochondria and each mitochondrion contains 2-10 identical copies of mtDNA, while each cell only contains one copy of the nuclear genome, 2) The mitochondrial genome is considered maternally inherited, with the paternal contribution being eliminated by an ubiquitin-dependent mechanism during fertilization (Sutovsky et al., 1999, 2000). However, this process of ubiquination has been questioned in the human as a paternal mitochondrial contribution has been detected in children in specific cases, thus leading to mitochondrial heteroplasmy (St John and Schatten, 2004). However, for the scope of this report, we will consider mtDNA to be only maternally inherited. 3) The mtDNA genome has considerably less efficient repair mechanisms than that of nuclear DNA. There is only one known mtDNA polymerase, gamma or PolG; and the only repair function is the 3'-5' exonuclease linked to the catalytic subunit of PolG. In all vertebrates, PolG is encoded in the nucleus and contains two subunits: a large catalytic subunit that harbors the 3'-5' exonuclease and 5'deoxyribose phosphate (dRP) lyase activities, and a smaller accessory subunit which increases both the catalytic activity and processivity of the catalytic subunit. PolG has conserved polymerase and exonuclease domains and the combination of the two domains is responsible for the high-fidelity of mtDNA replication with an average error rate in humans of approximately 1 error/500,000 bp *in vitro* (Lee and Johnson,

2006; Longley et al., 2005). Diseases such as progressive external ophthalmoplegia (PEO), Kearns Sayer syndrome (KSS), Alpers syndrome and ataxia, as well as symptoms of premature menopause, progressive muscle weakness, parkinsonism, and male infertility are coupled with mutations in human PolG (Luoma et al., 2004; Mancuso et al., 2004; Naviaux and Nguyen, 2004; Van Goethem et al., 2001; Zeviani et al., 1988; Zeviani et al., 1989). Reported mutations are primarily recessive and generally associated with other PolG mutations or with mutations in other genes that encode proteins involved in mtDNA replication, i.e. Twinkle.

Twinkle (PEO1) is an mtDNA helicase important for mtDNA replication and to date appears to be the rate limiting step in mtDNA replication initiation (Korhonen et al., 2003; Spelbrink et al., 2001; Tyynismaa et al., 2005). Mutations in Twinkle are associated with multiple mtDNA deletions and inhibition of Twinkle expression in cultured cells results in rapid mtDNA depletion, suggesting that it is required for mtDNA maintenance. In contrast, overexpression of Twinkle in transgenic mice results in increased mtDNA copy number (Tyynismaa et al., 2004). Furthermore, Twinkle helicase activity is stimulated by the mitochondrial single stranded DNA-binding protein (SSBP1), which acts as a stabilizer of mitochondrial chromosome and nucleoid structure (Korhonen et al., 2003). Together with PolG, these proteins form a minimal replisome (Figure P1) capable of synthesizing 16kb lengths of DNA in cell–free systems (Kaguni, 2004; Korhonen et al., 2003), since PolG alone is unable to support DNA synthesis. Twinkle also co-localizes with Tfam and SSBP1.

Mitochondrial Transcription

Transcription of mtDNA takes place following interaction between the nuclear-encoded regulatory proteins and regions within the mitochondrial D-Loop. Transcription requires mitochondrial RNA polymerase (Polmt) (Tiranti et al., 1997), Tfam (Fisher and Clayton, 1985, 1988), and at least one of the transcription factors Tfb1m or Tfb2m (Falkenberg et al., 2002).

Several studies suggest that transcription is initiated bi-directionally at two promoters, HSP and LSP for heavy and light strands, respectively, within the D-Loop regulatory region (Clayton, 2000; Shadel and Clayton, 1997). Light strand transcription commences from the LSP to the second conserved sequence block within the D-Loop region, generating a complementary RNA sequence (Xu and Clayton, 1995). Binding of PolG to the resulting sequence enables replication of the heavy strand to initiate within the D-Loop. Both promoters share a critical upstream enhancer that serves as the recognition site for Tfam, an HMG-box protein that stimulates transcription through specific binding to upstream enhancers. Tfam can bend and unwind DNA and is linked to its ability to stimulate transcription upon binding DNA (Fisher et al., 1992). Both Tfb1m and Tfb2m proteins work together with Tfam and Polmt to direct initiation from HSP and LSP. Knockdown of the Tfb2m isoform results in reduced mtDNA transcription and copy number, while knockdown of Tfb1m appears to have no effect on mtDNA transcription or replication (Matsushima et al., 2005). Termination of mitochondrial transcription is associated with the specific initiation site for H-strand transcription, via the mitochondrial transcription termination factor (mTERF) (Martinez-Azorin, 2005).

Mitochondrial Dynamics

In mammalian cells, the size and shape of mitochondria varies widely, ranging from long, interconnected tubules to individual, small spheres. In some cell types, the mitochondrial population consists mostly of tubules, which constantly migrate back and forth along microtubule tracks (Chen and Chan, 2004). In other cell types, the actin cytoskeleton is used for transport (Hollenbeck and Saxton, 2005). During migration along these tracks, individual mitochondria can encounter each other and undergo fusion, resulting in mixing of both lipid membranes and intra-mitochondrial content (Detmer and Chan, 2007). Conversely, individual mitochondria can divide by fission to yield two or more smaller mitochondria. In addition to

changes occurring during migration, the internal structure of mitochondria can change in response to their physiological state. The length, shape, size and number of mitochondria are maintained by fusion and fission. At steady state, dynamic events are balanced to maintain the overall morphology of the mitochondria. When this balance is disturbed, remarkable transitions in mitochondrial shape can occur.

Genetic studies have shown that cells with a high fusion:fission ratio have few, long and highly interconnected mitochondria (Bleazard et al., 1999; Chen et al., 2003; Sesaki and Jensen, 1999; Smirnova et al., 2001). Conversely, cells with a high fission:fusion ratio have higher numbers of smaller, spherical mitochondria referred to as fragmented mitochondria (Detmer and Chan, 2007). These fragmented mitochondria lead to apoptosis by regulating the release of cytochrome C and caspase into the inner membrane space (Detmer and Chan, 2007; Youle and Karbowski, 2005). Mitochondria proliferate on their own during the cell cycle in a manner similar to bacterial division. The dynamics of mitochondrial division are essential for mitochondrial function. A fundamental role of mitochondrial division in multiple cell types is the maintenance of appropriate distribution of mitochondria. Mitochondrial division can lead to a distorted mitochondrial network that allows areas of the cell to be devoid of mitochondria, resulting in altered cellular division. It has been shown in mammals that disruption of mitochondrial division can result in loss of membrane potential, increased ROS and oxidized proteins, as well as mtDNA loss (Lackner and Nunnari, 2008). The ability to identify areas of high and low mitochondrial density within cells may lead to the ability to identify normal mitochondrial distribution patterns.

It has been shown in pronuclear oocytes and early cleavage stage embryos in multiple species, including hamster (Barnett et al., 1996) and mouse (Van Blerkom, 1991), that mitochondria have a distinct perinuclear translocation and that perturbations from these patterns

result in embryonic lethality. In the human, mitochondrial localization patterns are significantly different between individual oocytes and between and among cohorts. However, pronuclear oocytes that showed a pronounced asymmetrical mitochondrial distribution during syngamy continued to show an asymmetrical distribution after the first cell division, and between blastomeres throughout embryo development. When analyzed individually, the blastomeres containing less mitochondria also had lower ATP content compared to blastomeres with higher mitochondrial content (Van Blerkom et al., 2000). It is well established that during the early stages of development, embryo competence depends upon ATP produced by mitochondria inherited from the mother (Gardner and Lane, 1998). During the early stages of embryo development, energy is produced by OXPHOS via amino acids, lactate and pyruvate, with the glucose pathway proving to be harmful at this stage; indicating that a threshold of mitochondrial activity is necessary to support energetic demands (Tarazona et al., 2006). Once embryonic genome activation begins, the metabolic activity of the embryo increases as it becomes capable of using alternative pathways such as anaerobic glycolysis, allowing mitochondrial activity to decrease. It has been shown that embryos that are developmentally incompetent have lower mitochondrial activity and inhibited cleavage. While the lack of mitochondrial organization has been correlated to poor implantation in the human (Scott and Smith, 1998), the exact influence of mitochondrial distribution on embryo competence remains to be determined.

The molecular analysis of mitochondrial dynamics began in 1997 with the identification of the *Drosophila* mitofusin gene Fzo (Hales and Fuller, 1997). Fzo is a mitochondrial outer membrane GTPase required for mitochondrial fusion during spermatogenesis. The core genes mediating mammalian mitochondrial fusion are Mfn1, Mfn2 and Opa1. Mfn1 and Mfn2, the mammalian homologues to Fzo, are large GTPases with similar homology and topologies that reside on the outer mitochondrial membrane (Chen and Chan, 2004). Opa1 is an inter membrane

space protein and is also essential for fusion (Detmer and Chan, 2007). The exact relationship between these proteins remains unclear; however cells lacking any of the three proteins have been shown to have inhibited respiratory capacity. Additionally, the contact between the inner and outer membranes, where these proteins reside, is essential to mitochondrial fusion (Detmer and Chan, 2007). During apoptosis structural changes in mitochondria, such as fragmentation, occur, leading to increased fission activity. Concurrently, mitochondrial outer membrane permeabilization (MOMP) causes the release of contents of the intermembrane space, including cytochrome c, which is sequestered in the cristae, into the cytoplasm. Once in the cytosol, cytochrome c activates caspases that propagate and execute apoptosis. Inhibition of fission blocks mitochondrial fragmentation, reduces cytochrome c release and can reduce or delay apoptosis (Detmer and Chan, 2007). The core genes mediating mitochondrial fission are Drp1, Fis1 and MTP18. Drp1 is a large dynamin-related GTPase located primarily in the cell cytosol. A portion of Drp1 moves to the mitochondrial tubule where it interacts with Fis1 (Parone et al., 2006). Fis1 is a transmembrane protein anchored to the mitochondrial outer membrane. Both Drp1 and Fis1 have been implicated in mitochondrial outer membrane permeability and mediate downstream apoptosis (Chen and Chan, 2004). Inhibition of Drp1 has been shown to delay mitochondrial division and partially inhibit apoptosis. MTP18 is a mitochondrial protein that contributes to the balance of mitochondrial fission and fusion. Changes in the expression levels of MTP18 interfere with the balance of mitochondrial fission and fusion; whereas loss results in increased mitochondrial fusion and an increase results in increased number of fragmented mitochondria (Tondera et al., 2005).

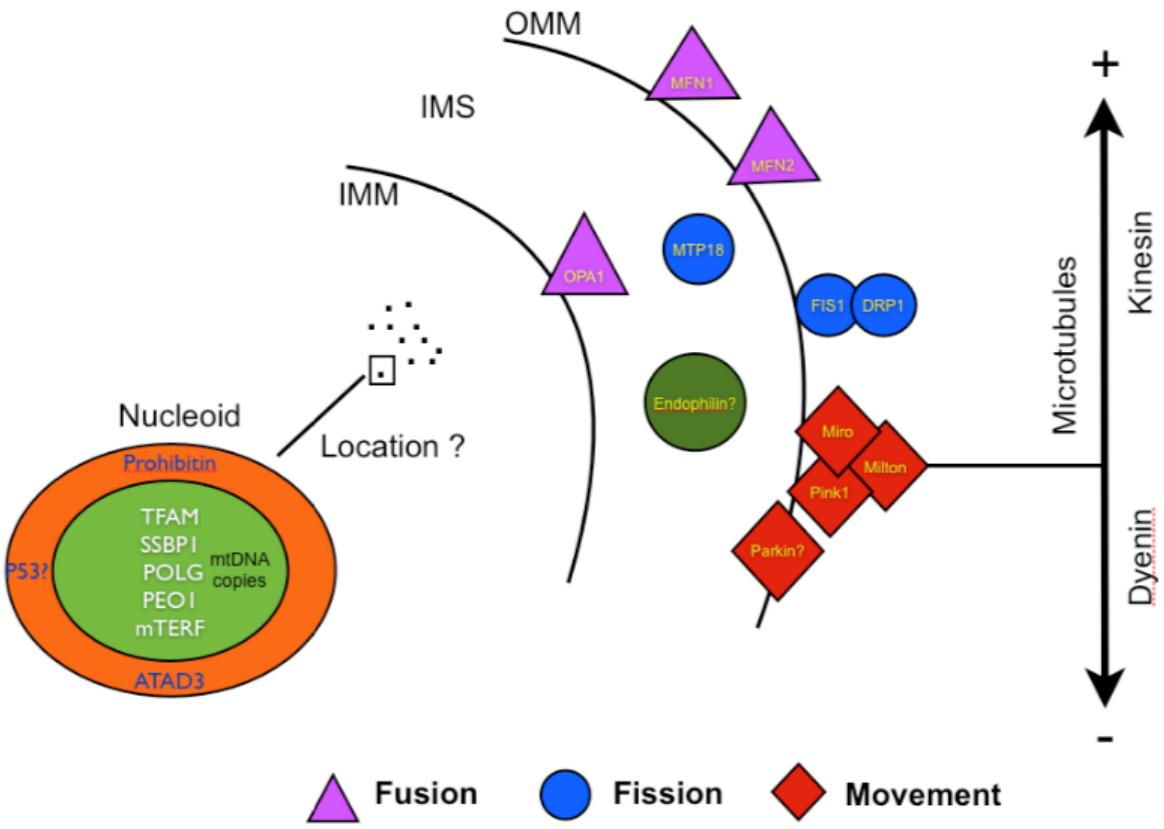

Figure P2: Proposed interaction of genes involved in the control of mitochondrial dynamics, movement and mtDNA replication.

Mitochondria, which are among the most abundant and mobile membrane-bound organelles, move along both microtubules and actin, using microtubule-based molecular motors for long distance movements (Morris and Hollenbeck, 1995) (Figure P2). Distribution of mitochondria varies in response to multiple regulatory cues such as energy requirements, growth factors and mitochondrial membrane potential (Chada and Hollenbeck, 2004; Miller and Sheetz, 2004; Morris and Hollenbeck, 1993). Conventional kinesin moves mitochondria to the plus ends of microtubules, while dynein moves them toward the minus ends (Pilling et al., 2006; Tanaka et al., 1998). Two research groups identified two different components important for transport; Milton (Stowers et al., 2002), involved with kinesin heavy chains, and Miro (Guo et al., 2005), a GTPase which is an integral outer mitochondrial membrane protein. Mutation in either of these genes appears to abolish anterograde mitochondrial transport. Glater et al. (Glater et al., 2006)

showed that kinesin, Milton, and Miro work together in anterograde transport and that Milton attaches kinesin to mitochondria through Miro. Pink1, a putative serine–threonine kinase that localizes to the outer mitochondrial membrane, has been shown to have a role in cellular protection against oxidative stress and to affect mitochondrial dynamics. Recent findings have shown that Pink1 interacts with the Miro/Milton complex and that overexpression of either Miro or Milton can suppress mitochondrial fragmentation induced after Pink1 silencing (Weihofen et al., 2009). Pink1 and Parkin, a gene associated with Parkinson's disease, are linked in a common pathway that is involved in the protection of mitochondrial integrity and function (Pridgeon et al., 2007). Our lab has recently found that monkey oocytes have an abundance of Miro1 and 2, Milton, Pink1, Parkin, Mfn1, Mfn2 and Drp1 (unpublished data). Therefore we speculate that mitochondrial trafficking is important for fusion and fission mechanisms and may impact oocyte and embryo quality.

Oocyte and Embryo Quality

In order to adequately assess oocyte and embryonic potential, both qualitative and quantitative biology of the structure must be considered. The morphological appearance or qualitative assessment of oocytes and embryos coupled with growth rates are heavily relied upon in ART programs. Basically, the oocyte or embryo that best fits the established standards of what it "should" look like is considered the most likely to develop normally. While it is generally accepted what a "bad" embryo or oocyte looks like, it is entirely subjective as to what a "good" embryo looks like and is not a definitive indication of embryonic viability. While there are likely many avenues to explore when developing better methods for evaluating oocyte and embryo quality, two approaches we are particularly interested in are the proper timing of the resumption of meiosis and mitochondrial distribution and dynamics.

In order for proper fertilization to occur, the GV-stage oocyte, which is arrested at the

diplotene stage of metaphase I, must first resume meiosis and arrest again a MII, with the extrusion of the first polar body. The use of standard ART protocols in human infertility treatment can result in the failure of meiotic resumption in 15 – 27% of oocytes within the standard 36 h after the administration of human chorionic gonadotrophin (hCG) (de Vos et al., 1999; Junca et al., 1995). Immature oocytes (GV or MI) may be collected at oocyte retrieval along with mature MII oocytes. Typically, oocytes are collected 32-34 hours post hCG and cultured for approximately 4 hours, after which some immature oocytes will extrude a polar body and may subsequently be used for IVF. Multiple studies reveal that immature human MI and GV oocytes maturing overnight or even within a few hours have lower fertilization rates in comparison to oocytes that were mature at retrieval (Balakier et al., 2004; de Vos et al., 1999; DeScisciolo et al., 2000; Huang et al., 1999; Nogueira et al., 2000; Shu et al., 2007; Strassburger et al., 2004). Embryos originating from immature GV and MI oocytes incubated overnight to attain maturity were qualitatively inferior when compared to those resulting from oocytes that were mature at the time of retrieval (DeScisciolo et al., 2000; Nogueira et al., 2000). It is fairly common for immature MI oocytes from human IVF cycles to be fertilized and develop into 'morphologically normal' embryos (de Vos et al., 1999; Huang et al., 1999; Junca et al., 1995; Strassburger et al., 2004), however these embryos generally lead to reduced pregnancy and live birth rates (de Vos et al., 1999; Vanhoutte et al., 2005).

Mitochondria as Indicators of Oocyte and Embryo Quality

When considering the quantitative aspects of mitochondria in oocyte and embryo development, it is well established that both oocytes and embryos require a certain threshold of OXPHOS capacity to function and develop normally. During mitosis, mitochondria are distributed randomly among the blastomeres in the embryo. If a particular blastomere does not acquire a sufficient number of ATP producing mitochondria, it may fragment, thus affecting the

qualitative assessment of the embryo (Cummins, 2004).

Because mitochondria play such a significant role in oocyte and embryonic development, oocytes and embryos are also subject to the same oxidative damage as any slow or non-dividing cell or tissue. The accumulation of mtDNA mutations due to ROS damage or the aging process affects oocytes and embryos in the same manner that they affect skeletal muscle or neuronal tissue. The time at which these mutations accumulate and how they are passed from one generation to the next are areas of extensive research. Slow and non-dividing cells such as neurons, skeletal muscle and oocytes appear to harbor a higher percentage of mitochondrial mutations than rapidly dividing cells, possibly due to the proximity of mtDNA to highly mutagenic ROS generated in the mitochondria (Brenner et al., 1998). Understanding why a high proportion of seemingly normal oocytes give rise to developmentally incompetent embryos following IVF is increasingly important in infertility research. Oocytes arrested at the GV stage are relatively anaerobic and subsist in a metabolically quiet environment, which may protect the genome from DNA damage and mutation. Their OXPHOS needs are likely quite minimal, as long as there is sufficient available glucose, anaerobic ATP production may satisfy all energy needs. The presence of mtDNA deletions and point mutations in oocytes may have no effect prior to a replication event. However, once ATP requirements increase, metabolic deficiencies or replication disorders in some infertile women and in women of increased reproductive age may cause qualitatively normal oocytes to develop abnormally. In other words, mitochondrial dysfunctions or genetic anomalies present in the oocyte may be critical determinants of developmental competence of the embryo (Barritt et al., 2000b; May-Panloup et al., 2005; Van Blerkom, 2004). Human IVF pre-implantation embryos show high frequencies of abnormal development and early demise, with further losses seen after intra-uterine transfer, when measured by outcome per embryo.

While a direct relationship between mitochondrial mutations in oocytes and embryos and reproductive success has not been demonstrated, it is generally assumed that there must be a sufficient number of functional mitochondrial genomes in the embryo in order for it to develop and implant successfully (May-Panloup et al., 2005). A high proportion of genetically abnormal mitochondria in the oocyte could potentially reduce the number of functional mitochondria, leading to embryonic arrest, failed implantation or mitochondrial disease. More than 150 mtDNA rearrangements, including deletions, insertions and duplications have been identified in various somatic cells (Wallace, 1993). These mutations are responsible for a number of catastrophic neuromuscular diseases such as Kearns Sayer Syndrome (KSS), chronic PEO, and Pearson's syndrome. MtDNA rearrangements also accumulate with age and can become more prevalent in post-mitotic tissues (Cortopassi and Arnheim, 1990).

Among the mitochondrial mutations that have been described in humans, one that is termed the "common deletion" or ΔmtDNA4977 entails the deletion of 4977bp (Barritt et al., 1999; Brenner et al., 1998; Chen et al., 1995; Keefe et al., 1995). This mutation can be detected at frequencies as high as 30% to 50% in human oocytes, although its frequency appears to be considerably lower in embryos that are generated from the same cohorts of oocytes (Barritt et al., 1999; Brenner et al., 1998). Other studies show that oocyte-specific mutations predominate in the regulatory control region of the mitochondrial genome (Barritt et al., 2000a; Barritt et al., 2000b). Age-dependent accumulations of these mutations in the mitochondrial control region may be responsible for impaired transcription and regulated replication of mitochondria in oocytes from older women (Barritt et al., 2000b; Michikawa et al., 1999). Support for age-dependent increases in mitochondrial mutations comes from studies of skeletal muscle in rhesus macaques that not only showed the presence of the ΔmtDNA4977 deletion, but also revealed that multiple deletions existed in all animals older than 13 years. In some cases, the deletions were

so extensive that large portions of the mtDNA genome were missing (Lopez et al., 2000; Mehmet et al., 2001; Schwarze et al., 1995). These data provide intriguing insights into mitochondrial mutations and aging in the human female gamete that warrant further investigation in appropriate primate models.

NHP as a Model to Study Oocyte and Embryo Quality

NHP models are particularly relevant to study due to their close resemblance to humans in anatomy and physiology compared to more commonly employed laboratory animals, such as rodents. The NHP, particularly the rhesus macaque, is the most appropriate animal model to study due to evolutionary closeness and the dramatic similarities in reproductive function and peri-implantation development. Rhesus macaque menstrual cycles are similar to those of women in length and in steroid and protein hormone profiles. Similarities in physiology between humans and NHP are also evident for spermatogenesis, embryo preimplantation development and implantation. The monkey oocyte is similar in size, shape and appearance to that of the human and does not show the pigmented appearance of some livestock species (Michaels et al., 1982). Moreover, the developing monkey embryo displays similar requirements to those of humans during *in vitro* embryo culture. Studies involving ovarian, hormonal, and pituitary functions of rhesus macaques have consistently shown biological changes mirroring those known to occur in women. Rhesus monkey ART has been quite successful and provides an excellent animal model for human IVF. Furthermore, because of NIH federal restrictions, studies utilizing human oocytes and embryos from infertility clinics remain prohibited. Therefore, the NHP has been established as a clinical translational model for understanding mechanisms of oocyte maturation, reproductive aging, pre- and peri-implantation development, and derivation of ESCs for differentiation and therapeutic and regenerative medicine (Steuerwald et al., 2000). We postulate that mitochondrial dysfunction in oocytes and embryos may occur due to the

breakdown of mtDNA repair machinery which alters processes related to mtDNA mutations, nucleoid stability and mitochondrial dynamics. The oocyte is an ideal model to study since it is associated with dysfunctions such as a decrease in mitochondrial membrane potential, increase of mtDNA damages, chromosomal aneuploidies, the incidence of apoptosis, and changes in gene expression. It has been suggested that these mitochondrial changes may arise from ROS that are closely associated with the oxidative energy production and calcium. Thus understanding aspects of mitochondrial dynamics, such as fusion, fission and transport in the oocyte may lead to a better understanding of important biological mechanisms and thus leading to new therapeutics.

The central goals of this study were to evaluate mitochondria in NHP oocytes and embryos, to determine how mtDNA mutations and mitochondrial function affect oocyte and embryo quality and to further establish the rhesus macaque as an excellent comparative model to study preimplantation development in the human. The major topics addressed in this dissertation were:

- determine if there is a large mitochondrial mutation present in rhesus macaque oocytes and embryos similar to the common deletion (mtDNA4977) seen in humans
- determine if mature oocytes and IVF produced embryos from gonadotrophin stimulated rhesus macaques harbor a higher proportion of specific mtDNA mutations than immature oocytes from non-stimulated monkeys
- determine if the rhesus common mutation is present in *in vitro* and in *in vivo* derived embryonic stem cell lines, as well as adult stem cell lines
- determine if polymorphisms in the mtDNA control region as well as polymorphisms in the mtDNA repair mechanism, PolG, are associated with aging
- determine if failure of oocytes to reach meiotic maturity is due to specific polymorphisms

in the mtDNA control region

- determine if mtDNA control region sequence differences exist between embryos
- identify methods to improve gonadotrophin stimulation protocols and IVF efficiency at the Caribbean Primate Research Center
- determine if, during syngamy, expression of genes involved in mitochondrial dynamics, transport and replication are influenced by meiotic stage of gonadotrophin stimulated oocytes at the time of collection
- determine if expression of genes involved in mitochondrial dynamics, transport and replication are influenced by mitochondrial distribution during syngamy
- determine expression profiles of genes involved in mitochondrial dynamics, transport and replication during syngamy and early embryo development

CHAPTER 2

SPECIFIC AIM 1

Introduction

Specific Aim 1.1: To determine if the rhesus macaque can be utilized as a clinically translational model for mitochondrial mutations identified in human IVF.

Specific Aim 1.2: To determine if the rate of mitochondrial mutations in rhesus macaque oocytes and IVF produced embryos is increased due to gonadotrophin stimulation.

Understanding why a high proportion of seemingly normal oocytes give rise to developmentally incompetent embryos following IVF is a high priority for human infertility clinics. Human IVF preimplantation embryos show high frequencies of abnormal development and early demise, with further losses seen after intra-uterine transfer, when measured by outcome per embryo. One hypothesis is that mitochondrial dysfunctions or genetic anomalies in the oocyte may be critical determinants of developmental competence of the embryo (Barritt et al., 2000a; May-Panloup et al., 2005; Van Blerkom, 2004). More than 150 mtDNA rearrangements, including deletions, insertions and duplications have been identified in various somatic cells (Wallace, 1993). These mutations are responsible for a number of catastrophic neuromuscular diseases such as Kearns-Sayre syndrome (KSS), chronic progressive external ophthalmoplegia (CPEO) and Pearson's syndrome. Mitochondrial DNA rearrangements also accumulate with age, and can become more prevalent in post-mitotic tissues (Cortopassi and Arnheim, 1990). Slow and non-dividing cells such as neurons, skeletal muscle and oocytes appear to harbor a higher percentage of mitochondrial mutations than rapidly dividing cells, possibly due to the proximity of mtDNA to highly mutagenic ROS generated in the mitochondria (Brenner et al., 1998). Perhaps these mutations are due to the lack of mitochondrial repair activity by the DNA polymerase gamma (PolG) during oogenesis.

While a direct relationship between mitochondrial mutations in oocytes and embryos and reproductive success has not been demonstrated, it is generally assumed that there must be a sufficient number of functional mitochondrial genomes in the embryo in order for it to develop and implant successfully (May-Panloup et al., 2005). A high proportion of genetically abnormal mitochondria in the oocyte could potentially reduce the number of functional mitochondria, leading to embryonic arrest, failed implantation or mitochondrial disease.

Among the mitochondrial mutations that have been described in humans, one that is termed the “common deletion" or ΔmtDNA4977 entails the deletion of 4977bp (Barritt et al., 1999; Brenner et al., 1998; Chen et al., 1995; Keefe et al., 1995). This mutation can be detected at frequencies as high as 30% to 50% in human oocytes, although its frequency appears to be considerably lower in embryos that are generated from the same cohorts of oocytes (Barritt et al., 1999; Brenner et al., 1998). This is intriguing, considering that surplus embryos available for analysis in clinical IVF programs are generally classified as developmentally defective. In addition to the ΔmtDNA4977 deletion, a further 23 novel mtDNA rearrangements have been described in human oocytes and embryos (Barritt et al., 1999). Using a nested PCR strategy, mtDNA rearrangements can be detected in 51% and 32% of human oocytes and embryos, respectively. Multiple rearrangements were detected in 31% of oocytes and 14% of embryos. Other studies show that oocyte-specific mutations predominate in the regulatory control region of the mitochondrial genome (Barritt et al., 2000a; Barritt et al., 2000b). Age-dependent accumulations of these mutations in the mitochondrial control region may be responsible for impaired transcription and regulated replication of mitochondria in oocytes from older women (Barritt et al., 2000a; Michikawa et al., 1999). These data provide intriguing insights into mitochondrial mutations and aging in the human female gamete that warrant further investigation in appropriate primate models. Support for an age-dependent increase in mitochondrial

mutations comes from studies of skeletal muscle in rhesus macaques that not only showed the presence of the ΔmtDNA4977 deletion, but also revealed that multiple deletions existed in all animals older than 13 years. In some cases, the deletions were so extensive that large portions of the mitochondrial genome were missing (Lopez et al., 2000; Mehmet et al., 2001; Schwarze et al., 1995). The specific aims of this study, therefore, were to determine if mature NHP oocytes and embryos generated by IVF have a higher frequency of the rhesus macaque ΔmtDNA4977 deletion (common deletion) than immature NHP oocytes.

Material and Methods

Ovarian Stimulation, Oocyte Recovery and In Vitro Fertilization

All animals were used with approval of the Tulane University's Institutional Animal Care and Use Committee. Ovarian stimulation and oocyte recovery were performed as previously described by Schramm (Schramm et al., 2002). Briefly, females were observed for signs of menstrual activity and were subjected to follicular stimulation via intramuscular injections of recombinant human follicle stimulating hormone (FSH) over an eight day period. On the 9th day of FSH injections, the animal was injected with recombinant human chorionic gonadotropin (hCG). Cumulus-oocyte complexes were collected from anesthetized animals by laparoscopic follicular aspiration (27-30 hours post hCG) and placed in HEPES buffered TALP (modified Tyrodes' solution with albumin, lactate and pyruvate) medium (Bavister et al., 1983b) at 37°C. The cohort of oocytes was randomly split into two groups that were either immediately used for mtDNA analysis or subjected to IVF. Oocytes to be fertilized were transferred to equilibrated CMRL medium (Gibco Cell Culture, Invitrogen, Carlsbad, CA, USA) in oil with 5% CO_2 at 37°C.

Rhesus semen was collected by penile electroejaculation as described by Lanzendorf (Lanzendorf et al., 1990). Following liquefaction for 15 min at room temperature, the sample

was washed twice in TALP-HEPES by centrifugation for 7min. at 350g. Sperm count and motility analyses were performed and the spermatozoa were resuspended in TALP-HEPES; then the sperm suspension was stored at room temperature. Approximately 1 hour before insemination, spermatozoa were exposed to 1 mM dibutyryl cyclic adenosine 3',5'-monophosphate (dbcAMP) and 1mM caffeine for sperm activation (Bavister et al., 1983b).

For IVF, oocytes were inseminated with $5x10^4$ activated spermatozoa and cultured in 50 µl drops of CMRL at 37°C in 5% CO_2. Oocytes were examined 10-16 hours post insemination for the presence of pronuclei. Oocytes with pronuclei were cultured for 48 hr (to approximately the 8-cell stage) in HECM-9 (Bavister et al., 1983b).

DNA Purification

Embryos and oocytes (denuded of cumulus cells using 10mg/ml hyaluronidase dissolved in TL-HEPES) were stripped of their zonae pellucidae with acid Tyrode's solution (Sigma-Aldrich, St. Louis, MO, USA). They were individually placed into 0.2 ml PCR tubes with 3 µl of 0.1% polyvinyl alcohol (PVA) in phosphate buffered saline (PBS). Samples were stored at -20°C until use. Prior to PCR, 3 µl of a mixture of $4x10^{-4}$ M sodium dodecyl sulfate (SDS; Sigma-Aldrich) and 125 µg/ml proteinase K (Roche Diagnostics Corp, Indianapolis, IN, USA) were added to each tube for cell lysis. The tubes were incubated at 37°C for 1 hr, and then heated to 95°C for 15 min to inactivate proteinase K.

The gene-specific oligonucleotide primers used in this study were synthesized by Sigma Genosys (The Woodlands, TX, USA). To amplify the internal control mtDNA region and the rhesus common deletion simultaneously, the DNA was divided into two 3µl aliquots prior to PCR amplification. Each aliquot was amplified using a MyCycler Thermal Cycler (Bio-Rad, Hercules, CA, USA) in a 25 µl reaction volume containing 1.5 mM $MgCl_2$, 1 IU *Taq* polymerase, 200 µM each dNTP and 0.5 µM gene-specific primers using the following

amplification profile: 1 cycle of 95°C for 1 min, 30 cycles at 95°C for 25 sec, 62°C for 25 sec and 72°C for 2 min, followed by 1 cycle at 72° C for 5 min and then a hold at 4°C. For the nested PCR, 3µl of the first reaction served as a template. The nested PCR was performed using the following profile: 1 cycle of 95°C for 1 min, 40 cycles at 95°C for 25 sec, 62°C for 25 sec then 72°C for 2 min followed by 1 cycle at 72° C for 5 min and then a hold at 4°C. The evaluation of PCR results was performed by agarose gel electrophoresis. Eight microliters of nested PCR product and 2 µl of blue/orange loading dye were separated on 1.5% gel and stained with ethidium bromide. The positive control for the rhesus common deletion was DNA isolated from skeletal muscle derived from a 31 yr old male rhesus macaque, amplified under the same conditions as the oocytes and embryos.

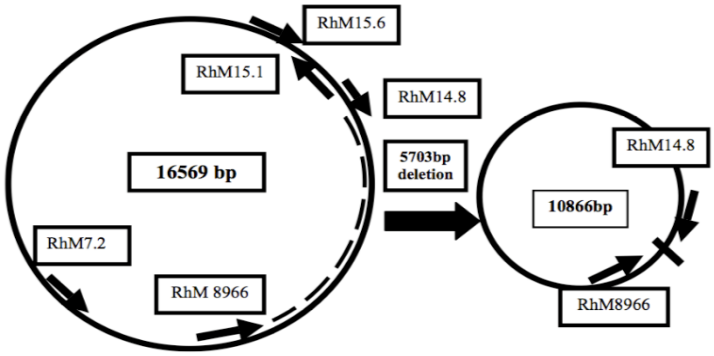

Figure 1: Nested PCR strategy used to detect the rhesus common deletion in the mitochondrial DNA genome. Primers RhM15.1 (inside arrow) and RhM15.6 (outside arrow) were used as an internal control for the presence of mtDNA. RhM15.6 (outside arrow) and RhM7.2 (inside arrow) were used in the first PCR amplification. Primers RhM14.8 (outside) and RhM8966 (inside) were used for the nested PCR reaction to identify the 5703 bp deletion. The dashed inside line represents the 5703 rhesus common deletion which is removed in the nested PCR reaction if mtDNA mutations are present in the sample. Black bar indicates deleted base pairs.

PCR Detection of the Rhesus Common Deletion

To detect the rhesus common deletion, a nested PCR strategy was applied. Oligonucleotide sequences were taken from mtDNA sequence analysis by Schwarze (Schwarze et al., 1995). PCR primers were designed based on the corresponding sites in the human mtDNA genome. Primer sequences and corresponding human mtDNA positions are shown in Table 1.

Primers RhM15.1 and RhM15.6 were used for amplification of a 599bp internal control mtDNA amplicon. Primers RhM15.1 and RhM7.2 were used in combination with RhM14.8 and RhM8966 using a nested PCR strategy to detect the rhesus monkey common deletion as shown in Figure 1.

Table 1. Polymerase chain reaction (PCR) primer sequences used to detect the rhesus common deletion in the mitochondrial genome

Name	mtDNA Location (nt)	Sequence (5'-3')
RhM15.1	15069	TCCTCCTAGAAACCTGAAACATTGG
RhM15.6	15643	AAGTATAGGGATGGCTGCTAGAATG
RhM7.2	7206	GGAATACCCCGACGCTACTCTG
RhM14.8	14849	AAAATTAGGCAGGCTGCAAGAAGTG
RhM8966	8966	TCAGTCTACTATTCAACCAGTGGC

Statistical Analysis

A 2x2 G test was performed to determine significant differences between percentages of non-stimulated oocytes vs. stimulated oocytes; non-stimulated oocytes vs. stimulated, unfertilized oocytes; non-stimulated oocytes vs. IVF embryos; and all non-stimulated vs. stimulated oocytes and embryos. The statistical formula used was

$$t_s = (\arcsin \sqrt{P_1}) - (\arcsin \sqrt{P_2}) / \sqrt{[820.1\ (1/n_1) + (1/n_2)]}$$

where P_1 and P_2 are the percent frequencies of the two groups being compared; n_1 and n_2 are respective sample sizes; and 820.1 is a constant representing the parametric variance of a distribution of arcsine transformations of percentages (Sokal and Rohlf, 1981). The arcsine is the angle, in degrees, whose sine corresponds to the value given and was determined from published tables (Rohlf and Sokal, 1981). The t_s values were compared to critical values of Student's t distribution using a two-tailed distribution.

Results

Characterization of the Rhesus Mitochondrial Common Deletion

To validate the assay for the detection of the rhesus common deletion, the nested PCR strategy shown in Table 1 and Figure 1 was employed. Primers RhM15.1 and RhM15.6 were

used to amplify DNA isolated from skeletal muscle tissue of a 31 year old rhesus monkey. A 599bp internal positive mtDNA control was observed in duplicate (Figure 2; lanes 1 and 2). The rhesus mitochondrial common deletion was amplified from the same tissue and showed a 180bp fragment (Figure 2; lanes 3 and 4). DNA sequencing of the 180bp amplicon demonstrated that a 5703bp deletion occurred in the rhesus mitochondrial genome. Using a nested PCR reaction as described in materials and methods, the remaining 180bp amplicon is the result of the deletion of the 5703bp, as shown in Figure 1.

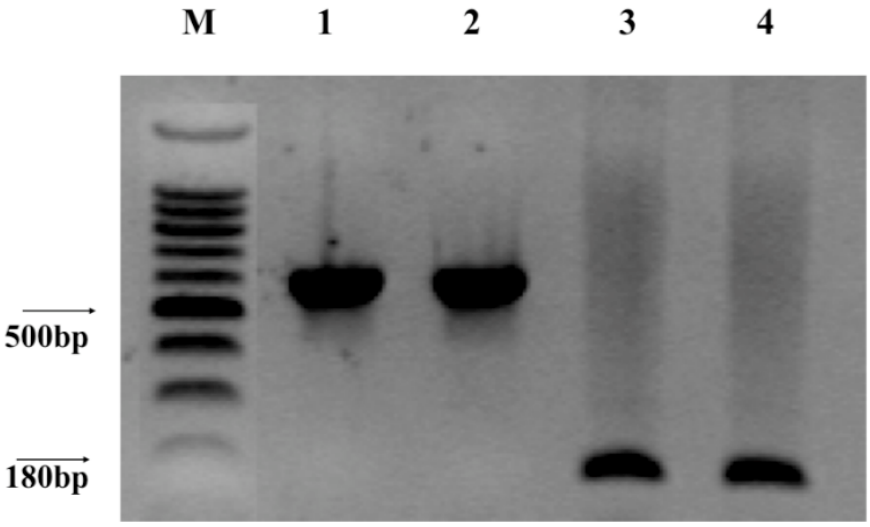

Figure 2: The rhesus common deletion was generated using DNA isolated from 31yr old rhesus skeletal muscle. Nested PCR products were separated on 1.2% agarose gel and stained with ethidium bromide. Lane M: 100 bp DNA Ladder. Lane 1 and 2 demonstrate the internal control region amplified with primers RhM15.6 and RhM15.1 showing a 599 bp band. Lanes 3 and 4 show the rhesus common deletion 180 bp amplicon generated using primers RhM15.1 and RhM7.2 followed by RhM14.8 and RhM8966.

Mitochondrial Deletions in Immature Oocytes

Using the same nested PCR strategy, a total of 127 immature oocytes excised from necropsied ovaries (n=13 animals) were examined for the presence of the rhesus common deletion. Only 27 of the 127 oocytes contained this deletion (21.3%). The amplification efficiency was over 90%. The intensity of the amplified PCR product varied, indicating different copy numbers of mtDNA among the oocytes analyzed. Skeletal muscle tissue from a 31 year old

monkey served as a positive control and showed the same 180bp amplicon illustrated in Figure 2

Mitochondrial Deletions in NHP Oocytes and Embryos

Using the same PCR amplification strategy, a total of 22 metaphase II (MII) oocytes (n=4 animals), 17 oocytes that failed to fertilize (n=3 animals) and 31 day three embryos (n=6 animals) were tested for presence of the common deletion. Surprisingly, 77.3% of the stimulated MII oocytes, 70.6% of failed fertilized oocytes and 67.7% of the embryos harbored this mutation (Table 2). Figure 3A (Lanes; 1-13) shows the positive mitochondrial control 599bp amplicon from each oocyte and embryo. Figure 3B (Lanes; 1, 2, 4, 5, 6, 8, 9) shows the 180bp common deletion. This mitochondrial deletion was not present in every oocyte and embryo in Figure 3B (Lanes; 3, 7, 6, 11, 12, 13). There was a substantial difference in the band intensity of the common deletion in oocytes and embryos. In addition, faint PCR products were observed with both the positive internal mitochondrial control primers and the nested primers.

Table 2. Detection of the mtDNA rhesus common deletion in rhesus oocytes and embryos by nested PCR.

Source	No. amplified	mtDNA Deleted	mtDNA Non-Deleted	Detected (%)
Necropsy oocytes	127	27	100	21.3[a]
Stimulated oocytes	22	17	5	77.3[b]
Stimulated UFOs[c]	17	12	5	70.6[b]
IVF embryos	31	21	10	67.7[b]

[ab] Statistically significant difference (P<0.001) by 2x2 G test and Students' t-Test distribution. A ratio of the number of rhesus common deletions amplified compared to the number of samples amplified indicates the amplification frequency. [c]UFO = failed to fertilized oocyte.

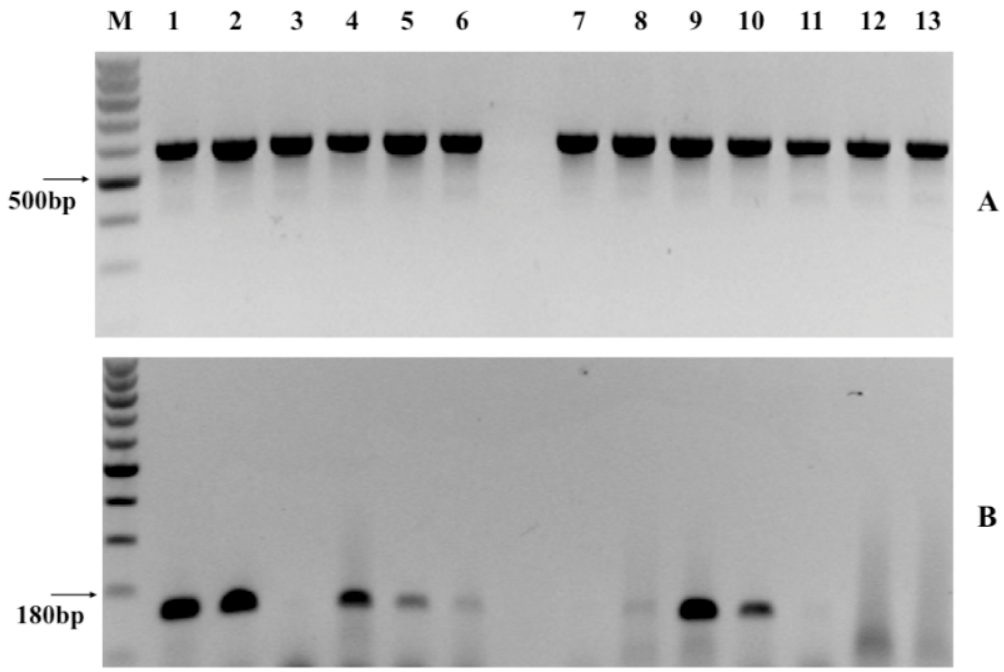

Figure 3: Rhesus control mtDNA and common deletion in rhesus gonadotrophin-stimulated oocytes and day 3 embryos. Lane M: 100 bp ladder. **A)** Lanes 1-6 demonstrates the internal control region amplified with primers RhM15.6 and RhM15.1 showing a 599 bp band in stimulated oocytes. Lanes 7-13 show a 599 bp band control amplicon in IVF day 3 embryos. **B**) Lanes; 1, 2, 4, 5, 6,7, 8, 9 show the rhesus common deletion or 180 bp amplicon generated in stimulated oocytes or day 3 embryos using primers RhM15.1 and RhM7.2 followed by RhM14.8 and RhM8966.

Comparison of Non-Stimulated Oocytes and Stimulated Oocytes and Embryos that Harbor the Mitochondrial Deletions

In the non-stimulated oocytes, 21.3% (27/127) contained the mitochondrial common deletion compared with a frequency of 71.4% (50/70) in stimulated oocytes and embryos ($P<0.001$, Tables 2 and 3). Additionally, there was a statistically significant difference ($P<0.001$) in the ratio of this mutation between non-stimulated oocytes and mature MII oocytes, embryos and oocytes that failed to fertilize, as a group.

Discussion

Over 100 mitochondrial mutation associated diseases have been identified. In addition, the accumulation of mtDNA deletions has been found in brain, cardiac muscle, skeletal muscle

and liver. It was imperative that the initial mtDNA analysis be done using somatic tissue, such as skeletal muscle, as the comparative baseline for mtDNA candidates in monkey oocytes and embryos. Skeletal muscle biopsies collected at 3-year intervals in rhesus macaques suggest that the increasing frequencies of mtDNA deletions are correlated with increasing age (Gokey et al., 2004). Specific Aim 1.1 of this study was to determine if the rhesus macaque can be utilized as a clinically translational model for mitochondrial mutations identified in human IVF. In order to do this, a nested PCR strategy was applied using skeletal muscle from a 31 year old monkey. Analysis by PCR generated a 180bp PCR mtDNA fragment, rather than the 930bp amplicon observed in human mtDNA (Figure 2; lanes 3 and 4). This indicates that the common deletion in rhesus monkeys entails the loss of 5703 base pairs, which is considerably larger than the 4977bp common deletion typically found in human KSS patients. One reason for this discrepancy might be that the rhesus mitochondrial genome has only 80.4% nucleotide sequence homology with the human mitochondrial genome and may contain altered sites of preferential replication errors (Gokey et al., 2004). Using the same nested PCR strategy shown in Figure 1, 127 immature oocytes and 70 stimulated oocytes and IVF embryos were evaluated for the rhesus mitochondria common deletion (Tables 2 and 3). Through these data, we conclude that the rhesus macaque is, in fact, an excellent model to study mtDNA mutations found in human oocytes and IVF produced embryos.

The second specific aim of this study was to determine if the rate of mitochondrial mutations in rhesus macaque oocytes and IVF produced embryos is increased due to gonadotrophin stimulation. We found that the frequency of common mtDNA mutation in immature germinal vesicle (GV) oocytes excised from necropsied ovaries was low (21.3%) when compared with gonadotrophin-stimulated MII oocytes and embryos (71.4%). The precise mechanisms responsible for this increase are unknown. It has been suggested that there is a

massive amplification of the mitochondrial genome during the process of oogenesis, presumably to support the initial period of embryonic development. The average mtDNA copy number of the human MII oocyte has been estimated to be 795,000 (+/- 243,000) (Barritt *et al*., 2002). However, it is not known whether the number of mtDNA molecules remains stable during primate preimplantation development. It has recently been suggested that mitochondrial replication occurs during a very short period of time from the 1-to 2-cell stage in the mouse embryo prior to fertilization (McConnell and Petrie, 2004). It is therefore unclear when mitochondrial transcription and replication actually begin in primate oocytes and embryos. It has been shown that preimplantation mouse embryos are dependent upon the energy produced from mitochondria derived solely from the oocytes, and that mitochondrial replication does not occur until the blastocyst stage (Piko and Taylor, 1987; Smith and Alcivar, 1993). In fact, the mtDNA copy numbers remain remarkably stable throughout preimplantation development. Moreover, it has been reported that there is a high variability of mtDNA copy numbers among individual mouse blastocysts, suggesting that some embryos may be able to initiate mtDNA replication before implantation (Thundathil et al., 2005). The increase in the mtDNA common deletion in stimulated rhesus MII oocytes and embryos shown here implies that mitochondrial replication may occur during either exogenous gonadotrophin stimulation, and/or during oocyte maturation in the rhesus macaque.

The mechanisms that regulate mitochondrial replication and function during oogenesis and preimplantation development are largely unknown but are likely dependent on nuclear-encoded factors, such as mitochondrial transcription factor (Tfam), nuclear respiratory factor 1 (Nrf1), mitochondrial RNA polymerase (Polmt) and TFB1M and TFB2M. It is unknown how mitochondrial replication processes might generate mitochondrial deletions and point mutations in aging tissues or non-dividing cells such as oocytes. One theory is that ROS in the vicinity of

the mtDNA can cause both single-stranded and double-stranded breaks before Tfam binds to and stabilizes the mtDNA (Trifunovic et al., 2004).

It has long been assumed that all of the mitochondria in the mature MII oocyte arise from the clonal expansion of an extremely small number of mitochondria during oogenesis, but it has never been determined precisely when these events take place (Van Blerkom, 2004). Perhaps during the clonal expansion of these mitochondria, the rapidity of the replication process causes mtDNA defects. Recent data show that oocyte ATP content increases significantly during porcine IVM similar to what has been reported in the cow (Brevini et al., 2005; Stojkovic et al., 2001). These data suggest that mitochondrial replication may also occur during porcine and bovine IVM.

One explanation for the high frequency of mtDNA defects in oocytes from gonadotrophin-stimulated humans and monkeys (Barritt et al., 1999; Brenner et al., 1998; Chen et al., 1995; Keefe et al., 1995) and present study) is that administration of high doses of exogenous FSH recruits many more follicles than in a natural cycle, so that numerous defective oocytes that were destined for atresia are aspirated along with normal oocytes. As a corollary, the proportion of oocytes with mtDNA defects increases in older primates because most of the "good" oocytes with normal mtDNA have already been recruited. If this were true, then we would expect GV stage oocytes in unstimulated ovaries to contain a high incidence of mtDNA defects, which would not change much, if at all, following gonadotrophin stimulation. However, our data contradict this explanation. We found that the frequency of mtDNA defects (the common deletion) was relatively low (21%) in GV oocytes from unstimulated rhesus ovaries, but increased more than 3-fold (71%) in oocytes from follicles recruited using FSH stimulation. This result suggests that potentially the administration of exogenous FSH causes the mtDNA defects in oocytes. It is very unlikely that the answers can be found from human clinical data

because of the inherent priority to obtain pregnancy rather than to examine different gonadotrophin stimulation approaches. Instead, the etiology of mtDNA defects in primate oocytes must be examined using NHP, such as the rhesus monkey, in which experiments can be designed to specifically evaluate possible effects of gonadotrophin stimulation on oocyte quality and competence. Such experiments are in progress in our laboratory to determine if extensive mitochondrial damage occurs during gonadotrophin stimulation and if this damage can be repaired via the nuclear mitochondrial transcription factor polymerase gamma (PolG) during early primate embryo development.

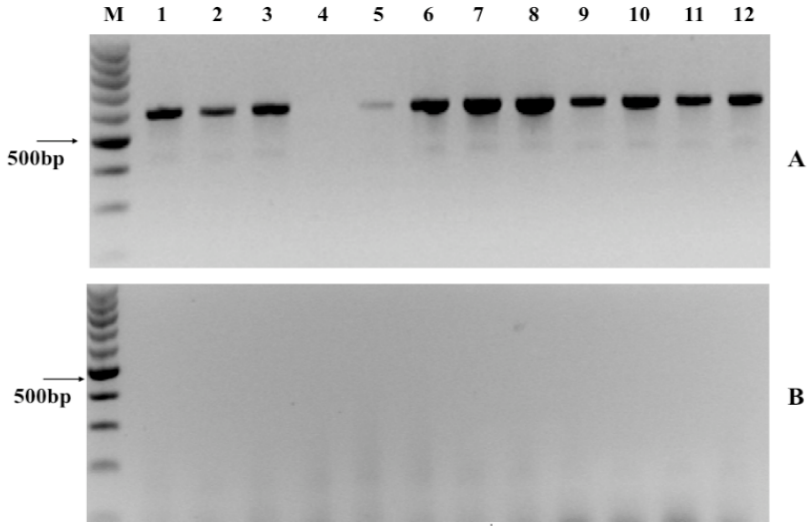

Figure 4: Rhesus mtDNA control and common deletion in non-stimulated germinal vesicle (GV) oocytes. Lane M: 100 bp DNA Ladder. **A**) Lanes 1-12: 590 bp mtDNA amplified with primers RhM15.1 and RhM15.6. **B**) Lanes 1-12 show that no PCR amplicons were generated in the same oocytes using primers RhM15.1 and RhM7.2 followed by RhM14.8 and RhM8966. ***NOTE:** Please see publication of this work for further detail (Gibson et al., 2005).

Table 3. Frequency of the mtDNA rhesus common deletion in stimulated oocytes and embryos vs. non-stimulated oocytes.

	No. amplified	mtDNA Deleted	mtDNA Non-Deleted	Detected (%)
Non-Stimulated	127	27	100	21.3[a]
Stimulated	70	50	20	71.4[b]

[ab] Statistically significant difference (P<0.001) by 2x2 G test and Students' t-Test distribution. A ratio of the number of rhesus common deletions amplified compared to the number of samples amplified indicates the amplification frequency.

CHAPTER 3

SPECIFIC AIM 2

Introduction

Specific Aim 2: Due to the frequency of the rhesus mtDNA common mutation in gonadotrophin stimulated, rhesus macaque MII oocytes and IVP embryos, we hypothesize that the common mutation will also be present in embryonic stem (ES) cells derived from IVP produced embryos.

Numerous laboratories have detected a specific mtDNA mutation, known as the common deletion (ΔmtDNA4977), in human oocytes and IVP embryos at a frequency of 30% to 50% (Barritt et al., 1999; Brenner et al., 1998; Chen et al., 1995; Keefe et al., 1995). These oocytes and embryos were obtained from infertile patients. Our laboratory has recently reported that the rhesus mitochondrial common deletion is present in fertile rhesus macaque oocytes and embryos (Gibson et al., 2005). Oocytes from necropsied ovaries of rhesus macaques show that the common deletion naturally occurs in 21% of GV oocytes (Gibson et al., 2005). Gonadotrophin stimulation increases the frequency of this deletion to 77% in MII oocytes, and 68% in day three IVP embryos (Gibson et al., 2005). These results imply that the administration of exogenous FSH may induce mtDNA defects in oocytes, embryos and their subsequent embryonic stem cell derivatives.

It is widely known that production of embryonic stem cell lines from the inner cell mass (ICM) of blastocysts is difficult and incurs considerable expense. At present, 18 rhesus macaque ES cell lines have been produced. Ten of these lines are derived from IVP blastocysts (ORMES series) and eight from *in vivo* produced blastocysts (R series) flushed from the uteri of monkeys after artificial insemination (Pau and Wolf, 2004).

Given that mtDNA mutations are present in gonadotrophin stimulated, rhesus macaque

MII oocytes and IVP embryos, it can reasonably be inferred that such defects also persist in the ES cells derived from them. Thus, the presence of numerous mtDNA deletions and point mutations may actually reflect the quality of affected oocytes or primate ES cells lines. Primate ES cell lines may harbor mutant mtDNA that may further accumulate during cell culture, and thus have consequences for long-term viability. Therefore, one objective of this study was to determine the prevalence of the rhesus mitochondrial common deletion in ES cells. Future medical applications of stem cell therapies include the use of cells originating not only from embryos but also from adult tissues (Bavister et al., 2005). Therefore, we investigated whether the common mitochondrial mutation was present in multiple passages of an adult bone marrow stromal stem cell line (BMSC). Finally, mtDNA from an adult adipose stromal stem cell line (ATSC) was compared with mtDNA from ATSC-TERT, the same line immortalized through transfection with a retroviral vector expressing telomerase.

Material and Methods

Animals

All animal procedures conformed to the requirements of the Animal Welfare Act and protocols were approved before implementation by the Institutional Animal Care and Use Committee (IACUC) of Tulane University and the Oregon National Primate Research Center.

DNA Extraction and PCR Analysis

To detect the rhesus common deletion, a nested PCR strategy was applied. Total cellular DNA, from TERT-ATSCs (passages (p) 10 and 20); ATSCs (p11 and 16); BMSCs (p1, 10, 20 and 30); as well as the ES cell lines ORMES 1 (p21), 2 (p11), and 7 (p24); and R4 (p20), was isolated using the Puregene Genomic DNA kit (Gentra Systems, Minneapolis, MN, USA). The gene-specific oligonucleotide primers used in this study were synthesized by Sigma Genosys (The Woodlands, TX, USA). Using a MyCycler Thermal Cycler (Bio-Rad, Hercules, CA,

USA), each sample was amplified in a 25 µl reaction volume containing 100 ng of DNA, 1.5 mM $MgCl_2$, 1 IU *Taq* polymerase, 200 µM each dNTP, and 0.5 µM gene-specific primers. For all cell lines, the following amplification profile was used: 1 cycle of 95°C for 1 min; 30 cycles at 95°C for 25 sec, 62°C for 25 sec and 72°C for 2 min; followed by 1 cycle at 72° C for 5 min; and a hold at 4°C. For the nested PCR, 2µl of the first reaction served as a template. The nested PCR was performed using the following profile: 1 cycle of 95°C for 1 min; 40 cycles at 95°C for 25 sec, 62°C for 25 sec then 72°C for 2 min; followed by 1 cycle at 72° C for 5 min; and a hold at 4°C. Duplicate PCR reactions were performed for each sample as well as a genomic GAPDH control for assessing PCR efficiency. The detection of all PCR results was detected by agarose gel electrophoresis, and stained with ethidium bromide

PCR Detection of the Rhesus Common Deletion

Oligonucleotide primers used for amplification of the target sequences of mtDNA and corresponding human mtDNA positions have been previously published (Gibson et al., 2005). Primers for rhesus GAPDH were: GAPDH forward ACCACCATGGAGAAG GCTGG; and GAPDH reverse TCAGTGTAGCCCAGGATGC, resulting in a 528bp DNA product after PCR amplification.

Results

Mitochondrial Deletions in NPH Oocytes and Embryos

Using a nested PCR amplification strategy (Gibson et al., 2005), all samples amplified a 599bp amplicon as a positive mtDNA control (Figure 5A). A high proportion of rhesus macaque MII oocytes and day 3 IVP embryos were found to harbor the rhesus mitochondrial common deletion (Figure 5B). Over 75% of MII oocytes (n=22) and greater than 65% of IVP embryos (n=31) harbored this mutation. Furthermore, differences in intensity of the common deletion amplicon were detected among individual oocytes and embryos.

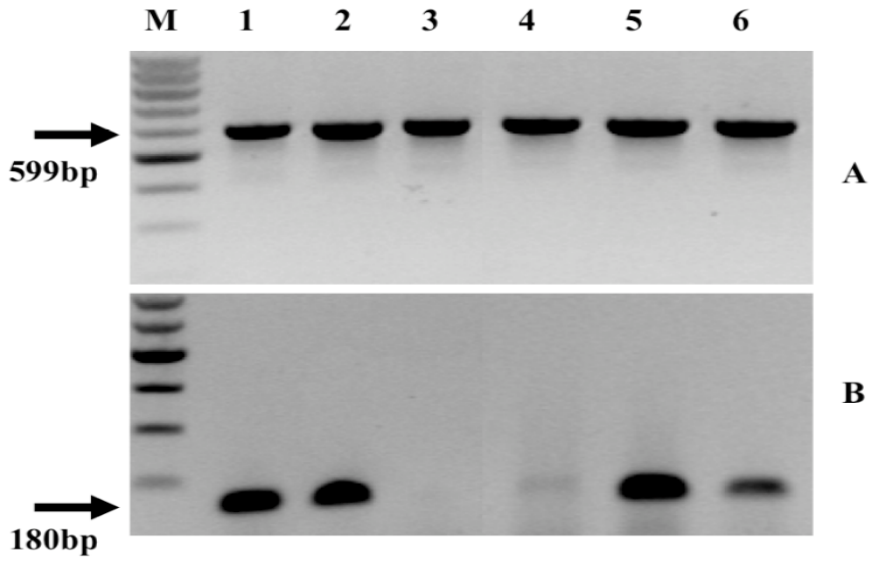

Figure 5: Rhesus control mtDNA and the common deletion in rhesus gonadotrophin-stimulated oocytes and day 3 embryos. Lane M: 100 bp ladder. Figure 1A, Lanes 1-3 demonstrate the internal control region showing a 599 bp amplicon in stimulated oocytes. Lanes 4-6 in 1A show a 599 bp band control amplicon in day 3 IVF embryos. Figure 1B, Lanes 1-3 show the rhesus common deletion, a 180 bp amplicon generated in stimulated oocytes. Lanes 4-6 in 1B show the common deletion generated in day 3 IVP embryos.

Mitochondrial Deletions in NHP ES Cell Lines

Using nested PCR, the rhesus mitochondrial common deletion was detected in all three IVP ESC lines; ORMES 1 (p21), 2 (p11) and 7 (p24), as well as from the R4 (p20) cell line, a rhesus ES cell line derived from an *in vivo* produced embryo (Figures 6 and 7, respectively). DNA sequencing of the 180bp amplicon (Figure 6, Lanes 2, 5 and 8; Figure 7, Lanes 3 and 4) demonstrated that it corresponded to the 5703bp deletion in the rhesus mitochondrial genome, as previously shown (Gibson et al., 2005). This mtDNA deletion corresponds to the same deletion found in rhesus macaque oocytes and day 3 embryos (Figure 5B). To confirm the presence of both mitochondrial and genomic DNA, a 599bp internal control (Figure 6, Lanes 1, 4 and 7; Figure 7, Lanes 1 and 2) and a 528bp rhesus GAPDH (Figure 6, Lanes 3, 6 and 9) were amplified and detected.

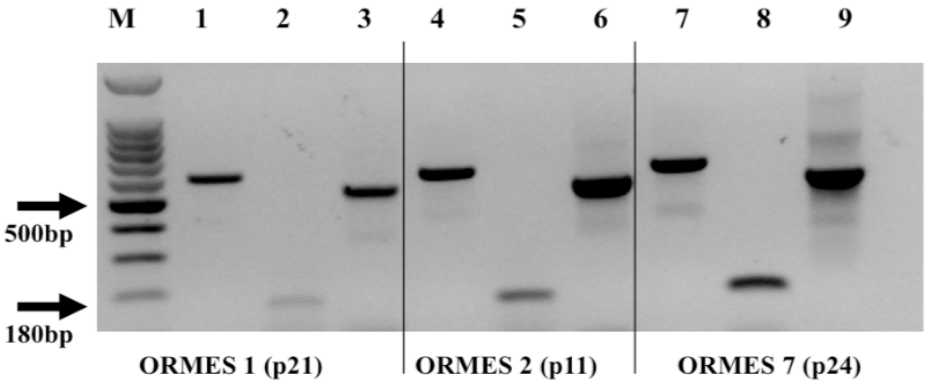

Figure 6: Rhesus control mtDNA, common deletion and genomic GAPDH in 3 *in vitro* derived ES cell lines ORMES 1, 2, and 7. Lane M: 100 bp ladder. Lanes 1, 4, and 7 demonstrate the internal control region showing a 599 bp amplicon in lines 1, 2 and 7, respectively. Lanes 2, 5 and 8 show the rhesus common deletion, a 180 bp amplicon generated in lines 1, 2, and 7, respectively. Lanes 3, 6 and 9 show a genomic GAPDH control, a 528 bp amplicon generated in lines 1, 2 and 7, respectively.

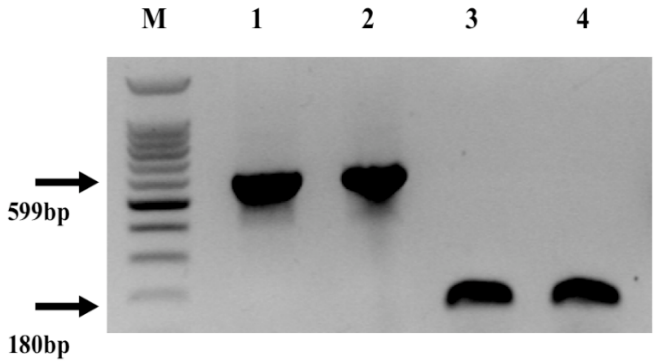

Figure 7: Rhesus control mtDNA and common deletion in the *in vivo* derived ES cell line R4. Lanes M: 100 bp ladder. Lanes 1 and 2 demonstrate the internal control region showing duplicate 599 bp amplicons. Lanes 3 and 4 show the rhesus common deletion, duplicate 180 bp amplicons.

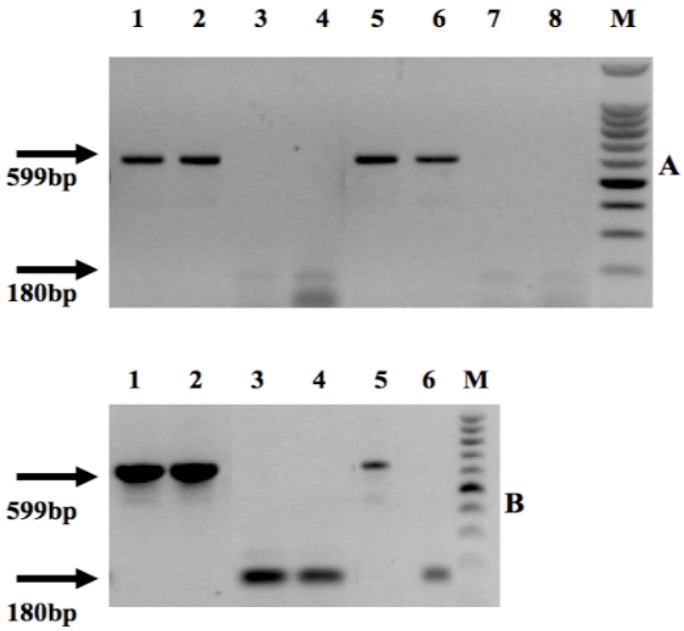

Figure 8: Rhesus control mtDNA and common deletion in the TERT-ATSC and the ATSC cell lines. Lane M: 100 bp ladder. **A**) Lanes 1, 2, 5 and 6 demonstrate the internal control region showing duplicate 599 bp amplicons from the TERT-ATSC cell line in passages 10 and 20, respectively. Lanes 3, 4, 7 and 8 show the rhesus common deletion, a very faint duplicate 180 bp amplicons from the TERT-ATSC cell line in passages 10 and 20, respectively. **B**) Lanes 1, 2 and 5 demonstrate the internal control region showing a 599 bp amplicon in duplicate (lanes 1 and 2) and a single replicate (lane 5) from the ATSC line, passages 11 and 16, respectively. Lanes 3, 4 and 6 show the rhesus common deletion, a 180 bp amplicon in duplicate (lanes 3 and 4) and a single replicate (lane 6) from the ATSC line, passages 11 and 16, respectively.

Detection of the Rhesus Common Deletion in Adult Stem Cells

A non-human primate adipose stromal cell line (ATSC), a telomerase induced adipose stromal cell line (TERT-ATSC) and a bone marrow stromal cell line (BMSC) were examined for the mtDNA common mutation. Interestingly, the TERT-transfected ATSCs grew continuously and the rhesus common mitochondrial mutation was only faintly detected, regardless of passage number (Figure 8A, Lanes 3, 4, 7, and 8). Non-transfected ATSC cells harbored the mtDNA mutation at passages 11 and 16 (Figure 8B, Lanes 3, 4 and 6). The BMSC cell line showed high levels of mitochondrial mutations in passages 3, 10 and 30 (Figure 9, Lanes 3, 4, 7, 8, 11 and 12). Both the internal control (see Figure Legends 8 and 9) and genomic GAPDH (data not shown) were detected in all adult stem cell lines.

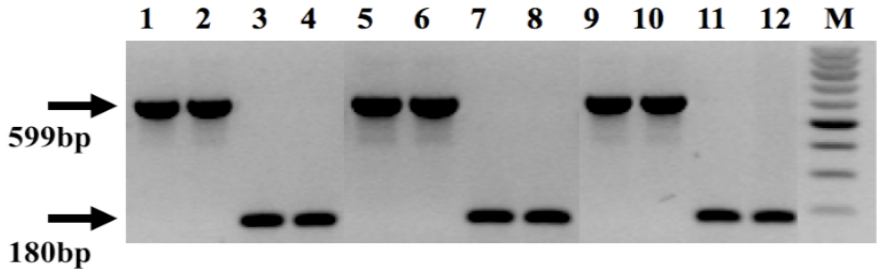

Figure 9: Rhesus control mtDNA and common deletion in the BMSC cell line. Lane M: 100 bp ladder. Lanes 1, 2, 5, 6, 9 and 10 demonstrate the internal control region showing duplicate 599 bp amplicons in passages 3 and 10 and 30, respectively. Lanes 3, 4, 7, 8, 11 and 12 show the rhesus common deletion, duplicate 180 bp amplicons in passages 3, 10 and 30, respectively. (***NOTE:** Please see Appendix B for this publication. (Gibson et al., 2006))

Discussion

This is the first study to examine mtDNA mutations in embryonic and adult NHP stem cell lines. In conjunction with our previous report (Gibson et al., 2005), it is now clear that mtDNA mutations are detectable in oocytes, embryos, adult and ES cells from fertile rhesus macaques. Over 60% of oocytes and embryos from gonadotrophin stimulated monkeys contain mitochondrial deletions, which carried into ES cell derivatives. It is likely that repeated passaging of ES cell lines derived from these oocytes increases the occurrence of deletions that may eventually render the cells metabolically impaired and, as a result, lead to developmental incompetence (Bavister et al., 2005), although this remains to be determined.

Increasing accumulation of mtDNA mutations may be linked to aging, which has been associated with functional competence of stem cells through changes in the activity of telomerase, the reverse transcriptase that elongates telomeres. Transfection of the catalytic subunit of telomerase, TERT, extends the lifespan of the transfected cell line, thus preventing senescense (Flores et al., 2005; Kang et al., 2004). In the present study, mtDNA from an adipose stromal cell line (ATSC) was compared with mtDNA from an immortalized (ATSC-TERT) cell line. Interestingly, the presence of the common deletion in the ATSC-TERT cell line was diminished compared with levels exhibited in the control (non-transfected) ATSC cell line

(Figure 8). Similarly, adult bone marrow stromal stem cells (BMSC) showed high levels of the rhesus mtDNA common deletion, regardless of passage (Figure 9). Furthermore, ongoing studies are in progress to determine whether additional deletions and point mutations are present in representative primate stem cell lines.

Disruption of ATP production in stem cell lines may not only alter cell division rates, but may also be related to premature apoptosis, mitochondrial disorders, the accumulation of chromosomal abnormalities such as aneuploidies, and early differentiation or possible failure to differentiate (Barnett et al., 1997; Chinnery et al., 2004; Eichenlaub-Ritter et al., 2004; McConnell and Petrie, 2004; Van Blerkom et al., 2000; Van Blerkom et al., 1995a). Preliminary results from our laboratory also indicate that mitochondrial properties, such as ATP production, may change in adult stem cell lines, supporting the results reported in the present study that adult stem cell lines may harbor defects in mitochondrial integrity and function (Lonergan, unpublished results). It has also been proposed that functional mitochondrial defects could also result from the asymmetric distribution of mitochondria during cell division, leading to disproportionate mitochondrial inheritance and a subsequent diminished ATP-generating capacity (Van Blerkom et al., 2000; Van Blerkom et al., 1995b).

The successful use of stem cell lines for therapeutic cell replacement or for drug development and testing will require that cells exhibit physiological and molecular stability over extended periods of culture. If stem cell lines accumulate mtDNA mutations with increased time in culture, the functional competence of these lines may be compromised. Our results, as well as others (Maitra et al., 2005), indicate that the characterization of mtDNA mutations in both non-human primate and human cell lines has important implications for establishing the normality of cell lines prior to use in future studies. A strong argument has previously been presented for the detailed characterization of NHP stem cell lines (Bavister et al., 2005).

At this time, there are no established molecular and cellular markers to ascertain which cell lines are defective or functionally competent. The key question is what functional characteristics, important for normal differentiation of these cell lines, should be used to select the most normal lines for further in-depth studies, particularly prior to organ and tissue derivation for use in human ES cell technologies? It is of concern that mtDNA mutations are found in a wide spectrum of cancers and aging tissues (Wallace, 2005). The possibility remains real that the use of aberrantly developing stem cells for therapeutic cell replacement could lead to the development of cancer. NHP ES cells, created from presumptively normal blastocysts, are derived from oocytes of young, fertile monkeys. However, results of the present study show that regardless of their origin, i.e., the *in vivo* derived cell line (R4) and the ES cell lines generated from IVP embryos, all lines exhibited the common deletion (Figures 6 and 7). These results suggest that the culture conditions under which the cell lines are maintained, or the derivation process, may alter the occurrence of this and possibly other deletion(s). However, whether the *in vivo* derived embryos exhibit the common deletion prior to generation of the stem cell line still needs to be determined.

It is clear from the present study that the existing stem cell lines, whether generated from *in vitro* or *in vivo* derived embryos, are not suitable for therapeutic purposes and perhaps not for drug testing. However, their characterization will enable identification of other useful stemness markers. It remains to be determined which lines should be selected for: (i) the development of improved, controlled culture conditions, free from serum and mouse feeder cell layers so that ES cells can be more reliably maintained in the undifferentiated state with karyotypic and mitochondrial stability; (ii) elucidation of control factors, (i.e., specific alterations to the culture milieu), growth factors, hormones, etc., to direct differentiation into derivative cell types and tissues; and (iii) comparisons of early versus late passage cells to establish mitochondrial

stability in prolonged culture. These studies will be considerably costly and time consuming. It is therefore imperative to select the best ES cell lines to be used as the starting material before committing major resources.

Mechanisms that regulate mitochondrial function during oogenesis and preimplantation development are largely unknown but are likely dependent upon nuclear-encoded mitochondrial transcription factor (Tfam), nuclear respiratory factor 1 (Nrf1), mitochondrial RNA polymerase (Polmt) and mitochondrial transcription factor B1 (Tfb1m) and B2 (Tfb2m). It is currently unknown how mitochondrial replication processes might generate mitochondrial deletions and point mutations in aging tissues or non-dividing cells, such as oocytes. Double stranded breaks may actually occur during the mitochondrial replication process. Furthermore, the DNA helicase (Twinkle) or replication stalling caused by mutations in polymerase gamma (PolG) may be partially responsible for these replication malfunctions. DNA PolG is required for both replication and repair of mtDNA. It has been shown that homozygous knock-in mice lacking PolG's exonuclease function harbored deletions and mutagenesis of mtDNA with a premature aging phenotype (Trifunovic et al., 2004). In further studies, we plan to investigate the molecular control of mitochondrial transcription and replication during IVP production of preimplantation embryos (Pei and Brenner, unpublished results).

We have shown that the common deletion occurs naturally in the rhesus oocyte; that the prevalence of this mutation is dramatically increased by gonadotrophin stimulation; and that there is continued presence of the deletion in the day 3 IVP embryo. Due to the existence of this same mutation in all 3 IVP derived ES cell lines examined, out of the 10 existing lines, it is logical to extrapolate that many, if not most, NHP ES cell lines may harbor this mutation. The rhesus common deletion was also found in two adult stromal stem cell lines. Additionally, in the presence of a subunit of telomerase known to extend the lifespan of cells, the occurrence and/or

replication of the mutation is limited. In both non-human primate and human stem cell lines, it is currently unknown what mutations exist and to what extent. It is imperative that ES cell lines be further characterized and that culture improvements be made. NHP ES cells present a valuable alternative to human ES cells in order to address issues of safety and efficacy of transplanted organs and tissues in NHP before any such clinical studies should be allowed in humans.

CHAPTER 4

SPECIFIC AIM 3

Introduction

Specific Aim 3.1: To determine if increases in the presence of mutations in the mtDNA control region, as well as the mitochondrial polymerase, PolG, are associated with aging in the rhesus macaque.

Specific Aim 3.2: To determine if increases in the presence of mutations in the mtDNA control region are associated with meiotic failure in oocytes.

It is thought that progressive damage to mtDNA during life contributes to the aging process. Mitochondrial DNA rearrangements accumulate with age and become predominant in post-mitotic, non-dividing tissues (Cortopassi and Arnheim, 1990). Similarly, in aging rhesus macaques, high levels of mtDNA deletions can accumulate in skeletal muscle (Schwarze et al., 1995). It has been shown that up to 50% of individuals over 65 years of age exhibit a T to G transversion at a base pair position (414) within the mitochondrial control region that was absent in younger individuals (Michikawa et al., 1999); as well as two other muscle-specific mutations that accumulate with age in the critical human mitochondrial control region (Wang et al., 2001). The tissues most commonly affected by mtDNA mutations and subsequent disorders are those that are normally affected by aging, i.e. brain, heart, skeletal muscle, kidney and the endocrine system (Wallace, 2005). It has been shown that there is a correlation between aging and the presence of the mitochondrial common deletion in skeletal muscle, however, to date mutations in the mtDNA control region have not been investigated. As previously stated, the mtDNA control region contains the origin of replication of the heavy strand, two initiation sites for transcription of the heavy strand mtDNA, the promoter for transcription of the light strand, and the primary and secondary initiation sites for heavy strand synthesis. In order to examine potential age

associated effects in skeletal muscle, we have established distinct age groups in the rhesus macaque that correlate to human reproductive ages. We compared the mtDNA control region sequences to a known rhesus macaque mtDNA sequence in order to determine if there were any significant changes associated with age.

One primary difference between nuclear DNA and mtDNA are the replication repair mechanisms present in the two genomes. It has been determined that the spontaneous mutation rate in mtDNA is more than two orders of magnitude higher than what is typically observed in nuclear DNA (Khrapko et al., 1997). Mitochondrial DNA do not have histones and there is only one known repair mechanism, the 3'-5' exonuclease function that is part of the only known mitochondrial polymerase, PolG. It is widely thought that the limited repair function is responsible for the high mutation rate seen in mtDNA. It has been shown that when the catalytic subunit of the mitochondrial polymerase, PolG, is knocked out it results in embryonic lethality in mouse embryos which causes early developmental arrest between embryonic days 7.5 and 8.5, associated with severe mtDNA depletion (Hance et al., 2005). Additionally, when homozygous knock-in mice that expressed a proof-reading-deficient version of PolGA, the catalytic subunit of PolG, were created, the mice developed an mtDNA mutator phenotype with a 3-5 fold increase in the levels of point mutations, as well as increased amounts of deleted mtDNA (Trifunovic et al., 2004). This increase in somatic mtDNA mutations is associated with reduced lifespan and premature onset of age-related phenotypes (Trifunovic et al., 2004). Considering how critical PolG is to mtDNA replication, we felt it was important to determine if mtDNA mutations in the catalytic subunit of PolG, as well as the region of such importance identified by Trifunovic et. al., are present in the same skeletal muscle samples used to determine if there are age related mutations in the mtDNA control region.

The final aim of this study was to determine if meiotic arrest of NHP oocytes is

correlated to mutations within the mtDNA control region. It has been proposed that in oocytes and preimplantation embryos accumulation of mtDNA mutations in the mitochondrial genome contributes to impaired metabolic function and thus to developmental incompetence (Barritt et al., 1999; Barritt et al., 2000b; Brenner et al., 1998; Chen et al., 1995; Keefe et al., 1995). Oocytes have large numbers of mitochondria, as discussed Chapter 1, that can support essential developmental processes such as oocyte growth and meiotic maturation. When tissues accumulate mtDNA rearrangements that reach a clinically significant threshold, the efficiency of oxidative phosphorylation is reduced (Cortopassi and Arnheim, 1990). It is generally assumed that there needs to be a sufficient number of normal mitochondrial genomes in order for the oocyte to mature and the subsequent embryo to develop and implant; however, a relationship between mitochondrial mutations and reproductive success is unproven. Therefore, we wanted to determine whether there is an increasing percentage of mtDNA mutational load within the control region corresponding to failure of oocytes to complete metaphase II of meiosis, enabling them to be fertilized and poor embryo development.

We compared mtDNA control region sequences from arrested GV, arrested MI, normal MII oocytes and preimplantation embryos within and among cohorts for mutations that may be causing meiotic arrest and poor embryo quality.

Materials and Methods

Experiment 1

Collection of material

Initially, we established age groups in which all animals were assigned: 1) <5 (juvenile), 2) 6-10 (early reproductive), 3) 11-20 (late reproductive) and 4) ≥21 (aged). These age groups were selected as they correlate to human reproductive ages. Establishment of these groups enabled us to examine any age effects that may be occurring during the aging process. The

rhesus macaque mtDNA control region, including the D-Loop, is approximately 1100bp long. MtDNA control region specific primers were designed and synthesized by Sigma Genosys (The Woodlands, TX, USA). Rhesus macaque skeletal muscle was collected by necropsy and flash frozen in liquid nitrogen (LN_2). Total DNA was extracted using a Puregene DNA purification system (Gentra, Minneapolis, MN, USA). Mitochondrial specific primers were used to detect the mtDNA control region, PolG catalytic subunit and PolG Exon 3. PCR reactions contained 100ng of total DNA in a 50µl reaction containing 1X Taq PCR buffer, 2.0mM MgCl2, 200µM each dNTP, 0.5µM each forward and reverse primers and 1.25U Taq DNA polymerase. PCR products were then submitted for direct sequencing. All sequences were performed using the BigDye Terminator sequencing reactions, purification and the analysis system by Applied Biosystems.

Experiment 2

Controlled Hormonal Ovarian Stimulation

The ovarian stimulation protocol has been previously described (Zelinski-Wooten et al., 1995). Briefly, rhesus macaques were hormonally stimulated with a sequential regimen of recombinant human gonadotrophins at either the Oregon National Primate Research Center (ONPRC) and the Caribbean Primate Research Center (CPRC). Female rhesus macaques at the ONPRC received rFSH (Organon, Oss, The Netherlands; 30 IU per injection, twice daily, IM) for 7 days, followed by 2 days of rFSH and rLH (EMD Serono, Rockland, MA; 30 IU rFSH and 30 IU rLH per injection, twice daily, IM). During the final 3 days of the rFSH treatment, animals also received the GnRH antagonist Acycline (0.075 mg/kg animal body weight) to prevent a spontaneous LH surge. Ovarian stimulation of rhesus macaques at the CPRC was performed by administration of rFSH (Organon, Roseland, NY; 37.5 IU per injection, twice daily, IM) for 10 days. At both primate centers, on the final day of rFSH treatment, 32–33 hours

before follicular aspiration, oocyte maturation was induced with a single injection of recombinant hCG (750–1,000 IU, IM).

Oocyte and Sperm Collection, Insemination, and Embryo Culture

Procedures for oocyte recovery, sperm collection, insemination, and embryo culture have been described previously (Bavister et al., 1983a; Boatman and Bavister, 1984; Wolf et al., 1989). Briefly, follicular fluid was collected in TALP-HEPES containing 0.3% bovine serum albumin (BSA). The aspirates were sifted through a cell strainer and oocytes were collected after rinsing the filter. After cumulus removal using hyaluronidase (0.03%), oocytes were rinsed, meiotic stage was determined (GV, MI or MII) and placed in these groups in TALP medium culture drops supplemented with BSA, (Bavister and Yanagimachi, 1977), and incubated in 5% CO_2 in air at 37°C. GV, MI and half of the cohort of MII oocytes were stripped of their zonae pellucidae with acid Tyrode's solution (Sigma-Aldrich, St. Louis, MO, USA). They were individually placed into 0.2 ml PCR tubes with 3 µl of 0.1% polyvinyl alcohol (PVA) in phosphate buffered saline (PBS). Samples were stored at -20°C until use. Semen was obtained by electroejaculation (Mastroianni and Manson, 1963) and seminal plasma was removed according to standard protocols. Spermatozoa were activated with dbcAMP and caffeine (1 mM each) and used for insemination of the remaining MII oocytes at a final concentration of 1.5–2.0×10^5 sperm/mL. The presence of pronuclei was assessed 18–20 hours after insemination. Presumptive zygotes were transferred to amino acid-supplemented HECM-9 culture drops (McKiernan and Bavister, 2000) and incubated under a 5% CO_2 in air atmosphere at 37°C until day 3. Approximately 72hrs post insemination embryos were frozen in the same manner as the rest of the cohort of oocytes.

Embryos and oocytes (denuded of cumulus cells using 10mg/ml hyaluronidase dissolved in TL-HEPES) were stripped of their zonae pellucidae with acid Tyrode's solution (Sigma-

Aldrich, St. Louis, MO, USA). They were individually placed into 0.2 ml PCR tubes with 3 µl of 0.1% polyvinyl alcohol (PVA) in phosphate buffered saline (PBS). Samples were stored at -20°C until use. Prior to PCR, 3 µl of a mixture of $4x10^{-4}$ M sodium dodecyl sulfate (SDS; Sigma-Aldrich) and 125 µg/ml proteinase K (Roche Diagnostics Corp, Indianapolis, IN, USA) were added to each tube for cell lysis. The tubes were incubated at 37°C for 1 hr, and then heated to 95°C for 15 min to inactivate proteinase K.

The gene-specific oligonucleotide primers used in this study were synthesized by Sigma Genosys (The Woodlands, TX, USA). To amplify the internal control mtDNA region and the rhesus common deletion simultaneously, the DNA was divided into two 3µl aliquots prior to PCR amplification. Each aliquot was amplified using a MyCycler Thermal Cycler (Bio-Rad, Hercules, CA, USA) in a 25 µl reaction volume containing 1.5 mM $MgCl_2$, 1 IU *Taq* polymerase, 200 µM each dNTP and 0.5 µM gene-specific primers using the following amplification profile for all reactions (annealing temperature specific for each primer pair): an initial denaturation of 95ºC for 2 min, followed by 30 cycles of 95ºC for 30s, annealing for 30s and primer extension for 1min at 72º C, with a final extension of 72ºC for 5min. PCR products were then submitted for direct sequencing. All sequencing was performed using the BigDye Terminator sequencing reactions, purification and the analysis system by Applied Biosystems.

Sequence Analysis

Sequence analysis was performed on all rhesus macaque skeletal muscle, oocytes and embryos from IVP. Only sequences with a quality of Q20 or greater were used for analysis. For control region and PolG sequence analysis, consensus sequences were created for each skeletal muscle sample for both regions of interest. For oocyte and embryo sequence analysis, consensus sequences were created for each animal and oocyte group, (i.e. Animal 18879 GV) and compared to each other and the mtDNA control region of the Macaca Mulatta mitochondrion

complete genome (NCBI Accession AY612638) using Sequencher Software by Gene Codes Inc.

Results

Rhesus Macaque Skeletal Muscle Sequencing

A total of 18 rhesus macaque skeletal muscle samples were collected and assigned to each of the four age groups: 1(n=7), 2(n=4), 3(n=4) and 4(n=3). Due to the limited number of aged animals in captivity, group 4 had the lowest number of samples available. MtDNA consensus sequences from both skeletal muscle and reproductive material were compared to the mtDNA control region of the Macaca Mulatta mitochondrion complete genome (NCBI Accession AY612638) and to each other. One area within the mtDNA control region became an area of interest once all samples were compared.

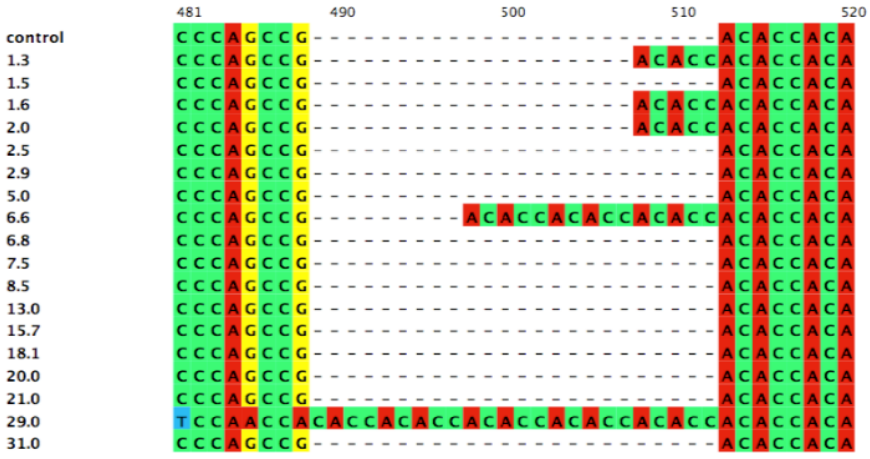

Figure 10: Region of interest within the mtDNA control region. All sequences are identified by the age of the animal at the time of death and aligned to control sequence.

From mtDNA nucleotides 251-252 insertion of an ACACC repeat ranging from 1-5 repeats were found in 5 samples (Figure 10). This region corresponds to the H-Strand promoter region and a TFAM binding site. This insertion was seen in three samples in age group 1, one sample in age group 2 and one sample in age group 4 (Table 4). There was no correlation between advanced aging and this insertion. In fact, three out of seven samples in the youngest age group (group 1) were found to harbor this insertion (Table 4). The muscle sample from the

29 y.o. animal had an extremely high rate of point mutations, insertions and deletions (Appendix 1).

Table 4: Number of skeletal muscle samples analyzed in each of the four age groups and number of samples that exhibited the insertion in the TFAM binding site in each of the four age groups.

	Group 1	Group 2	Group 3	Group 4
# samples	7	4	4	3
# samples with insertions	4	1	0	1

All 18 skeletal muscle samples analyzed for mtDNA control region inserts were also analyzed for mutations within the catalytic subunit of the mitochondrial polymerase, PolG. We found no consistently exhibited mutations in the catalytic subunit and no correlation between advance age and accumulation of mutations (Appendix 2).

Rhesus Macaque Oocyte and Embryo Sequencing

Oocytes from 5 rhesus IVF cycles and embryos from 2 rhesus IVF cycles were analyzed (Table 5).

We found no correlation between meiotic arrest during oocyte maturation and mtDNA control region mutations (Appendix 3). We believe the lack of correlation between aging and oocyte maturation to mtDNA control region mutations indicates there are other factors influencing the aging phenotype as well as meiotic arrest during oocyte maturation.

Table 5: Number of oocytes and embryos sequenced from each animal.

	18879	19733	21129	21176	22031	Total
GV	-	7	3	-	-	10
MI	-	8	5	4	3	20
MII	5	7	5	4	5	26
Day 3 Embryos	-	8	4	-	-	12

Discussion

The purpose of this specific aim was threefold: 1) to determine if there are mutations in the mtDNA control region associated with the aging phenomenon, 2) to determine if there are mutations in the catalytic subunit of the mitochondrial polymerase, PolG, associated with aging,

and 3) to determine if there are mutations in the mtDNA control region associated with failure of oocytes to resume meiosis and develop to a fully mature state, (MII), enabling them to be fertilized. We sequenced the mtDNA control region of 18 rhesus macaque skeletal muscle samples from animals ranging in age from 1-31 year. We found no correlation between aging and the accumulation of mtDNA point mutations within the mtDNA control region. One muscle sample from an aged animal (29 y.o.) that we analyzed did contain numerous deletions, insertions and point mutations within the mtDNA control region (Appendix 1). This sample was from the same 29 year old male rhesus macaque that was analyzed by Gokey (Gokey et al., 2004). They too found that this animal contained numerous mtDNA breakpoints and deletions throughout the mtDNA genome. However, we also analyzed 2 other skeletal muscle samples from aged animals (31 y.o. and 21 y.o.) and found no point mutations in common with the 29 y.o. This indicated that point mutations and mtDNA control region deletions can accumulate with age, however it may be due to susceptibility of the individual animal and not due to the aging phenomenon. This hypothesis is supported by the fact that three out of seven samples analyzed that were from animals under 5 years of age showed multiple insertions and point mutations within the same region (bp 251-252), to those seen in the sample from a 29 y.o. animal (Appendix 1). These animals may have been susceptible to mtDNA damage from a very young age and therefore likely would have accumulated more mutations as they aged due to clonal expansion of mutant mtDNA genomes, not necessarily due to the aging process causing mtDNA damage over time.

We sequenced the catalytic subunit of the mitochondrial polymerase, PolG, of all 18 samples sequenced for the mtDNA control region. PolG is encoded in the nucleus and contains two subunits: a large catalytic subunit that harbors the 3'-5' exonuclease and 5'deoxyribose phosphate (dRP) lyase activities, and a smaller accessory subunit which increases both the

catalytic activity and processivity of the catalytic subunit. Diseases such as PEO, KSS, Alpers syndrome and ataxia, as well as symptoms of premature menopause, progressive muscle weakness, parkinsonism, and male infertility are coupled with mutations in human PolG (Luoma et al., 2004; Mancuso et al., 2004; Naviaux and Nguyen, 2004; Van Goethem et al., 2001; Zeviani et al., 1988; Zeviani et al., 1989). Two particular studies led us to examine the catalytic subunit of PolG in the rhesus. First, Trifunovic found that homozygous knock-in mice that expressed a proof-reading-deficient version of the catalytic subunit of PolG developed an mtDNA mutator phenotype with a 3-5 fold increase in the levels of point mutations, increased amounts of deleted mtDNA as well as reduced lifespan, weight loss, reduced subcutaneous fat, hair loss, curvature of the spine, osteoporosis, anaemia, reduced fertility and heart enlargement; all factors seen due to aging (Trifunovic et al., 2004). Secondly, Hance found that when the PolG catalytic subunit is knocked out it results in embryonic lethal and PolG deficiency in mouse embryos which causes early developmental arrest between embryonic days 7.5 and 8.5, associated with severe mtDNA depletion (Hance et al., 2005). While both of these studies were in mice and knock-out primates have yet to be developed, we felt it relevant to determine if there were any mutations in the rhesus catalytic subunit of PolG, associated with aging, that were detectable in tissue from rhesus macaques at different ages. We found no correlation between accumulation of mutations in the catalytic subunit of PolG and the aging phenomenon (Appendix 2).

We sequenced the mtDNA control region of 10 arrested GV oocytes, 20 arrested MI oocytes and 28 normally maturing MII oocytes from 5 rhesus macaques (Table 5). We found no consistently exhibited point mutations or deletions in either GV, MI or MII oocytes, indicating that the failure of oocytes to complete MII of meiosis is not caused by alterations in the mtDNA control region (Appendix 3). Furthermore, we found no consistently exhibited point mutation or

deletions in day 3 embryos that originated from normal MII oocytes, indicating that there are likely no changes or mutations to the mtDNA control region during fertilization and early embryo development. Due to the limited number of samples analyzed, we agree that these findings are not statistically significant and do not lead to conclusions about either the involvement, or lack there of, of either the mtDNA control or PolG to the aging process or meiotic failure. The DNA sequencing methods available at the time of this study were limited, especially considering the complex, technologically advanced methods that have recently been developed. Furthermore, we did not sequence the entire mtDNA genome of any sample, however we feel the analysis of the control region, which is the location where numerous process involved in mtDNA replication and transcription (Table 6), may indicate that the specific sequence of the mtDNA control region may not be critical to the process of meiotic maturation of oocytes.

Table 6: Sites within the mtDNA control region and base pair locations.

Function	Location
Control Region with D-Loop	16024-576
Hypervariable Region I	16024-16383
Termination Sequence	16157-16172
Control Element	16194-16208
L-strand Control Element	16499-16506
Hypervariable Region II	57-372
H-strand Replication Origin	110-441
Conserved Sequence Block I	213-235
TFAM Binding Site	233-260
TFAM Binding Site	276-303
Conserved Sequence Block II	299-315
Replication Primer	317-321
Conserved Sequence Block III	346-363
H-strand Control Element	371-379
H-strand Control Element	384-391
L-strand promoter	392-445
TFAM Binding Site	418-445
TFAM Binding Site	523-550
Major H-strand Promoter	545-567

CHAPTER 5

SPECIFIC AIM 4

Introduction

Specific Aim 4: To determine if gonadotrophin stimulation efficiency can be improved through optimization of gonadotrophin stimulation protocols.

Our laboratory has been working with the Caribbean Primate Research Center (CPRC) for approximately 4 years in an effort to establish a reputable rhesus macaque IVF program in order to pursue developmental biology grants and research projects using the CPRC as a resource to provide high quality rhesus oocytes, embryos and sperm. In order to obtain rhesus macaque reproductive material of the highest quality, we worked onsite with the current staff at CPRC in order to improve the efficiency of the IVF program. Through previous collaboration with other primate research centers and upon working with CPRC, it became apparent that a significant inefficiency of the IVF program was the gonadotrophin stimulation regimen to which the monkeys were subjected. Individual NHP respond differently to gonadotrophin stimulation in the same manner that individual humans respond differently. The previous gonadotrophin stimulation protocol that was employed at the CPRC was as follows: 37.5IU rFSH twice daily for 10 days with 1000IU recombinant (rhCG) given on day 10 and oocyte collection 34 hours later. While this protocol worked intermittently, it generally resulted in a mediocre rate of mature oocytes (MII) upon collection and a high rate of GV oocytes. Therefore the objective of this specific aim was to identify and implement methods of gonadotrophin stimulation in order to improve upon the efficiency of rhesus macaque IVF at the CPRC.

Material and Methods

Controlled Hormonal Ovarian Stimulation

Ovarian stimulation and oocyte recovery was adapted from previously described

protocols (Schramm and Bavister, 1999). Briefly, rhesus macaque females were observed for signs of menstrual activity and subjected to follicular stimulation via intramuscular injections of (rFSH). Prior to this study, monkeys at the CPRC received 37.5 IU of rFSH twice daily, for 10 days. On the final day of rFSH treatment, 34 hours before follicular aspiration, oocyte maturation was induced with a single injection of 1000 IU recombinant hCG.

Oocyte Collection and Meiotic Stage Determination

Procedures for oocyte recovery, sperm collection, insemination, and embryo culture have been described previously (Bavister et al., 1983a; Boatman and Bavister, 1984; Wolf et al., 1989). Briefly, follicular fluid was collected in TALP-HEPES containing 0.3% bovine serum albumin (BSA). The aspirates were sifted through a cell strainer and oocytes were collected after rinsing the filter. After cumulus removal using hyaluronidase (0.03%), oocytes were rinsed, meiotic stage was determined (GV, MI or MII) and placed in these groups in TALP medium culture drops supplemented with BSA, (Bavister and Yanagimachi, 1977), and incubated in 5% CO_2 in air at 37°C.

Experiment 1: Reduction of FSH dose

Due to the high cost of rFSH used for gonadotrophin stimulation protocols, we wanted to determine if there was a significant reduction in the number of oocytes collected when the total daily dose of rFSH was reduced by half. Animals continued to receive injections twice daily, however instead of 37.5IU rFSH in each injection (75IU daily), animals received 18IU rFSH in each injection (36IU daily). Follicular aspiration was performed as described above and numbers of oocytes collected was recorded. Data from a total of 40 stimulation cycles was included in this comparison; 21 cycles using a high rFSH dose (75IU daily) and 19 cycles using a low rFSH dose (36IU daily). Comparisons were made using a paired t-test and results were considered statistically significant at ≤ 0.05.

Experiment 2: Addition of rLH

Recombinant luteinizing hormone (rLH) is commonly used in both human and NHP gonadotrophin stimulation protocols in an effort to mimic the acute rise and fall of LH seen in the natural cycle, which initiates ovulation. In this study, monkeys were given 15IU rLH on the evening of day 9 of rFSH stimulation and 15IU rLH on both the morning and evening of day 10 (30IU total on day 10). Follicular aspiration was performed as described above and numbers of GV, MI and MII oocytes collected was recorded. Data from a total of 40 stimulation cycles was included in this comparison: 24 cycles without LH and 16 cycles with LH. Comparisons were made using a paired t-test and results were considered statistically significant at ≤ 0.05.

Experiment 3: Replacement of recombinant hCG with biological hCG

Human chorionic gonadotrophin (hCG) is widely used in both human and NHP IVF to induce ovulation during gonadotrophin stimulation. Generally, a synthetically derived form of hCG (Ovidrel) is used instead of biologically derived forms. Multiple NHP IVF programs have had great success with recombinant hCG (rhCG), however, at the CPRC, cycles using rhCG typically produced inconsistent numbers of mature oocytes, with a consistently large number of oocytes remaining at the GV phase of meiosis. As previously discussed, oocytes must reach the meiotic stage of MII in order for fertilization to occur, thus, GV oocytes are not capable of being used for IVF related studies. Therefore, we replaced recombinant hCG with a biologically derived form (Pregnyl). The same dose (1000IU) and timing of injection were used in all cycles. Follicular aspiration was performed as described above and numbers of GV, MI and MII oocytes collected was recorded. Data from a total of 40 stimulation cycles was included in this comparison: 24 cycles using recombinant hCG and 16 cycles using biological hCG. Comparisons were made using a paired t-test and results were considered statistically significant at ≤ 0.05.

Results

Experiment 1: Reduction of rFSH dose: The average number of oocytes collected in the high rFSH group was 20.4 oocytes per collection, while the average number of oocytes collected in the low rFSH group was 20.8 oocytes per collection. Figure 11 shows that the reduction of the previously used dosage of rFSH (75IU) to approximately half (36IU) resulted in no significant difference in the average number of oocytes collected per stimulation.

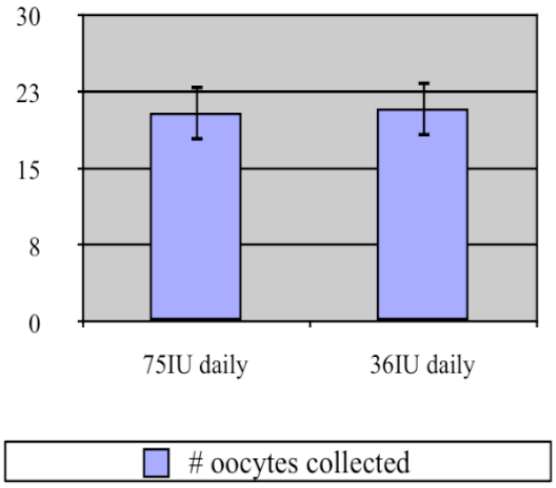

Figure 11: Comparison of average number of oocytes collected per stimulation using either high FSH (75IU daily) (n=21) or low FSH (36IU daily) (n=19).

Experiment 2: Addition of LH: When comparing the average percentage of MII, MI and GV oocytes collected per stimulation, we found no significant differences between stimulations with LH or without LH. While there was no significant difference found in the number of MII, MI or GV oocytes collected, there was an observable trend in the number of MII and GV oocytes collected (Figure 12) that we feel will become significant with the inclusion of data from more cycles.

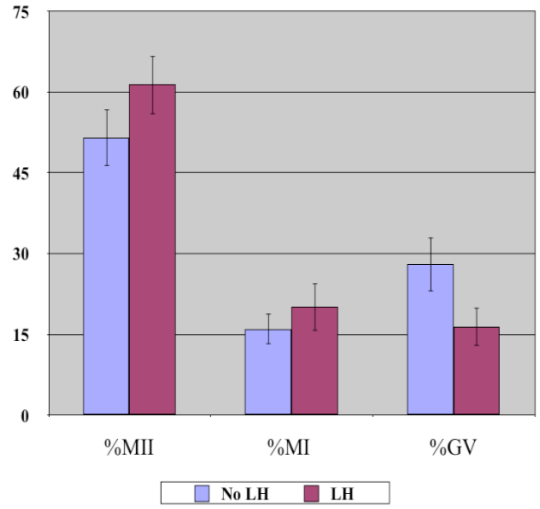

Figure 12: Average percentage of MII, MI and GV oocytes collected, per stimulation, without LH (n=24) compared to the average percentage of MII, MI and GV oocytes collected, per stimulation, with LH (n=16). Standard error bars are shown.

Experiment 3: Replacement of recombinant hCG with biological hCG: We found that the use of a biological hCG resulted in a significantly higher average percentage of oocytes resuming meiosis and completing germinal vesicle breakdown (GVBD). In Figure 13, we show a statistically significant increase in the average percentage of MII oocytes collected, as well as a statistically significant decrease in the average percentage of GV oocytes collected due to the replacement of R-hCG with B-hCG.

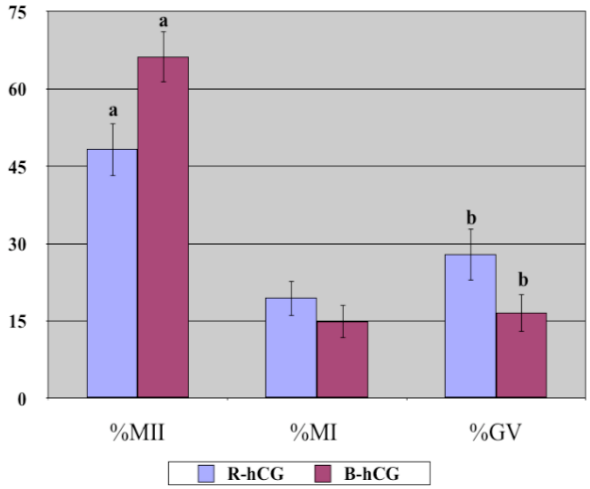

Figure 13: Average percentage of MII, MI and GV oocytes collected, per stimulation, with recombinant hCG (n=24) compared to the average percentage of MII, MI and GV oocytes collected, per stimulation, with biological hCG (n=16). Standard error bars are shown. Statistical significance: a,b ≤ 0.05.

Finally, we found that the combination of the protocol changes in Experiment 2 and 3, i.e. the addition of rLH and the use of biological hCG, resulted in a statistically significant increase in the average percentage of MII oocytes collected, as well as a statistically significant decrease in GV oocytes collected (Figure 14).

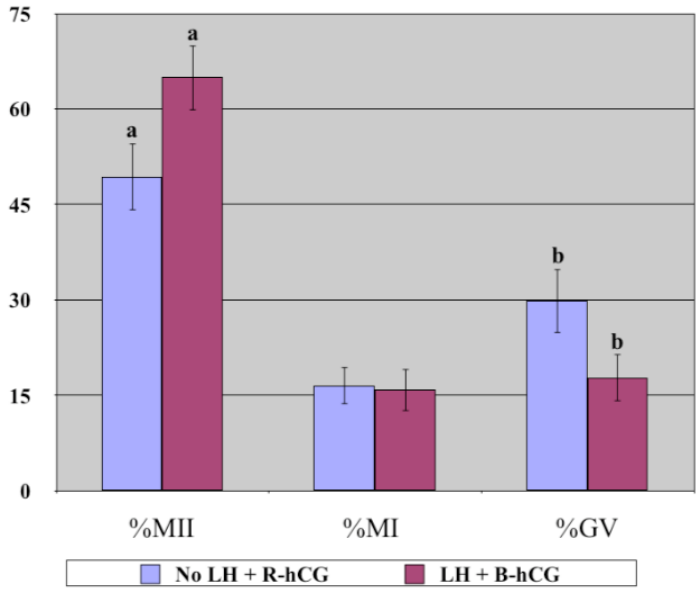

Figure 14: Average percentage of MII, MI and GV oocytes collected, per stimulation, without LH and using recombinant hCG (previous standard protocol) (n=22) compared to the average percentage of MII, MI and GV oocytes collected, per stimulation, with LH and using biological hCG (n=14). Standard error bars are shown. Statistical significance: a,b ≤ 0.05.

Discussion

The objective of this study was to identify and implement methods of gonadotrophin stimulation in order to improve upon the efficiency of rhesus macaque IVF at the CPRC. This was accomplished by 1) reducing the dose of rFSH, yet maintaining the number of oocytes collected per stimulation, thus reducing the overall cost per stimulation cycle; 2) by significantly increasing the number of meiotically "normal" (MII) oocytes collected that can be used in IVF related studies; and 3) by significantly reducing the number of meiotically "abnormal" (GV) oocytes collected that are unable to be used for IVF studies. The high dose of rFSH (75IU) used in the previous protocol, while occasionally resulting in higher oocyte numbers (data not shown),

also resulted in a significantly larger number of immature oocytes, which generally cannot be used for IVF experiments. While the trend in human IVF has been to collect as many oocytes as possible, embryologists and scientists are beginning to recognize the importance of quality oocytes over simply large cohorts. We have shown that reducing the rFSH dose by half has no significant difference on the average number of oocytes collected per stimulation (Figure 11).

The inclusion of rLH in gonadotrophin stimulation protocols is a common practice in both human and NHP IVF programs. However, until this study, it was not used at CPRC and it was unknown if there would be any benefit to adding it to the current protocol. While we were not able to find that the addition of rLH alone significantly increased the number of MII oocytes collected, we did find that the combination of the inclusion of rLH, as well as replacing recombinant hCG with biological hCG, did result in significantly increased numbers of MII oocytes collected, as well significantly decreased numbers of GV oocytes collected (Figure 14). This is the first study to show that, not only does B-hCG increase the number of oocytes collected after gonadotrophin stimulation that reach meiotic maturity (MII), thus allowing more oocytes to be used for IVF experiments, but this is also the first study to show that monkeys can be subjected to more than one dose of B-hCG without becoming refractory to the drug itself. It is well known that rhesus macaques can only be subjected to one stimulation cycle of biological FSH before becoming refractory to FSH stimulation, resulting in a complete lack of ovarian response and follicle development. This is the reason why most protocols use a recombinant form of FSH, as we have used throughout this study, in order to stimulate each animal multiple times. The practice of using an animal for multiple stimulation protocols is well established and reduces the number of animals needed for experimentation. Concomitantly, it has been assumed that a typical refractoriness would be seen with biological hCG as has been historically seen with biological FSH. However, we have found that animals can be given biological hCG up to three

times with no detrimental effect to the process of ovulation (data not shown). Furthermore, we have shown here that biological hCG significantly increases the number of meiotically "normal" oocytes (MII) collected as well as reducing the number of unusable oocytes (GV). The changes to standard IVF protocols we have used in this study will enable the collection of higher quality reproductive material to be used for future research projects as well as reducing unusable material and the overall cost of reproductive research in the rhesus macaque.

CHAPTER 6

SPECIFIC AIM 5

Introduction

Specific Aim 5.1: To determine if expression levels of genes controlling mitochondrial dynamics are correlated to the failure of oocytes to resume meiosis.

Specific Aim 5.2: To determine if expression levels of genes controlling mitochondrial dynamics are correlated to important preimplantation development time points.

Interest in the mitochondrion and its role in the preimplantation mammalian embryo has had a recent resurgence (Bavister and Squirrell, 2000; Van Blerkom, 2000). Prior studies have shown that mitochondria are initially morphologically immature; spherically shaped rather than elongated, with relatively few cristae; and undergo a maturation process prior to blastocoel formation. The general process of mitochondrial maturation has been described for multiple species; mouse (Hillman and Tasca, 1969); pig (Hyttel and Niemann, 1990); bovine (Plante and King, 1994) and primates (Panigel et al., 1975). It is also understood that mitochondria undergo a variety of species-specific reorganizations during early preimplantation development. Mitochondria in hamster embryos reorganize from a homogeneous distribution in the oocyte to a distinct perinuclear organization late in the pronucleate and two-cell stages (Barnett et al., 1996). Mitochondrial distribution has also been associated with developmental competence in multiple species including hamster (Barnett et al., 1997; Lane and Bavister, 1998; Squirrell et al., 2001); mouse (Muggleton-Harris and Brown, 1988); pig (Hyttel and Niemann, 1990) and cattle (Van Blerkom et al., 1990). It has been shown that in oocytes and early cleavage stage embryos in multiple species, including hamster and mouse (Van Blerkom, 1991), that mitochondria have a distinct pronuclear translocation and that perturbations from these patterns result in embryonic lethality. Human zygotes have shown a pronuclear accumulation of mitochondria (Noto et al.,

1993; Van Blerkom et al., 2000), as well as significantly different mitochondrial localization patterns between individual oocytes between and among cohorts. Furthermore, pronuclear oocytes that showed a pronounced asymmetrical mitochondrial distribution during syngamy continued to show an asymmetrical distribution after the first cell division, and between blastomeres throughout embryo development. When analyzed individually, the blastomeres containing less mitochondria also had lower ATP content compared to blastomeres with higher mitochondrial content (Van Blerkom et al., 2000). Additional studies in the rhesus have shown that zygotes exhibit a distinct organization of mitochondria that accumulate between the male and female pronuclei, similar to the human (Squirrell et al., 2003). There is also marked difference is the heterogeneity of mitochondrial organization among oocytes, which may reflect the heterogeneity of oocyte quality and developmental competence in primates, unlike homogeneity in hamsters (Barnett et al., 1996). It is possible that the localization of mitochondria in rhesus zygotes is a result of mitochondria undergoing fusion and fission processes during preimplantation resulting in what appears to be the microtubule network guiding the mitochondria to their pronuclear location. It has also been shown that the pronuclear accumulation of mitochondria in rhesus oocytes is transient, on the order of a few hours, although the exact length of time remains uncertain (Squirrell et al., 2003).

It is well established that during the early stages of development, embryo competence depends upon ATP produced by mitochondria inherited from the mother. During the early stages of embryo development, energy is produced by OXPHOS via amino acids, lactate and pyruvate, with the glucose pathway proving to be harmful at this stage; indicating that a threshold of mitochondrial activity is necessary to support energetic demands (Tarazona et al., 2006). Once embryonic genome activation begins, the metabolic activity of the embryo increases as it becomes capable of using alternative pathways such as anaerobic glycolysis,

allowing mitochondrial activity to decrease. While the lack of mitochondrial organization has been correlated to poor embryo development and poor implantation in the human (Scott and Smith, 1998), the exact influence of mitochondrial distribution on embryo competence remains to be determined.

The molecular analysis of mitochondrial dynamics began in 1997 with the identification of the *Drosophila* mitofusin gene Fzo (Hales and Fuller, 1997). Fzo is a mitochondrial outer membrane GTPase required for mitochondrial fusion during spermatogenesis. The core genes mediating mammalian mitochondrial fusion are Mfn1, Mfn2 and Opa1. Mfn1 and Mfn2, the mammalian homologues to Fzo, are large GTPases with similar homology and topologies that reside on the outer mitochondrial membrane (Chen and Chan, 2004). Opa1 is an inter membrane space protein and is also essential for fusion (Detmer and Chan, 2007). The exact relationship between these proteins remains unclear, however cells lacking any of the three proteins have been shown to have inhibited respiratory capacity. Additionally, the contact between the inner and outer membranes, where these proteins reside, is essential to mitochondrial fusion (Detmer and Chan, 2007). The core genes mediating mitochondrial fission are Drp1, Fis1 and MTP18. Drp1 is a large dynamin related GTPase located primarily in the cell cytosol. A portion of Drp1 moves to the mitochondrial tubule where it interacts with Fis1 (Parone and Martinou, 2006), which is a transmembrane protein anchored to the mitochondrial outer membrane. Both Drp1 and Fis1 have been implicated in mitochondrial outer membrane permeability and mediate downstream apoptosis (Chen and Chan, 2004). Inhibition of Drp1 has been shown to delay mitochondrial division and partially inhibit apoptosis. MTP18 is a mitochondrial protein that contributes to the balance of mitochondrial fission and fusion. Changes in the expression levels of MTP18 interfere with the balance of mitochondrial fission and fusion; whereas loss results in increased mitochondrial fusion and an increase results in increased number of fragmented

mitochondria (Tondera et al., 2005). As previously stated, mitochondria move along both microtubules and actin, using microtubule-based molecular motors for long distance movements (Morris and Hollenbeck, 1995). Distribution of mitochondria varies in response to multiple regulatory cues such as energy requirements, growth factors and mitochondrial membrane potential (Chada and Hollenbeck, 2004; Miller and Sheetz, 2004; Morris and Hollenbeck, 1993). Mutations in either of two of the components identified to be important for transport; Milton (Stowers et al., 2002) and Miro (Guo et al., 2005) appears to abolish anterograde mitochondrial transport. It has been shown (Glater et al., 2006) that kinesin, Milton, and Miro work together in anterograde transport and that Milton attaches kinesin to mitochondria through Miro. Pink1, a putative serine–threonine kinase that localizes to the outer mitochondrial membrane, has been shown to have a role in cellular protection against oxidative stress and to affect mitochondrial dynamics. Recent findings have shown that Pink1 interacts with the Miro/Milton complex and that overexpression of either Miro or Milton can suppress mitochondrial fragmentation induced after Pink1 silencing (Weihofen et al., 2009). Pink1 and Parkin, a gene associated with Parkinson's disease, are linked in a common pathway that is involved in the protection of mitochondrial integrity and function (Pridgeon et al., 2007). Our lab has recently found that monkey oocytes have an abundance of Miro1 and 2, Milton, Pink1, Parkin, Mfn1, Mfn2 and Drp1 (unpublished data). Therefore we speculate that mitochondrial trafficking is important for fusion and fission mechanisms and may impact oocyte and embryo quality.

Materials and Methods

Ovarian Stimulation, Oocyte Recovery and In Vitro Fertilization

All supplies were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated. Ovarian stimulation and oocyte recovery was adapted from previously described protocols by Schramm et al (Schramm and Bavister, 1999). Briefly, rhesus macaque females

were observed for signs of menstrual activity and subjected to follicular stimulation via intramuscular injections of (rFSH) as follows; Days 1-10 rFSH twice daily; day 9 (P.M.), (rLH) once and day 10 rLH twice (A.M. and P.M.). On the night of the 10th day, the animal was injected with bhCG. The employment of rLH in this manner was to mimic the endogenous surge of LH followed by hCG. Procedures for oocyte recovery, sperm collection, insemination, and embryo culture have also been described previously (Bavister et al., 1983a; Boatman and Bavister, 1984; Wolf et al., 1989). Briefly, follicular fluid was collected in TALP-HEPES containing 0.3% bovine serum albumin (BSA). The aspirates were sifted through a cell strainer and oocytes were collected after rinsing the filter. After cumulus removal using hyaluronidase (0.03%), oocytes were rinsed, meiotic stage was determined (GV, MI or MII) and oocytes were placed in these groups in modified CMRL medium and incubated in 6% CO_2 in air at 37°C. Rhesus semen was collected by penile electroejaculation (Lanzendorf et al., 1990). Sperm count and motility analyses were performed, spermatozoa cleaned with TL-HEPES medium, resuspended and stored in TALP and allowed to equilibrate in the incubator at 6% CO_2 at 37°C for at least two hours. Just before insemination, MI oocytes were examined for maturation and oocytes were inseminated in three groups; MII, MI and MI matured to MII (MI-MII) before insemination. This second assessment of oocyte maturation established a group of oocytes that matured from MI to MII between the time of collection (33-34 hrs post hCG) and the time of insemination (37-38 hours post hCG). Previous work in our laboratory (Dupont et al., 2009) has shown that this period of time is important in chromosomal aneuploidy and mosaicism. All oocytes were placed into equilibrated, modified TL within the three groups. Spermatozoa were activated with dbcAMP and caffeine (1mM each) and used for insemination of all MII, MI and MI-MII oocytes at a final concentration of $1.5–2.0\times10^5$ sperm/mL. Approximately 12-14 hours later, presumptive zygotes were cleaned of sperm and placed into fresh TL for fluorescent

imaging. To guarantee the removal of all granulosa cells and sperm, oocytes were aspirated with plastic stripper tips (Mid Atlantic Diagnostics, Marlton, NJ, USA).

For oxygen concentration studies, all oocytes were inseminated under high O_2 levels (6% CO_2/20% O_2). Fourteen hours after insemination, presumptive zygotes were randomly placed in amino acid-supplemented HECM-6 culture drops (Schramm and Bavister, 1996) and incubated under either high O_2 (6% CO_2/20% O_2) or low O_2 (6% CO_2 /5% O_2 /89% N_2) at 37°C until day 3. All embryos used for the oxygen concentration study originated from MII oocytes at the time of collection in order to eliminate the possibility of maturation stage differences.

Imaging

For zygotes: 13 hours post-insemination, zygotes exhibiting 2 pronuclei (2PN) were placed into MitoTracker Orange CMTMRos (Molecular Probes), which passively diffuses across the plasma membrane and accumulates in active mitochondria, as well as a nucleic acid stain, Hoechst 33342. After incubation for 45 minutes, zygotes were rinsed through 3 drops of fresh modified TL to remove excess stain, and immunofluorescence detection of mitochondrial localization and nuclei was assessed using fluorescence microscopy.

For embryos: approximately 72 hours post-insemination, digital images of each embryo was taken for assessment of quality grade.

Fluorescent and brightfield imaging was performed as quickly as possible in order to prevent damage caused by exposure to light.

RNA Analysis and cDNA Synthesis

Immediately after either fluorescent or brightfield imaging, zygotes and embryos were individually placed into cell lysis buffer according to the published protocol from the Stratagene Absolutely RNA Nanoprep Kit (Stratagene, La Jolla, CA, USA), snap frozen in LN_2 and stored at -80°C to preserve RNA for later analysis. Following thawing on ice and according to the

manufacturer's instructions, RNA from the zygotes and embryos was extracted using the Absolutely RNA Nanoprep Kit (Stratagene, La Jolla, CA, USA). Recovered RNA was reverse transcribed into cDNA using random hexamers and Superscript III reverse transcriptase (Invitrogen, San Diego, CA, USA) following instructions from the manufacturer.

Multiplex target specific preamplification of cDNA

Custom TaqMan gene expression assays for genes encoding mitochondrial fusion (Mfn2), fission (Drp1), mitochondrial transport (Miro1 and Pink1) and mitochondrial replication (SSBP1) that were previously designed in our laboratory were employed in this study. In addition to these custom-made gene expression assays, a commercially available primer pair and probe for eukaryotic 18S rRNA was purchased (Applied Biosystems, Foster City, CA, USA).

A multiplex PCR preamplification of 5 cDNA targets was performed in a final volume of 25µl using a commercial PreAmplification Mix (Applied Biosystems, Foster City, CA, USA) according to manufacturer's instructions. Because of its high abundance, the endogenous control 18S rRNA was not preamplified. Reaction mixtures were initially held at 95°C during 10 min before 14 preamplification cycles of 15 sec at 95°C and 4 min at 60°C were executed. The linearity of the preamplification was tested following quantitative real-time PCR of both unamplified and diluted (1:20) preamplified 0.1 ng/µl rhesus macaque testis cDNA. Within the unamplified and preamplified testes cDNA group, C_T values from all the transcripts of interest were compared to a randomly chosen reference gene out of the 5 genes of interest. The ΔC_T values for each gene were subtracted from each other and resulting values ($\Delta\Delta C_T$) were indicative of the linearity of the preamplification. The $\Delta\Delta C_T$ values approximating zero were considered linear, whereas values exceeding two were regarded as undesirable. To guarantee the linearity of the reference gene, the complete calculation was repeated using another reference gene out of the 5 preamplified targets.

Quantitative Real-Time PCR

Triplicate amplifications were conducted in a 10µl final reaction volume using the 7900HT Fast Real-Time PCR System. The reaction mixture contained 1x TaqMan Universal Master Mix (Applied Biosystems, Foster City, CA, USA), 1x TaqMan Gene Expression Assay (Applied Biosystems, Foster City, CA, USA) and 2.5 µl of preamplified cDNA. After an initial hold at 95°C for 10 min, reaction mixtures underwent 40 cycles of 15 sec at 95°C and 1 min at 60°C. In order to create the standard curves, ten-fold dilutions of diluted (1:1) 0.1 ng/µl preamplified rhesus macaque testis cDNA were quantified on the same plate as diluted (1:20) preamplified zygote cDNA. Following the amplification, the threshold and baseline values for each gene were empirically determined and remained constant over all plates. The threshold values from each transcript of interest were used to approximate RNA equivalents from the standard curve. Following this extrapolation, each RNA equivalent was normalized to the non-preamplified 18S RNA equivalent.

Results

Zygote Data Analysis

Zygotes originating from the three maturation groups (MI, MII, MI-MII) from eight rhesus macaque females were analyzed in this study. Details of monkey oocytes from the three maturation groups are summarized in Table 7.

Our initial goal was to correlate mitochondrial movement throughout the embryo during syngamy with expression of genes known to control mitochondrial dynamics and movement. While we were able to collect images of mitochondrial localization at the zygote stage that show the heterogeneity of mitochondrial distribution in a population of NHP zygotes (Figure 15) regardless of maturation status of the original oocyte (MI, MII or MI-MII), we were not able to correlate these images with gene expression profiles.

Table 7: Animal identification and numbers of zygotes used from each animal.

Animal ID	MI	MII	MI-MII
98H	3	-	-
53O	2	-	-
15S	-	3	5
12S	4	-	-
80C	1	5	1
76P	-	5	-
M598	2	-	5
88J	-	5	1
Total	12	18	12

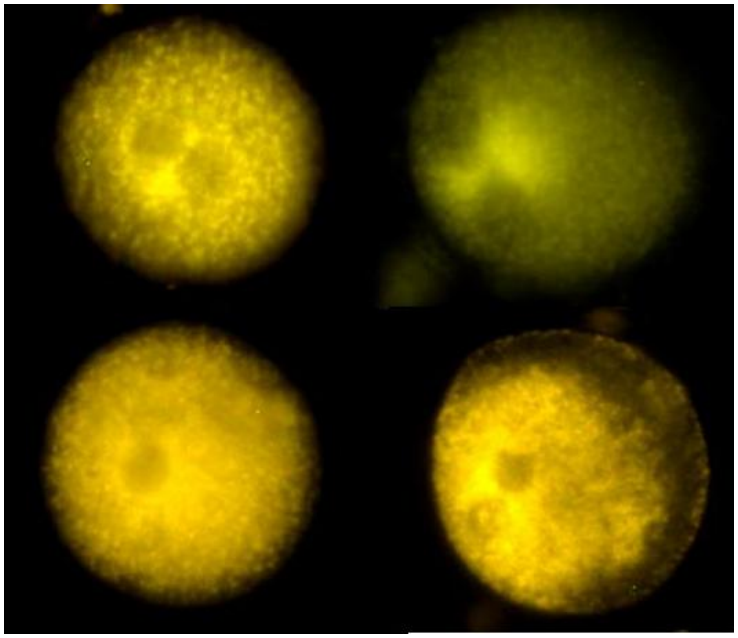

Figure 15: Mitotracker staining of 4 rhesus macaque zygotes, from 4 different animals, 14 hours post insemination. Note the heterogeneity between the individual zygotes

Previous studies by Squirrell (Squirrell et al., 2003) employed the use of confocal microscopy and multi-photon laser scanning microscopy (MPLSM). Neither of these technologies were available at the CPRC and therefore all images were taken using epifluorescense, which does not allow pinpoint images of the cell to be taken. Because of the limitations of epifluorescense and the available microscope, we cannot definitively say that mitochondrial localization, i.e. perinuclear localization during syngamy, is correlated to the expression of genes controlling mitochondrial dynamics and movement. However, mitochondria

are most certainly going through fusion, fission and movement processes during fertilization and syngamy as can be seen by the differences in mitochondrial morphology and localization in the images. Further studies need to be performed using confocal or MPLS microscopy.

Due to the success of the gonadotrophin stimulation protocol (Chapter 5) and the high percentage of MII oocytes upon collection, it was not possible to use equivalent numbers of oocytes from each female for each maturation group. Therefore no comparisons were made between animals and each zygote was treated individually within the three maturation groups; MI (n=12), MII (n=18) and MI-MII (n=12) (Table 7). Using the Student-Neuman-Keuls Test, that compares the means of all three maturation groups to each other, for each individiual gene of interest, we found no significant differences in the relative expression levels of all 5 genes between the three maturation groups (Table 8).

Table 8. Means and number of samples analyzed (N) for each maturation group for each gene of interest

Maturation Group	N	Mfn2	Pink1	Drp1	Miro1	SSBP1
MII	12	81.60	27.86	103.48	175.12	153.33
MI	12	72.54	15.17	62.16	90.05	103.58
MI-MII	18	34.53	11.34	35.26	43.63	52.67
Significance		0.414	0.356	0.295	0.234	0.196

Student-Neuman-Keuls Test was used to compare the means of the 3 maturation groups to each other, for all 5 genes of interest. No significant differences found.

Expression analyses revealed all genes analyzed were highly correlated; indicating that mitochondrial fusion (Mfn2), fission (Drp1), movement (Pink1, Miro1) and replication (SSBP1) are all strongly ($p<.001$) associated with each other during syngamy and preimplantation development (Table 9).

Table 9: Pearson correlation analysis showing strong correlation between all 5 genes of interest for all zygotes analyzed, regardless of maturation group.

		Pink1	SSBP1	Mfn2	Drp1	Miro1
Pink1	Pearson Correlation	1	.873**	.827**	.933**	.980**
	Sig. (2-tailed)		.000	.000	.000	.000
	N	41	41	41	41	41
SSBP1	Pearson Correlation	.873**	1	.939**	.893**	.871**
	Sig. (2-tailed)	.000		.000	.000	.000
	N	41	42	42	42	42
Mfn2	Pearson Correlation	.827**	.939**	1	.884**	.789**
	Sig. (2-tailed)	.000	.000		.000	.000
	N	41	42	42	42	42
Drp1	Pearson Correlation	.933**	.893**	.884**	1	.909**
	Sig. (2-tailed)	.000	.000	.000		.000
	N	41	42	42	42	42
Miro1	Pearson Correlation	.980**	.871**	.789**	.909**	1
	Sig. (2-tailed)	.000	.000	.000	.000	
	N	41	42	42	42	42

**. Correlation is significant at the 0.001 level (2-tailed).

Pearson Correlations with Sig.(2-tailed) indicating all $p<0.001$ and N = total number of samples analyzed regardless of maturation group.

Embryo Data Analysis

Embryos from 3 rhesus macaque females were used for this study. Fourteen hours after insemination, embryos were places in either low O_2 (n=12) or high O_2 (n=12). Real-Time PCR gene expression analysis of the same 5 genes analyzed in zygotes revealed no significant difference between day 3 embryos cultured under low O_2 versus high O_2 (Figure 16 & Table 10).

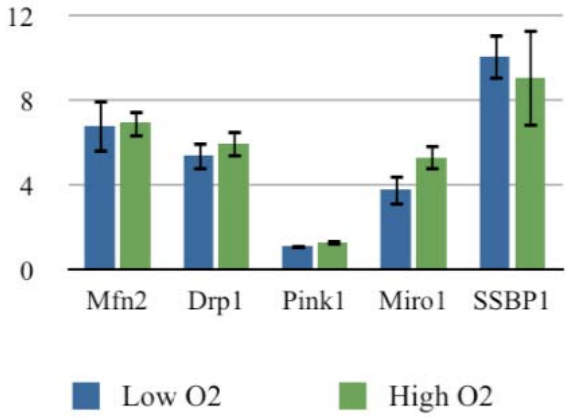

Figure 16: Comparison of relative expression levels of genes involved in mitochondrial dynamics and replication in day 3 embryos cultured under either low or high oxygen concentration. Each gene is normalized to the internal control, ribosomal 18S. No significant differences shown.

Table 10: Genes involved in mitochondrial dynamics and replication in day 3 embryos cultured under either low or high oxygen concentration.

	Oxygen Concentration	N	Mean	Std. Deviation	Std. Error
Pink1	Low	11	1.09214	0.466822	0.140752
	High	10	1.25179	0.393131	0.124319
SSBP1	Low	11	10.05574	3.627983	1.093878
	High	11	9.06683	7.760284	2.339814
Mfn2	Low	11	6.79198	4.248865	1.281081
	High	11	6.92026	2.357120	0.710698
Drp1	Low	11	5.37615	2.296540	0.692433
	High	11	5.93824	2.239771	0.675316
Miro1	Low	10	3.77506	2.374944	0.751023
	High	11	5.30295	2.076477	0.626081

Showing mean, samples analyzed (N), standard deviation and standard error of relative expression levels of genes involved in mitochondrial dynamics and replication in day 3 embryos cultured under either low or high oxygen concentration. Each gene is normalized to the internal control, ribosomal 18S.

Preimplantation Development Analysis

Mature MII oocytes that were previously analyzed in our lab for the same 5 genes of interest were included in this analysis. The inclusion of this data allowed comparison of three distinct time points during preimplantation development; oocyte, zygote and day 3 embryo. All material used for this analysis originated from mature MII oocytes at the time of collection in order to eliminate the possibility of maturation stage differences. The data for all three groups of interest were non-normally distributed and therefore a Krustal-Wallis one-way ANOVA by ranks was used. Krustal-Wallis is a non-parametric method for testing the equality of population medians among groups. It is identical to a one-way ANOVA with the data replaced by their ranks. As shown, MII oocyte gene expression is at basal levels for all genes analyzed except for Pink1 (Table 11). After fertilization and during syngamy (zygote stage) all genes increase 2-3 fold from levels seen in the oocyte (Table 11). By day 3 of embryo development, all genes have decreased to levels still significantly higher than those seen in the oocyte (except for Mfn2 and

Pink1), but also significantly lower than what is seen in the zygote. Table 11 and Figure 17 show the mean rank comparisons of each gene for the 3 developmental groups. All comparisons are statistically significant ($p<0.001$) except for Mfn2 expression in oocytes vs. embryos.

Table 11: Mean rank using the Krustal-Wallis Test for each of the three maturation groups for each gene.

	Oocyte	Zygote	D3 Embryo	Oocyte vs Zygote	Oocyte vs D3 Embryo	Zygote vs D3 Embryo
Mfn2	14.93	46.72	22.33	< 0.001	0.172	< 0.001
Drp1	10.87	46.67	25.92	< 0.001	0.002	< 0.001
Pink1	28.07	44.67	14.58	< 0.001	0.004	< 0.001
Miro1	12.8	45.67	21.18	< 0.001	0.186	< 0.001
SSBP1	8.4	46.39	30.67	< 0.001	< 0.001	< 0.001
N	15	18	12			

Each sample analyzed, regardless of developmental time point (N=45) is ranked, using the relative expression normalized to ribosomal 18S, from highest to lowest, and the mean of each time point is compared to the mean of every other time point, for each individual gene. Each comparison is statistically different for each comparison except for Mfn2 and Miro 1 in oocyte vs day 3 embryo.

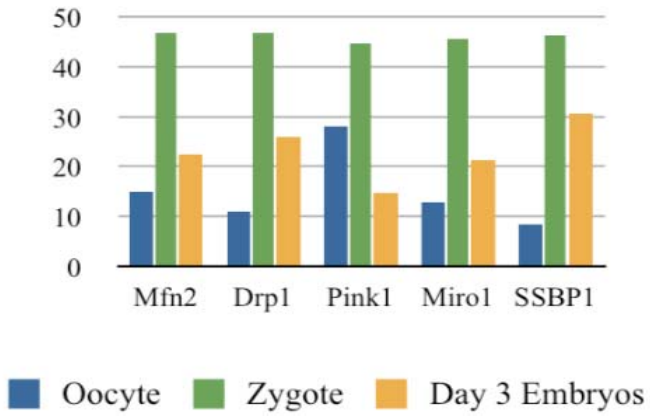

Figure 17: Data depicting the Krustal-Wallis test using mean rank.

Finally, a small subpopulation of zygotes and embryos were found to have dramatically higher levels of gene expression of all 5 genes of interest. As shown in Figure 18, scatterplots reveal this is especially true in zygotes. Raw data from this subpopulation is shown in Table 12. Expression of all five genes is highly correlated as shown in Table 9.

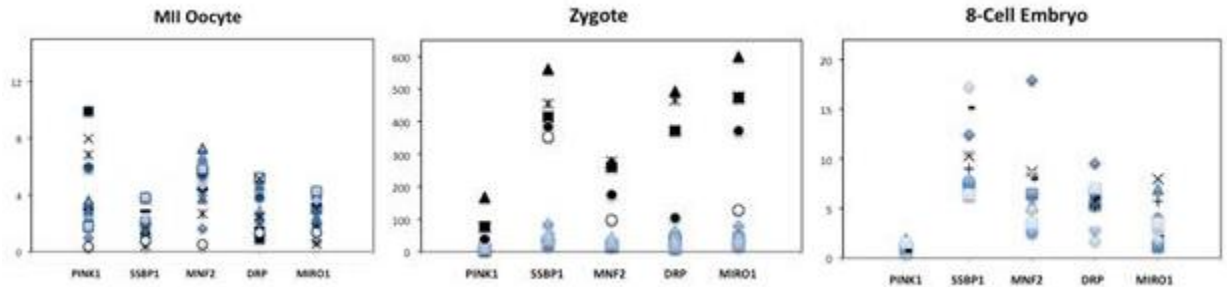

Figure 18: Scatterplots of MII oocytes, zygotes and day 3 embryos that express dramatically higher expression levels.

Table 12: Relative expression levels

Animal ID	Pink1	SSBP1	Mfn2	Drp1	Miro1
80C	8.417	25.048	25.944	11.157	31.397
80C	77.336	414.239	260.978	372.071	473.345
76P	5.908	89.272	39.87	42.226	80.255
76P	19.728	352.451	97.429	44.904	127.845
88J	10.218	37.299	32.213	23.881	32.37
88J	37.8	383.488	174.988	103.61	371.384
15S	10.963	48.645	41.01	29.486	29.973
15S	167.599	561.784	274.333	493.693	1,183.635
15S	77.312	454.633	277.142	465.973	477.528
73J	1.546	6.428	3.494	7.101	3.514
73J	17.592	482.643	111.949	220.513	385.024

Shown are samples with typical expression levels (white) and matched samples from the same animal that fall within the subpopulation of samples with dramatically higher expression levels (green).

Discussion

The suppositions of this aim were primarily based on previous work that showed the accumulation of mitochondria between the male and female pronucleus in rhesus macaque zygotes and the heterogeneity of mitochondrial localization in rhesus oocytes, zygotes and embryos (Squirrell et al., 2003). While that study did confirm the transient nature of mitochondrial localization during NHP preimplantation development, it did not examine what factors may be contributing to these mitochondrial movements. In the present study, we have been successful in correlating expression of genes involved in mitochondrial dynamics and movement, as well as mtDNA replication, with specific time points in preimplantation development that have been previously shown to be important in mitochondrial activity.

The hypothesis of this specific aim were twofold. Our initial hypothesis was that meiotic stage and mitochondrial distribution after fertilization influence expression of genes involved in mitochondrial dynamics. As previously discussed, in order for proper fertilization to occur, the GV-stage oocyte, which is arrested at the diplotene stage of MI, must resume meiosis and reach MII, with the extrusion of the first polar body. The use of standard ART protocols in human infertility treatment can result in the failure of meiotic resumption in 15 – 27% of oocytes within the standard 36 h after the administration of hCG (de Vos et al., 1999; Junca et al., 1995). Immature oocytes (GV or MI) are generally collected at oocyte retrieval along with mature MII oocytes. Typically, oocytes are collected 32-34 hours post hCG injection and cultured for approximately 4 hours, after which, some MI oocytes will extrude a polar body, achieve MII and subsequently be used for IVF. It is fairly common for immature MI oocytes from human IVF cycles to fertilize and develop into morphologically normal embryos (de Vos et al., 1999; Huang et al., 1999; Junca et al., 1995; Strassburger et al., 2004), however these embryos generally lead to reduced pregnancy and live birth rates (de Vos et al., 1999; Vanhoutte et al., 2005). Furthermore, previous studies have shown that embryos developed from oocytes with delayed polar body extrusion display high rates of chromosomal abnormalities regardless of morphology (Dupont et al., 2009). In this study, we determined expression levels of genes involved in mitochondrial fusion (Mfn2), fission (Drp1), movement and transport (Pink1, Miro1) and replication (SSBP1) in zygotes originating from mature MII oocytes, immature MI oocytes and oocytes that matured during the window of time between collection and insemination (MI-MII) (Table 7). We found no differences in gene expression levels in any of the five genes analyzed between the three maturation groups (Table 8). It is assumed that oocytes that had not extruded the first PB by the time of insemination (MI group) must have extruded it sometime while in the presence of sperm, in order for fertilization and PN formation to have occurred. With these data,

we propose that oocyte maturation to MII, from the span of time between collection (33-34 hrs post hCG) and insemination (37-38 hrs post hCG), while involved in chromosomal normality and stability (Dupont et al., 2009), does not influence the mitochondrial contribution to the processes of fertilization and syngamy. Interestingly, we did find very strong correlations between all genes analyzed. Indicating, for the first time, that mitochondrial fusion (Mfn2), fission (Drp1), movement (Pink1, Miro1) and transcription (SSBP1) are all strongly ($p<0.001$) associated with each other during syngamy and preimplantation development (Table 9).

Our secondary hypothesis in this specific aim was that oxygen concentration during embryo culture influences expression of genes involved in mitochondrial dynamics. Cohorts of zygotes from 3 different animals that all originated from MII oocytes upon collection were randomly assigned to embryo culture in either high O_2 (6% CO_2 / 20% O_2) or low O_2 (6% CO_2 / 5% O_2 / 89% N_2) until day 3. The same five genes that were analyzed in zygotes above were analyzed in day 3 embryos in order to determine if differences in gene expression were influenced by oxygen concentration during *in vitro* embryo culture. We found no significant differences in gene expression for any of the five genes of interest between low and high O_2 concentration (Figure 16 and Table 10). Due to the low number of embryos analyzed (Table 10) in each group, we agree these data are not conclusive. However, we propose that the paucity of differences between the low and high oxygen culture conditions may be due to the fact that all of these embryos were inseminated under high O_2, and only split into different O_2 treatments 14-16 hours post insemination. We propose future studies in which cohorts of oocytes are randomly split into low or high O_2 groups, inseminated and cultured to blastocyst stage of development and then analyzed for the same genes analyzed here. We predict that non-physiological O_2 concentrations (20% O_2) will negatively impact mitochondrial dynamics gene expression in blastocysts. The blastocyst stage of development is the first formation of two distinct cell lines,

the ICM and the trophectoderm, as well as the time of mitochondrial replication and transcription in most species. Clearly, this is an extremely important stage of development. The impact of mitochondria at this stage has been well established; however, the exact role played by mitochondrial dynamics and movement has yet to be determined.

Additionally, with the inclusion of similar gene expression data collected in our laboratory from MII oocytes, we have been able compile gene expression levels from three distinct time point in preimplantation development. For the first time, we are able to show that expression of genes involved in the control of mitochondrial fusion, fission, movement and replication fluctuates during preimplantation development in NHP, depending upon the stage of development. In the MII oocyte, gene expression is at basal levels for all genes analyzed except for Pink1 (Table 11), while in the zygote, all genes increase 2-3 fold from levels seen in the oocyte (Table 11). By day 3 of embryo development, Drp1, Miro1 and SSBP1 levels have decreased, but remain significantly higher than those seen in the oocyte, but also significantly lower than what is seen in the zygote. There were no significant differences observed for Mfn2 between the oocyte and embryo time points. Interestingly, Pink1, a serine–threonine kinase that localizes to the outer mitochondrial membrane that has a role in cellular protection against oxidative stress, was actually statistically higher in the oocyte than in the day 3 embryo. The reason for this expression level in the oocyte is unknown. These data show that throughout preimplantation development, certain time points are metabolically quiet (oocyte and day 3 embryo), while other time points require dramatic increases in metabolism (zygote). It is likely that increased ATP is needed in order to meet the energetic demands of the embryo for the processes occurring during these developmental stages (i.e. PN formation, polar body extrusion, syngamy and perhaps even mitochondrial replication, as discussed in Specific Aim 1). Future studies that will determine gene expression in the blastocyst will be of great importance in

establishing a true profile of mitochondrial metabolic activity during preimplantation development.

Interestingly, in both zygotes and day 3 embryos, there emerged a subset of samples with dramatically higher expression of all genes (Figure 18). As shown in Table 12, expression levels of these samples can exceed two and even three standard deviations beyond the mean of the total cohort. The subpopulation is not revealed in the oocyte, however, these samples were not from the same group of animals used for the zygote and embryo studies and the quality of the oocytes analyzed is questionable. Through the use of exogenous gonadotrophin stimulation, oocytes that would have normally become atretic in monovulatory species such as humans and NHPs actually become dominant follicles due to the excess FSH available. While these oocytes may reach MII status and ovulate, the quality of these oocytes is suspect. As discussed in Chapter 1, there is not a benchmark method of identifying poor or good quality oocytes based on anything besides morphology assessment by the embryologist and the achievement of MII. Furthermore, poor or bad embryos can be identified based upon morphology and embryonic arrest during the cleavage stages; however, it remains difficult to identify a high quality embryo based upon anything beyond development rate and morphological quality score. We propose the subpopulation of zygotes and embryos seen in Figure 18 and Table 12 were derived from oocytes that were of the highest quality and naturally selected for ovulation. The dramatically higher levels of gene expression is not seen in all samples because cohorts are largely comprised of lower quality oocytes, zygotes and embryos. This hypothesis is supported by human IVF data from 2007 that revealed only 39.6% of cycles performed resulted in a live birth with an average of 2.2 embryos used per transfer (2007 SART National Summary). While the number of embryos produced was not revealed in the SART data report, there were most certainly a significantly larger number of embryos produced that arrested at some point during development, indicating that a

subpopulation of embryos were likely of much higher quality than the majority of the cohort. Furthermore, as previously discussed, a high proportion of genetically abnormal mitochondria in the oocyte can potentially reduce the number of functional mitochondria, leading to embryonic arrest and failed implantation. We believe this hypothesis to be true as seen by the low expression of genes involved in mitochondrial dynamics, movement and replication in the majority of the samples analyzed. Further analysis of a larger sample size for all time points, as well as other critical time points in preimplantation development (blastocyst), will likely reveal even further the impact of mitochondria on preimplantation development.

CHAPTER 7

CONCLUSION

The central goals of this study were to evaluate mitochondria in NHP oocytes and embryos, to determine how mtDNA mutations and mitochondrial function affect oocyte and embryo quality and to establish the rhesus macaque as an excellent comparative model to study preimplantation development in the human. The major topics addressed in this dissertation were:

- determine if there is a large mitochondrial mutation present in the rhesus macaque similar to the common deletion (mtDNA4977) seen in humans
- determine if mature oocytes and IVF produced embryos from gonadotrophin stimulated rhesus macaques harbor a higher proportion of specific mtDNA mutations than immature oocytes from non-stimulated monkeys
- determine if the rhesus common mutation is present in *in vitro* and in *in vivo* derived embryonic stem cell lines, as well as adult stem cell lines
- determine if polymorphisms in the mtDNA control region as well as polymorphisms in the mtDNA repair mechanism, PolG, are associated with aging
- determine if failure of oocytes to reach maturity is due to specific polymorphisms in the mtDNA control region
- determine if mtDNA control region sequence differences exist between embryos
- determine how to improve gonadotrophin stimulation methods and IVF efficiency at the Caribbean Primate Research Center
- determine if, during syngamy, expression of genes involved in mitochondrial dynamics, transport and replication are influenced by meiotic stage of gonadotrophin stimulated oocytes at the time of collection
- determine if expression of genes involved in mitochondrial dynamics, transport and

replication are influenced by mitochondrial distribution during syngamy

- determine expression profiles of genes involved in mitochondrial dynamics, transport and replication during syngamy and early embryo development

We determined that the rhesus common deletion entails the loss of 5703 base pairs, which is considerably larger than the 4977 bp common deletion typically found in humans. Using the same experimental strategy we found that the rate of detection of the rhesus common mutation in immature GV oocytes was dramatically lower when compared with gonadotrophin-stimulated MII oocytes and embryos (Chapter 2). It has been suggested that there is a massive amplification of the mitochondrial genome during the process of oogenesis, presumably to support the initial period of embryonic development, and that mitochondrial replication occurs during a very short period of time from the 1-to 2-cell stage in the mouse embryo prior to fertilization (McConnell and Petrie, 2004). Our data, as well as others', indicates that a mitochondrial replication event may occur during either exogenous gonadotrophin stimulation, and/or during oocyte maturation (Chapter 2).

Once we had established that there is, in fact, a rhesus common deletion and that this deletion is present in both mature oocytes and IVF embryos, we felt it important to determine if this mutation potentially persists into ESC lines derived from IVF produced embryos. This was the first study to examine mtDNA mutations in embryonic and adult NHP stem cell lines and we determined that the rhesus common deletion is present in adult and ES cells from fertile rhesus macaques. In conjunction with Chapter 2 we have shown that oocytes and embryos from gonadotrophin stimulated monkeys contain mitochondrial deletions, which carry into ES cell derivatives. Furthermore, we have shown that adult stem cell lines harbor high levels of the rhesus mtDNA common deletion, regardless of passage, as well as ESC derived from both *in vivo* and *in vitro* produced rhesus embryo (Chapter 3).

We agree that further studies that both characterize other mutations that may have occurred in conjunction with the rhesus common mutation in oocytes and embryos, as well exploring the mechanisms behind the transfer of mutations from the embryo to ESC, should have been performed. However, immediately following the data collection and analysis used in the two publications that became Specific Aim 1 and 2, Hurricane Katrina ravaged New Orleans, as well as the University of New Orleans, and all material collected for further analyses was destroyed. It eventually became imperative for our laboratory to relocate to Wayne State University. During that time, the field of determining mitochondrial impact on primate development changed dramatically and we were no longer in collaboration with Tulane Primate Research Center, the provider of all the material used in Specific Aim 1 and 2. Upon becoming reestablished at WSU, we began to focus primarily on the original specific aims described in the RO1 research grant written by Carol Brenner and Barry Bavister, R01-HD045966-01: Defects in Mitochondria Impacting Primate Oocyte Quality. The specific aims of this grant were: to determine the frequency and percentage of mtDNA deletions in primate oocytes and early embryos, and to determine the localization of active mitochondria in primate oocytes and early embryos, measure the activity of these mitochondria, and correlate these measurements with oocyte competence. Previous studies involving aging effects of mitochondria on oocytes were also included in the direction taken with experiments at that time. Therefore, it became important that we proceed in two directions in order to encompass previous work performed by our laboratory; aging and preimplantation development. In Specific Aim 3, we set out to determine; 1) if there were mutations in the mtDNA control region associated with the aging phenomenon, 2) if there were mutations in particular regions of interest within the mitochondrial polymerase, PolG, associated with aging, and 3) if there were mutations in the mtDNA control region associated with failure of oocytes to resume meiosis prior to fertilization. We found no

correlation between aging or failure of meiotic resumption prior to fertilization with mutations in either the mitochondrial control region or the only known mitochondrial polymerase, PolG (Chapter 4). While it is clear that mtDNA damage and repair mechanisms most certainly have an impact on mitochondrial replication and transcription in skeletal muscle and other post-mitotic tissues, other factors must be contributing to oocyte and embryo quality and preimplantation development.

Therefore, in order to improve the quality of oocyte and embryonic material used for further analyses, we began onsite collaborations with the Caribbean Primate Research Center. Through improvements made to the existing gonadotrophin stimulation regimens used at the CPRC, involving the reduction of total rFSH used per stimulation cycle, the addition of rLH to all cycles and the use of biological hCG in lieu of recombinant hCG, we were able to dramatically improve the number of MII oocytes collected as well as significantly reduced the number of unusable GV oocytes collected, as well as reducing the hormone cost of every cycle by reducing the dose of FSH by half, without any reduction in the number or quality of oocytes collected (Chapter 5).

With improvements made to the rhesus macaque IVF cycles, and the collection of reliable, quality material, we have been able to correlate, for the first time, expression of genes involved in mitochondrial dynamics and movement, as well as mtDNA replication, with specific time points in preimplantation development that have been previously shown to be important in mitochondrial activity. Unfortunately, due to the limitations of equipment available at the CPRC, we were not able to correlate mitochondrial movement and localization with expression analysis of genes involved in mitochondrial dynamics and movement. However, we do believe these processes to be linked and future studies employing confocal microscopy have been proposed for correlation. We have shown that while critically involved in chromosomal

normality and stability (Dupont et al., 2009), oocyte maturation from the time of collection (33-34 hrs post hCG) to the time of insemination (37-38 hrs post hCG) does not influence the mitochondrial contribution to the processes of fertilization and syngamy. Furthermore, we have shown the processes of mitochondrial fusion, fission, movement and transcription are all strongly associated with each other throughout critical time points of preimplantation development (Chapter 6).

We determined that embryos inseminated under high O_2, and then split into either low or high *in vitro* O_2 conditions after fertilization exhibited no differences in levels of gene expression (Chapter 6). In conjunction with the high levels of gene expression seen in zygotes at the time of syngamy, these data indicate that oxygen concentration conditions may influence embryo culture at an earlier time point than day 3, namely during fertilization and syngamy when there is a higher metabolic demand for ATP. We propose other time points of high metabolic demand (blastocyst stage) may also be influenced by oxygen concentration during *in vitro* culture. Similar studies where oocytes are fertilized under either low or high O_2 concentration have been proposed in order to determine at what point oxygen tension becomes critical.

Furthermore, not only have we been able to show, for the first time, that expression of genes involved in the control of mitochondrial fusion, fission, movement and replication fluctuates depending upon the stage of development; but that a subpopulation of high quality oocytes, zygotes and embryos may be identified based upon level of mitochondrial fusion, fission movement and replication gene expression (Chapter 6)

Due to the ethical limitation of using human oocytes and embryos for invasive studies, we have shown here that the rhesus macaque is an excellent model used in lieu of human material. Destructive analyses used in these studies will likely never be allowed to be employed on human material. Without the use of clinically translational models, such as the rhesus

macaque, the methods used to determine why fertility and reproductive problems exist as well as methods to identify, prevent and treat issues and diseases related to fertility and mitochondria, would be profoundly inadequate.

APPENDIX 1

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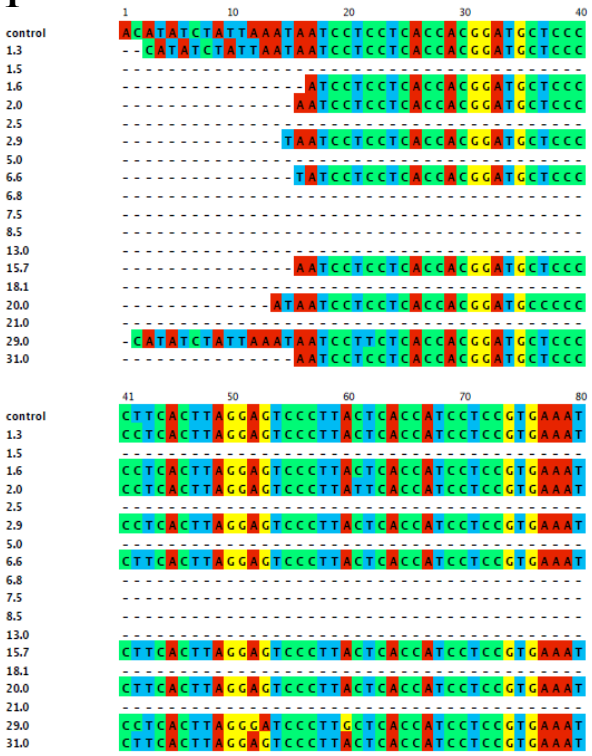

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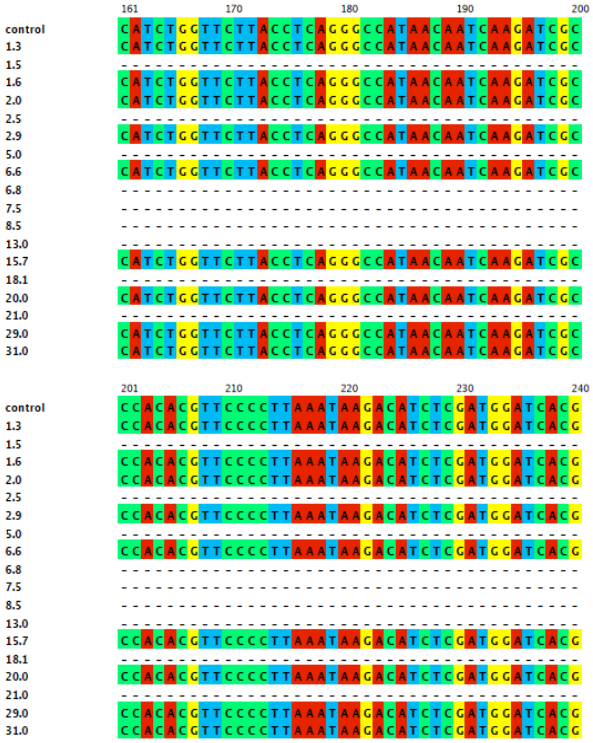

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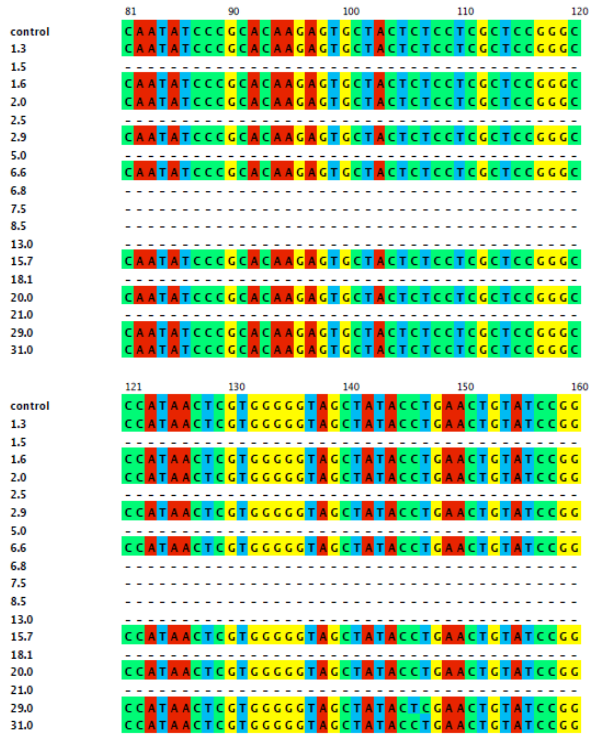

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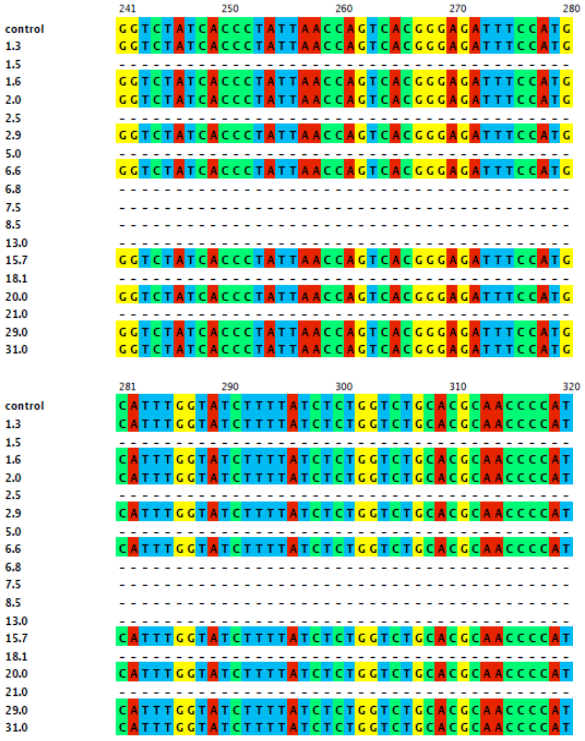

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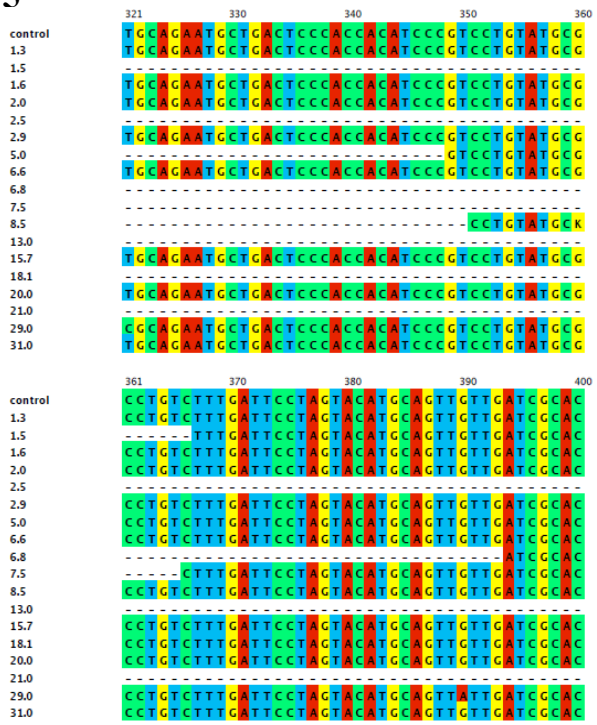

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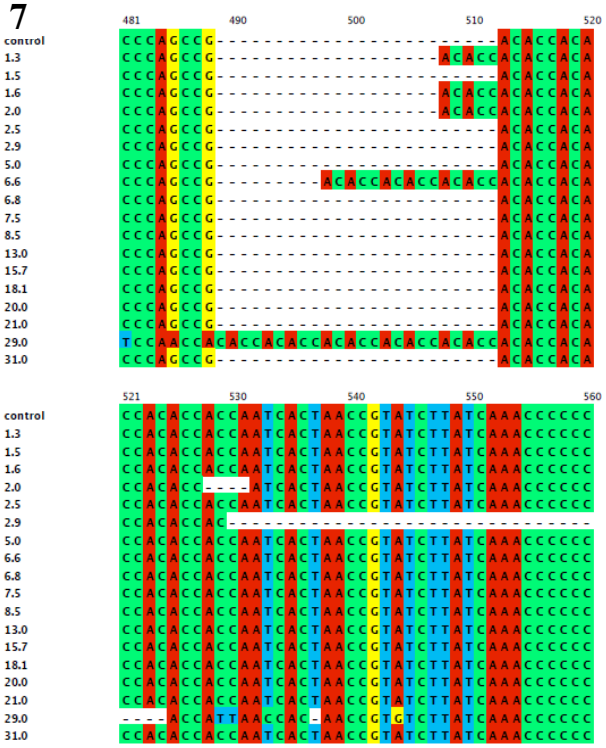

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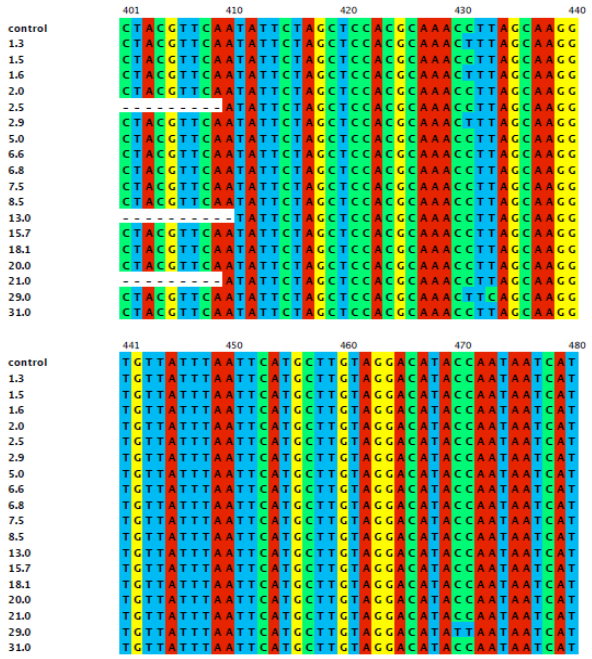

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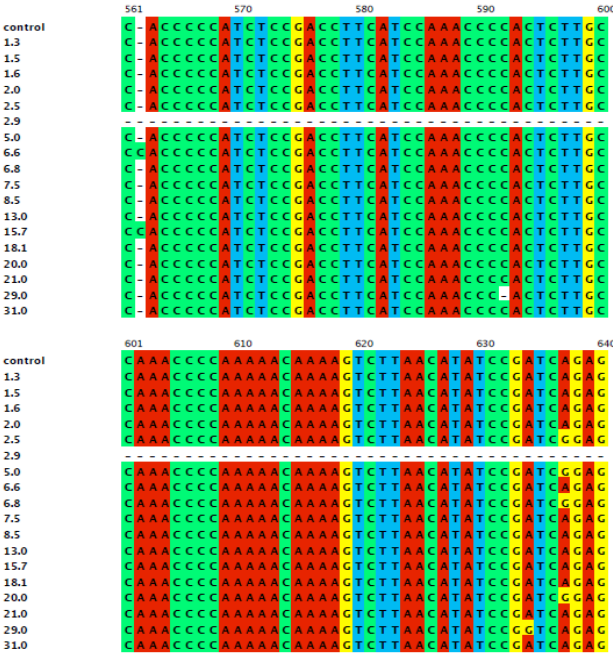

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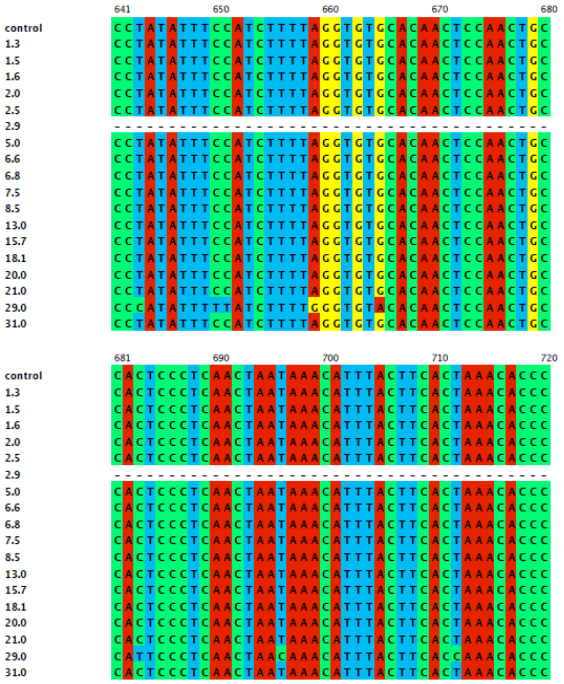

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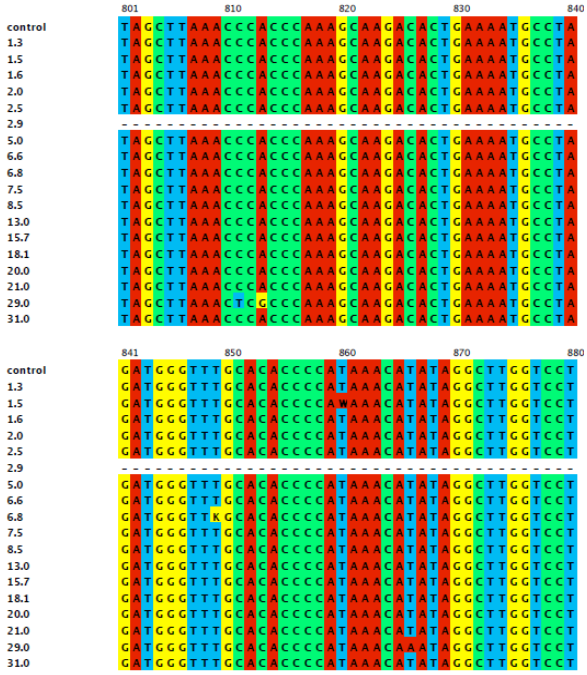

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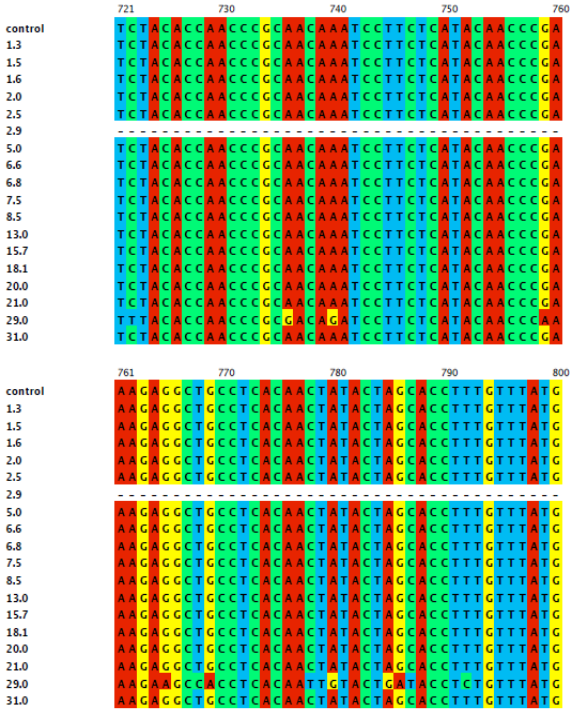

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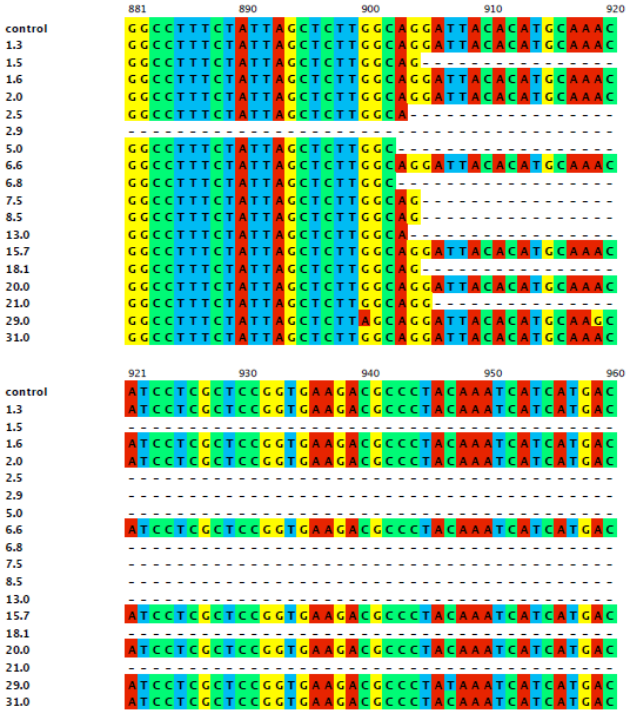

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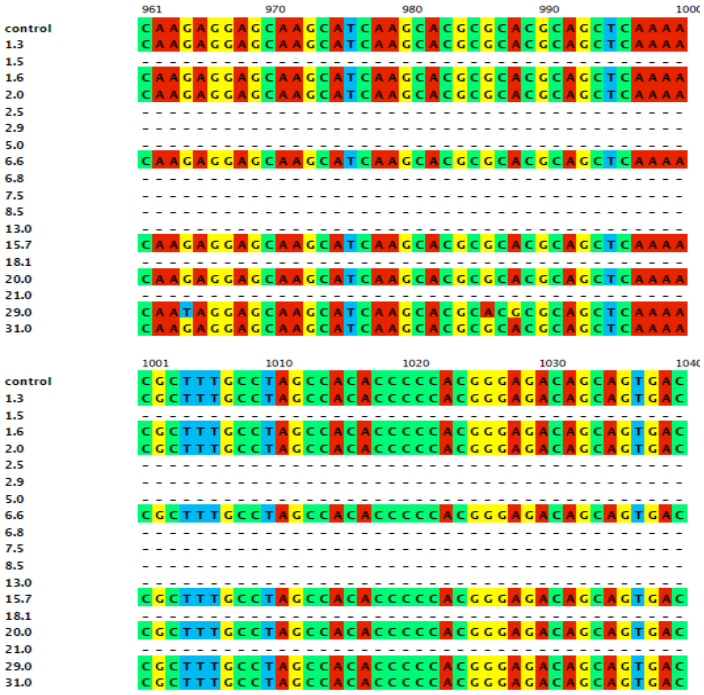

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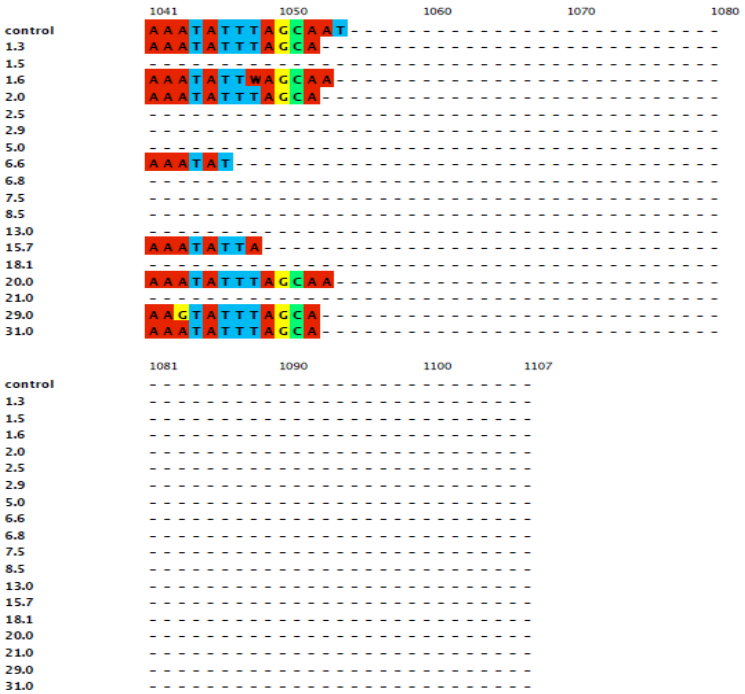

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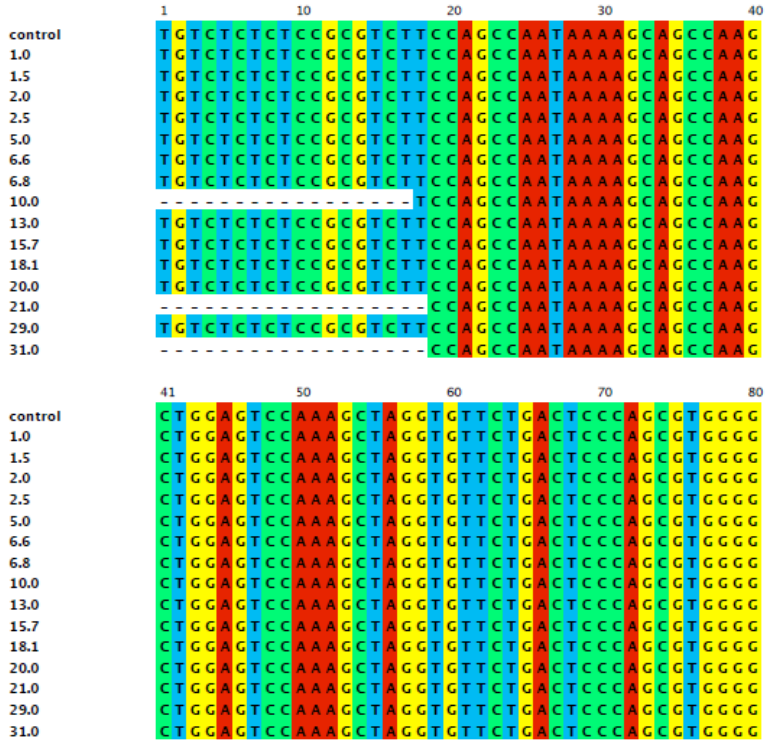

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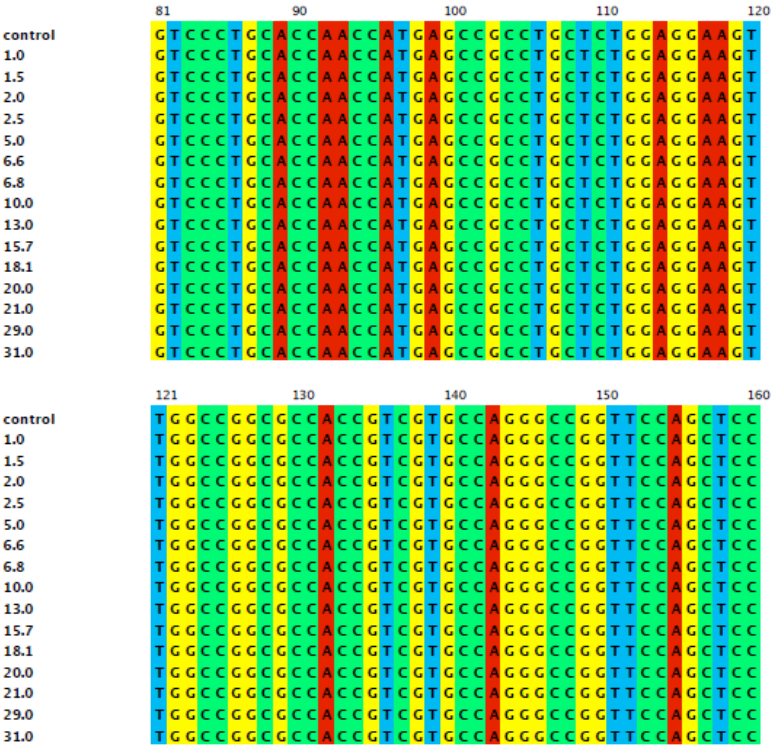

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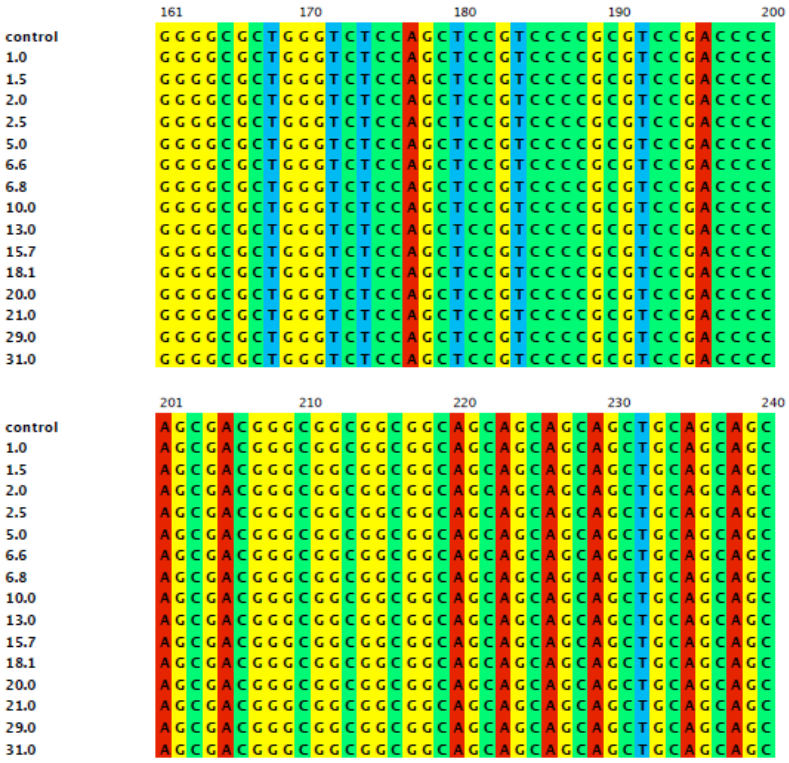

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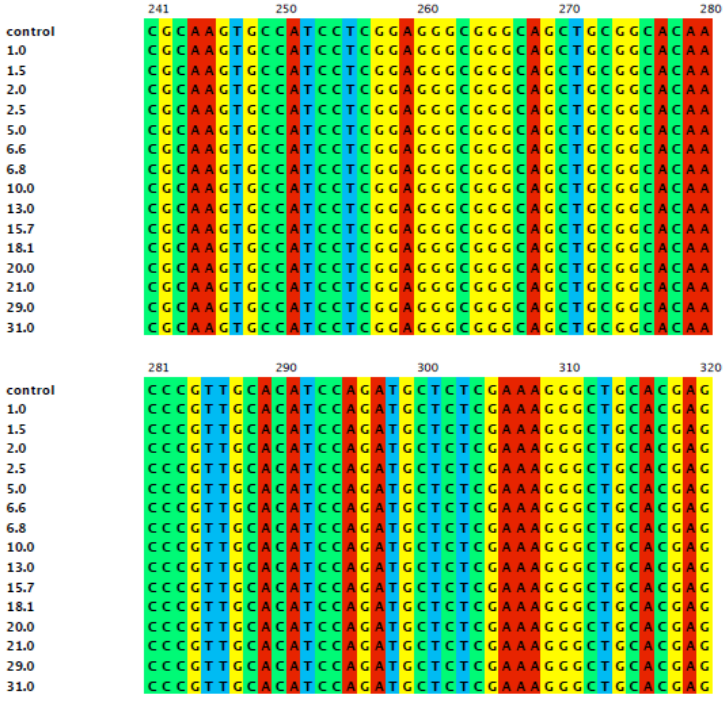

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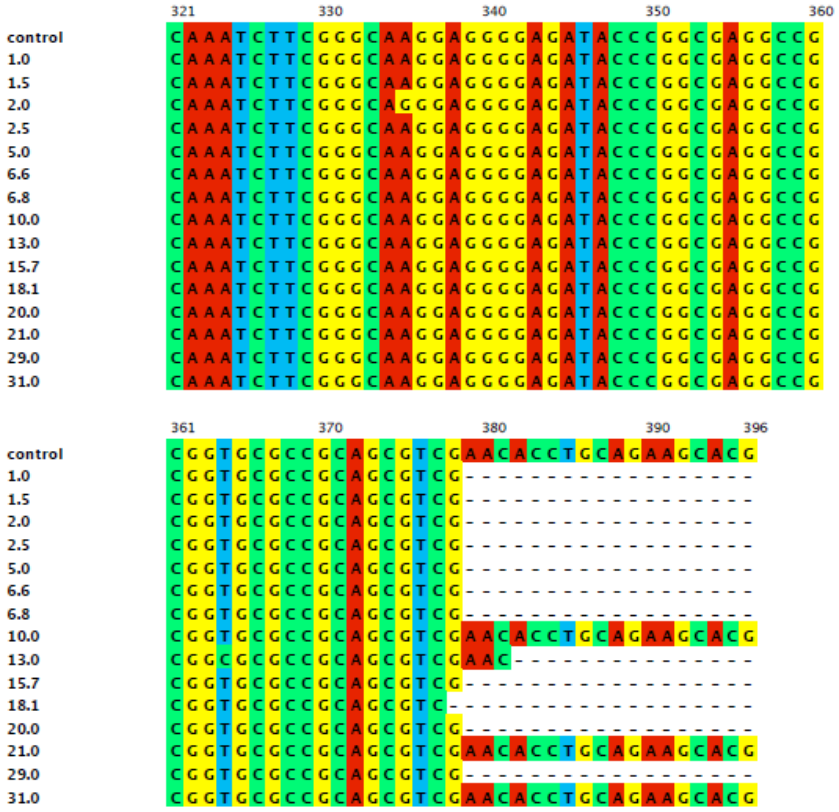

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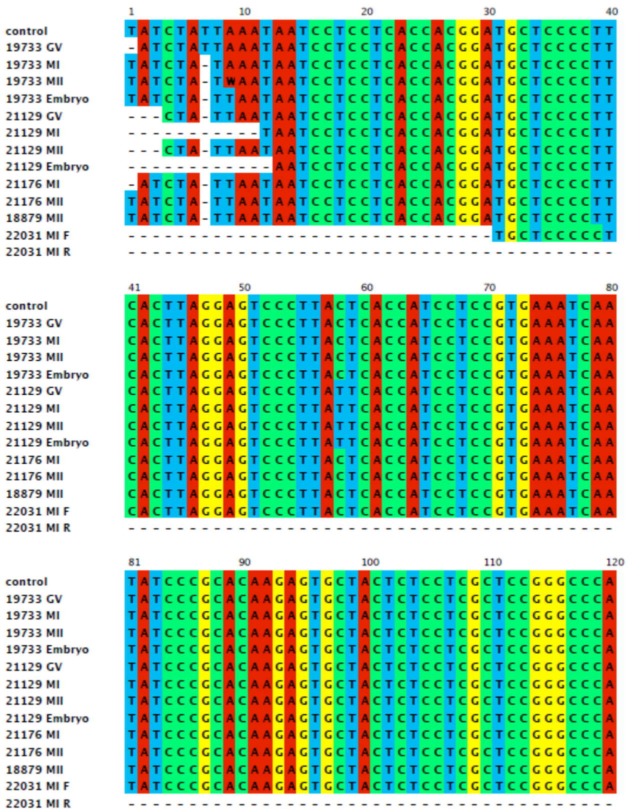

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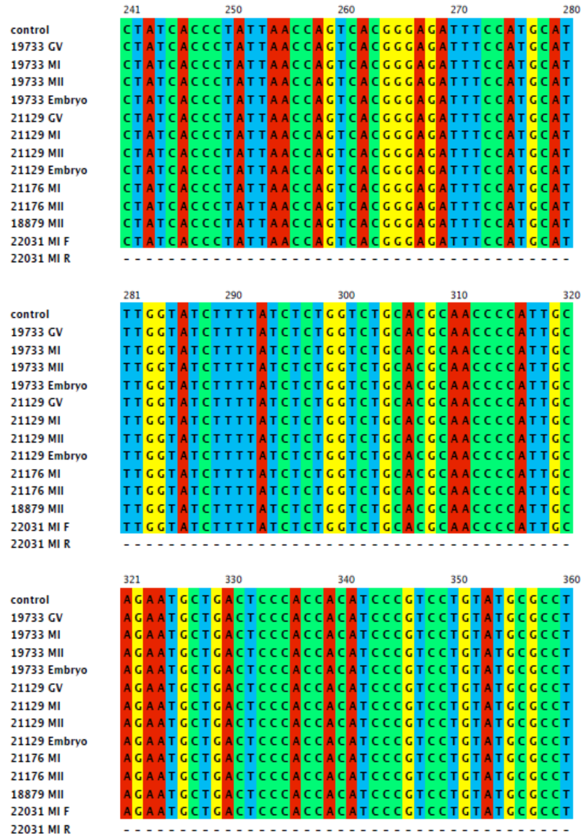

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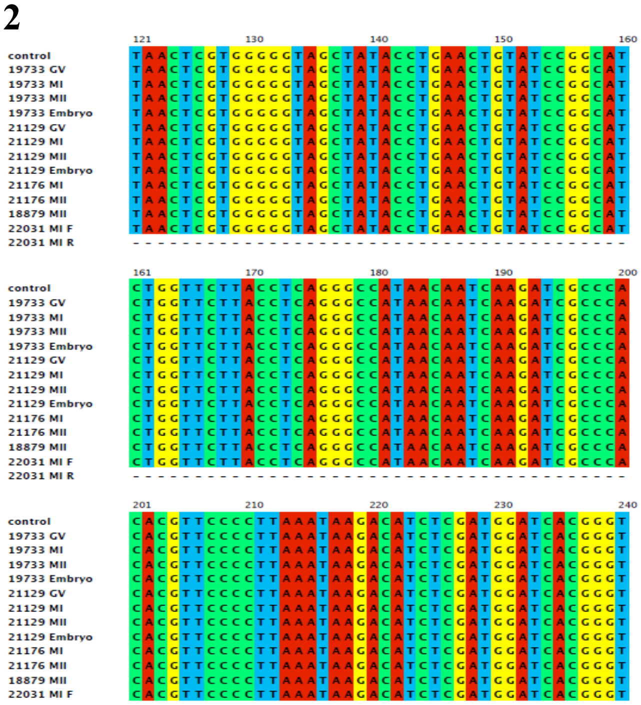

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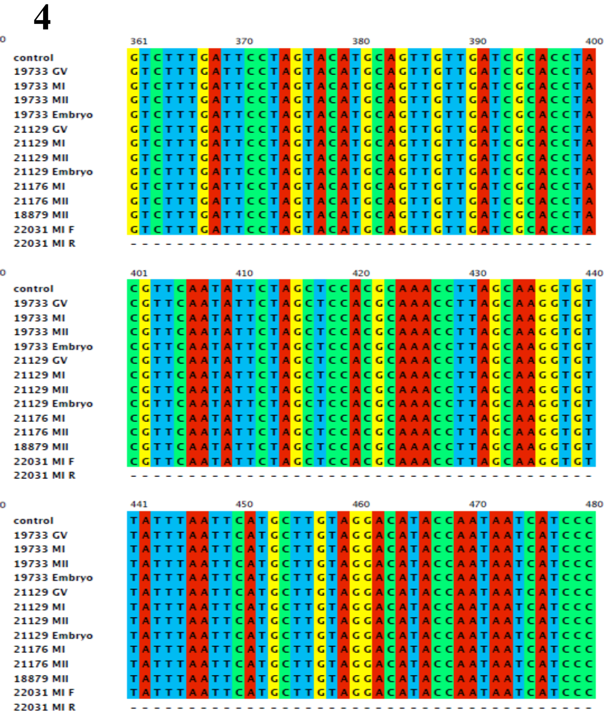

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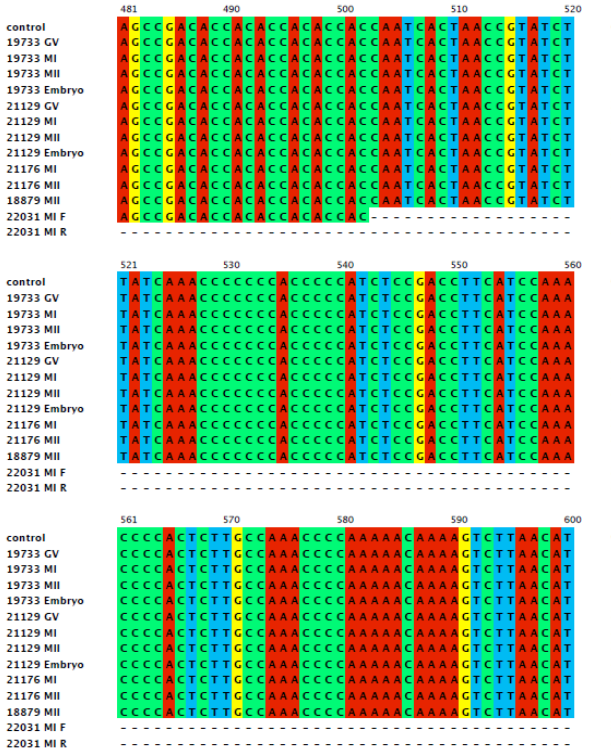

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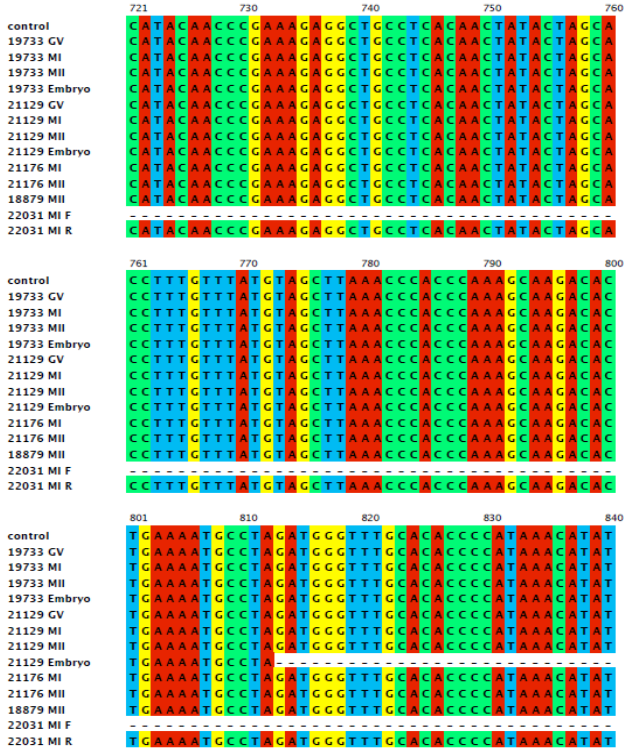

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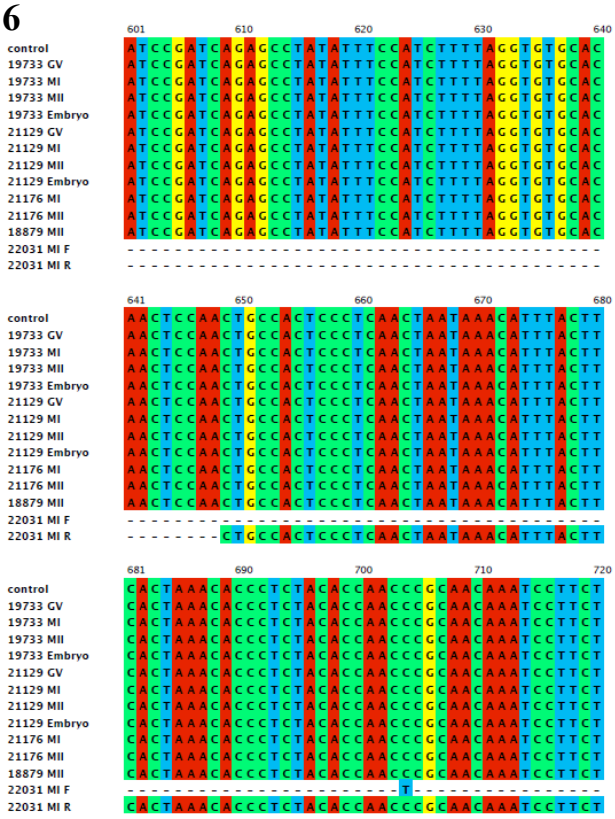

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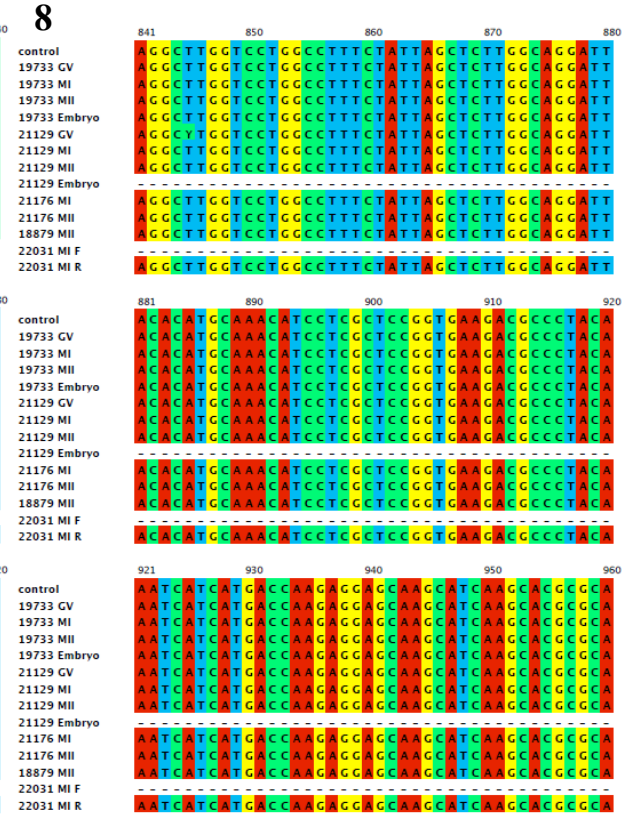

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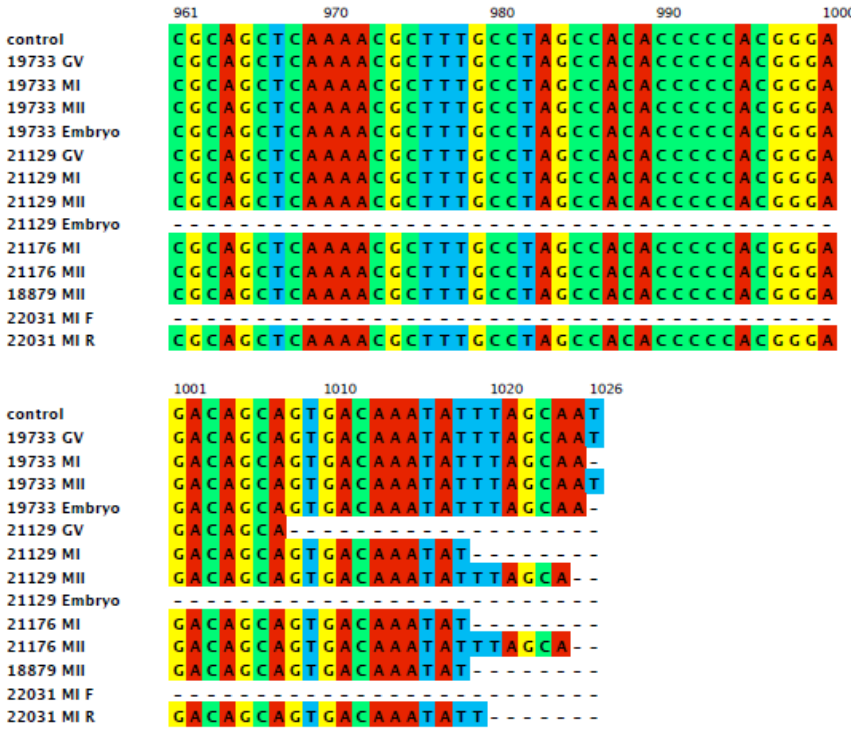

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ABSTRACT

THE IMPACT OF MITOCHONDRIA ON PREIMPLANTATION DEVELOPMENT IN THE RHESUS MACAQUE

by

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Assisted Reproduction Technologies (ART) have been used to treat infertility since 1790. In the centuries following, dramatic advances have been made in the field of infertility research through the employment of a variety of species. Despite these advances, determination of oocyte and embryo quality remains largely subjective. Ascertainment of methods to measure oocyte and embryo quality to improve selection in ART clinics and increase pregnancy success rates is imperative. These methods are only possible with a more comprehensive understanding of the cellular and molecular properties of oocytes and preimplantation embryos. A key component of all cells, including oocytes and embryos, is the mitochondrion, which produces energy and maintains its own DNA for encoding proteins necessary for energy production. Defects in either the proteins responsible for energy production, the mitochondrial DNA (mtDNA) genome, or other contributing factors can lead to loss of oocyte competence and embryo quality. Human oocytes and embryos would be the optimal material to use for performing studies on preimplantation development. However, practical and ethical constraints prevent the use of human material for intensive, potentially terminal studies; thus, compelling researchers to find and use suitable animal models instead. The non-human primate (NHP) is the most befitting

animal model for human reproduction due to evolutionary closeness and the dramatic similarities in reproductive function and preimplantation development. Furthermore, because studies in human preimplantation development are restricted to the use of discarded oocytes from infertility clinics, the use of the NHP has established the rhesus macaque as an excellent research model for human reproduction due to the availability of normal, viable oocytes and embryos from fertile animals. Therefore, the overall goals of this study were to evaluate mitochondria in NHP oocytes and embryos and to determine how mtDNA mutations and mitochondrial function affect oocyte and embryo quality.

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