MITOCHONDRIAL DISTRIBUTION IN MAMMALIAN CELLS

APPROVED BY:

________________________
Dr. Shirley Wright, Thesis Advisor

________________________
Dr. Mark Nielsen, Committee Member

________________________
Dr. Carl Friese, Committee Member

________________________
Dr. Jayne Robinson, Department Chair
ABSTRACT

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Name: Jiang, Lei
University of Dayton

Advisor: Dr. Shirley Wright

The mitochondrion is an important and essential cell organelle which provides about 90% energy for the organism and plays a number of important roles in cell functions. Recent research found that mitochondrial distribution in somatic cells is homogenously scattered throughout the cytoplasm, while the distribution in some stem cells and mature oocytes is perinuclear. These findings suggested that the spatial distribution of mitochondria may be related to their normal functions. In addition, Bavister has hypothesized that this perinuclear localization of mitochondria may indicate the pluripotency of stem cells. But mitochondrial distribution has not been examined in embryonic stem cells to date. My thesis investigates the distribution of mitochondria during development in mammalian cells and proposes experiments to determine and compare the spatial distribution of mitochondria in embryonic stem cells and differentiated cells using
fluorescence staining of MitoTracker Green to test Bavister’s hypothesis. In addition, research suggested that mitochondrial distribution is mediated by the cytoskeleton in higher eukaryotes. Therefore, an additional goal of my research was to address the effects of cytoskeletal disruptors on mitochondrial arrangement.
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LIST OF ABBREVIATIONS

ATP:  adenosine-5'-triphosphate

ATSC:  adult rhesus macaque stromal cell line

BSA:  bovine serum albumen

DAPI:  4',6-diamidino-2-phenylindole

DMEM:  Dulbecco’s modified eagle medium

ER:  endoplasmic reticulum

ES cells:  Embryonic stem cells

FBS: fetal bovine serum albumen

GSH:  glutathione

GV:  germinal vesicle

GVBD:  germinal vesicle breakdown

ICM:  inner cell mass

IVF:  in vitro fertilization

iPSCs:  induced pluripotent stem cells

LIF:  Leukemia inhibitory factor

MES:  mouse embryonic stem cells
Met I: metaphase I
Met II: metaphase II
MFs: microfilaments
MLE-15: mouse lung epithelial cells
MPF: mitosis-promoting factor or maturation-promoting factor
mtDNA: mitochondrial genome or DNA
MTs: microtubules
NEAA: non essential amino acids
NFAT: nuclear factor of activated T cells
NO: nitric oxide
OXPHOS: oxidative phosphorylation
PBS: phosphate-buffered saline
ROS: reactive oxygen species
TNF: tumor necrosis factor
CHAPTER I
INTRODUCTION
A. Focus of Thesis

Mitochondria are known as “the power house of the cell,” because they provide energy for the cell. Mitochondria generate adenosine-5′-triphosphate (ATP) from carbohydrates, proteins and fats that are obtained by organisms in their diet to provide energy for biological activities. Normal mitochondrial distribution is critical for several cell functions including intracellular calcium homeostasis, cell signaling and apoptosis (Hales et al., 2004; Katayama et al., 2006; Cerveny et al., 2007).

As “the power house for cells,” mitochondria play critical roles during gametogenesis, fertilization and embryonic development (Smith et al., 2005; Katayama et al., 2006; Dumollard et al., 2009; Van Blerkom et al., 2009). Recently, researchers found there were translocations of mitochondria during early embryonic developmental stages, which implicate mitochondrial distribution, in playing an important role during oogenesis and early embryonic development (Barnett et al., 1996; Sun et al., 2001; Katayama et al., 2006; Wang et al., 2009). These data lead to the questions: Is the spatial distribution of mitochondria important for mitochondrial function? What role does mitochondrial spatial distribution play in gametogenesis and embryogenesis? My research will review the current literature to address these questions. Data on mitochondrial distribution during gametogenesis and development will be collected and analyzed.

In 2006, Bavister et al. hypothesized that mitochondrial distribution is
different in stem cells and somatic cells, and that perinuclear localization of mitochondria may be an indicator for "stemness." Therefore, my research objectives are to determine and compare the spatial distribution of mitochondria in an undifferentiated cell type such as mouse embryonic stem cells (MES cells), and a differentiated cell type such as mouse lung epithelial cells (MLE-15). In addition, research suggests in most higher eukaryotes that mitochondrial distribution is mediated by the cytoskeleton (Haggeness et al., 1978). Interestingly, the cytoskeleton influences mitochondrial activity including mitochondrial respiratory activity, fusion and fission (Boldogh and Pon, 2007). While studies have focused on mitochondrial-cytoskeletal interactions, less focus has been on their role in differentiation and development of disease. Therefore, an additional goal of my research is to review the literature to examine the effects of cytoskeletal disruptors on mitochondrial arrangement. This study will help elucidate the behaviors of mitochondrial distribution during differentiation; and demonstrate the impact of the cytoskeleton on mitochondrial distribution.

My thesis investigates the distribution of mitochondria during development in mammalian cells. To provide background information for my thesis so that the mitochondrial distribution during stages of gametogenesis, fertilization and preimplantation development could be understood better, first a literature review of gametogenesis and development will be presented in Chapter II. Next a summary of the mitochondrion including its structure, biochemistry and function will be discussed in Chapter III. Chapter IV
addresses how the distribution of mitochondria changes during oogenesis and development. Chapter V discusses Dr. Barry Bavister’s hypothesis that mitochondrial distribution may be an indicator of "stemness," and proposes experiments that could be used to address this hypothesis. Lastly, Chapter VI addresses conclusions and future directions.

B. Significance

Embryonic stem cells (ES cells) are unique cell populations with the ability to undergo both self-renewal and differentiation (Odorico et al., 2001). They have the potential to be used to treat a variety of diseases (Levy et al., 2004; Faulkner and Keirstead, 2005). Although mitochondria play important roles in normal cell function, their distribution, interaction with the cytoskeleton as well as their role during differentiation have not been examined in ES cells. Therefore, my research to understand the spatial distribution of mitochondria in gametes, undifferentiated and differentiated cells may reveal specific differences among these cell types that will allow us a better understanding in stem cell biology, and may provide novel targets for stem cell therapy. Besides addressing the distribution of mitochondria during differentiation, this study will also demonstrate the impact of the cytoskeleton on mitochondrial spatial arrangement. This study may also lead to a better understanding of mitochondria in development, so that a suitable therapy for mitochondria-related disorders can be developed. Finally yet importantly, this study tests Bavister’s hypothesis that the arrangement of mitochondria
changes after fertilization to meet the energy needs of cells as development progresses (Lonergan *et al.*, 2007). If Bavister’s hypothesis holds true, mitochondrial distribution can be an exciting, brand new indicator for “stemness” of cells.
CHAPTER II
INTRODUCTION TO GAMETOGENESIS AND DEVELOPMENT
Gametogenesis is the process in which primordial germ cells undergo meiosis and differentiate to become mature gametes. Spermatogenesis is the formation of the male gamete, the sperm. Oogenesis is the formation of the female gamete, the ovum or egg cell (Gilbert, 2006). Both of these processes will be described in detail below.

A. Spermatogenesis

In mammals, sperm develop within seminiferous tubules in the testes and are stored in the epididymis (Heller and Clermont, 1963). Several steps occur during spermatogenesis (Gilbert, 2006). First, diploid male germ cells called spermatogonia (a type of stem cell) undergo mitosis during spermatocytogenesis to become primary spermatocytes. Then each primary spermatocyte undergoes two rounds of meiosis during spermatidogenesis to form four spermatids. After that, the spermatids undergo spermiogenesis. During this process, each spermatid produces a flagellum, condenses the nucleus, develops a mid-piece, and differentiates into a mature sperm.

Mitochondria translocate during spermatogenesis. In germ cells and spermatocytes of mammals, mitochondria are scattered all throughout the cytoplasm. However, along with formation of the flagellum, the mitochondria begin to accumulate around the flagellum in the mid-piece of the sperm. Finally, a mature sperm has formed with a flagellum that has grown from the centriole pair in the neck, a mid-piece containing mitochondria that form a ring
around the base of the axoneme, and a head containing a highly condensed nucleus with an acrosome at the anterior end. The mitochondria located in the mid-piece of the sperm provide ATP needed to whip the flagellum and propel the sperm (Gilbert, 2006).

During spermiogenesis in *Drosophila*, mitochondria in early spermatids aggregate and fuse into two large organelles that wrap around each other to form a giant elaborate structure called the Nebenkern (Benard and Karbowski, 2009). The protein mitofusin (Fzo1) is required for mitochondrial fusion during Nebenkern formation. Mutations in the *fzo* gene prevent mitochondrial fusion during Nebenkern formation and the mutant flies are sterile.

**B. Oogenesis**

In mammals, oocytes develop within follicles in the ovary (Fig. 1; Eppig and O’Brien, 1996). Primary oocytes are formed before birth in the fetus by mitosis of primordial germ cells called oogonia (Gilbert, 2006). Each diploid primary oocyte is contained in the primary follicle and arrests in prophase I of meiosis. During this period, the primary follicle enlarges due to synthesis of RNA and organelles for later oocyte growth, fertilization and preimplantation development. The large nucleus of the primary oocyte is known as the germinal vesicle (GV). When the follicle cells grow and divide to from a larger follicle, the primary oocyte also grows larger. Then germinal vesicle breakdown (GVBD) occurs during first meiosis (Meiosis I). The primary
Meiotic competence is the oocytes’ ability to complete meiosis (Motlik et al., 1984). It will determine whether oocytes can mature. Meiotic competence may depend on the spatial arrangement of mitochondria. Abnormal distribution of mitochondria was found to lead to arrest of oocyte development (Wang et al., 2009). Therefore, proper mitochondrial distribution during oocyte maturation is necessary for further development and is a determinant for oocyte quality (Wang et al., 2009). The first and second polar bodies disintegrate later to discard the extra chromosomes. As in spermatogenesis, both processes of mitosis of primordial germ cells and follicular cells, and meiosis of oocytes require ATP. Besides that, mitochondria also provide ATP for glutathione (GSH) production, which helps detoxify the cell during oocyte maturation (Dumollard et al., 2009).

C. Fertilization

Fertilization is the fusion of haploid gametes to reconstitute a diploid cell with the potential to become a new individual (Gilbert, 2006). Fertilization is
not a moment or an event, but a series of events from contact of gametes to the activation of development (Gilbert, 2006). When the oocytes are mature, they will be ovulated from the ovary and enter the oviduct (Fig. 1). The sperm also travel from the vagina to the oviduct to meet the oocytes. During this trek, capacitation of sperm happens so the sperm gain the capacity to fertilize the egg. The major driving force for the trip of sperm from the vagina to the oviduct is the muscular activity of the uterus (Gilbert, 2006). Sperm motility is important once sperm arrive within the oviduct (Gilbert, 2006). ATP provided by mitochondria are critical for sperm motility. Recent research found that mtDNA mutation can lead to impaired spermatogenesis and impaired sperm motility (Shamsi et al., 2008).

Fertilization occurs in the upper third of the oviduct (Fig. 1). The fertilized egg cell is called a zygote (Gilbert, 2006). Fertilization begins from the contact of sperm and egg. Sperm have a cap-like structure at their anterior part of their head called the acrosome. Once the sperm reaches the zona pellucida an extracellular matrix which surrounds the oocyte, it will trigger the acrosome reaction. Acrosomal enzymes like acrosin and hyaluronidase are released to digest the zona pellucida so that the sperm can penetrate the zona pellucida. After that, part of the sperm’s plasma membrane (at the equatorial segment) fuses with the oocyte’s plasma membrane, and the contents of the sperm including the sperm nucleus and sperm mitochondria are delivered into the ooplasm. An exception is the Chinese hamster: the tail and mid-piece of the sperm remain outside the oocyte after fertilization.
(Ankel-Simons and Cummins, 1996). In most species, the nuclei in mature sperm are highly condensed and genetically inactive. During fertilization, the sperm nucleus decondenses and is reactivated (Wright, 1999). After fertilization, the activated sperm nucleus undergoes a series of morphological and biochemical transformations and becomes the male pronucleus, while the oocyte chromosomes develop into the female pronucleus. These transformations including decondensation of the sperm nucleus and assembly of the pronuclear envelope are ATP-dependent (Raskin et al., 1997; Collas and Poccia, 1998). Once decondensed, the sperm DNA can begin transcription and replication immediately (Gilbert, 2006). By 15 hours after fertilization, the two pronuclei migrate together; the pronuclear envelopes gradually disappear and the chromosomes from the sperm and oocyte intermix (Gilbert, 2006). Pronuclear migration and apposition require participation of the cytoskeleton including microtubules and actin (Maro, 1985; Branzini et al., 2007).

This whole process of fertilization, from ovulation, the movement of the sperm, the transformations of the sperm nucleus and pronuclear DNA replication require a considerable amount of ATP, which is supplied by mitochondria. It has not been determined whether sperm mitochondria produce ATP when in the ooplasm. This is an issue because sperm mitochondria are thought to degrade in the ooplasm, and thus do not contribute their mtDNA to the embryo.
There are a couple of cases of maternal inheritance of mtDNA found in mammals (Hutchison et al., 1974). The typical mammalian sperm mid-piece contains about 50-75 mitochondria with one copy of mtDNA in each. In contrast, the oocyte contains around $10^5$ to $10^8$ mitochondria which exceed that of sperm by a factor of at least $10^3$. Therefore, a simple explanation of maternal inheritance is the paternal contribution of mtDNA is diluted by that of the maternal (Ankel-Simons and Cummins, 1996).

D. Preimplantation Development

After fertilization, the zygote enters preimplantation development which includes formation of the zygote, cleavage, activation of the embryonic genome, and the beginning of cellular differentiation (Kanka, 2003). This stage takes 3 to 4 days in mice, 5 to 7 days in humans (Lanza, 2006). Upon activation of mitosis-promoting factor (MPF), cleavage is initialized in the zygote (Gilbert, 2006). The zygote undergoes first mitosis and divides into a 2-cell stage, 4-cell stage, 8-cell stage and 16-cell stage embryo which is called morula (Fig. 1). The morula is a solid ball of cells. The embryos undergo cleavage stages in the oviduct (Fig. 1). As the zygote divides, the cells become smaller. The early embryo does not increase in size during these first few cleavages. Therefore, the morula is the same size as the zygote (Gilbert, 2006).
An important event called compaction occurs at the 8-cell stage (Maro et al., 1990; Gilbert, 2006). During compaction, cells increase their adhesion by forming tight junctions, adherent junctions and gap junctions between the individual blastomeres. As a result, the blastomeres flatten upon each other. The cytoskeleton plays a role during compaction. Microfilaments facilitate microtubule redistribution during compaction and the organization of the actin also changes during preimplantation development (Albertini et al., 1987; Maro et al., 1990). After compaction, the embryo emerges as the morula (Fig. 1).

The next stage is the blastula which in mammals is called a blastocyst (Gilbert, 2006). During the blastocyst stage, a process named cavitation happens, during which fluid is transferred across the outer blastomeres to form a fluid filled cavity called the blastocoel. The blastocyst is the stage at which differentiation of the embryo starts. Two cell types are present in the blastocyst: inner cell mass (future embryo) and trophoblast (future placenta) (Fig. 2).

Expression of several genes including Cdx2, eomesodermin, Oct4, and Nanog leads to differentiation of the two cell types in the blastocyst (Gilbert, 2006). Oct4 stimulates the morula cells to become inner cell mass and not trophoblast, while Nanog works at the next differentiation event, promoting the ICM cells to become the pluripotent embryonic epiblast and preventing the ICM cells from differentiating into hypoblast (Gilbert, 2006). In addition, Oct4, Nanog and Sox2 are key factors in maintaining pluripotency in ES cells (Pan and Thomson, 2007). Oct4 expression is initiated at the late 4-cell
stage and becomes restricted to the inner cell mass (ICM) of the blastocyst. In contrast, Nanog transcripts are first detected in the compacted morula and become restricted to the ICM. Homozygous Nanog mutant embryos give rise to an ICM, but they fail to maintain pluripotency in the cells of the epiblast which instead differentiate into primitive endoderm cells causing embryo death (Facucho-Oliveria and St. John, 2009). These data suggest that Nanog cooperates with Oct4 and Sox2 during late preimplantation and early post-implantation embryogenesis to maintain the pluripotency of the epiblast cells (Facucho-Oliveria and St. John, 2009).

Cdx2 and eomesodermin are involved in trophoblast formation (Gilbert, 2006). At the 8-cell stage, Oct4, eomesodermin and Cdx2 are expressed in each blastomere. At the blastocyst stage, Cdx2 and eomesodermin expression is restricted to the trophoblast cells. Eomesodermin stimulates expression of genes that form trophoblast (Gilbert, 2006). Cdx2 blocks Oct4 and Nanog expression in the trophoblast, thereby maintaining the trophoblast cells.

Developmental competence is the capacity of the zygote to undergo normal development. The activation of the embryonic genome called zygotic gene activation is an important event that must occur following fertilization. Zygotic gene activation occurs at different developmental stages in various species (Schultz et al., 1995). For example, it occurs at the 2-cell stage in the mouse and 4-cell stage in the human (Gilbert, 2006). Microarray analysis
identified 4,562 genes that were differentially expressed in the preimplantation embryo, such as Cdk2, Cap1 and Ube2j2 (Jeong et al., 2006).

Developmental competence depends on a normal mitochondrial distribution. Research studies with mice have found a slight but significant decrease in ATP content during development from the 1-cell or 2-cell stage to 4-cell and 8-cell embryos. The ATP content remained constant in morulae and early blastocysts, but was reduced significantly in late blastocysts just before implantation (Spielmann et al., 1984). These results suggest a need for ATP during preimplantation development, which is not surprising to see since mitochondria provide energy for nearly all cell activities in eukaryotes (Smith et al., 2005).

E. Implantation

After about 4-5 days of development, the embryo emerges from the oviduct at the blastocyst stage (Fig. 1). After the blastocyst hatches from the zona pellucida, it is ready to implant in the endometrium of the uterus (Fig. 1; Gilbert, 2006). Gastrulation occurs during later stages of implantation. During gastrulation, the three embryonic germ layers are formed by differentiation of the ICM and cell migration. Implantation and gastrulation need actively functioning mitochondria since they require a lot of energy in the form of ATP.
Figure 1. From human oogenesis to implantation. In mammals, oocytes develop within follicles in the ovary. When the oocytes are mature, they will be ovulated from the ovary and enter the oviduct. During ovulation, the secondary oocyte is released from the ovary and enters the oviduct. The secondary oocyte is arrested in metaphase II (Met II) until fertilization. Fertilization occurs in the upper third of the oviduct. After fertilization, the zygote enters preimplantation development. The zygote undergoes first mitosis and divides into a 2-cell stage, 4-cell stage, 8-cell stage and 16-cell stage embryo which is called morula. The next stage is the blastula which in mammals is called a blastocyst. After about 4-5 days of development, the embryo emerges from the oviduct at the blastocyst stage. The blastocyst is now ready to implant in the endometrium of the uterus.
Figure 2. Mammalian blastocyst. Two cell types are present in the blastocyst: inner cell mass (future embryo) and trophoblast (future placenta).
CHAPTER III

THE MITOCHONDRION AND ITS FUNCTIONS
A. Structure

Mitochondria are important and essential organelles in eukaryotic cells. There can be hundreds to thousands of mitochondria per cell (Freitas, 1999). For example, there are 1000-2000 mitochondria per cell in liver cells (Alberts et al., 1994). Some cells such as sea urchin sperm have a single mitochondrion (Ardon et al., 2009). Mitochondria range from 1–10 micrometers in diameter (Freitas, 1999; Campbell and Reece, 2005). They are surrounded by a double-membrane system consisting of inner and outer membranes (Fig. 3). These two membranes are composed of a phospholipid bilayer and proteins similar to that of the eukaryotic plasma membrane (Alberts et al., 1994). The outer membrane contains porin proteins which form channels that are permeable to water-soluble molecules that are 5 kDa or less (Ha et al., 1993). The inner membrane has a high protein content and contains the phospholipid, cardiolipin, but lacks porins (Alberts et al., 1994; Cooper and Hausman, 2006). The inner and outer membranes are separated by an intermembrane space. It has a similar ionic concentration to the cytosol (Alberts et al., 1994). The space enclosed by the inner membrane is called the matrix. The matrix contains the mitochondrial genome (mtDNA) and enzymes responsible for oxidative phosphorylation (OXPHOS). It is rich in protein. The inner membrane is folded inward to the matrix cristae. This increases the surface area of the inner mitochondrial membrane enhancing the function of this organelle. The cristae are numerous and prominent when mitochondria are active (Alberts et al., 1994).
Mitochondria are unique among the cytoplasmic organelles because they contain their own DNA (Cooper and Hausman, 2006). The fission and fusion of mitochondria are under the control of both the nuclear genome and their own genome (Benard and Karbowski, 2009). Mitochondria are semi-autonomous and self-producing organelles.

The mtDNA has several characteristics. First, it is circular like bacteria, which could be evidence for the origin of mitochondria (Cooper and Hausman, 2006). Secondly, it has a few genetic code variants. Mitochondria use a slightly different genetic code from the “universal” genetic code (Cooper and Hausman, 2006). For example, AGA and AGG are universal for arginine in mammals; but in mtDNA, they are for a stop codon. In another example, UGA is standard for a stop codon, but encodes for tryptophan in mitochondria. Third, there are multiple copies of mtDNA (2-10) per mitochondrion (Wiesner et al., 1992).

Finally, the size of mtDNA can vary considerably between different species. For instance, the size of mtDNA is 6 kb to 77 kb in protists, 19 kb to 100 kb in fungi, 187 kb to 570 kb in plants, usually more than 200 kb in humans, and less than 40 kb (usually 13 kb to 22 kb) in most other animals (Bullerwell and Gray, 2004). Despite enormous variations in genome size, the coding function of the mtDNA has remained relatively stable. Plants have larger mtDNA. An example is the largest sequenced plant mtDNA to date: Arabidopsis thaliana mtDNA, which is 367 kb and encodes for 57 proteins (Unseld et al., 1997). However, they do not appear to contain significantly
more genetic information. The genome expansion is accounted for primarily by large intergenic regions, repeated segments, introns, as well as by incorporation of foreign DNA (plastid, nuclear and plasmid DNAs). In general, mtDNA codes only for genes involved in the mitochondrial translation apparatus, electron transport and OXPHOS (Bullerwell and Gray, 2004). For example, human mtDNA is relatively small and has 16,569 bp. It has 37 genes with no introns and encodes 13 peptides (Anderson et al., 1981). The 13 peptides localize to the inner mitochondrial membrane. Human mtDNA also encodes for two rRNA (16S and 12S rRNA) and 22 tRNAs which are required for translation of the proteins encoded by mtDNA (Pérez-Martínez et al., 2008). The D loop contains a transcriptional promoter sequence. The rest of the proteins found in mitochondria are encoded by the nuclear genome which has about 1,500 mitochondrial-related genes (Lonergan et al., 2007).

B. Origin

The origin and evolution of mitochondria remains controversial. However, the most popular hypothesis is the endosymbiotic theory of Lynn Margulis (Sagan, 1967). It is thought that primordial eukaryotic cells were unable to metabolically use oxygen. At some point, they were colonized by primitive aerobic bacteria that provided oxidative metabolism to the primordial eukaryotic cells. In return, the primitive eukaryotic cells provided for the bacteria which eventually evolved into mitochondria. Endosymbiotic theory posits mitochondria are the direct descendants of a bacterial endosymbiont.
(alpha-Proteobacteria) that became established at an early stage in a nucleus-containing host cell (Gray et al., 1999). This symbiotic relationship is believed to have developed 1.7-2 billion years ago (Feng et al., 1997). The ability of these bacteria to conduct respiration benefited the host cells, so it is considered as an evolutionary advantage.

Several pieces of evidence support the endosymbiotic theory (Gray et al., 1999). First, mtDNA is circular which is different from nuclear DNA and is similar to DNA of bacteria. Besides that, the mitochondrion is about the same size as a bacterium. Also, mitochondria carry several enzymes and transport systems similar to those of prokaryotes. The last piece of evidence is from the genome. In recent years, the genomes of a large variety of mitochondria and bacteria were sequenced. DNA sequence analysis and phylogenetic construction data support a monophyletic origin of the mitochondria from an alpha-proteobacteria ancestor (Bullerwell and Gray, 2004).

C. Biogenesis

Binary fission is the process by which mitochondria reproduce (Benard and Karbowski, 2009). Their reproduction is not necessarily timed with the cell cycle. Instead, energy demands of the cell appear to dictate mitochondrial replication in that more mitochondria are present when energy needs are high such as during exercise. New mitochondria can also form by
fusing together (Benard and Karbowski, 2009). The significance of this not understood.

D. Inheritance

Mitochondrial inheritance is unusual and unlike that of the nuclear genome. The nuclear genome exhibits biparental inheritance in which each daughter cell receives a copy of the nuclear genome. During fertilization, the ovum and sperm contribute to the genome equally. In contrast, mitochondria exhibit uniparental inheritance in most organisms. Although the sperm contributes one or more mitochondria to the zygote, they are ubiquitinated and actively degraded. Therefore, sperm mitochondria do not contribute genetic information to the embryo (Sutovsky et al., 1999). Instead, mitochondria are maternally inherited.

E. Biochemistry

The primary function of mitochondria is to generate useful energy in the form of ATP for cellular activities (Fig. 4). They do this by the process of OXPHOS in which carbohydrates, proteins and fats that are obtained by organisms in their diet are broken down and converted into ATP (Cooper and Hausman, 2006). About 90% of the energy the organism uses for various activities is produced by mitochondria (Pike and Brown, 1984).
Mitochondria produce ATP by a chemiosmotic mechanism (Cooper and Hausman, 2006). In this process, carbohydrates and fats obtained by the organism are used for energy production. The carbohydrates first split into pyruvate in the cytosol through glycolysis (Fig. 4). Then the pyruvates are transported into the mitochondrial matrix where they are oxidized into acetyl-CoA and enter the citric acid cycle (also known as the TCA cycle). Fats are converted to fatty-acyl-CoA and transported into the matrix where they are oxidized to Acetyl-CoA and enter the TCA cycle.

The enzymes for the citric acid cycle in the matrix oxidize the acetyl-CoA to carbon dioxide, and produce three molecules of NADH and one molecule of FADH2, which are used as a source of electrons for the electron transport chain. The electrons released by NADH are transferred through the electron transport chain by complex I, cytochrome c, and complexes III and IV. Electrons released by FADH2 are transferred through the electron transport chain by complexes II and III, cytochrome c, and complex IV. A proton gradient is created in the intermembrane space which is pH 7 compared to pH 8 in the matrix. The energy stored in the proton gradient then drives the synthesis of ATP when the protons flow back to the matrix through complex V that is also called ATP synthase. This coupling of ATP synthesis and proton flow is called chemiosmosis (Cooper and Hausman, 2006).
F. Mitochondrial Polarity

Mitochondrial polarity ($\Delta \Psi m$) refers to the potential difference across the inner mitochondrial membrane (Van Blerkom et al., 2002). The magnitude of mitochondrial polarity is a determinant of several mitochondrial functions, such as regulation of ionic fluxes and ATP liberation, and therefore reflects mitochondrial activity (Van Blerkom et al., 2002; Wang et al., 2009). It is also the driving force for other activities, including mitochondrial protein translocation and modification, material transport, energy transition, and intercellular contact and communication (Huang et al., 2002; Van Blerkom et al., 2006). Mitochondria that look morphologically homogenous in differentiated cells and in mouse and human oocytes can be distinguished based on $\Delta \Psi m$ as visualized with mitochondria-specific potentiometric fluorescent probes such as JC-1 (Van Blerkom et al., 2002, 2009). The mitochondria with high $\Delta \Psi m$ are often pericortical (Van Blerkom et al., 2002). Therefore, although morphologically homogenous, mitochondria can be functionally heterogeneous. The significance of changes in mitochondrial $\Delta \Psi m$ is not well understood; however, it may relate to the presence of contact points between cells, or the need for high activating mitochondria in the cortex in preparation for cytokinesis.
G. Function

Besides this conventional function of energy production as described above, recent research suggests that mitochondria are involved in calcium homeostasis, cell signaling, oxygen sensing and apoptosis (Duchen, 1999; Chandel and Schumacker, 2000). For instance, mitochondria also participate in calcium storage and the transport of Ca2+ (Berridge et al., 1998; Hanjnoczky et al., 2007). When mitochondria are near the plasma membrane, they can regulate Ca2+ entry. When mitochondria are near the endoplasmic reticulum (ER) which is the major site of calcium storage, the mitochondria are involved in Ca2+ propagation. Recent research revealed that mitochondria regulate calcium signaling and the Ca2+-dependent nuclear factor of activated T cells (NFAT) pathway whose target genes are essential for embryonic heart development (Cao and Chen, 2009).

Mitochondria also play a role in programmed cell death – apoptosis (Duchen, 1999). When the Bcl-2 proteins on mitochondrial membranes detect DNA damage, they activate Bax proteins, which cause the mitochondrion to release cytochrome C, and other proteins, which trigger downstream apoptosis signals like caspases. This finally leads to destruction of the cell. Thus mitochondrion – mitochondrion interactions are important for apoptotic signals. In addition, mitochondria are found to translocate during the tumor necrosis factor (TNF)-induced apoptosis in HeLa cells (Domnina et al., 2002). This suggests mitochondrial location may play a role in the process of apoptosis.
Mitochondrial diseases and disorders of the mitochondrial respiratory chain can be caused by mutations of either mtDNA or the nuclear genome (DiMauro, 2004). There are various symptoms of mitochondrial diseases with various causes (Wallace, 1999). They can be present in various body regions and the disease varies from person to person. Many organs could be affected in mitochondrial diseases such as the cells of the brain, nerves, muscles, kidneys, heart, liver, eyes, ears, or pancreas. One or more organs could be affected in different patients, so the disorder can range in severity from mild to fatal. The symptoms might include poor growth, muscle weakness, visual or hearing loss, mental retardation, heart, liver or kidney disease, diabetes, respiratory or gastrointestinal disorders, obesity, Leber’s hereditary optic neuropathy, Leigh syndrome and even infertility (Wallace, 1999; Schapira, 2006).

Most mitochondrial diseases are inherited and the inheritance patterns vary from Mendelian to maternal inheritance as well as a combination of the two (Wallace, 1999). The major causes of mitochondrial diseases are nuclear DNA defects, mtDNA defects, or a combination of both defects. For example, deletions in mtDNA and tRNA point mutations cause cardiomyopathies (Arbustini et al., 1998) and diabetes (Suzuki et al., 1997; Liou et al., 2003). The genotype-to-phenotype correlations in mitochondrial diseases are complex since the same mutation can result in multiple
phenotypes, and the same phenotype can result from several different mutations. In addition, the distribution of mtDNA mutations varies from being present in all tissues to only being found in specific cells or tissues (Schapira, 2006).

Besides DNA defects, mitochondrial $\Delta \Psi m$ declination can also lead to mitochondrial-related disorders (Wang et al., 2009). In Alzheimer’s disease, cyclical mitochondrial $\Delta \Psi m$ fluctuation linked to electron transport, $F_0F_1$ ATP-synthase and mitochondrial $Na^+/Ca^{+2}$ exchange are found to be reduced (Thiffault and Bennett, 2005). In some circumstances, mitochondrial diseases can be triggered by medicines or toxic substances (Schapira, 2006).

The mitochondrion is such a crucial organelle. Defects in mtDNA or dysfunction of OXPHOS or other functions of mitochondria prevent the cell from functioning normally. For example, if one of the multi-subunit complexes on the electron transportation chain such as ATP synthase has defects in structure, chemiosmosis cannot happen and ATP is not produced. A recent study found that defects in human complex I result in energy generation disorders, and also lead to neurodegenerative disease (Lazarou et al., 2009). The cells suffer from energy crisis. What is more, the incompletely burned food might accumulate inside the cell as poison to harm the body even further. For example, free radicals such as reactive oxygen species (ROS) might be produced and cause oxidative stress (Schapira, 2006; Dumollard et al., 2009). The principal location for ROS generation is complex III (Chen, 2003). However, since a great number of genes are involved, the biochemical
mechanisms of mitochondrial diseases are diverse.

Mitochondrial dysfunction is also involved in mammalian aging and carcinogenesis (Wallace, 1999). Several types of cancers have mtDNA mutations. The progressive accumulation of mtDNA mutations during a lifetime has been suggested to contribute to aging and carcinogenesis (Chinnery et al., 2002; Trifunovic and Larsson, 2008). At least 271 cancer mutations were observed in conserved positions of mtDNA, and 70 of them appeared in more than one tumour (Santos et al., 2008). As mentioned earlier, excessive ROS generation can cause oxidative stress, which is one of the important reasons for DNA damage. Therefore, it is easy to understand the progressive accumulation of mtDNA mutations in mitochondrial disorder patients which can be even worse. Besides causing aging and cancer, the progressive accumulation of mtDNA mutations also cause some age-related dysfunctions which decrease oocyte quality so that mitochondrial function can be seen as a parameter to evaluate oocyte quality (Wang et al., 2009).

One notable characteristic in mitochondrial diseases caused by mtDNA mutations is that they can cause ultrastructural changes of mitochondria, like reduction of the amount of cristae membranes caused by depletion of mtDNA (Gilkerson et al., 2000) and swelling mitochondria with sparse cristae in transmission electron microscopic studies of cultivated human skin fibroblasts harboring three different pathologic mtDNA point mutations (Brantová et al., 2006). When the clinical characteristics and ultrastructural features of skeletal muscle in mitochondrial cytopathies were examined with a
transmission electron microscope, there was an excess proliferation and abnormal shape of mitochondria. Some mitochondria were enlarged or contained multiple granules or paracrystalline structures (Zhang et al., 2009). These ultrastructural changes are valuable in the diagnosis of mitochondrial disorders. Additionally, these results also revealed the tight relationship between mitochondrial structure and function.

In addition to the tight relationship of mitochondrial structure and mitochondrial diseases, recent studies found mitochondrial diseases are also related to mitochondrial dynamics. The balance of mitochondrial fusion and fission which are opposing forces are critical for cell survival (Benard and Karbowski, 2009). Mutations in two mitochondrial fusion genes MFN2 and OPA1 cause prevalent neurodegenerative diseases. In addition, impaired MFN2 expression also causes other diseases such as type 2 diabetes or vascular proliferative disorders (Liesa et al., 2009).

Currently, mitochondrial-related diseases cannot be cured and the treatment for these diseases is still very limited. The treatment for mitochondrial diseases is highly individualized due to the complexity of various causes. Certain vitamin and enzyme therapies could be helpful for some patients like Coenzyme Q10, vitamin B family, vitamin C, biotin, vitamin E and other antioxidants (Przyrembel, 1987). However, the use of antioxidants in mitochondrial diseases has not been tested in clinical trials yet (Schapira, 2006). Because many mitochondrial disorders are maternally inherited, a recently proposed treatment for inherited mitochondrial disease is embryonic
mitochondrial transplantation and gene therapy (Kyriakouli et al., 2008). These approaches have only been attempted in cell cultures and have showed promise. However, they are still far from clinical application.

I. Role in Gametogenesis and Development

Mitochondrial fusion is important for formation of the Nebenkern and mutations which inhibit mitochondrial fusion lead to sterility of mutant flies (Benard and Karbowski, 2009). ATP production is important for sperm motility. As described in Chapter II, sperm carry mitochondria in their midpiece to provide energy for fertilization. A recent research study found that mtDNA mutation can lead to impaired spermatogenesis and impaired sperm motility (Shamsi et al., 2008).

Mitochondria play a central role in oogenesis and early embryonic development since they provide ATP for the processes from oogenesis to implantation. In addition, mitochondria also provide ATP for the processes of mitosis and meiosis in the events of DNA replication, breakdown and formation of the nuclear membrane, and mitotic spindle movements. Mitochondria appear to be required for oocyte and embryo maintenance and development (Wang et al., 2009). For example, the mitochondrial fusion proteins, Mfn1 and Mfn2 are vital for embryo survival in Mfn1-/- and Mfn2-/- knockout mice (Benard and Karbowski, 2009). There are several examples that mitochondrial dysfunction causes infertility. In mouse oocytes, mitochondrial
dysfunction can result in preimplantation embryo arrest after in vitro fertilization (Thouas et al., 2004).

The number of mitochondria changes during development from the oocyte stage to implantation (Van Blerkom, 2009). The actual number of mitochondria per oocyte or blastomere is highly controversial, and to date has not been established so that there is a consensus among researchers (Van Blerkom, 2009). Estimates of mitochondrial number based on mtDNA copy number per mitochondrion (which also appear to vary with meiotic competence and development) do not agree with estimates based on morphometry using tissue sections and transmission electron microscopy (Van Blerkom, 2009). What is consistent, however, is that mitochondrial numbers decline in unhealthy oocytes, and oocytes from women of advanced maternal age. This may relate to the maternal-age associated decline in meiotic and developmental competence (Van Blerkom, 2009). The assisted reproductive technique of cytoplasmic transfer has been shown to restore oocyte health (Von Blerkom, 2009). In this technique, cytoplasm containing mitochondria from the oocyte of a younger, healthy individual is transferred to an oocyte of advanced maternal age, and oocyte health is restored (Wang et al., 2009). This indicates that there is an age-related difference in cytoplasm, which may be based on mitochondrial activity.

Facucho-Oliveira et al. (2007) claim that there is no mtDNA replication until postimplantation. This implies that mtDNA copy number does not increase, but could decline due to mtDNA mutation or damage. This would
leave oocytes highly vulnerable to mitochondrial dysfunction (Facucho-Oliveira et al., 2007; Wang et al., 2009).

In addition to a threshold number of mitochondria being critical for meiotic competence and developmental competence, defects in actual mitochondrial structure result in a decline in meiotic competence. For example, defects in mitochondrial structure including swelling or disrupted cristae cause a decrease in meiotic competence and increase in infertility in women of advanced maternal age (Wang et al., 2009). Moreover, mitochondria change shape during development and this is important for developmental competence (Van Blerkom, 2009).

The relative level of mitochondrial ΔΨm is important for normal fertilization and early embryonic development (Van Blerkom et al., 2006, 2007). Mitochondria in oocytes and preimplantation embryos may have different mitochondrial ΔΨm. High-polarized pericortical mitochondria may have a role in the acquisition of oocyte competence and the regulation of early developmental processes (Van Blerkom et al., 2002), while low-polarized mitochondria are associated with mitochondrial dysfunction and abnormal embryos (Wilding et al., 2001). A recent study found mitochondrial ΔΨm in human and mouse oocytes can be regulated by competition between oxygen and nitric oxide (NO) (Van Blerkom et al., 2008). It has been suggested that NO from cumulus cells depresses ATP production in mitochondria, and this is important for ovulation to occur (Wang et al., 2009).
Mitochondria also regulate intracellular calcium and proapoptotic factors during these processes (Wang et al., 2009). A local transient increase in free Ca\textsuperscript{2+} can lead to an increase or decrease in mitochondrial respiration locally in mouse oocytes (Van Blerkom, 2009). Intracellular Ca\textsuperscript{2+} (in addition to ATP) is important for nuclear envelope breakdown during meiotic maturation in oocytes (Wang et al., 2009). This is consistent with a role of mitochondria in maintaining intracellular calcium homeostasis (Duchen, 1999).

Another example that mitochondria play a central role during early embryonic development is that mammalian embryonic cells have a low glucose metabolism at the earliest stages of development. Both glycolysis and the pentose phosphate pathway are suppressed, so the citric acid cycle of mitochondria is the major source to reduce equivalents (or electron donors, such as NADH) that are used in antioxidant defense (Dumollard et al., 2009). Therefore, mitochondrial dysfunction in embryos is very likely to cause developmental retardation.

The functions of mitochondria decline as the organism ages. It has been proposed that ROS may cause oxidative stress in mitochondria (Schapira, 2006). This may result in mutations in mtDNA, resulting in a reduction of copies of mtDNA, and the reproduction and increase of mtDNA damage and misfolded enzymes. When the ratio of normal mitochondria to dysfunctional mutant mitochondria falls below the threshold, the cell cannot get enough energy from mitochondria and programmed cell death will be activated.
In support of this idea, ROS can make the oocyte unhealthy (Wang et al., 2009).
Figure 3. Schematic diagram of mitochondrial structure.
Figure 4. Schematic diagram of bioenergetic pathways in mitochondria.
CHAPTER IV

MITOCHONDRIAL DISTRIBUTION
A. Role of the Cytoskeleton

The cytoskeleton is involved in the distribution of mitochondria (Katayama et al., 2006; Kabashima, 2007). The cytoskeleton is a network of protein filaments extending throughout the cytoplasm, and is found in all eukaryotic cells. The cytoskeleton provides a structural framework for the cells, maintains cell shape, and positions of organelles (Pollack and Kopelovich, 1979). In addition, it is involved in a variety of cellular activities such as cell movements, transportation, exchange of energy, cell division, cell differentiation, and even signal transduction (Liu et al., 2002).

The cytoskeleton has three principal types of protein filaments: microfilaments (MFs), intermediate filaments, and microtubules (MTs). MFs, which are also called actin filaments, are made by polymerization of actin monomers to form F actin. In contrast, MTs are made by polymerization of proteins called tubulin. MTs are assembled from reversible polymerization of dimers of two types of tubulin proteins, α-tubulin and β-tubulin. MTs in most cells extend outward from a microtubule-organizing center called centrosome. MTs play a role in a variety of cellular processes. Mitochondrial movements and morphology are regulated through MTs (Linden et al., 1989). During mitosis, MTs form the mitotic spindle for chromosome separation (Cooper and Hausman, 2006). Both MFs and MTs are required for completion of cytokinesis (Larkin and Danilchik, 1999).
Actin and tubulin polymerization have been extensively studied in vitro. There are specific drugs to block their assembly. For example, colchicine and nocodazole lead to disassembly of MTs by binding to tubulin thereby inducing depolymerization (Leach et al., 2005), whereas cytochalasin B and cytochalasin D disrupt MFs by binding to the barbed end of an actin filament thereby preventing monomer addition and slow the rate of filament polymerization (Bonder and Mooseker, 1986). These drugs have been used to gain insight into the functions of the cytoskeleton.

B. Distribution in Somatic Cells

Mitochondria are dynamically distributed in cells and utilize cytoskeletal tracks and motor proteins for their movements (Frederick and Shaw, 2007). Appropriate mitochondrial distribution is essential for cell survival and developmental competence of embryos (Katayama et al., 2006; Nagai et al., 2006). The spatial organization of mitochondria has been shown to depend on MTs in neurons and pig embryos (Sun et al., 2001). Several cell functions need accurate mitochondrial distribution. For example, during cytokinesis, equal mitochondrial distribution is critical to ensure viability of daughter cells, since they are essential organelles that provide energy for all cellular activities (Hales, 2004). On the other hand, mitochondrial distribution is restricted by the arrangement of the cytoskeleton. These interactions of mitochondria with the cytoskeleton are crucial for normal mitochondrial function (Boldogh and Pon, 2007).
In differentiated somatic cells, mitochondria are distributed homogeneously. For example, mitochondria are scattered throughout the cytoplasm of these differentiated cells (Figure 5). In porcine fetal fibroblast cells, mitochondria are not scattered throughout the cytoplasm, but form a meshwork filling the entire cell. In addition, this localization depends on microtubules, since nocodazole treatment leaves mitochondria tightly located around the nucleus in a dot-like pattern (Katayama et al., 2006). Using different cytoskeletal modulators, other studies have shown mitochondrial distribution depends on microfilaments. In two-cell embryos of golden hamsters, microtubules and microfilaments were found to control mitochondrial distribution mutually (Kabashima, 2007). In two-cell embryos of golden hamsters, normal mitochondrial distribution is perinuclear with a few mitochondria at the cell cortex. After nocodazole treatment, mitochondria were found to extend into the subcortical region and aggregated in patches. After cytochalasin D treatment, there were less mitochondria at the cell cortex, suggesting that mitochondria moved back around the nucleus. After a treatment of both inhibitors, mitochondrial distribution was found to be similar to the pattern after cytochalasin D treatment (Kabashima, 2007).

C. Distribution during Oogenesis, Fertilization and Preimplantation Development

Several studies have tried to clarify the relationship between mitochondrial distribution and oogenesis, fertilization and preimplantation
development. The studies found the distribution patterns of mitochondria are stage- and cell-cycle-specific (Fig. 6). Homogeneous and heterogeneous spatial arrangements are two major mitochondrial distribution patterns in oocytes (Wang et al., 2009).

Research on mitochondrial distribution in pig oocytes using MitoTracker Green FM stain showed that during oogenesis, GV and Met II oocytes have scattered mitochondrial distribution. However, during fertilization and preimplantation development, pronuclear embryos, 4-cell embryos and blastocysts have a perinuclear mitochondrial arrangement (Sun et al., 2001). Therefore, mitochondria translocate during normal development. The dramatic translocation of mitochondria upon fertilization suggests that the change may be triggered by the fertilization event.

Even primary oocytes have a different mitochondrial distribution than mature oocytes. A recent research study on mitochondrial distribution in canine oocytes found that the primary oocyte in the GV stage has a different mitochondrial distribution than Met II stage oocytes (Valentini et al., 2009). The GV stage oocytes have three types of mitochondrial distribution. The first type of distribution is small aggregates diffused throughout the cytoplasm; the second type is diffused tubular networks; the third type is pericortical tubular networks. However, most Met II stage oocytes showed a diffused tubular mitochondria network. Besides the mitochondrial distribution pattern difference, they provided a possible explanation for it: the changes in the
mitochondrial distribution pattern in immature canine oocytes is related to the reproductive cycle stage (Valentini et al., 2009).

Similar to pig and canine oocytes, evidence of stage- and cell cycle-specific mitochondrial distribution were also found in mouse and human oocytes (Van Blerkom, 2009). In GV stage mouse oocytes, mitochondria are largely uniformly distributed throughout the cytoplasm. However, from GVBD to metaphase I (Met I), the mitochondria begin to surround the newly condensed chromosomes as a sphere and form a perinuclear distribution. In Met II oocytes mitochondria are randomly and uniformly distributed. The perinuclear distribution returns after fertilization. It becomes more obvious after fertilization in two-cell embryos (Van Blerkom, 2009).

In addition to changes in density of mitochondria, mitochondrial membrane potential can also vary. Recent studies showed the transition of mitochondria from homogeneous to heterogeneous is correlated with the cumulus apoptosis during oogenesis and different developmental stages (Wang et al., 2009).

Mitochondria are even involved with developmental competence. An example is abnormal mitochondrial distribution in Met II mouse oocytes leads to reduced developmental competence. In a recent study, a treated group of Met II oocytes of mtGFP-tg mice were frozen in liquid nitrogen for 5 minutes and thawed (Nagai et al., 2006). This treated oocytes showed a significantly lower developmental competence when they were compared with normal,
untreated control oocytes. The control oocytes showed perinuclear mitochondrial staining, and these oocytes when fertilized had a high developmental competence. The treated oocytes had randomly clumped mitochondria in the cytoplasm, and poor developmental competence. The meiotic arrest in the oocytes with abnormal mitochondrial distribution may be due to abnormal distribution of ATP (Nagai et al., 2006). Moreover, another study found the loss of developmental competence due to oocyte mitochondrial dysfunction. This might be overcome by cytoplasmic transfer of normal mitochondria into the oocyte (Nagai et al., 2004). In the same manner, abnormal mitochondrial distribution also causes loss of meiotic competence in human oocytes. During in vitro fertilization (IVF), abnormal mitochondrial distribution that had dense clusters of mitochondria in human oocytes arrested meiosis prematurely (Van Blerkom, 2009).

The spatial translocation of mitochondria during development leads to the question: Is the spatial distribution of mitochondria important for their function? This is not well understood so far. Several possible explanations were proposed based on the results from research, which will be addressed in more detail in Chapter V.
Figure 5. Diagram showing location of mitochondria in a typical somatic cell. A bovine pulmonary artery endothelial cell was triple-labeled for the nucleus (blue) with 4’,6-diamidino-2-phenylindole (DAPI), microtubules (green) with anti–α-tubulin mouse IgG<sub>2b</sub> monoclonal antibody prelabeled with the Zenon® Alexa Fluor® 488 Mouse IgG<sub>2b</sub> labeling kit and the mitochondria (red) with anti-OxPhos Complex V subunit a, mouse IgG<sub>2b</sub> monoclonal antibody (A21350) prelabeled with the Zenon® Alexa Fluor® 555 Mouse IgG<sub>2b</sub> labeling kit. Mitochondria are scattered throughout the cytoplasm of this differentiated cell. Diagram based on fluorescence micrographs from www.invitrogen.com.
Figure 6. Distribution of mitochondria during oogenesis and fertilization in mammalian oocytes. The black spots represent mitochondria. GV, germinal vesicle stage; HPM, high potential (polarized) mitochondria; GVBD, germinal vesicle breakdown stage; M I, metaphase I; M II, metaphase II; PN, pronuclear stage. Based on Sun et al., 2001; Valentini et al., 2009; and Van Blerkom, 2009.
CHAPTER V

MITOCHONDRIAL DISTRIBUTION IN EMBRYONIC STEM CELLS
A. Embryonic Stem Cells

ES cells are cultures of cells derived from the inner cell mass of a blastocyst or earliest morula stage embryos (Wright, 1999; Fig. 7). They have the ability to undergo self-renewal and differentiation, and have the potential to give rise to an entire organism and to all cell lineages (Abbondanzo et al., 1993; Odorico et al., 2001). Thanks to the characteristic of immortality, ES cells are capable of unlimited, undifferentiated proliferation in vitro and can be maintained for years in continuous culture (Thomson et al., 1998; Hoffman and Carpenter, 2005).

ES cells have their specific gene expression. The key pluripotent factors are Oct4 and Sox2. Oct4 is the master regulator and needs to be maintained at a specific expression level in order to maintain pluripotency (Niwa, 2000). Besides the two fundamental key factors, Nanog is also an important transcription factor which is regulated by Oct4 and P53, and at the same time works together with Oct4 and Sox2 to control the downstream gene regulation and maintain pluripotency. These key factors form a regulatory network to limit each other’s expression level, which is essential to maintain the properties of ES cells (Pan and Thomson, 2007). Therefore, these pluripotent factors Oct4, Sox2 and Nanog are often used as marker genes in charactering ES cells.

Besides their specific gene expression, the ability to form a teratoma, which is a tumor made of cells from all three germ layers, is another
characteristic of ES cells (Thomson et al., 1998). Recent studies suggest perinuclear mitochondrial distribution could be another characteristic of ES cells (Lonergan et al., 2006; Facucho-Oliveria and St. John, 2009). This interesting finding will be discussed more in the next section.

ES cells have medical significance for they could potentially provide an unlimited supply for tissue regeneration and tissue transplantation (Wright, 1999) and apply in cell replacement therapies (Hoffman, 2005). Conversely, when a stem cell mutates, it could become a cancer cell sustaining its growth and spreading rapidly (Reya et al., 2001).

B. Bavister’s Hypothesis

Although the spatial distribution of mitochondria with respect to the cytoskeleton has been studied in several cell types, it has not been examined in ES cells. The contribution of mitochondria to stem cell viability and differentiation must be vitally important in view of their critical roles in all other cell types. Recent research on translocation of mitochondria in the oocyte during fertilization has shown that mitochondria cluster around the pronuclei and can remain in a perinuclear pattern during embryonic development (Sun et al., 2001). This strongly suggests a key role for mitochondria in ES cells, because this clustering appears to be essential for normal embryonic development. Moreover, ES cells are derived from blastocysts. Bavister and associates have hypothesized that mitochondrial perinuclear clustering
persists through preimplantation embryonic development into the stem cells, and that this localization is indicative of stem cell pluripotency (Lonergan et al., 2006). My preliminary data using the protocols described below in Section D showed that mitochondrial distribution in MLE-15 cells is a meshwork around the nucleus. This is supportive of Bavister’s hypothesis. However, the actual mitochondrial distribution in ES cells needs to be investigated. If the results also support Bavister’s hypothesis, mitochondrial distribution could possibly be an exciting, brand new marker for ES cells. Bavister’s group has suggested that because zygotes and cleavage stage embryos including blastocysts have perinuclear staining of mitochondria, ES cells may also have perinuclear staining, indicating that the perinuclear localization of mitochondria may be an indicator of “stemness.”

When they examined mitochondrial arrangement in an adult stem cell line, Bavister et al. found perinuclear staining, indicating that the hypothesis holds true for adult stem cells (Fig. 8; Lonergan et al., 2006). They also said that since only two stem cell lines Rhesus & human have been examined, many more cell lines will need to be investigated to see if the hypothesis holds. It is important to be able to detect good stem cells with confidence especially since many are suboptimal since they are derived from “leftovers” from IVF clinics, and human ES cells may one day be used therapeutically.

Recently, studies agree that pluripotency seems to be associated with anaerobic metabolism (Facucho-Oliveria and St. John, 2009). In addition, small and immature mitochondria with a perinuclear distribution is an important
characteristic of ES cells (Facucho-Oliveria and St. John, 2009). This characteristic can even apply for the novel method of generating human and mouse induced pluripotent stem cells (iPSCs). Results of Oct4, Sox2 and Nanog expression might not be sufficient to conclude they are pluripotent. The mitochondrial properties should be investigated before concluding iPSCs are pluripotent (Facucho-Oliveria and St. John, 2009).

C. Possible Reasons for Perinuclear Mitochondria in Stem Cells

There are a number of potential reasons for the stem cells to have a perinuclear distribution of mitochondria. First, perinuclear distribution matches the OXPHOS demands of differentiating cells. The ATP content in human ES cells and an adult rhesus macaque stromal cell line (ATSC) were lower when cells were stem-like, but increased four-fold in human ES cell line and five-fold in the monkey ATSC cell line upon differentiation. Another benefit of perinuclear mitochondrial distribution at the stem cell stage is that the mitochondrion and nucleus interaction could be easier (e.g., the nuclear genome codes approximately 1500 mitochondrial-related genes). Moreover, import and export of macromolecules across the nuclear pores involve the energy-dependent Ran monomeric G protein transport system (Cooper and Hausman, 2006). Positioning mitochondria near the nucleus might provide the energy for this process in an efficient manner. Finally, perinuclear
arrangement of mitochondria might buffer the nucleus from fluctuations in Ca2+ levels occurring in the cytoplasm (Lonergan et al., 2007).

Since the main functions of mitochondria are ATP synthesis and calcium supply, the mitochondrial distribution in the oocyte may result from the high demand of ATP and calcium during cytoplasmic maturation (Wang et al., 2009). The perinuclear arrangement of mitochondria during embryonic development is very likely to due to the high energy demand around nucleus (Wang et al., 2009). Therefore, it is highly possible that the redistribution of mitochondria from perinuclear in stem cells to scattered throughout the cytoplasm in somatic cells is due to the localized change of energy demand.

The iPSCs generated by defined factors from mouse or human fibroblast cells were verified to have ES cell morphology, specific gene expression such as Oct4, Sox2 and Nanog and were capable of teratoma formation (Takahashi and Yamanaka, 2006; Yu et al., 2007). However, another important characteristic of pluripotency has not been tested yet -- whether iPSCs have immature mitochondria with a perinuclear distribution. For the considerable amount of data mentioned above, there are reasons to believe that iPSCs indeed are pluripotent. Therefore, if we carry out the described experiments in iPSCs, the staining of mitochondria should exhibit perinuclear distribution just as ES cells do.
D. Hypothetical Experiments Designed to Test Bavister’s Hypothesis

Given the need to identify healthy ES cells, it is important to know the mitochondrial arrangement in healthy ES cells, and whether this is an indicator of “stemness.” The following experiments are proposed to test Bavister’s hypothesis.

Research Objectives

1. Determine and compare the spatial distribution of mitochondria with respect to the nucleus and cytoskeleton in MES cells and a differentiated cell line, MLE-15 cells. To investigate pluripotency of iPSCs with regards to mitochondrial properties, mouse iPSCs generated from fibroblast cells as described in Takahashi and Yamanaka (2006) could be used.

2. Understand the spatial arrangement of mitochondria during differentiation by staining of mitochondria in MES cells and MLE-15 cells.

3. Determine whether mitochondrial distribution is cytoskeletal-dependent after disruption of the cytoskeleton in MES cells and MLE-15 cells.

Materials

For undifferentiated cells, MES cells could be used. For differentiated cells, MLE-15 (mouse lung epithelial) cells could be used. For iPSCs, mouse
iPSC-MEF could be used.

**Research Method**

To analyze the spatial arrangement of mitochondria during differentiation and disease, the following techniques are proposed.

a) Cell culture. MLE-15 cells will be cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 100 U/mL penicillin-streptomycin. MES cells will be cultured in DMEM containing 90 mL ES-qualified FBS, 6 mL non-essential amino acids (NEAA), 6 mL GlutaMax, 6 mL 100 U/mL penicillin-streptomycin, 6 uL β-mercaptoethanol, and 500 uL recombinant eukemia inhibitory factor (LIF) in 500 mL DMEM. Both cell types will be cultured at 37°C in 5% CO₂ in humidified air.

b) Immunofluorescence microscopy. To visualize mitochondria, nuclei and the cytoskeleton, fluorescence staining will be used. Cells attached to microscope slides (in a thin glass bottom sterile culture dish) will be fixed in 70% ethanol for 10 minutes, and rehydrated in blocking solution containing phosphate-buffered saline, (PBS, pH 7.2) and 0.3% bovine serum albumen (BSA). Tubulin will be detected with a mouse monoclonal anti-tubulin antibody (E7 antibody, Developmental Hybridoma Studies Bank, University of Iowa), and actin will be detected using a mouse monoclonal anti-actin antibody (Mab-5; LabVision Corporation Fremont, CA). Both antibodies will be diluted
1:100 in blocking solution and detected using a TRITC-conjugated goat-anti-mouse secondary antibody. Fixed cells will be incubated for 1 hr at 37°C in primary antibody (either anti-tubulin or anti-actin antibody), washed twice in PBS, incubated 1 hr at 37°C in secondary antibody, and mitochondria stained for 30 min with 1 mM MitoTracker (Molecular Probes, Invitrogen) in PBS followed by staining for 30 min in 5 µg/ml DAPI in PBS to reveal the nucleus. Cells will be mounted in Vectashield (an anti-fade agent) and viewed with a conventional fluorescence microscope using the UV, FITC and Rhodamine filter sets, or an Olympus Fluoview FV1000 confocal laser scanning microscope (Olympus America Inc., Melville, NY). The negative control will include omission of the primary antibody from the protocol, and the immunostaining data compared with the negative control to decide antigen localization.

c) Mitochondrial localization during cytoskeletal disruption. To disrupt the cytoskeleton MES and MLE-15 cells will be transferred into DMEM media supplemented with 10-100 µM colchicine to disrupt microtubules (or cytochalasin B to disrupt microfilaments), and cultured for 6 hr. After fixation as above, samples will be stained as follows. For samples treated with colchicine, anti-tubulin antibody will be used for first antibody. For samples treated with cytochalasin B, anti-actin antibody will be the first antibody, followed by secondary antibody as above, and MitoTacker and DAPI staining. Cytoskeletal and mitochondrial patterns in treated and untreated cells will be compared to reveal the role of the cytoskeleton in mitochondrial arrangement.
during differentiation.

**Expected Results**

To demonstrate feasibility of using Mitotracker FM Green and DAPI to localize mitochondria and nuclei, respectively, MLE-15 cells were double-labeled with these dyes (Fig. 9). Mitochondria appear restricted to the cytoplasm and are absent from the nucleus. This is consistent with Bavister's hypothesis. Thus the fluorescent dyes are feasible for use with MLE-15 cells.

Staining for mitochondria using MitoTracker FM Green is expected to reveal differences in mitochondrial distribution in mouse undifferentiated and differentiated cells. Treatment with the cytoskeletal disrupters (nocodazole, colchicine, cytochalasin B) should show mitochondrial distribution is mediated by the cytoskeleton. MES cells are expected to exhibit perinuclear mitochondrial distribution, and cytoskeletal disrupters are expected to further concentrate mitochondria around the nucleus. MLE-15 cells will exhibit perinuclear or random, scattered mitochondrial distribution, and cytoskeletal disrupters should concentrate mitochondria around the nucleus. The iPSCs will exhibit perinuclear mitochondria distribution just as the MES cells do. This would demonstrate a role of the cytoskeleton in maintaining the spatial distribution of mitochondria.
Figure 7. Preparation of cultured embryonic stem (ES) cells. When inner cell mass cells are removed from the blastocyst and placed in culture, the cells survive and are known as ES cells.
Figure 8. Measurement of mitotracker fluorescence intensity. Passage 11 adult rhesus macaque stromal cells (ATSC) were stained with 50 nM Mitotracker to show mitochondrial location. Cells were viewed with fluorescence microscopy at 600 x magnification. The cell periphery is outlined with a black line. The cell nucleus is shown in blue. The black dots around the nucleus represent mitochondrial staining. The red lines are the axes of the graph showing the extent of fluorescence intensity (green line) across the cell. The intense mitochondrial fluorescence around the nucleus drops precipitously toward the cell periphery, indicating the ATSC cell has a perinuclear mitochondrial arrangement. (Recreated from Lonergan et al., 2006)
Figure 9. Mitochondrial distribution in mouse lung epithelial (MLE-15) cells. Cells stained with 1 mM MitoTracker to localize mitochondria (A) and 5 μg/ml DAPI to visualize DNA (B) were viewed at 60x with conventional fluorescence microscopy. C Shows mitochondria (green) and nuclei (blue) simultaneously. Mitochondria appear restricted to the cytoplasm and are absent from nuclei.
CHAPTER VI

CONCLUSIONS AND FUTURE DIRECTIONS
A. Conclusions

Mitochondria play a vital role in oogenesis and development in mammals. This is because mitochondria not only provide ATP for these developmental processes, but mitochondria also regulate calcium signaling and apoptosis, which are critical in embryonic development. There is a subcellular mitochondrial heterogeneity. Morphologically homogenous mitochondria can be functionally heterogeneous, since they can still be distinguished on the basis of mitochondrial $\Delta \Psi m$. The significance of this is not understood.

Patients with mitochondrial-related diseases have various symptoms with various causes. Due to the complex causation of mitochondrial diseases, there is no cure for them so far, and the treatments are highly individualized and still very limited. Currently, embryonic mitochondrial transplantation and gene therapy are the two proposed methods of treatment. However, they are both experimental in vitro. In addition, mitochondrial transplantation is not feasible in adults.

Mitochondrial distribution patterns in oocytes are stage- and cell-cycle-specific. There are two major mitochondrial distribution patterns in oocytes, homogeneous and heterogeneous based on mitochondrial spatial distribution and polarity. Perinuclear mitochondrial distribution is suggested to indicate the maturation of the oocyte. There is growing evidence in several different mammalian species (mouse, dog, pig, human) supporting a
mitochondrial distribution change from scattered to perinuclear during maturation of oocytes so that development can be proceed further, otherwise there will be a meiotic arrest. Therefore, mitochondrial distribution plays a central role in establishing meiotic competence. Due to the correlation of mitochondrial distribution and oocyte meiotic competence, mitochondria can be seen as signs for evaluation of oocyte quality in reproduction.

In summary, from the data of recent studies on mitochondrial distribution, Bavister’s hypothesis is very likely to hold true. So the perinuclear mitochondrial distribution could possibly be used as a brand new marker for "stemness." My proposed research on comparison of mitochondrial distribution in mouse MLE-15 and MES cells is designed to test Bavister’s hypothesis and lead to a better understanding of mitochondrial behavior in development. Understanding the role of the cytoskeleton will also be important. This research will be of value in finding therapy for various mitochondrial diseases and cancer, and will aid in understanding ES cell function.

B. Future Directions

The spatial distribution of mitochondria in MES cells needs to be determined and compared with the distribution of mitochondria in MLE-15 cells to test if Bavister’s hypothesis holds true. Experiments need to be carried out to examine effects of the cytoskeletal modulators, nocodazole, colchicine or
cytochalasin on mitochondrial arrangement in order to determine the interaction of mitochondrial distribution and the cytoskeleton.

After clarifying the mitochondrial distribution during development in mammalian cells, the reasons for mitochondrial translocation during development and the mechanisms regulating mitochondrial translocation during differentiation deserve further investigation so that we can get a better understanding of developmental processes with regards to mitochondria. The more we understand about these mechanisms, the more likely for us to find therapy or even a cure for mitochondrial disorders. Some research has suggested that mitochondrial translocation is related to reproductive cycle stages. Recent studies find that not only the mitochondrial distribution, but the heterogeneity of shape and $\Delta \Psi m$ of mitochondria can be important for mitochondria to function normally. These claims need further confirmation in a variety of cell types.
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