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DNA damage response in in vitro matured oocytes

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BOSTON UNIVERSITY
SCHOOL OF MEDICINE

Thesis

DNA DAMAGE RESPONSE IN IN VITRO MATURED OOCYTES

by

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DNA DAMAGE IN IN VITRO MATURED OOCYTES

ELISA ATAMIAN

ABSTRACT

The reproductive lifetime of a woman is limited primarily by her age. The state of an oocyte represents the central determinant of the fate of an ovarian follicle as well as embryo development throughout maturation. Oocyte reserve and oocyte quality are two major determinants of the likelihood of achieving pregnancy for a woman. Assisted Reproductive Technology (ART) has provided a valuable alternative for women attempting to conceive at an older age, however even with ART the likelihood of a live birth also decreases with increased age.

Mammalian oocytes undergo meiotic maturation in preparation for ovulation and fertilization. Throughout most of its lifetime the oocyte remains arrested in the dictyate stage of prophase of meiosis I (MI), also called the germinal vesicle (GV) stage, until the follicle receives a hormonal signal to progress through meiosis. Only a small fraction of the follicles present in the ovaries receive this signal, while the rest remain unresponsive.

The DNA damage response (DDR) is activated in the presence of double stranded breaks (DSBs) in DNA and can induce various cellular responses including senescence or cell cycle arrest, and/or apoptosis, also known as programmed cell death. Telomeres mediate senescence in most cells. Telomeres consist of tandem DNA repeats and associated proteins, which cap and protect chromosome ends. Telomeres and their associated proteins form a loop at the ends of chromosomes,

which buries them. This telomere complex is called shelterin. Shelterin prevents the ends of chromosomes from triggering a DNA damage response. However, with each round of DNA replication chromosomes lose small segments of their telomeres. Telomere attrition also can arise in non-dividing cells via the action of oxygen radicals.

We hypothesize that germinal vesicle arrest, which occurs in some oocytes retrieved for ART that fail to progress through meiosis, is associated with telomere attrition and the associated cellular senescence pathway induced by DNA damage. Previous studies have identified higher levels of DNA damage foci in isolated GV arrested oocytes compared to those that progress through the meiotic cell cycle. Our studies confirm the presence of the DNA damage response (DDR) regulator, ATM, at higher levels in GV stage oocytes versus those that have matured to later stages. Immunostaining shows a near 50% increase in presence of ATM in arrested oocytes.

Confirming the role of the DDR in cell cycle arrest during oocyte maturation could highlight a new target for strategies to improve ART technology and increase the likelihood of achieving pregnancy later in life.

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LIST OF ABBREVIATIONS

ART	Assisted Reproductive Technology
AMH	Anti-Mullerian Hormone
ATM	Ataxia Telangiectasia Mutated
ATM-P	Ataxia Telangiectasia Mutated Phosphorylated
COH	Controlled Ovarian Hyperstimulation
DDR	DNA Damage Response
DSBs	Double Stranded Breaks
FSH	Follicle Stimulating Hormone
GVBD	Germinal Vesicle Breakdown
ICSI	Intracytoplasmic Sperm Injection
IVF	In Vitro Fertility
LH	Luteinizing Hormone
MDC1	Mediator DNA Damage Checkpoint Protein
MRN	MRE11, RAD50 and NBS1
NYUFC	New York University Fertility Center
OHS	Ovarian Hyperstimulation Syndrome
SASP	Senescence Associated Secretory Phenotype
SART	Society of Assisted Reproductive Technology

INTRODUCTION

Reproductive Aging

The reproductive lifespan of a woman is limited primarily by her age. The female reproductive capacity peaks by her early 20s after which it begins to decline from her 30s, until it eventually leads to sterility about ten years before menopause. The mechanisms driving the age related decline in fertility, while women still have primary follicles in their ovaries, remains poorly understood.

One factor contributing to age related reproductive decline is a decrease in ovarian reserve, i.e. the total number of available oocytes, throughout a woman's lifetime. The oogonial stem cell population halts proliferation prior to birth leading to a fixed amount of primordial follicles available throughout the reproductive lifetime (Faddy & Gosden, 1996). Of the nearly 7 million follicles available during the second trimester of a female fetus's life, only around 500,000 survive to puberty. Around 500 follicles ovulate throughout a woman's lifetime. The rest undergo atresia. Fertility peaks during the mid-twenties, and then declines steeply beginning at age 35 paralleling the exponential decrease in follicle number (Hansen et al., 2008) (Figure 1). Menopause begins when only a few hundred follicles remain (Thomford, Jelovsek, & Mattison, 1987). Moreover, marker of ovarian reserve, such as basal follicle stimulating hormone (FSH), antral follicle count and anti-mullerian hormone (AMH), only poorly predict fertility. Thus, the age related decrease in infertility cannot be explained solely by diminished ovarian reserve.

While the decline in female fertility precedes menopause by nearly 10 years, menstrual cycles continue to occur monthly. However the developmental capacity of oocytes, called oocyte quality, declines. This becomes apparent when women experience increasing difficulty achieving pregnancy after their late 30s. Between ages of 35 and 40, a woman’s chances of achieving pregnancy within a year declines from 66% to 44%, leading to most women meeting the criteria for the definition of “infertility”. (Leridon, 2004) (Figure 1)

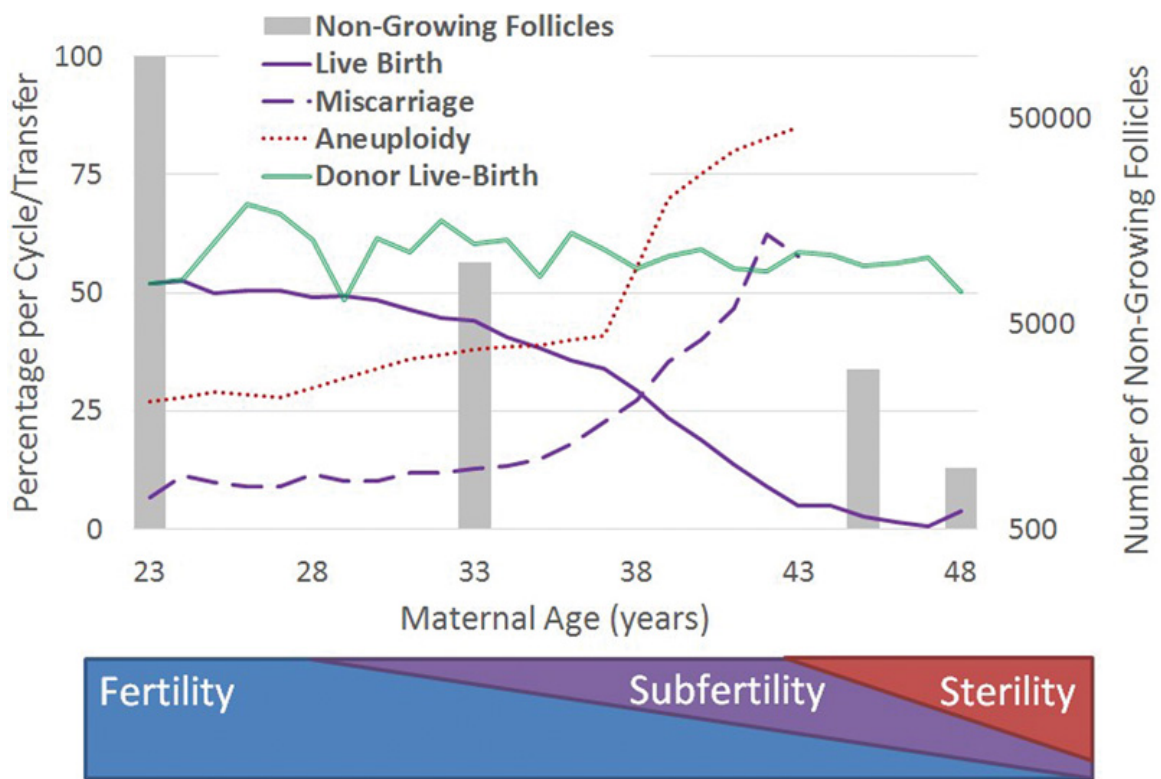


Figure 1: Varying factors reflecting decline in fertility in aging women. As maternal age increases the number of non-growing follicles available for maturation decreases, along with the chances of a live birth. An increased rate of miscarriage and aneuploidy is also seen. Donor pregnancy rates are shown to restore pregnancy

rates to those of younger aged women. (Kalmbach, Antunes, Kohlrausch, & Keefe, 2015)

One of the major causes of ovarian aging is increased aneuploidy, or abnormal number of chromosomes. Lethal aneuploidies lead to failed implantation and miscarriage. Consequently, the likelihood of achieving a live birth declines dramatically with age, until age 43 when most women are sterile (Hassold, Hall, & Hunt, 2007) (Figure 1).

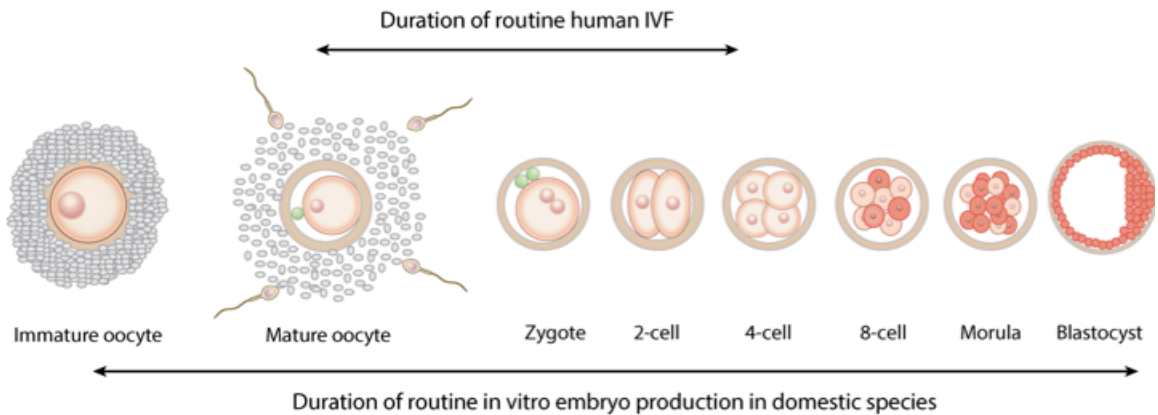
Women increasingly postpone attempts at childbearing. The average age of becoming a first time mother increased in recent years. Also, the percentage of women seeking alternative methods of achieving pregnancy has increased significantly (Matthews & Hamilton, 2014). Increasingly women turn to Assisted Reproductive Technology (ART) to achieve pregnancy. In some developed countries, such as Denmark and Israel, up to four percent of babies are conceived by ART.

Assisted Reproductive Technology

The average age of first-time mothers has increased significantly over the past 3 decades and the proportion of first births to women over the age of 35 has increased nearly eight times since 1970. (Matthews & Hamilton, 2009) As an increasing proportion of women in industrialized countries delay their first attempts at achieving pregnancy, more women are using Assisted Reproductive Technology (ART) procedures to address their decreased fertility rates. (SART)

Currently most fertility centers, including the New York University Fertility Center (NYUFC), utilize controlled ovarian hyperstimulation (COH) to maximize the number of mature oocytes available for retrieval per cycle. These protocols utilize exogenous gonadotropins, to monitor and control the ovarian environment by promoting follicle development and increasing the number of eggs available for fertilization. (Pacchiarotti et al., 2016) An appropriate dosage is required to avoid either Ovarian Hyperstimulation Syndrome (OHS) or insufficient number of oocytes available. (La Marca et al., 2012)

Two methods are currently used to fertilize eggs. The first method, in vitro fertilization (IVF), incubates sperm and eggs in a culture dish. The second method, intracytoplasmic sperm injection (ICSI), injects sperm directly into the egg cytoplasm. After several days of culture in vitro the embryos either are transferred to the uterus with careful timing or frozen for future transfer. The fertilized oocyte begins its first rounds of mitotic division. Transfer of the embryo is conducted either during the early stages of cleavage (day 3 post fertilization) or at the blastocyst stage of development (day 5 post fertilization) (Figure 2) (Lonergan & Fair, 2016).



Lonergan P, Fair T. 2016.
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Figure 2: Duration of routine human IVF: Processes occurring during human IVF including oocyte maturation, fertilization, and culture prior to embryo transfer or freezing. (Lonergan & Fair, 2016)

A woman's age is the most important factor determining her chances of achieving pregnancy using ART procedures. The average age of women who had ART cycles during 2013 using fresh non-donor eggs was around 35, while 66% were ages 30-39, and 23% were ages 40 or older. As a woman ages, the likelihood of a successful response to ovarian stimulation, progression to egg retrieval, and embryo development all decrease. Additionally, embryos that implant are more likely to miscarry with advancing maternal age. (Figure 3) (CDC, 2013)

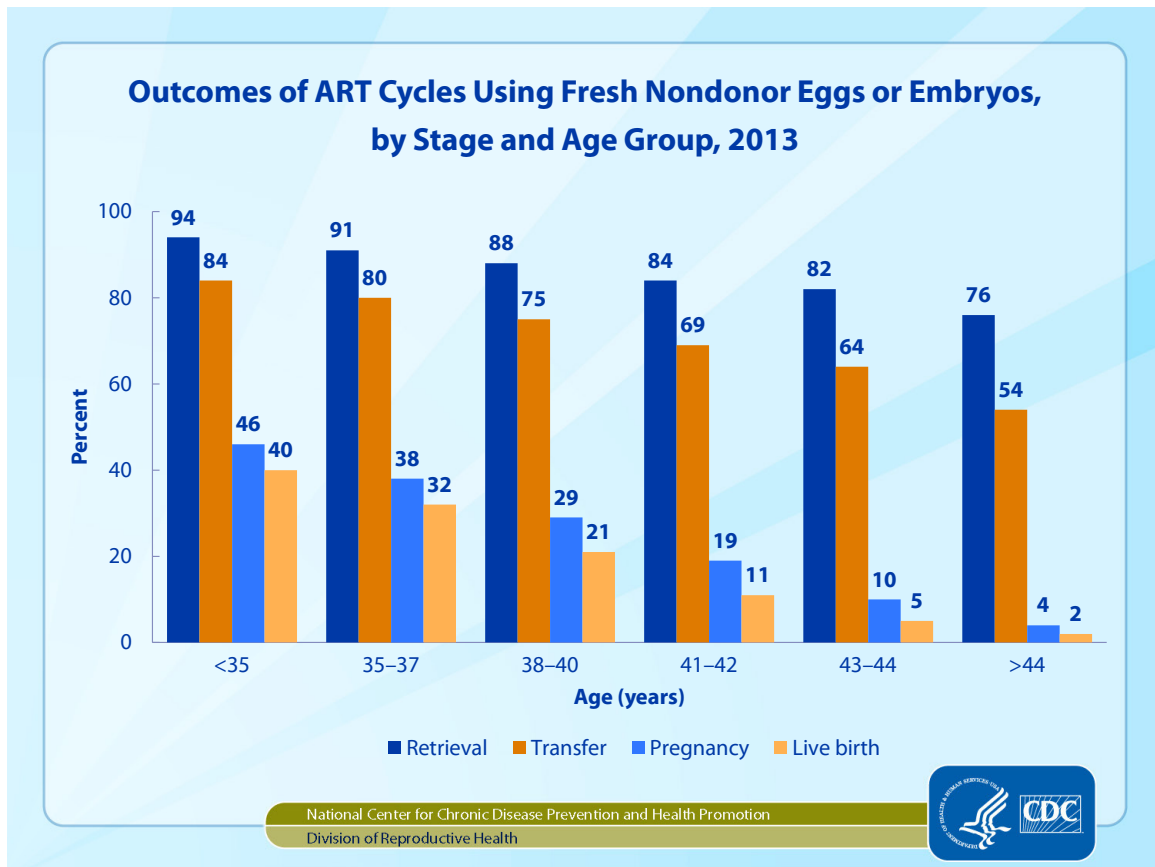


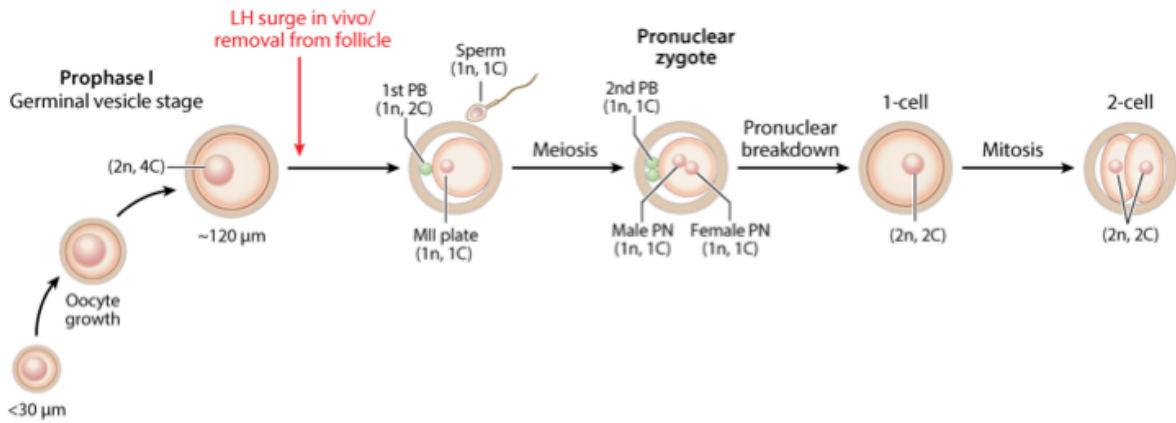
Figure 3: Outcomes of ART Cycles using Fresh Nondonor Eggs or Embryos, by Stage and Age Group: The likelihood of a successful outcome of ART decreases with every step of the process as a woman ages from below the age of 35 to above the age of 44. (CDC, 2013)

Oocyte Maturation

Prior to successful fertilization the oocyte must undergo meiotic maturation. Meiosis is a process exclusive to gametes, which consists of one round of replication and two rounds of segregation. The first round separates homologous chromosomes. The second round separates chromatids, to produce haploid gametes. (Whitaker, 1996) During meiosis the oocyte arrests at two different steps.

These cell cycle arrests are broken by the LH surge triggering ovulation, and by the fertilizing sperm. (Whitaker, 1996)

Meiosis begins when oogonia stop dividing and enter meiosis. These primary oocytes arrest at the germinal vesicle (GV) stage or prophase of meiosis I (Lei & Spradling, 2013). The oocytes can remain arrested at this diplotene stage for up to 50 years, until they are committed to either ovulation or undergo atresia. (Picton, Briggs, & Gosden, 1998) At this stage a single layer of pregranulosa cells on a basement membrane surrounds the oocyte. This complex is termed the primordial follicle. At this stage the oocyte DNA content is $2n$ but $4C$, i.e. it contains four times the haploid complement of DNA. (Figure 5) The action of maturation-promoting factor (MPF), a complex consisting of cyclin-dependent kinase 1 (CDK1) and cyclin B, controls the arrest of oocytes at the GV stage. Dephosphorylation of MPF by *cdc25* induces MPF activation, which peaks during meiosis I. (Figure 4)



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Figure 4: Oocyte Maturation in Vivo. During development oocytes become arrested at the germinal vesicle stage with a $2n$, $4C$ DNA content. The LH surge leads to progression through meiosis and arrest at the metaphase II (M2) stage, as the first polar body (PB) is extruded. Penetration of the sperm results in extrusion of the second PB, and establishment of a $1n$, $1C$ DNA state in the oocyte. Fertilization results in a diploid embryo ($2n$, $2C$). (Lonergan & Fair, 2016)

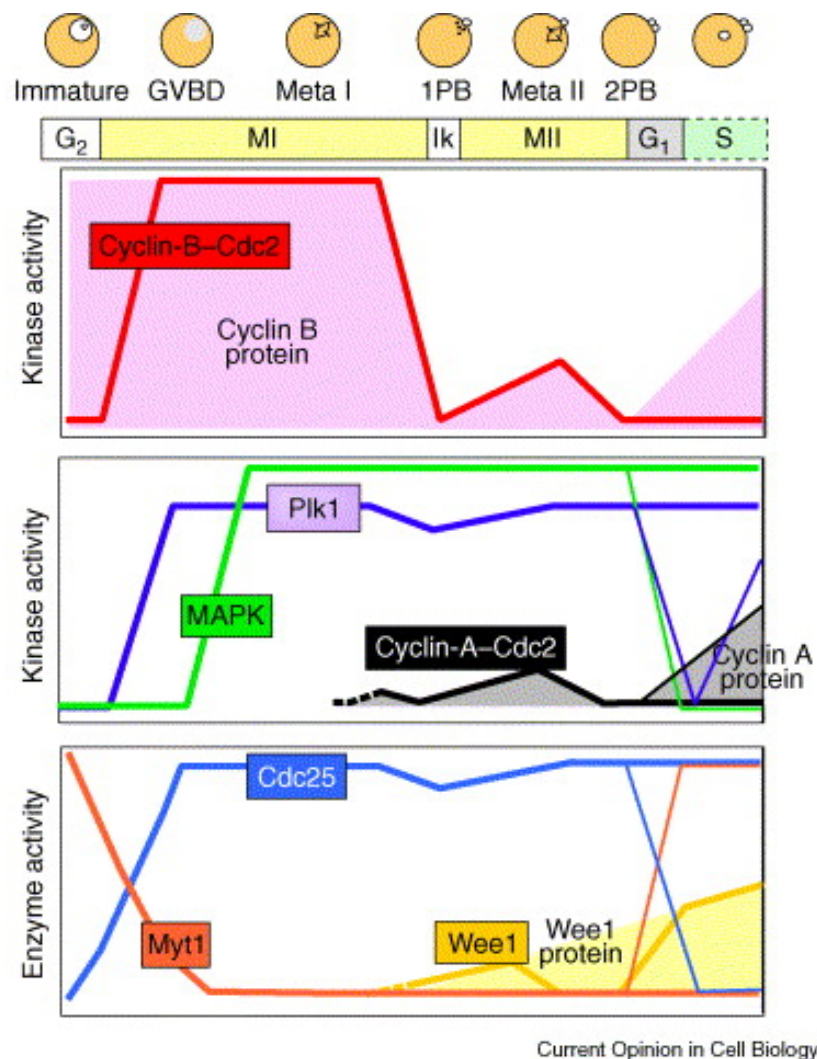


Figure 5: Interaction of regulators of cell cycle during meiotic maturation. Immature oocytes characterized by a GV, are arrested at prophase of meiosis I (MI). Meiosis is reinitiated by a hormonal signal, leading to GVBD and two phases of

meiosis (MI and MII) resulting in haploid cells due to the lack of an S phase between. (Kishimoto, 2003)

Meiosis is resumed *in vivo* following the preovulatory surge of Luteinizing Hormone (LH). This LH surge results in the breakdown of the nuclear envelope of the oocyte or germinal vesicle breakdown (GVBD), as well as various other cytological changes. In addition, the first polar body, which contains one of each pair of homologous chromosomes, is extruded. The oocyte progresses to metaphase II of meiosis and arrests again. Around one day later the oocyte is ovulated and ready for fertilization. If it is fertilized the oocyte completes meiosis and begins to divide continuously to initiate embryonic development. (Picton, Briggs, & Gosden, 1998) (Lonergan & Fair, 2016) (Figure 5)

Oocyte maturation is a prolonged process that occurs successfully only for a small portion of the oocytes available in the ovaries. Immature oocytes, characterized by the presence of a GV, limit reproductive performance in both spontaneous and IVF cycles (Figure 5). Oocytes arrested at the GV stage, premature GVBD, and persistence of meiotic arrest in prophase I lead to a decreased chance of pregnancy. (Aydin, Celik, Celik, Gungor, & Haberal, 2015)

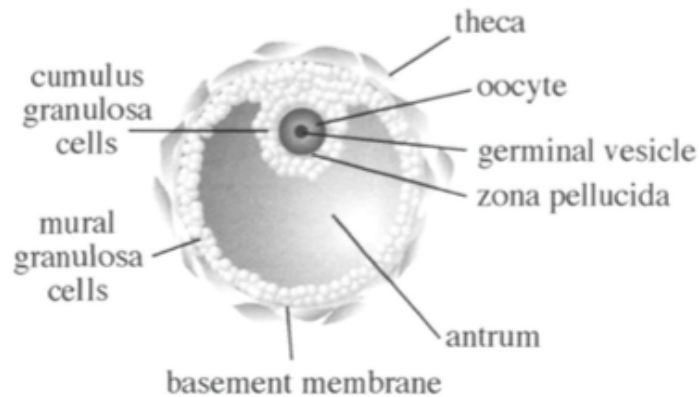


Figure 6: Antral follicle containing primary oocyte arrested in prophase of Meiosis I. Immature oocyte arrested at germinal vesicle stage of maturation.

A study of the factors involved in the senescence of oocytes at the immature GV stage can provide insight into factors affecting ovarian aging and the decline in fertility rates after the age of 30. Cellular senescence has been linked to aging and age-related diseases. Senescence is induced by multiple factors, one of which is nuclear DNA damage creating stress on the cell. (Campisi & d'Adda di Fagagna, 2007)

Oocyte Developmental Competence

The marked decline in natural fertility as well as in ART pregnancy rates after the age of 35 can be explained by poor oocyte quality rather than uterine contribution to implantation failure. Various factors, including chromosomal integrity, mutations and deletions in mitochondrial DNA, meiotic spindle

abnormalities, incomplete separation of sister chromatids, and telomere DNA attrition, have been shown to influence oocyte quality.

As women age rates of meiotic nondisjunction increase markedly, resulting in increased aneuploidy during the early stages of embryo development. This is characterized not only by atypical number of chromosomes in pre-implantation embryos, but also in offspring. For example Down's syndrome, caused by trisomy 21, results from meiotic non-disjunction in over 80% of cases and increases with maternal age. For older women nearing the end of their reproductive lifespans, typically all embryos are aneuploid. Genetic abnormalities in the early stages of meiosis are much more detrimental than faults at later stages, resulting in much more extensive aneuploidy and more extensive damage. (Nagaoka, Hassold, & Hunt, 2012)

Donation of oocytes from younger women, overcomes age-related infertility thus establishing that poor oocyte quality rather than uterine factors is responsible for the age-related reduction in fertility. ART using the woman's own oocytes, however, only partly reduces the fertility gap caused by postponing attempts at pregnancy past age 35. (Leridon, 2004) Transfer of an euploid embryo following preimplantation genetic screening (PGS) produces fertility rates comparable to oocyte donation, suggesting that chromosomal nondisjunction is centrally involved in the decline in oocyte competence with aging. (Forman, Hong, Franasiak, & Scott Jr., 2014)

In addition to chromosomal abnormalities, the oocyte is highly susceptible to various levels of DNA damage, both at the nuclear and mitochondrial level. The arrest of the oocyte in prophase of meiosis I makes it increasingly susceptible to DNA damage, which can lead to infertility or genetic abnormalities. (Jacquet, Adriaens, Buset, Neefs, & Vankerkom, 2005) The body's DNA damage response (DDR) protects the genome integrity of cells, however little is known of this mechanism in oocytes. If DNA damage is not successfully repaired, cells enter senescence or undergo apoptosis.

DNA Damage Response and Cellular Senescence

During the cell's lifespan various stresses challenge its genomic integrity. Cells have evolved a complex and organized pathway to amend genomic stress called the DNA damage response (DDR). The DDR senses DNA damage, arrests the cell cycle, employs repair mechanisms, and/or eliminates cells with permanently damaged genomes. The DDR signal transduction pathway is driven primarily by phosphorylation of various proteins that carry out the pre-determined downstream processes. (Maréchal & Zou, 2013)

Double-stranded breaks in DNA (DSBs) arise from incorrect DNA replication, cell-cycle deregulation, cellular metabolism, especially oxygen radicals, or exogenous genotoxic stress such as ionizing irradiation. (Bzymek, Thayer, Oh, Kleckner, & Hunter, 2010) At a low level, cells may adapt to the damage created by DSBs, trigger apoptosis or inactivate an essential gene necessary for cellular

senescence, which predisposes to malignant transformation (Khanna & Jackson, 2001). DSBs in DNA occur physiologically during meiotic recombination in oocytes prior to their arrest at the GV stage. DDR mechanisms have evolved to ensure that when DNA damage takes place at other times, the damage is detected and the cell cycle is arrested to allow the repair process to occur.

A complex consisting of MRE11, RAD50 and NBS1 (MRN) initially senses DSBs. This complex plays an essential role in DNA damage repair. MRN is responsible for sensing and binding to DSBs and recruiting and activating Ataxia telangiectasia mutated (ATM) kinase. (Figure 5) (Carson, 2003) ATM phosphorylates histone H2AX at serine 139 at the sites of damage. (Burma, Chen, Murphy, Kurimasa, & Chen, 2001) Following phosphorylation, mediator DNA damage checkpoint protein (MDC1) interacts with γ H2AX to recruit more MRN complexes to the site of damage.

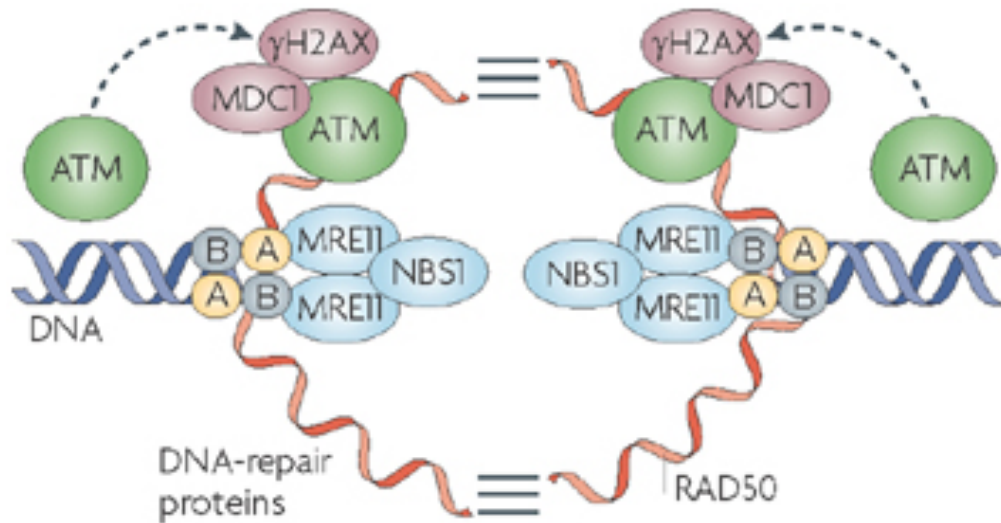
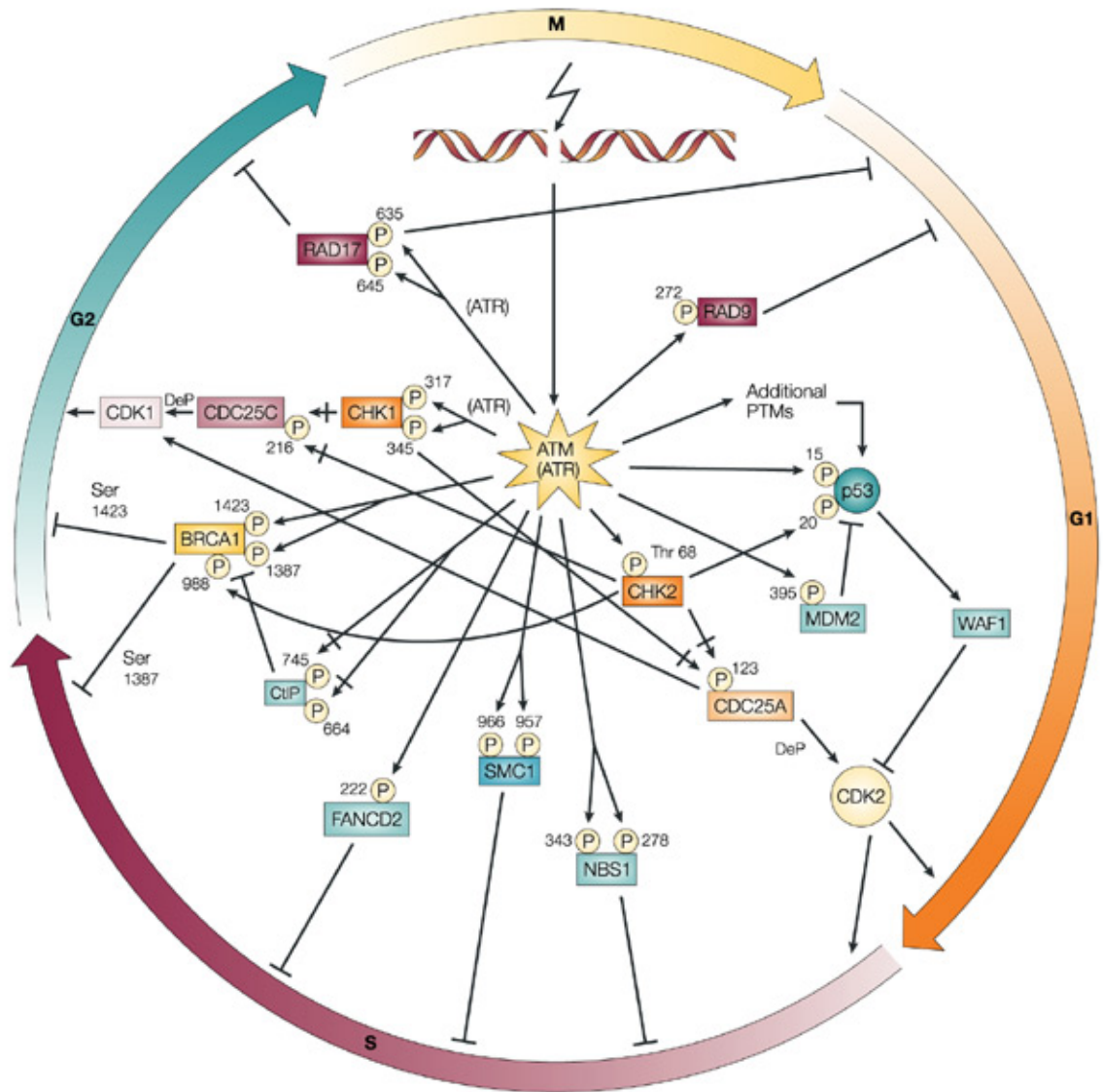


Figure 7: The MRN complex senses and binds to DSBs: MRE11, Rad50, and NBS1 work as a complex to sense and bind to broken ends of DNA subsequently recruiting ATM and other DDR proteins. (Lavin, 2008)

MRN promotes the repair of DSBs during meiotic recombination through multiple pathways including homologous recombination, non-homologous end joining, and alternative non-homologous end joining. (Mayer et al., 2016) MRN also plays a structural role in the DDR, by creating a bond between the tethered strands of DNA, and by promoting the resection of the DSB ends. (Stracker & Petrini, 2011)

ATM activation is known to be the primary step in the establishment of the G2 phase checkpoint in the presence of DSBs. (Maréchal & Zou, 2013) ATM proceeds to phosphorylate and activate transducer checkpoint kinases 1 and 2 (CHK1 and CHK2), which in turn phosphorylate various effector proteins involved in DNA repair and cell cycle arrest. (Ciccia & Elledge, 2010)

One main target of ATM in the DDR pathway is p53, which is activated and stabilized via phosphorylation on many sites. By enhancing the activity of p53 as a transcription factor, the CDK2-cyclin-E inhibitor gene is transcribed, also known as p21. (Figure 6)(Shiloh, 2003) Both p53 and p21 are hallmarks of DNA damage induced cellular senescence or growth arrest. (Campisi & d'Adda di Fagagna, 2007) Senescent cells are characterized by nuclear DNA damage foci, senescence-associated heterochromatin foci (SAHF), increase in cell size, loss of nuclear HMGB1, and decreased expression of lamin B1. Additionally they express the senescence-associated secretory phenotype (SASP), which collectively includes pro-inflammatory cytokines, growth factors, and matrix metalloproteinases. (Coppé et al., 2008) Cellular senescence may have beneficial effects by preventing cancerous or damaged cells from dividing. However senescence can also be deleterious and has been linked to age-related pathologies. Thus senescence has emerged as an appealing target for therapeutic strategies. (Childs, Durik, Baker, & van Deursen, 2015)



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Figure 8: ATM mediated activation of cell-cycle checkpoints in response to DSBs. ATM very precisely controls a multitude of substrates that are responsible for activating the cell-cycle checkpoints. Complex pathways of phosphorylation and dephosphorylation

DNA Damage Response in Oocytes

DNA integrity in oocytes is critical for successful reproduction. Previous experiments in mouse oocytes have shown that H2AX is phosphorylated during the DDR in response to DSBs, forming γ H2AX foci. However, increased DNA damage typically does not prevent entry of the oocyte into M phase of meiosis I due to a limited DDR in oocytes. Despite the presence of DNA damage, one experiment found that oocytes were able to undergo GVBD and enter M phase almost normally. The lack of an effective DNA damage checkpoint suggests that oocytes may not depend upon the ATM kinase. (Marangos & Carroll, 2012)

Recent data shows that during MI, the phosphorylation of H2AX is independent of ATM. This suggests that other effector proteins may be responsible for regulation of H2AX and its role in cell-cycle progression. MRE11 has been found to be involved in H2AX phosphorylation, and maintenance of genome integrity during oocyte maturation. (Mayer et al., 2016)

The DDR in human oocytes have been much less studied. It is unclear whether permanent arrest at the GV stage in human oocytes is even linked to the cellular senescence pathway. The role of ATM activation, as occurs in somatic cells in response to increased DNA damage, has been even less studied in the human oocyte.

SPECIFIC AIMS

The current lack of knowledge regarding the permanent arrest of oocytes at the GV stage presents a critical barrier to understanding ovarian aging and the decline in fertility in older women. ART technology has provided a groundbreaking alternative for women attempting to achieve pregnancy, but also it provides access to study human oocytes.

The DDR affects the ability of mouse oocytes to progress past cell cycle checkpoints during meiosis in response to DSBs. As the majority of oocytes remain arrested in their immature state throughout most of a woman's reproductive lifetime, the DDR may be involved in the maintenance of cell senescence. ATM, a central regulator of cell cycle progression in somatic cells, may be involved in this arrest by activating the cellular senescence pathway. Previous findings have shown that other factors besides ATM are responsible for the detection of DSBs in mouse oocyte DNA, including MRE11 and ATR, but the story in human oocytes remains to be told.

Our current study aims to test the hypothesis that GV arrest in isolated human oocytes is associated with an increased amount of DNA damage compared to oocytes in later stages of maturation, which activates the cellular senescence pathway. To test the hypothesis we will:

1. Compare presence of DDR marker, ATM, during latest stages of oocyte development with those that did not successfully mature in vitro past the GV stage.
2. Evaluate levels of ATM-P, the phosphorylated and active version of ATM, in GV arrested oocytes versus M1/M2 oocytes.
3. Examine the expression of DDR markers at chromosomes versus in the cytoplasm.

These studies will define a role of the DDR, particularly the serine/threonine protein kinase ATM, in cellular senescence at prophase of meiosis I.

METHODS

Sample Collection

The Fertility Center (FC) of New York University Langone Medical Center (NYULMC) routinely performs IVF for numerous patients with infertility. Patients underwent gonadotropin/ antagonist cycles for in vitro fertilization (IVF) with intra-cytoplasmic sperm injection (ICSI) or elective oocyte freezing with vitrification. Retrieved oocytes stripped of cumulus cells were assessed for maturity prior to use for ICSI or oocyte vitrification. Rather than discard oocytes found to be at GV stage(N= 28), we cultured them in Global for Fertilization Media (G2, Life Global®) and monitored them in culture for 48 hours post retrieval. Oocytes were cultured in a CO₂ incubator that maintained cells at 37 degrees in 5% oxygen. (Table 1) Early oocyte development was examined and morphological patterns of in vitro maturation (IVM) were noted. Oocytes arrested at the GV stage after a 24-hour period were separated from those that progressed to either the M1 or M2 stage. Samples were processed separately based on patient and stage of maturation.

Table 1. Conditions for Oocyte Incubation:

Temperature	37°C
CO ₂ Levels	5%

Fixation

Both GV and M1/M2 samples were first fixed in a paraformaldehyde (PFA) solution. This solution consists of 0.1 g of PFA in 10 mL of 0.1% PBS-PVP solution. The solution was incubated in a waterbath heated to 50-60°C for 20 minutes or until the PFA was completely dissolved. The solution was then filtered with a 0.45 μ g filter to remove any undissolved PFA. After washing the oocyte twice in 0.1% PBS-PVP stock, the GV/MII sample was placed into the PFA solution for 15 minutes in the first well of a 96 well plate and placed on ice. The zona pellucida was not removed.

Permeabilization

After a brief wash, oocytes were placed in a blocking solution consisting of 3% goat serum, 0.1% Bovine Serum Albumin (BSA) in PBS. Oocytes were transferred to a permeabilization solution for 30 minutes. This solution consists of 0.1% Triton X-100 in blocking solution, to permeabilize the nuclear membrane to allow the reagents to have access to the DNA contained within. Oocytes were then washed in three separate wells of blocking solution for 15 minutes each.

Immunofluorescence and Confocal Microscopy

After three consecutive washes in blocking solution, oocytes were transferred into the appropriately diluted primary antibody dissolved in PBS. Various primary antibodies were used to assess the level of DNA Damage Response occurring within the GVs or MIIs. (Table 1) The samples were incubated overnight in the antibody at 4°C. After washing the oocytes in blocking solution, three times

for 15 minutes each, the oocytes were incubated with secondary antibody for one hour at room temperature and away from light.

ATM was detected by rabbit polyclonal antibody (Abcam, ab82512), and ATM-Phosphorylated (ATM-P) by rabbit monoclonal antibody (Abcam, ab81292). All primary antibodies were used at a dilution of 1:200. Anti-rabbit antibodies were conjugated with Texas Red Goat Anti-Rabbit antibody (Vector Labs, TI-1000).

Control oocytes were stained with Texas Red, in a 1:200 dilution, without prior incubation with a primary antibody, to serve as negative controls. Control images showed no signs of red foci.

The oocytes were then washed in blocking solution again, three times for 15 minutes each. After an ultimate wash in 0.1% PBS-PVP the oocyte was placed on a slide in a 5- μ l drop of Vectashield, a mounting medium used for preserving fluorescence, (Vector H1000) and Vectashield with DAPI, a nuclear counterstain, (Vector H-1200) in a 4:1 ratio for DNA staining. A spacer made of a wax and Vaseline mixture was used to place the cover slip on the drop to prevent the disruption of the oocyte structure.

Microscope slides were imaged using Zeiss LSM710 confocal microscope, with Zeiss Plan-Apochromat 63x/1.4 Oil DIC lens. Various images were taken using immunofluorescent lights detecting Texas Red, and DAPI.

Table 2. Antibodies and their associated species and dilutions used for immunostaining of GV and M2 oocytes. All antibodies diluted in PBS:

Primary Antibody	Description	Species Reactivity	Dilution
ATM	Rabbit Polyclonal	Mouse, Human	1:200
ATM-Phosphorylated	Rabbit Monoclonal	Human	1:200
Secondary Antibody	Description	Species Reactivity	Dilution
TexasRed	Goat	Rabbit	1:200

Image Analysis

Image analysis was performed using the software ImageJ. (Rasband WS, 1997) Manual assessment of ATM, ATM-P, and MRE-11 foci was performed after adjusting the brightness/contrast levels for optimal viewing. The overlapping colors of the blue DAPI and Texas Red associated with the primary antibodies resulted in a purple-like color indicating the presence of DNA damage response markers at chromosomes. Images were evaluated for both the overall presence of ATM and ATM-P by counting the total Texas Red foci, as well as the presence of ATM and ATM-P at chromosomes by counting the number of DAPI and Texas Red foci overlapping.

Statistical Analysis

The study was single blind between measurements of DDR foci and clinical data. After prospective sample collection was complete, de-identified accession numbers were used to obtain any relevant clinical data. Data analysis was conducted using GraphPad Prism Version 6 Software. (2016 GraphPad Software, Inc) The data was collected from 2 different experiments using ATM antibody and ATM-P antibody. Differences in foci number between GVs and M1/M2 samples were evaluated using paired t-tests, as well as an evaluation of the mean. Samples with a P value of less than 0.05 were considered statistically significant.

RESULTS

The role of ATM was investigated in GVs and M1/M2 oocytes by comparing the difference in foci number in each. GV (n=6) and M1/M2 (n=6) samples were stained with anti-ATM antibody to reveal the amount of expression within the cells. (Figure 7) Following image analysis and statistical analysis using a paired t-Test it was found that the difference in total expression of ATM between GVs and M1/M2 oocytes was not significant (P= 0.2177).

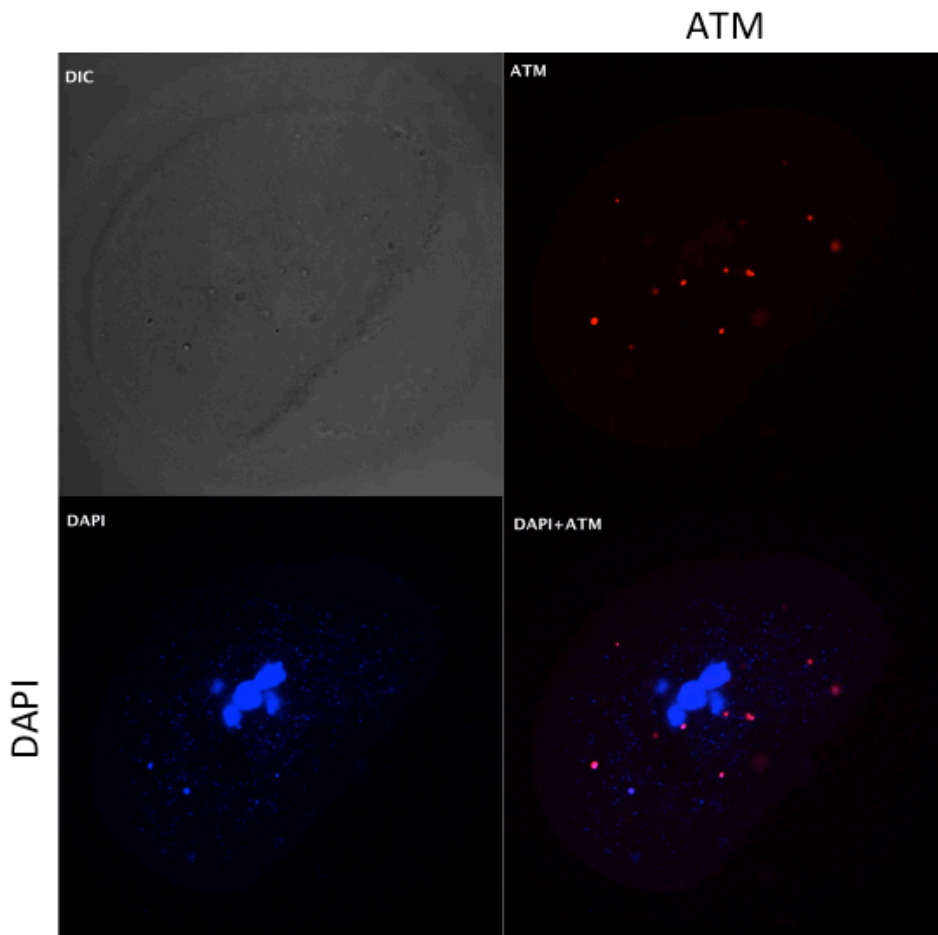


Figure 9: GV oocyte stained with anti-ATM antibody and DAPI. Red foci indicate presence of ATM within the cell and blue foci indicate the location of DNA within the nucleus of the cell. The lack of overlapping red and blue foci indicates that ATM is only being expressed within the cytoplasm.

Additionally the appearance of ATM at the chromosomes, indicated by overlapping red and blue immunofluorescence was found to be minimal. GV arrested oocytes showed an average of 1.33 overlapping foci while M1/M2 oocytes showed an average of 0.67 overlapping foci. (Figure 8) The difference in the number of co-localized ATM and DAPI foci between arrested GV oocytes and M1/M2 oocytes was also not significant. (P= 0.1381) (Figure 9)

The expression of ATM-P between GVs (n=5) and M1/M2 (n=11) samples was evaluated after immunostaining and image analysis. The number of red foci within the cell represented total ATM-P expression. The average number of ATM-P foci in GVs was found to be significantly higher than M1/M2 oocytes. (Figure 9) GVs had an average of 11.8 total ATM-P foci, while M1/M2s had an average of 7.9 total foci. (P=0.0496) (Figure 8) GVs showed a 49% increase in ATM-P from M1/M2 oocytes.

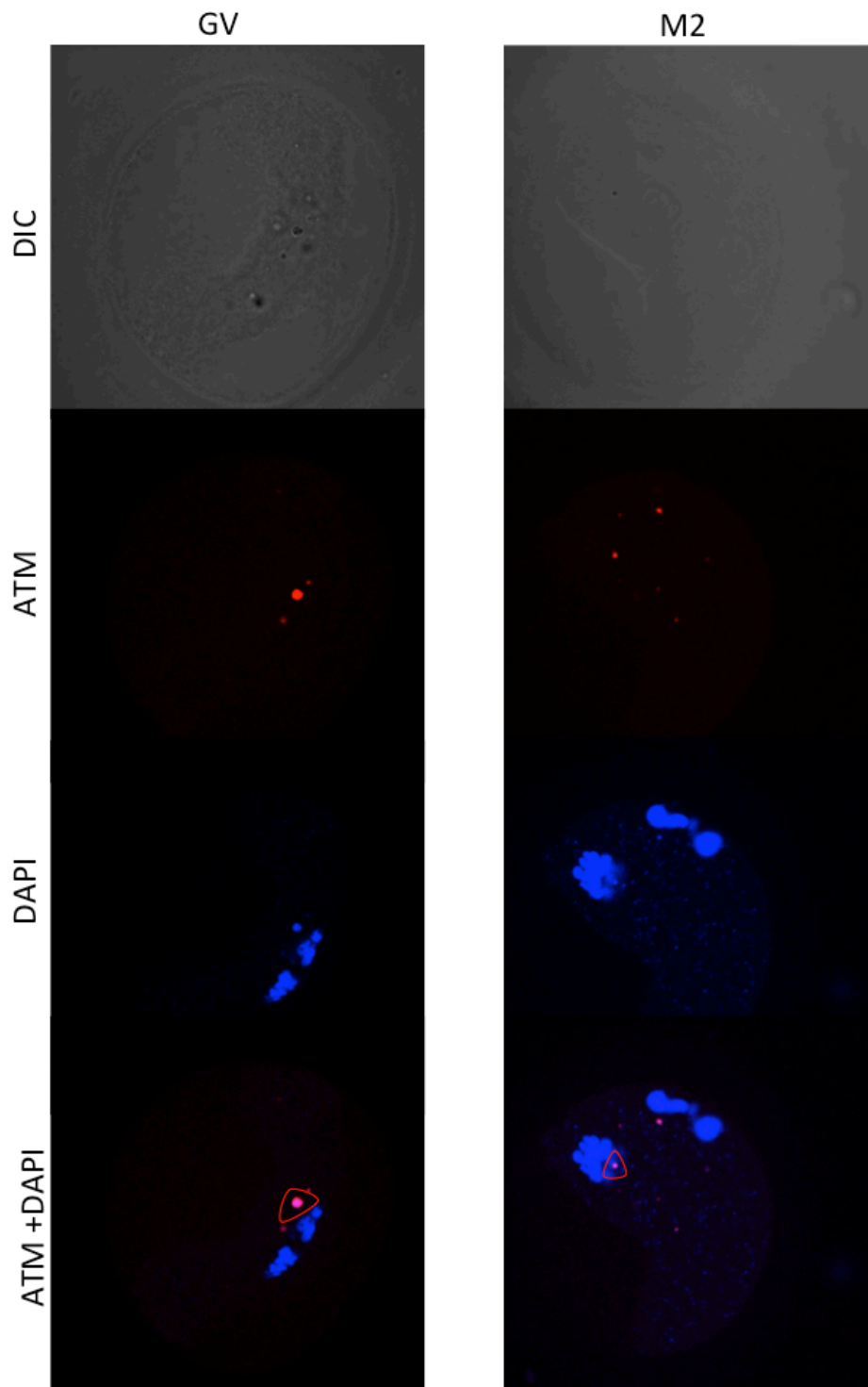


Figure 10: Co-localized immunostaining of anti-ATM antibody and DAPI. Red circles indicate areas of both ATM and DAPI staining. Foci are represented by a pink/purple color.

Appearance of ATM/ATM-P Foci In Human Oocytes

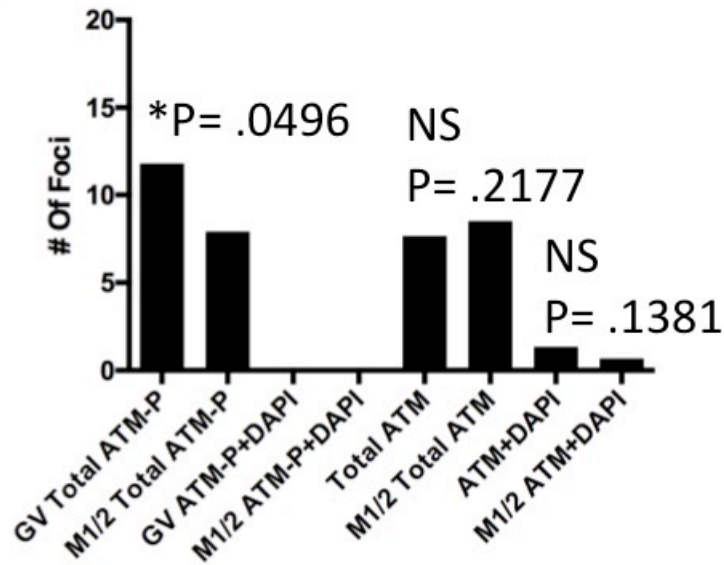


Figure 11: Appearance of ATM/ATM-P Foci in Human Oocytes with statistical P values. X-axis represents both maturation stage of oocyte and the evaluated staining. Y-axis represents average number of foci in each category. Statistical P values are represented above compared categories (Paired T-test). GV arrested and M1/M2 oocytes showed no overlapping staining of ATM-P and DAPI.

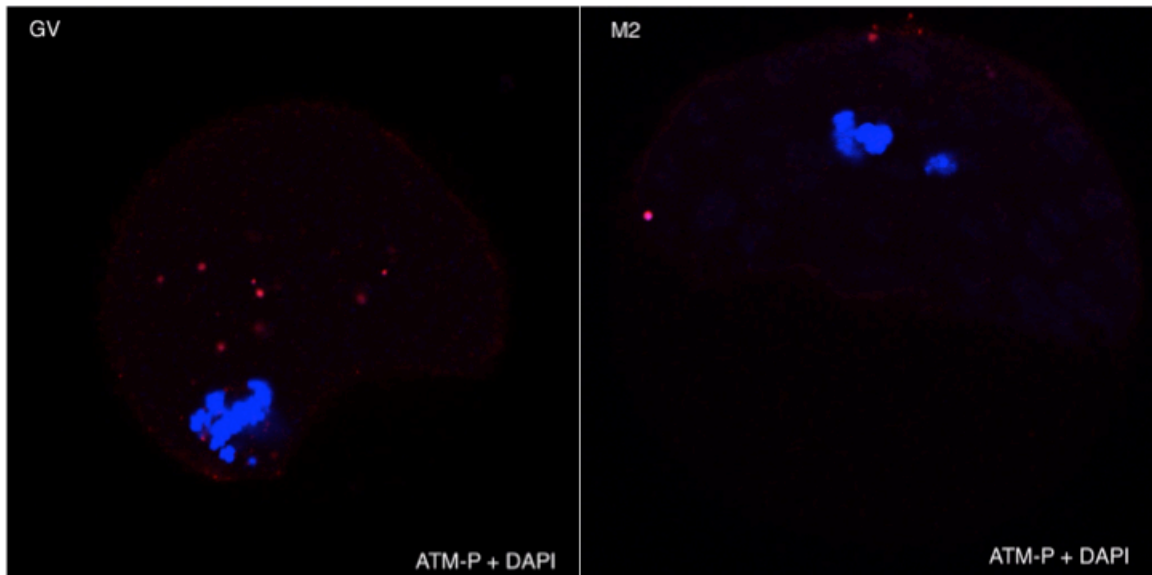


Figure 12: Confocal Microscopy Images of GV vs. M2 Staining with anti-ATM-P antibody and DAPI. GV oocytes show a significantly higher number of ATM-P related foci compared to M2 oocytes.

The total number of oocytes stained with ATM-P and DAPI was 16. None of the images showed signs of overlapping foci in either GV arrested (n=5) or M1/M2 oocytes (n=11). All red foci were located in the cytoplasm. This resulted in a mean value of 0 for the total number of foci. (Figure 11) (Figure 12)

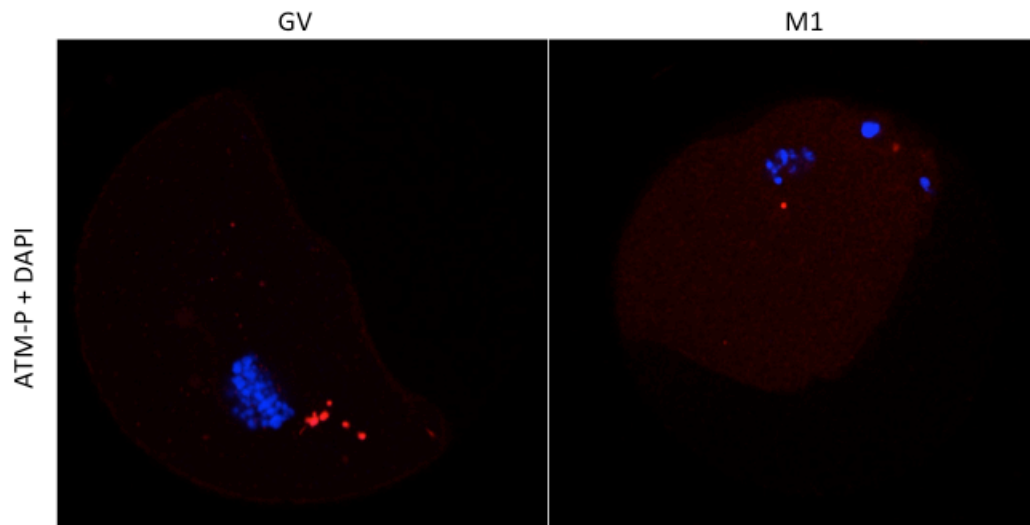


Figure 13: Absent colocalization of ATM-P antibody and DAPI. All samples stained with ATM-P showed no signs of staining at the nuclear DNA. Blue staining represents nuclear DNA and red staining represents ATM-P.

Appearance of ATM/ATM-P Foci In Human Oocytes

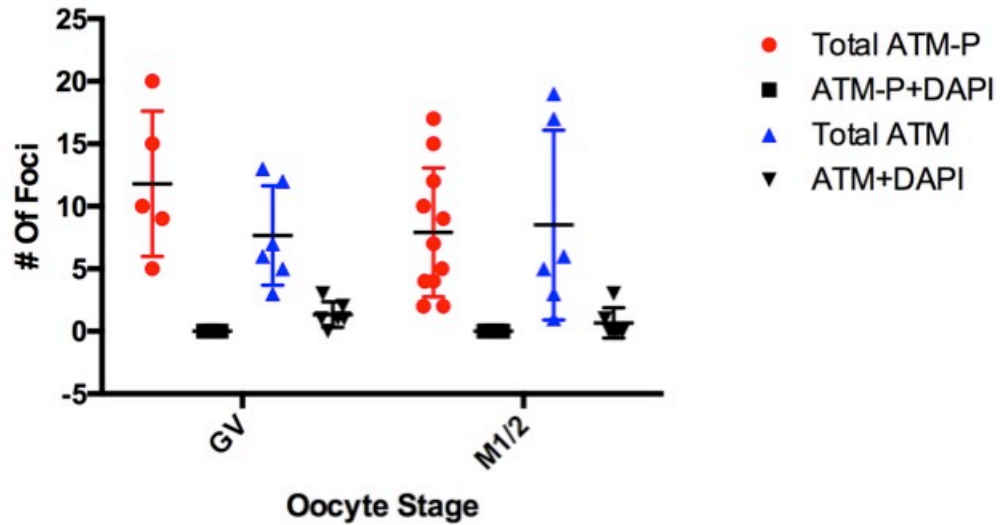


Figure 14: All values of Individual Number of ATM/ATM-P Foci in Human Oocytes. Representation of all values of number of foci representing ATM, ATM-P, and ATM colocalized with DAPI, and ATM-P colocalized with DAPI for both GVs and M1/M2 oocytes. All values for ATM-P at DAPI for both stages are 0.

DISCUSSION

As women increasingly delay attempts at achieving pregnancy, an increasing amount of women have turned to ART methods to successfully conceive. While ART may increase the chances of fertility, as a woman ages past 35, the likelihood of achieving a successful pregnancy from non-donor oocytes declines significantly.

Oocyte competence plays a major role in the likelihood of progression through maturation, achieving successful fertilization and ultimately pregnancy. Oocyte competence is affected by various factors including DNA damage from exogenous stressors on the cell. DSBs are the most common and debilitating of the types of DNA damage, and induce a specific and complex response pathway to either repair the cell, induce apoptosis, or cellular senescence. GV cells are arrested at prophase of meiosis I, and are particularly susceptible to DNA damage. Cellular senescence at the GV stage can be detrimental to reproduction, preventing oocytes from progressing the mature stage capable of fertilization.

While many studies have evaluated the DDR in mouse oocytes, few studies have assessed the DDR markers that may play a role in cell cycle arrest in human oocytes. By comparing the presence of DDR markers during the latest stages of oocyte development, we are able to identify certain markers that may play a role in cell cycle arrest at the GV stage of oocyte maturation. As ATM is a major regulator of the DDR pathway and cell cycle checkpoint progression, we studied its presence within oocytes at various stages of maturation. ATM is a ubiquitously expressed

protein amongst cells, while ATM-P, the phosphorylated version of ATM, is expressed in response to DNA damage.

Ubiquitous expression of ATM between GVs and M1/M2 Oocytes

As we expected, ATM was expressed universally throughout meiosis. It was found both in GVs and M1/M2 oocytes. The number of foci representing the total expression of ATM showed no significant difference between GVs and those that had matured to further stages. DNA damage signal amplification results in the phosphorylation and activation of ATM, which is localized at the site of the DSB. Our data is thus consistent with the idea that levels of ATM should not differ depending on the meiotic stage of the oocyte.

In addition, the number of foci representing overlapping ATM and DAPI also did not show a significant difference between the various stages.

Significant Difference in ATM-P expression between GVs and M1/M2 Oocytes

Our data showed that the total expression of the phosphorylated version of ATM was higher in GVs than in oocytes in more mature stages. This suggests that ATM-P exists at higher levels in oocytes that have been arrested for over 24 hours. ATM-P is a central regulator of the cell cycle, thus our data suggests that it may play a negative role in the maturation of GVs to meiosis I in human oocytes.

As ATM-P localizes at sites of DNA damage our results suggest a higher level of DSBs in GVs compared to M1/M2 oocytes. This would explain the permanent arrest of GVs and the limitation they place on reproductive performance.

Lack of ATM-P Foci at Chromosomes

We observed that the total level of ATM-P was higher in GVs compared to M1/M2 oocytes, but there was a lack of ATM-P foci at chromosomes at all stages of maturation. While ATM-P localizes at sites of DNA damage, its absence on sites of DAPI staining suggest that the existing ATM-P foci may be associated with other cytoplasmic structures. Neither GVs nor M1/M2 oocytes showed signs of ATM-P at chromosomes, which suggest that the role ATM-P plays in the DDR is consistent for all stages of maturation.

Previous studies in mouse oocytes have found that ATM expression was low in response to induced DSBs. Our data is consistent with these findings in human oocytes. These studies also found other DDR factors to be responsible for the phosphorylation of H2AX at sites of damage, and initiating the DDR. Our data also suggests that H2AX phosphorylation is ATM-independent in human oocytes due to the lack of ATM presence at the nuclear DNA.

Other DDR Markers to Evaluate

Past studies in mice have found that while ATM is ubiquitously expressed in oocytes, a significant amount of DSBs must occur to result in its activation. ATM

inhibition in mouse oocytes has been found to have no effect on the number of phosphorylated H2AX foci along with MDC1 in GV stage oocytes and M1, concluding that ATM is unessential for maturation during meiosis. This is seemingly consistent with our findings.

Various other factors have been found to play essential roles in H2AX phosphorylation in mouse oocytes. These include ATR, which phosphorylates H2AX in response to hindered DNA replication in somatic cells. (Ward & Chen, 2001) ATR has been found to be involved in H2AX phosphorylation during meiotic maturation. Another essential factor in the DDR in mouse oocytes is MRE11. MRE11 recently has been shown to be essential for H2AX phosphorylation in M1 mouse oocytes, but not M2 oocytes. Additionally, the inhibition of MRE11 in mice increases the observed number of DSBs in M2 eggs. (Mayer et al., 2016) Future studies should examine the role of MRE11 in the DDR of human oocytes.

As our data indicate that ATM expression and activation is low in human oocytes, consistent with recent findings in mice that H2AX phosphorylation in M1 is ATM-independent. Future studies of the relative presence of factors such as ATR and MRE11 will reveal the role they play in H2AX phosphorylation and cell cycle arrest at the GV stage.

Additionally the study of p21 and p53, novel markers of the cellular senescence pathway, would provide insight to the pathway leading to the permanent arrest of certain GVs. A high presence of these factors would suggest that the DDR is in fact responsible for cell cycle arrest.

Limitations of Study

Limitations of this study include its small sample size. This is due to using discarded human oocytes collected from IVF cycles in a fertility center. Even a large fertility center, such as that at NYULMC, makes it difficult to access oocytes.

Additionally as this study was performed throughout the months of November to February, it overlapped with a period when the IVF laboratory was closed for three weeks during the period of holiday cleaning.

Future Investigations

To continue this course of study it would be beneficial to further characterize the difference between DNA damage in GV's and M1/M2 oocytes. Previous experiments have found increased telomere attrition in GV oocytes. (Kalmbach, Antunes, Kohlrausch, & Keefe, 2015) Telomere shortening due to the loss of telomere DNA repeats throughout the lifetime of an organism results in senescence, apoptosis, and genomic instability. (de Lange, 2010) Telomere shortening is mediated by several mechanisms, one of which employs the DDR to excise telomere repeats in response to oxidative stress. The Telomere Theory of Reproductive Senescence suggests that telomere shortening is enhanced during the prolonged time between birth and ovulation due to chronic exposure to reactive oxygen species. (Keefe, Marquard, & Liu, 2006)

It would be valuable to assess the signs of DDR at chromosome ends in GV arrested versus M1/M2 oocytes. Since arrested oocytes have deprotected telomeres susceptible to DNA damage, DDR markers such as ATM, ATR or MRE11 would target DSBs at telomere DNA. This would result in colocalization of γ H2AX with telomere-specific proteins.

In addition a study of the average telomere length of GV arrested versus M1/M2 oocytes alongside immunostaining for DDR markers would conclude if the DDR is more active in cells with shorter telomeres leading to cell cycle arrest.

Immunostaining using antibodies against the various other markers of DSBs and cell cycle arrest, including MRE11, ATR, p21, p53 would provide insight into the complex DDR occurring in GVs compared to oocytes that successfully mature in vitro. In addition, inhibition of these proteins and measuring levels of H2AX phosphorylation would clarify which are more active in the various stages of maturation.

Further study of the level of expression of SASPs in GV stage oocytes in contrast with matured oocytes can provide more insight to the connection between early meiotic arrest and the cellular senescence pathway.

Overall these studies along with our own have shown that GV arrested oocytes present a difficulty to achieving pregnancy. A further understanding of the complex mechanisms involved in early meiotic arrest is vital to improving the current methods of ART and consequently increasing rates of successful pregnancies in both spontaneous and IVF cycles. Increased knowledge on the

mechanisms behind GV arrest can provide insight into methods of recovering immature oocytes for potential use in IVF cycles. This would provide an attractive alternative to current IVF treatment due to decreased gonadotropin administration and thus decreased risk of OHS, decreased cost, and increased convenience.

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- B.S. **Boston University Sargent College of Health and Rehabilitation** **May 2014**
Bachelor of Science in Human Physiology
- SIT World Learning: International Honors Program** **Spring 2013**
Health and Community Studies – India, Argentina, South Africa

Research Experience

- New York University Langone Medical Center** August 2015-Present
Research Fellow – Department of Ob/Gyn
- Conducted research project studying reproductive aging in women
 - Studied connection between DNA damage in oocytes and embryo outcome
 - Gained laboratory skills including micro-manipulation, immunostaining, and microscopy
- Beth Israel Deaconess Medical Center** January 2014-May2014
Research Intern – Center for Resuscitation Science
- Assist a team in conducting research in the department of emergency medicine
 - Work in laboratory setting and utilize various methods of blood analysis
 - Familiarized with multiple databases and methods of data entry
- SIT World Learning: International Honors Program** January 2013 – May 2013
Student Researcher – Health and Communities
- Conducted research in rural and urban settings on the prevalence of cardiovascular disease in India, Argentina, and South Africa
 - Presented research findings in a lecture setting
 - Engaged with doctors, politicians, patients, community leaders to discuss current healthcare policies

Work Experience

Lululemon Athletica – SoHo Broadway; New York, NY October 2015-Present

Educator

- Worked in retail environment providing guests with proper education on products, community, and health and wellness
- Organized community events within the NYC fitness scene

New York Presbyterian Hospital; New York, NY May 2013-August 2013

Observership and Medical Assistant

- Assisted a head orthopedic surgeon, Dr. Nercession during his day to day activities
- Engaged with patients during routine consultations
- Observed high intensity surgeries

Volunteer Experience

Brigham and Women’s Hospital; Boston, MA Summer 2012

Volunteer Ambassador

- Volunteered in Patient Access Services and Central Transport
- Became familiar with hospital environment and logistics

Global Medical Brigades; Honduras Summer 2011

Volunteer and Medical Assistant

- Provided medical care and attention to a community in Honduras with a team of 30 students
- Shadowed doctors and dentists while treating patients with varying diagnoses
- Improved health care of underprivileged areas

HONORS/AWARDS

Dean’s List 2012-2013, 2013-2014

CLUBS/ORGANIZATIONS

Delta Gamma Sorority January 2011-Present

Boston University Armenian Student’s Association 2010-2014

AGBU Young Professionals September 2015-Present