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Preimplantation genetic screening during in vitro fertilization, clinical applications and insight into embryological development

A thesis submitted to the University of Kent for the degree of

DOCTOR OF PHILOSOPHY

in the Faculty of Science

2017

Tyl H. Taylor, MS

School of Biosciences

Declaration

No part of this thesis has been submitted in support of an application for any degree or qualification of the University of Kent or any other University or Institute of learning.

Tyl H. Taylor, MS

30 April 2017

Acknowledgements

First, I need to thank Dr. Darren Griffin (if he passes me) for his guidance and for pretty much everything. At times you frustrate me but in the end, I've learned not to take things so seriously. You actually made this whole process fun, which is more than I can say for the next person I'm going to thank.

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To my parents who ask when graduation will be…EVERY MONTH. The graduation might be in July (if I pass).

To Harper and Gavin, you two didn't do anything, which I guess is good considering how much trouble you could have caused. Your mom and I love you.

To my wife, the most important part of my life. You have spent many nights alone because I have been working. I have done this in hopes that our lives will improve and yet I have realized that my life has improved even without this. Before I started this, I thought I needed a PhD to be successful. Now I know, I don't need a PhD, but I do need you. Without you, this means nothing. I love you.

Incorporation of published work

This thesis largely incorporates published work from several papers and has been submitted as a “thesis by publication” under the ordinances of the University of Kent. The following is a comprehensive list of my published works that has been performed during my registration period (2012-2017).

Peer Reviewed Journal Articles

Taylor TH, Griffin DK, Katz SL, Crain JL, Johnson L, Gitlin SA. 2016. Technique to isolate individual cells of the human blastocyst and reconstruct a virtual image of their location. J Clin Embryo. 18;2:31-5.

Taylor TH, Griffin DK, Katz SL, Crain JL, Johnson L, Gitlin SA. 2016. Technique to ‘map’ chromosomal mosaicism at the blastocyst stage. Cyto genet Genome Res. 149(4):262-6.

Taylor TH, Patrick JL, Gitlin SA, Crain JL, Wilson M, Griffin DK. Blastocyst euploidy and implantation rates in a young (<35 years) and old (≥35 years), presumed fertile and infertile patient population. Fertil Steril. 102;5:1318-23.

Taylor TH, Gitlin SA, Wilson JM, Crain JL, Patrick JL, Griffin DK. 2013. The origin, mechanisms, and incidence of chromosomal mosaicism in humans. *Hum Reprod Update.* 20(4):571-81.

Taylor TH, Patrick JL, Gitlin SA, Wilson JM, Crain JL, Griffin DK. 2013. Pregnancy and implantation rates of blastocysts that were thawed, are warmed, biopsied, vitrified, and rewarmed for euploid blastocysts transfer. *Reprod Biomed Online*. 29(1):59-64.

Taylor TH, Patrick JL, Gitlin SA, Wilson JM, Crain JL, Griffin DK. 2014. Comparison of aneuploidy, pregnancy and live birth rates between day 5 and day 6 blastocysts. *Reprod Biomed Online*. 29;3:305-10.

Submitted

Taylor TH, Divic N, Katz SL, Wing RW, Griffin DK. Reanalysis of blastocysts previously diagnosed as aneuploidy demonstrates that one in four are rediagnosed as euploid.

Taylor TH, Griffin DK, Wilson JM, Wing RL, Johnson L, Katz S. A comparison of aneuploidy rates and ongoing pregnancy rates between blastocysts screened using quantitative

polymerase chain reaction or array comparative genomic hybridization, results from a single center.

Taylor TH, Patrick JL, Das D, Crain JL, Wilson JM, Griffin DK. Implantation and live-birth rates of poor quality embryos transferred on day 6 during a fresh in-vitro fertilization cycle.

Invited Works

Taylor TH. 2016. Mosaicism in Humans. ASRM certificate course.

Taylor TH. 2016. Embryo biopsy during IVF. ASRM certificate course.

Abstracts

Strecker MN, Sahoo T, Dzidic N, Commander S, Taylor TH, Hovanes K. 2016. Evolution of chromosomal abnormalities from conception to pregnancy loss: a comparative study of PGS and miscarriages samples. Fertility Steril. 106;3:e150.

Stankewicz-McKinney Tl, Taylor TH, Glassner MJ, Orris JJ, Brasile DR, Griffin DK. 2015. Preliminary assessment of aneuploidy rates between the polar, mid and mural trophectoderm. PGDIS. Oral.

T.H. Taylor, M. Adamowicz, N. Dzidic, B. Acacio. Mosaicism in the trophectoderm is present, albeit at a low rate of clinical significance. *ASRM 2015*.

T.H. Taylor, R.L. Wing, D.K. Griffin. A Technique to "map" mosaicism at the blastocyst stage using comprehensive chromosome screening. *ASRM 2015.*

Taylor TH, Patrick JL, Das D, Crain JL, Wilson JM, Griffin DK. 2014. Euploid live births from diagnosed aneuploid cleavage stage embryos. *PGDIS*. Canterbury, England. Oral.

Das D, Taylor TH, Patrick JL, Crain JL, Teaff N, Wing R. 2014. Effect of paternal age on blastocyst aneuploidy rates. Fertil Steril. 102;3:e352.

Taylor TH, Welch LR, Katz S, Crain JL, Wilson JM, Patrick JL. 2013. Comparison of aneuploidy, pregnancy, and implantation rates between day 5 and day 6 blastocysts, a sibling oocyte study. *ASRM Boston*. Oral.

Taylor TH, Welch LR, Das D, Crain JL, Wilson JM, Patrick JL. 2013. Pregnancy, implantation, and live birth rates of blastocysts that were thawed, are warmed, biopsied, vitrified, and rewarmed for euploid blastocysts transfer. *ASRM Boston*. Oral.

Taylor TH, Welch LR, Wing R, Crain JL, Wilson JM, Patrick JL. 2013. Pregnancy, implantation and live birth rates of fertile and infertile patients following transfer of euploid blastocysts. *ASRM Boston*. Poster.

Where I was the primary author of the work then the text is left largely unchanged. Where I was not the primary author, I have changed the text to emphasise my personal contribution. In order to allow for a coherent narrative, rather than simply annexing the typeset manuscripts, I have added passages to link the pieces of research together. In so doing I intend that rather than appearing as several loosely related studies, the work appears as a coherent "whole."

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Abbreviations

aCGH array comparative genomic hybridization

AH assisted hatching

Bub benzimidazole

Ca^{2+}/Mg^{2+} Calcium/Magnesium free

CCS comprehensive chromosome screening

CENP centromere proteins

CPM confined placental mosaicism

CSC continuous single culture

CSF cytostatic factor

CVS chorionic villi sampling

DNA deoxyribose nucleic acid

EEM extraembryonic mesoderm

FCA fetal cardiac activity

FET frozen embryo transfer

FISH fluorescence in-situ hybridization

FSH follicle stimulating hormone

G1 Gap 1

G2 Gap 2

GV Germinal Vesicle

GVBD germinal vesical breakdown

hESC human embryonic stem cells

ICM inner cell mass

ICSI intracytoplasmic sperm injection

IVF in-vitro fertilization

LH luteinizing hormone

Mad mitotic arrest-deficient

MAPK maturation promotion factor

MI metaphase I

MII metaphase II

MTOC microtubule organization centers

NGS next generation sequencing

PCOS polycystic ovarian syndrome

PGC primordial germ cells

PGS preimplantation genetic screening

qPCR quantitative polymerase chain reaction

RPL recurrent pregnancy loss

SNP single nucleotide polymorphism

SPS serum protein supplement

SSC saline sodium citrate

SSS serum substitute supplement

S Synthesis

UPD uniparental disomy

WGA whole genome amplification

ZP zona pellucida

2PN two pronueclei

Abstract

Aneuploidy (extra or missing individual chromosomes) is the leading cause of miscarriage, embryo wastage and in-vitro fertilization (IVF) failure. Aneuploidy increases with maternal age and is widespread in human preimplantation embryos. Thus, aneuploidy screening before implantation during an IVF cycle (preimplantation genetic screening or PGS), to increase pregnancy rates and decreasing miscarriage rates, is also widespread. Despite this, PGS faces challenges in terms of both biological and technical limitations that may impede its full potential. Biologically, the phenomenon of chromosomal mosaicism (the presence of two or more cell lines - typically, one aneuploid and one euploid) may lead to false positives or false negatives, and the discard or transfer of euploid or aneuploid embryos, respectively. Technically, it is uncertain whether diagnosis on the biopsied piece is representative of the remaining embryo. Because these dilemmas it is unknown if PGS will only benefit a few selected groups of patients or potentially the entire IVF patient population. In a series of published works, this thesis demonstrates a significant contribution to field of preimplantation genetics, provides insight into technical and biological limitations of PGS, and into the etiology of aneuploidy and mosaicism.

Specifically, I introduce a novel technique to “map” chromosomal mosaicism, by reconstructing a virtual image of the blastocyst with the approximate location of individual cells and their corresponding chromosomal makeup. I also demonstrate the

ability of PGS to be performed on blastocysts that were previously frozen; thus, blastocysts have to be thawed/warmed, biopsied, vitrified and rewarmed prior to use.

From a clinical standpoint, I present evidence of the differences in PGS outcomes between day 5 and day 6 blastocysts: The data suggests that day 6 blastocysts are less likely to be euploid than day 5 blastocysts. Furthermore, day 6 euploid blastocysts exhibit similar pregnancy and implantation rates when compared to their day 5 counterparts. I also published on a study examining differences in PGS outcomes in those patients that are defined as "presumed fertile" as opposed to those that are "infertile". Another study examined pregnancy and implantation rates between two competing platforms, quantitative polymerase chain reaction (qPCR) and array comparative genomic hybridization (aCGH). I also examined the pregnancy rates of poor quality embryos on day 6 that would have been discarded.

From a biological standpoint, I examined the mechanisms through which embryos diagnosed as aneuploid on day 3 could develop to a euploid blastocyst, demonstrating that euploid blastocysts can develop from aneuploid cleavage stage embryos. I also demonstrated differences in aneuploidy rates between polar, mural, and a piece defined as "mid" trophectoderm, and blastocysts diagnosed as aneuploid may not reflect the chromosomal constitution of the whole embryo proper.

This work herein presented provides a deeper understanding of the technical limitations of PGS and into the etiology of the chromosomal basis of early human development.

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1.0 General Introduction

1.1 Chronology/Timing

1.1.1 8-20 Week Gestation

Fetal oogenesis begins around the 8th week gestation with the migration of primordial germ cells (PGC) into the germ layer, specifically the gonadal primordi (De Felici, 2010). Between weeks 8 to 11 of gestation, the PGCs multiply and are now referred to as oogonia (Garcia *et al.*, 1987). By week 20, clusters of oogonia have formed within the fetus' ovary. Oogonia within the ovary have to undergo DNA replication followed by two reduction divisions referred to as meiosis I and meiosis II in order to produce a haploid gamete(s) necessary for reproduction.

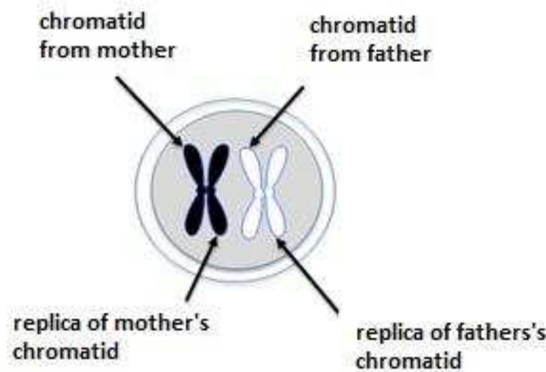
Meiosis I is divided into five stages: interphase, prophase, metaphase, anaphase, and telophase. Unlike mitosis, which is explained later and can take only a few hours, meiosis can take anywhere from 10-40 years to complete. All these processes involve the replication and division and chromosomes. Chromosomes will be explained in more detail later but they carry the traits and genes from each parent and enable the passing of genetic information from generation to generation.

The first stage of meiosis, or interphase, is a simple replication of the parental chromosomes. Thus, the chromosomes are replicated from 46 to 92. The oogonium becomes a primary oocyte consisting of 46 pairs of chromosomes. At this stage the oocyte contains four chromatids of each chromosome: 1. a chromatid from the

mother, 2. a duplication of mother's chromatid (sister chromatid), 3. a chromatid from the father, 4. a duplication of the father's chromatid (sister chromatid; Figure 1).

All of this occurs approximately at 20 weeks gestation. Once DNA is replicated and interphase is completed, the primary oocyte progresses to the diplotene stage of prophase of meiosis I where it will remain with all duplicated 46 chromosomes until puberty.

Figure 1: An arrested germinal vesicle with the chromosomes and their complement.

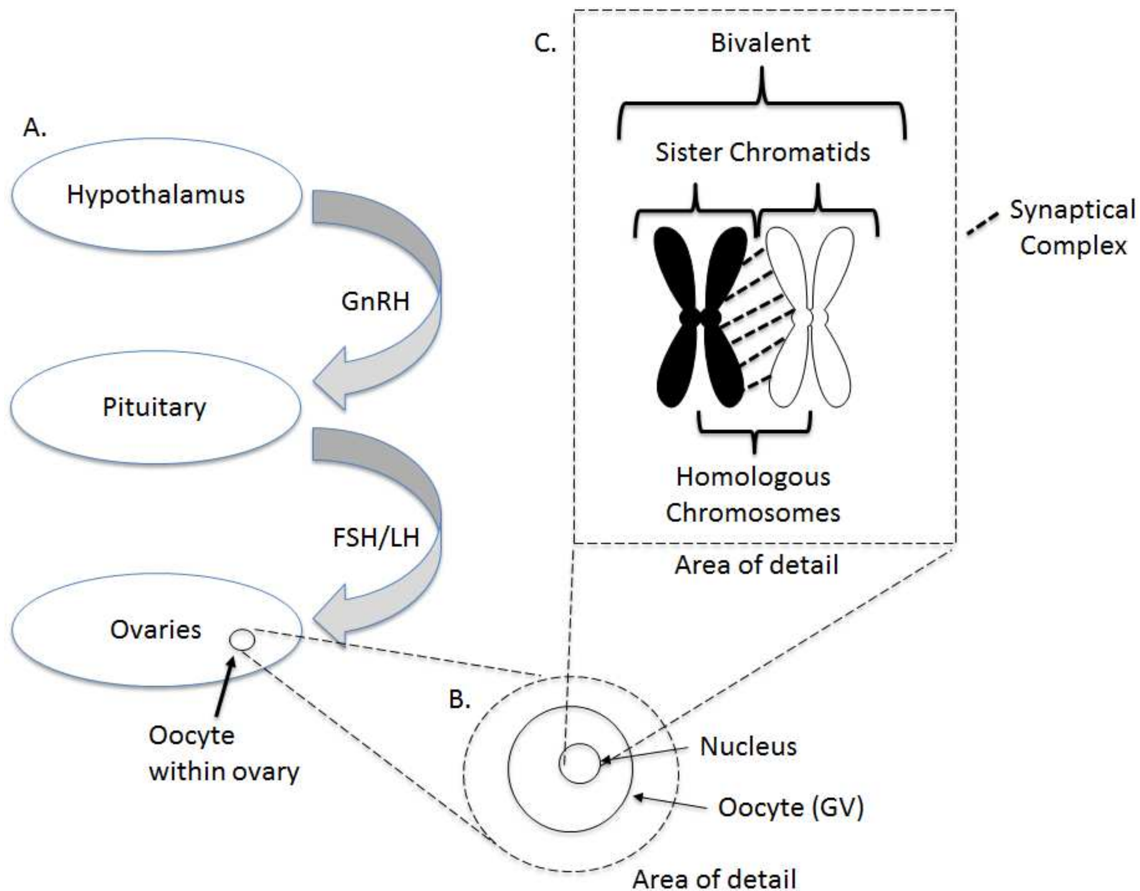


1.1.2 Puberty

Upon puberty, monthly hormonal signals in the form of luteinizing hormone (LH) and follicular stimulating hormone (FSH) are produced by the pituitary gland and act on the follicles where the oocyte is arrested. The combinations of these hormones stimulate the primordial follicle to undergo changes leading to the simultaneous maturation of the follicle and oocyte and the resumption of meiosis I (Figure 2).

During this time, the oocyte within the follicle are undergoing changes that increase the ribosomal and messenger ribonucleic acid that will be used in the development of the embryo. The zona pellucida (ZP) and gap junctions between the oocyte and granulosa cells also start to form in response to FSH and LH. The oocyte is still arrested at the diplotene stage of prophase at meiosis I.

Figure 2: Illustration of the events involved in oocyte maturation. A) Release of various hormones leading to oocyte maturation within the follicle. B) Resting stage of an oocyte at the onset of maturation. C) Chromosome configuration of each chromosome within the oocyte at the onset of maturation.



With a surge of LH, the GV oocyte will begin a process known as haploidization, reducing the total number of chromatids from 92 or 4 pairs of 23 chromatids (present in the GV)

to 46 or 2 pairs of 23 chromatids present in the mature oocyte. The maturation of the oocyte and reduction division of chromosomes needed for haploidization can only occur properly with aid from the spindle apparatus.

In response to the LH surge, numerous factors are activated by the oocyte aiding in the process of maturation and the reduction division of chromosomes. Maturation promotion factor (MPF) activity increases and in turn, activates mitogen activated protein kinase (MAPK). MAPK is needed in conjunction with cytostatic factor (CSF) for spindle stability and chromosome alignment during the meiotic divisions (Masui and Markert, 1971). The increase in MPF is the signal to begin the construction of the first meiotic spindle (Sobajima *et al.*, 1993). As the GV progresses, the nuclear envelope begins to break down (GVBD), allowing the microtubules access to the condensed chromosomes. Although still at prophase I, the oocyte now becomes known as a MI oocyte.

As previously discussed, during meiosis I chromosomes are arranged as a bivalent. In this configuration, genetic recombination can occur at places where two chromosomes cross, referred to as a chiasma. The molecule referred to as cohesin holds the sister chromatids together while the chiasmata and synaptical complex hold the homologous chromosomes together allowing for the formation of the bivalent. This process assures the genetic variation amongst offspring, but also helps control the process of chromosome segregation. During the MI division, not only do chiasmata serve as crossing over points, they also supply a force that opposes the pull on the chromosomes by the spindle. A decrease in number of chiasmata would decrease the force opposing the pull of the

spindle. In the case of no force opposing the pulling of the spindle, chromosome segregation may occur prior to the attachment of microtubules at the kinetochore, leading to premature separation of the chromosomes (Lamb *et al*., 1996).

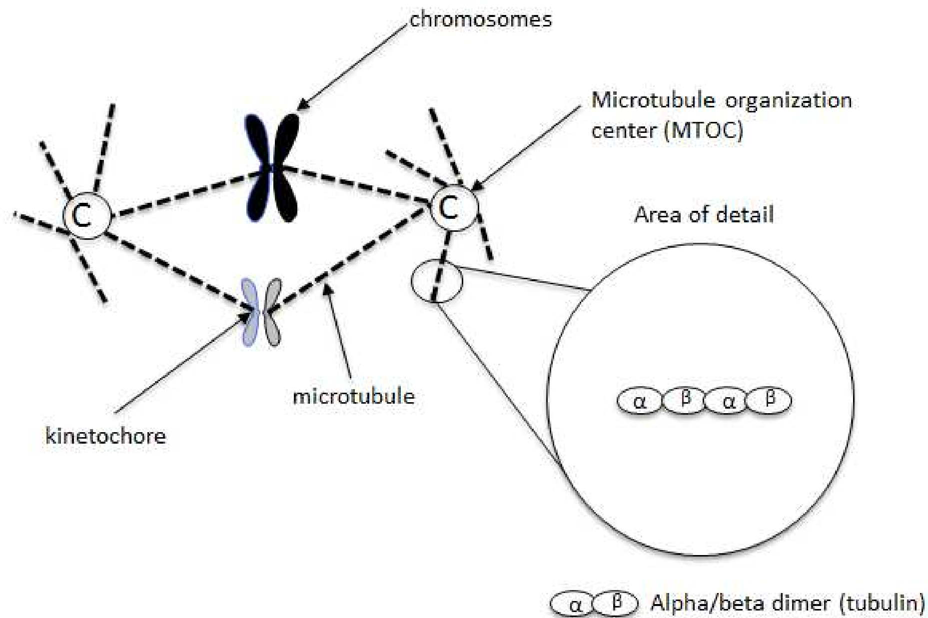
1.2 The Spindle Apparatus

Centrioles are organelles within the oocyte that define the poles of the spindle apparatus and begin the production of microtubules used to capture chromosomes. Research however, suggests that centrioles are non-existent in human oocytes (Szollosi *et al*., 1972). Instead, in mammals, including humans, the microtubules project from MTOCs (microtubule organization centers) referred to as centrosomes (Carabatsos *et al.*, 2000). Microtubules consist of alpha and beta monomers bundled together to form a dimer (Compton, 2000; Meng *et al.*, 2004). These microtubules randomly attach to the chromosomes on the centromere at a site called the kinetochore (Figure 3).

The kinetochore complex on the centromere of the chromosomes is composed of multiple proteins that are involved in the capture and movement of chromosomes along the microtubules. Centromere proteins (CENP's) are a class of proteins that are present on the centromere (more specifically the kinetochore of chromosomes) and are involved in chromosome capture and chromosome segregation (Ma *et al.*, 2003). CENP's include mitotic arrest-deficient (Mad) 1, Mad2, and benzimidazole (Bub; all present at the kinetochore) that act as signals for chromosome division.

When microtubules are not attached to the kinetochore, the kinetochore is phosphorylated and contains large amounts of CENP's (Chen *et al.*, 1996; Burke, 2000; Gardner and Burke, 2000). These proteins inhibit the premature division of chromosomes and sustain the spindle at the metaphase stage. During the MI stage, microtubules originating from the MTOC's begin to polymerize. Tubulin is added from the negative end at the spindle pole to the positive end that will extend into the cytoplasm (Eichenlaub-Ritter *et al.*, 2003). When deploymerization (removal of alpha/beta dimer) occurs, tubulin is removed from the negative end, effectively shortening the tubulin and pulling the chromosomes from each other towards the MTOC's.

Figure 3. Composite picture of the spindle, chromosomes, and microtubules.



To extend into the cytoplasm, the addition of tubulin to microtubules is facilitated by GDP/GTP hydrolysis (Palacios *et al.*, 1993; Combelles and Albertini, 2001). The microtubules extend in all directions from the MTOC's. By random chance, the

microtubules come in contact and attach to the chromosomes at the kinetochore. Once both MTOC's have captured the chromosome, tension is created by one MTOC pulling in one direction and the other MTOC pulling in the other. The bivalents are held together by chiasmata, creating a force opposing the pull of the microtubules (Smith and Nicolas, 1998). Tension created by the pulling of the chromosomes in opposite directions causes a loss of CENPs from the kinetochore, effectively dephosphorylating the chromosome (Nicklas *et al.*, 1997).

1.2.1 Spindle and Meiosis

As previously mentioned, the oocyte is arrested at the diplotene stage of prophase I of meiosis I. As oocyte maturation resumes, the GV envelope breaks down, the spindle condenses, and chromosomes migrate to periphery of the MI oocyte, thus signaling the end of the prophase I and the beginning of metaphase I. At metaphase I all chromosomes align on the metaphase plate and microtubules are attached to the kinetochore, the spindle apparatus and chromosomes rotate 90^0. Once rotation is complete, the oocyte leaves metaphase I and proceeds to anaphase I. At anaphase I, MPF activity decreases thereby allowing separation of the bivalents to occur and the chromosomes to proceed to the opposite poles. The arrival of the chromosomes to the opposite poles is referred to a telophase I and signals the end of the meiosis I (Figure 4). With the extrusion of the first polar body, the oocyte reduces its chromosome number by half thereby progressing to a MII oocyte (Figure 4).

At the end of telophase, the MII oocyte has completed meiosis I and contains one polar body with 46 chromatids and one oocyte with 46 chromatids. From here, the oocyte enters meiosis II. Immediately, the oocyte enters prophase II and the spindle begins formation again by increasing MPF, thereby activating MAPK that in turn activates CSF. With the activation of these factors, the spindle reforms and oocyte enters metaphase II as sister chromatids migrate to the MII plate where they remain arrested by CSF and MAPK until fertilization. These two are responsible for holding the chromosomes on the MII plate (Masui and Markert, 1971).

The oocyte will remain arrested at the metaphase II phase until penetration of the oocyte by the sperm causes a rise in calcium levels. The increase in calcium ions inactivates CSF and MAPK, allowing the MII oocyte to undergo its second meiotic division. The loss of the CENPs activates cyclin B, which degrades the cohesin between the sister chromatids, allowing for chromosome separation and enter anaphase II (Evans *et al.*, 1983; Figure 5). As the second meiotic division starts, microtubules begin to depolymerize. Starting at the negative end (spindle pole), the microtubules are depolymerized and actively shortened, effectively pulling the chromosomes towards the spindle poles. As the chromosomes arrive at opposite poles (23 in the second polar body and 23 in the oocyte), the oocyte has entered telophase II and completed both meiosis I and meiosis II. The meiosis II reduction separates the sister chromatids and reduces the total chromosome content of the oocyte from 23 pairs of chromatids to 23 individual chromosomes.

Figure 4. Meiosis I in the human oocyte. A) An oogonium prior to DNA replication containing a chromosome from the mother and father. B) Primary oocyte (germinal vesicle) containing a copy of both of the mother (black) and father (white) chromosomes. C) The metaphase I oocyte with the alignment of all chromosomes along the metaphase plate. The chromosomes are rotating and being captured by microtubules for expulsion into the first polar body. D) A metaphase II oocyte immediately after the expulsion of the first polar body. E) A metaphase II oocyte with chromosomes aligned on the metaphase plate, with microtubule attachment, and awaiting fertilization.

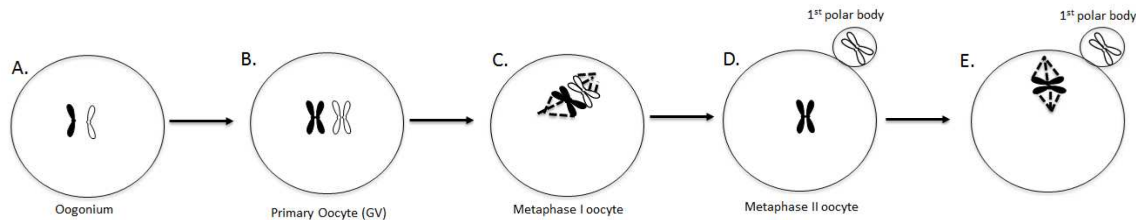
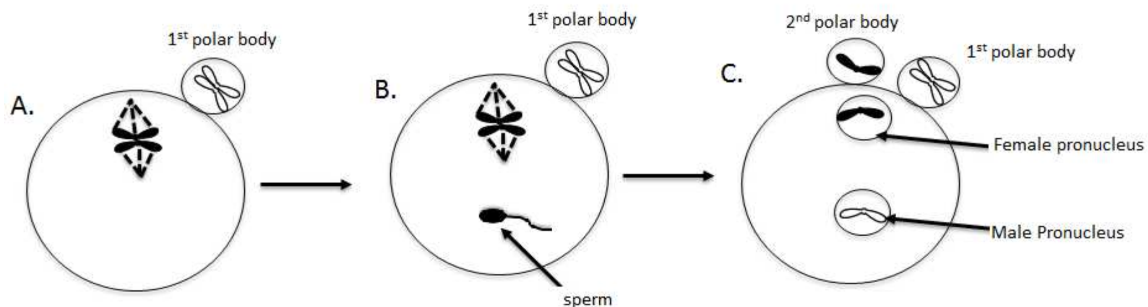


Figure 5. Meiosis II in the human oocyte. A) The mature metaphase II oocyte upon completion of meiosis I. B) Entry of a single sperm into the mature oocyte triggers meiosis II and the expulsion of the 2nd polar body. C) The male and female pronucleus within the mature oocyte.



1.2.2 Spindle Checkpoint

In some mammals and invertebrates, research suggests that a spindle checkpoint exists to protect the cell from dividing its chromosomes unequally (Li and Nicklas 1995; Stern

and Murray 2001; Homer *et al*., 2005; Brunet *et al.*, 2003; Wassmann *et al.*, 2003). If a chromosome(s) is not attached to the microtubules, the cell will prevent itself from dividing until the chromosome(s) is captured by the microtubules. This mechanism assures proper segregation of chromosomes; however, in human oocytes this mechanism seems non-existent. Advanced maternal age may be a reason for the lack of a spindle checkpoint, as evidenced by the increase incidence of aneuploidy in women of advanced maternal age. Or, it may be that other, age independent, factors are the cause (Liu and Keefe, 2002). LeMaire-Adkins *et al.* (1997) bred mice with Turner syndrome (45, XO) with euploid mice. If a checkpoint exists in the mouse, then no offspring with the karyotype 45, XO should be produced. Since multiple mice with the karyotype 45, XO were conceived, these data suggests that there is no spindle checkpoint. Yin *et al.* (1998) conducted a study where mouse oocytes were subjected to low levels trichlorfon. This drug disrupted spindle formation and prevented chromosome alignment, however the oocyte continued to progress to the MII stage. Hassold and Hunt (2001) estimate that 10-30% of fertilized human oocytes contain an unequal amount of chromosomes. Although this may not seem very high, but when compared to rates in yeast (0.01%) and in the fruit fly (0.02% - 0.06%), an apparent lack of a meiotic spindle checkpoint seems probable (Sears *et al.*, 1992; Koehler *et al.*, 1996).

The lack of a spindle checkpoint could allow chromosomes to divide unequally, therefore creating extra or missing chromosomes in the oocyte and subsequent embryo. This might explain why the majority of aneuploidy in humans originates in the oocyte during the meiotic reduction divisions (Hassold and Hunt, 2001). Furthermore, research suggests

that abnormalities in spindle structure of human oocytes can lead to an increase incidence of aneuploidy. Battaglia *et al.* (1996) found an increased rate of abnormally shaped spindles, microtubules, and chromosome misalignment in older women compared to the spindles of younger women, which appeared to have normal configurations.

Reproductive age plays a significant role in the production of aneuploidy due to the fact that the oocytes of older women have been arrested since prenatal development. An increase in reproductive age correlates to a loss in cohesin, which binds sister chromosomes together. Moreover, research has indicated that the loss of cohesin leads to frequent aneuploidy (Guacci *et al.*, 1997; Michaelis *et al.*, 1997; Losada *et al.*, 1998). Steffensen and colleagues (2001) demonstrated severe chromosome segregation in *Drospholia* when cohesin is not present. In mice with a mutated cohesin gene that 62% of oocytes had unpaired chromosomes (Hodges *et al.*, 2005). This percent increased with the age of the mouse; at 6 months of age, >80% of chromosomes were unpaired. If cohesion is decreased, there would be no force to oppose the pulling of the microtubules causing unequal separation of chromosomes at the MII phase. Furthermore, an increase in reproductive age also correlates with a decrease in chiasmata (Henderson and Edwards, 1968). A decrease in chiasmata would also decrease the force opposing the pulling of the microtubules and therefore would increase the rate of chromosomal malsegregation and subsequent aneuploidy. The spindle not only plays a role in the reduction divisions of meiosis but also in chromosome segregation of mitosis.

1.2.3 Spindle and Mitosis

In order to propagate, the fertilized oocyte must replicate the entire genome and equally divide into multiple blastomeres, a process referred to as mitosis. Similar to meiosis, mitosis equally divides chromosomes with the aid of the spindle apparatus. Mitosis is divided into five distinct processes: interphase, prophase, metaphase, anaphase, and telophase or cytokinesis.

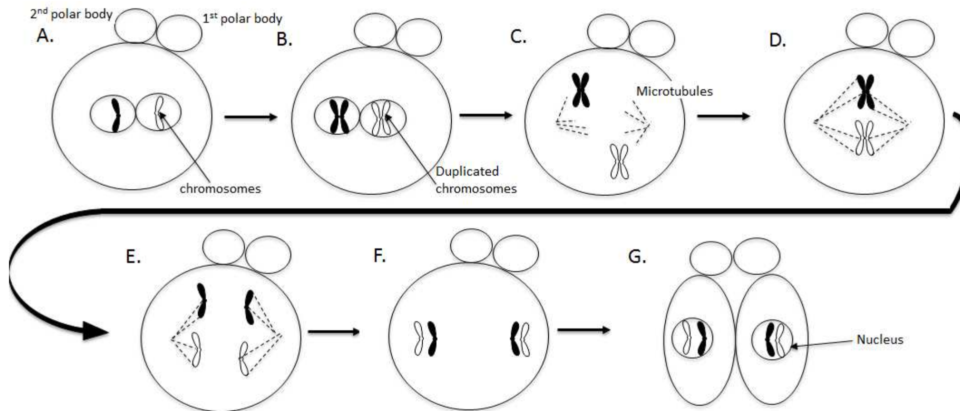
Interphase is the first step of mitosis and is subdivided into three phases Gap 1 (G1), Synthesis (S), and Gap (G2). During interphase, specifically G1, the cell is preparing itself for mitosis by producing the necessary proteins and energy stores needed for division. During the S phase, chromosomes are replicated. After replication, the cell enters G2 and awaits for prophase. During preimplantation development, interphase occurs shortly after the male and female pronucelus are joined in the zygote. The migration of the male and female pronucleus to the middle of the oocyte is aided by a network of actin microfilaments referred to as the sperm aster. Along these microfilaments, protein motors referred to as dynein, enable the movement of the pronucleus within the cytoplasm (Deng *et al.*, 2007; Wuhr *et al.*, 2009; Kimura and Onami, 2005; Kimura and Kimura, 2011; Schatten *et al.*, 1986; Kim *et al.*, 1996). The maternal and paternal DNA join together in syngamy producing a two pronuecli (2PN) embryo, now referred to as a zygote. At syngamy, DNA from every chromosome is replicated forming a sister chromatid (S phase; Figure 6B).

Prophase is the second step during mitosis and involves the degradation of the nucleus, condensation of chromosomes, and the formation of the spindle apparatus by MTOC's. As the chromosomes condense, proteins referred to as cohesins keep the sister chromatids connected (Tachibana-Konwalksi *et al.*, 2010). Simultaneously, the nuclear envelope starts to degrade, exposing the condensed chromosomes to the cell (Figure 6C).

Metaphase refers to the migration of the condensed chromosomes to the middle of the cell to an area referred to as the metaphase plate. While the chromosomes are in the metaphase plate, the MTOCs eject microtubules randomly into the cell. Once attached, tension occurs between the sisters chromatids by the pull of the microtubules. Due to proteins and phosphorylation, the separation of the chromosomes typically does not commence unless both MTOC's are connected the kinetochore (Chen *et al.*, 1996; Burke, 2000; Gardner and Burke, 2000). Once all microtubules are attached, tension is created between sister chromatids by the pull of the microtubules (Figure 6D).

At anaphase, tension is created by the pulling at the kinetochore in opposite directions by the MTOC's. This tension signals a protein referred to as separase to cleave cohesion; consequently, the sister chromatids separate and are pulled to opposite ends of the cell by the MTOC's (Nasmyth and Haering, 2009). The microtubules are shortened by the removal of the alpha and beta monomers at the MTOC. Thus, the chromosomes do not actually migrate to the opposite poles but are rather pulled by the shortening microtubules (Figure 6E). Once at opposing poles, the nuclear envelope begins to form around both sets of chromosomes and the chromosomes begin to decondense.

Figure 6: Zygote undergoing the first mitotic cleavage division. A) The zygote showing the 1st and second polar body and maternal and paternal chromosomes. B) The zygote with duplicated chromosomes (S phase of Interphase). C) Zygote with the formation of microtubules and decondensed nucleus, chromosomes are free floating (Prophase). D) Zygote with chromosomes that are captured by microtubules and aligned on the metaphase plate (Metaphase). E) Chromosomes being pulled to opposite poles (Anaphase). F) Chromosomes at opposite poles. G) The chromosomes decondense and form a nucleus and the cell splits into two; thus, the completion of mitosis (cytokinesis).



During this time, the cell starts to "pinch" itself along the metaphase plate. This "pinch" creates a cell membrane and separates the one cell into two daughter cells (Figure 6G). Once cleaved, the chromosomes are pulled to opposite ends of the cell and the cell is divided into two equals. Instead of "resting", the cells enter directly into another DNA replication and mitotic event (Boulding and Kimelman, 2014). Thus, the first cleavage stage divisions occur as fast as DNA can be replicated.

1.3 Preimplantation Embryonic Development

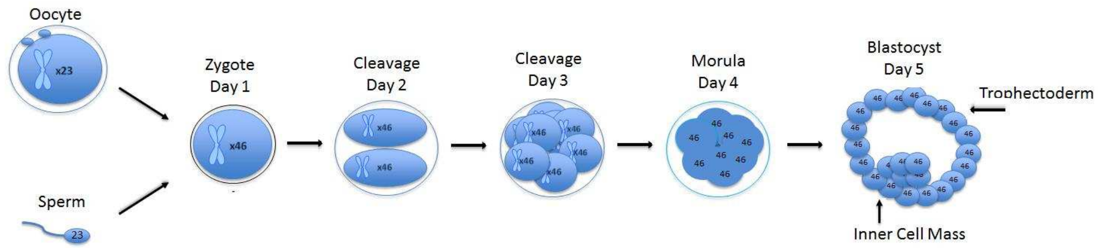
Preimplantation embryonic development constitutes a multitude of processes that start at the joining of the female (oocyte) and male (sperm) gametes in a process known as

fertilization. Once fertilization occurs, preimplantation embryonic development commences followed by implantation and live birth of a hopefully healthy individual.

Fertilization occurs in the fallopian tubes when the oocyte and sperm join together forming the zygote, the first cell of the new individual (Figure 7). The zygote is encased in a protein thick layer referred to as the ZP and compromised of two pronuclei, one from each parent (Figure 7). Each pronuclei contains 22 non-sex determining chromosomes referred to as autosomes and one of two sex determine chromosomes, either X (female) or Y (male). Thus, the zygote contains 44 autosomes and two sex chromosomes for a total of 46 chromosomes.

While traveling down the fallopian tube, the zygote undergoes multiple mitotic divisions, replicating the chromosomes (2N → 4N) and then dividing the chromosomes equally (4N → 2N) into two daughter cells (Figure 7). By the third day, the zygote has undergone four mitotic divisions and contains a total of six to eight cells referred to as blastomeres (Figure 7). The junctions between the blastomeres breaks down and they begin to form a tight ball of cells referred to as a morula (Figure 7). On day 4 the morula, while still undergoing mitotic divisions, starts to form a fluid filled cavity referred to as a blastocoel. The blastomeres start to differentiate into two different cell lines, the trophectoderm cells, which will become the placenta are located on the outside of the embryo, and the inner cell mass (ICM) ,which will become the fetus, form on the inside of the embryo (Barlow *et al.*, 1972; Figure 7, Figure 8). With the differentiation of the two cells lines, the embryo has progressed from the morula stage to the blastocyst stage (day 5; Figure 7).

Figure 7: Developmental stages of a human preimplantation embryo.



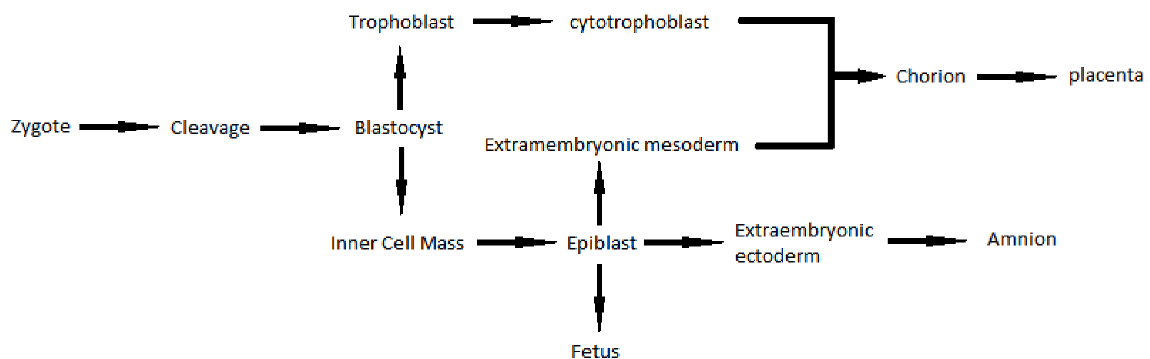
As the blastocyst exits the fallopian tubes and heads into the uterus, the blastocoel cavity continues to expand, thinning out the ZP. Ultimately, the blastocyst hatches out of the ZP and attaches itself to the uterine wall in a process known as implantation. Once attached, the blastocyst continues to increase in cell number while the trophectoderm invades the uterine wall in an attempt to attach itself to the mother's blood supply. Once implanted, the blastocyst continues to expand and develop into both the placenta and fetus.

1.4 Developmental Biology

The placenta is responsible for supplying nourishment and oxygen to the fetus. All of the cells and the derivatives from the trophoblast are considered extra embryonic tissue and do not constitute the embryo proper. The internal cells of the morula are destined to become the ICM (Pedersen *et al.*, 1986; Fleming, 1987). These cells will make up what will become the fetus proper. However, not all these cells are destined to become the fetus and thus, from the ICM, both extraembryonic and embryonic tissue persists. From

the ICM multiple cell lines deviate to form the fetus proper, yolk sac, and amnion (Figure 8). A recent study by De Paepe and colleagues (2013) demonstrated that isolated human trophectoderm cells can integrate into the ICM and express markers associated with ICM cells, indicating that they are not yet committed to becoming extraembryonic tissue. Hogan and Tilly (1978) dissected mouse ICM from the trophectoderm and left the ICM in culture. Within 5 days, some of the individual ICM's had the appearance of a blastocyst. Moreover, the individual ICM's derived trophoblast giant cells. These studies suggest that cells from the ICM feed the trophectoderm. Ultimately, the majority of the cells from the ICM will become the epiblast with further differentiation to extraembryonic mesoderm, extraembryonic ectoderm, and amnion, all of which are extraembryonic tissue (Figure 8; Crane and Cheung, 1988; Delhozizer-Blanchet, 1991). All of the above can only happen with the proper division of chromosomes.

Figure 8: Cell lineage from zygote (1 cell) stage to the fetus, including extraembryonic materials of the extraembryonic mesoderm and cytotrophoblast (adapted from Crane and Cheung, 1988; Delhozizer-Blanchet, 1991).



1.5 Chromosomes

Chromosomes were first discovered by Flemming (1882) and are molecular structures that contain genetic information in the form of deoxyribose nucleic acid (DNA) needed to create an individual, in essence they are the blueprint for life. DNA is a combination of four molecules: adenine, thymine, guanine, and cytosine. DNA forms a ladder like structure with adenine only connecting to thymine and guanine only connecting to cytosine; this ladder folds in on itself creating a double helix. DNA and the chromosomes reside in the nucleus of the cell. The complete DNA genome of the human is much longer than a single cell and thus the DNA must condense around molecules known as histones (Margueron and Reinberg 2010; Zhou *et al.*, 2011).

The condensed DNA forms a chromosome. The chromosome is composed of three different pieces: the centromere, telomere, and origin of replication. The centromere serves two major functions: allowing the sister chromatids to join together and to act as a site for the kinetochore to capture the microtubules during chromosome segregation (Yunis and Yasmineh 1971; Willard 1990; Schueler *et al.*, 2001). Centromeres can be located anywhere along the chromosome. In fact, where the centromeres are located dictates whether the chromosome is referred to as metacentric, submetacentric, or acrocentric. Metacentric refers to a chromosome where the centromere is in the middle giving the appearance of two chromosomes arms that are equal in length (i.e. = chromosomes 1, 3, 16, 19, and 20). Submetacentric refers to a chromosome that has two arms of unequal length (i.e. = chromosomes 2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 17, 18, X, Y).

Lastly, acrocentric refers to chromosomes where the centromere is located towards the very end of the chromosome (i.e. = chromosomes 13, 14, 15, 21, 22).

Telomeres are located at the ends of the chromosomes and consist of repeat sequences of DNA (Zakian, 1989). These repeat sequences of DNA protect the end of the chromosome from degradation during mitosis (Greider and Blackburn, 1985; Greider and Blackburn, 1989). Each round of cell division shortens the telomeres. So, as the cell ages, chromosomes become more susceptible to degradation via shortening of telomeres (Harley *et al.*, 1990). To combat this, chromosomes use an enzyme called telomerase that continually extends the telomeres to appropriate lengths (de Lange, 2005).

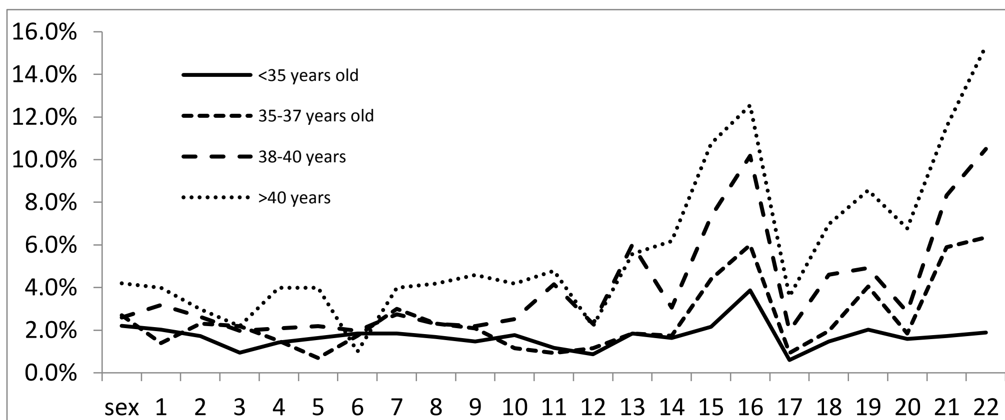
The origin of replication are all the areas outside of the telomeres and centromeres. These areas contain all of our genes and functional genetic makeup that make us who we are. These are areas within the chromosomes that are actively undergoing transcription and translation, allowing for the development of proteins that serve for cell functions.

The human genome contains a total of 46 chromosomes; 22 pairs of autosomes and two sex determining chromosomes. An individual with 22 pairs of autosomes and two sex determining chromosomes is considered euploid. Chromosomes are paramount during MI, MII, mitosis, and pre and post implantation development.

1.6 Aneuploidy during Reproduction

The majority of preimplantation embryo wastage and miscarriages are caused by aneuploidy (Hassold and Hunt, 2001). These errors can occur at all stages and involve any chromosome (Fragouli and Wells, 2011). It is believed that the low success rates of IVF could be attributed to aneuploidy as the older a woman gets, the incidence of aneuploidy increases (Figure 9). For example, at the age of 28 the average incidence of aneuploidy at the blastocyst stage is approximately 25% whereas the incidence at the age of 44 is greater than 80% (Frasnasiak *et al.*, 2014). Interestingly, the incidence of aneuploidy stays relatively constant from 25 to 35 years old, but after 35, the incidence increases approximately 5% year over year (Frasnasiak *et al.*, 2014).

Figure 9: Analysis of 4,500 blastocysts screened for aneuploidy and the rate of individual chromosomal abnormalities stratified by maternal age (Data courtesy of Alison Coates from Oregon Reproductive Medicine).



It should be noted that some chromosomes are more likely to malsegregate than others during preimplantation development. For example, in a large dataset of 15,169 blastocysts, Frasnasiak and colleagues (2014) demonstrated that chromosomes 13, 15,

16, 18, 19, 21, and 22 were the most common chromosomal aneuploidy present at the blastocyst stage. Interestingly, these are also the most common in product of conceptions, meaning that these abnormalities may not be selected against until after implantation (Sahoo *et al.*, 2016). Certain chromosomes are more prone to aneuploidy simply due to the location of the centromere along the chromosome. Chromosomes with the centromere located near the end of the chromosome are more prone to aneuploidy, followed by chromosomes with the centromere in the middle, and then chromosomes with a centromere in-between the middle and the end are least susceptible to aneuploidy (Frasnasiak *et al.*, 2014). It is not just the incidence of aneuploidy during preimplantation development but also mosaicism that may influence embryological development.

1.7 Chromosomal Mosaicism during Reproduction

It is not just aneuploidy that can impact reproduction. Another phenomena known as chromosomal mosaicism that is defined as the presence of two more chromosomally distinct lines can also influence embryological development. At its core, chromosomal mosaicism is the failure of chromosomes to properly segregate during mitosis, leading to aneuploidy. Chromosomal mosaicism has been implicated in genetic diseases, miscarriages, and preimplantation embryo wastage (Hassold and Hunt, 2001). Moreover, mosaicism has been shown in cancer (Lengauer *et al*, 1998) and associated with ageing (Ly *et al.*, 2000). Although prevalent, the exact threshold at which mosaicism switches from clinically irrelevant to relevant is unknown and differs depending on the stage and

severity at onset. Due to the prevalence and significance of mosaicism in the human species, it is important to understand the origins, mechanisms, and incidences of mosaicism throughout development.

1.7.1 General Mosaicism

General mosaicism is the presence of a two or more cell lines throughout the entire organism. In order for the mosaic cell lineage to be present within the entire organism, the aneuploidy in question must derive from a mitotic event during the first days of embryonic development, prior to any cellular differentiation. At this stage, mosaicism has been found to range between 65-70% (Mertzanidou *et al.*, 2013; Wells and Delhanty, 2000). However, this incidence may be overstated due to technical limitations, sampling error, and cell phase of sample collection. Simply because preimplantation embryos are mosaic does not mean that the abnormal cell line(s) will continue to propagate during development. Research has shown that euploid cells proliferate at a higher rate than aneuploidy cells (Ruangvutilert *et al.*, 2000). Scott and colleagues (2012) describe live births from diagnosed aneuploid cleavage stage and blastocyst stage embryos, although at a significantly lower rate than from euploid embryos. This indicates that aneuploidy and mosaicism may play a limited role in development, and its influence during development may be dependent on the degree of mosaicism and what chromosome is involved.

Chromosomal mosaicism is common during preimplantation development. However, due to the low number of cells present during preimplantation development, any abnormality can have much more of an impact as development continues. Furthermore, mosaicism can become isolated during embryonic development, leading to mosaicism confined to a particular area.

1.7.2 Confined Mosaicism

Confined mosaicism refers to chromosomal mosaicism that is only present in a particular area and has been reported in the brain (Yurov *et al*, 2007), placenta (Kalousek and Dill, 1983), and gonads, amongst other places. A major aspect of research about confined mosaicism deals with the relationship between the placenta and the developing fetus.

Confined placental mosaicism (CPM) is defined as chromosomal differences between the fetus and placenta. CPM was first reported in the human placenta by Warburton and colleagues (1978). They discovered that roughly 10% of trisomic conceptions contained a mosaic cell line. CPM has been linked to intrauterine growth retardation (Kalousek and Dill, 1990), spontaneous abortions, intrauterine death, still birth (Benn, 1998), and abnormal placental function (Koplan *et al.*, 1991). CPM is believed to occur in roughly 1-2% of all placental tissue analyzed (Kalousek *et al.*, 1991).

There are two types of invasive procedures utilized in prenatal testing, chorionic villus sampling (CVS) and amniocentesis. Both procedures determine the chromosomal

constitution of the fetus by sampling embryonic tissue rather than the fetus proper. CVS involves the sampling of the chorionic villus from either the cytotrophoblast or extraembryonic mesoderm (EEM). Alternatively, amniocentesis involves the removal of a sample of amniotic fluid, which is a product of extraembryonic ectoderm. Both the extraembryonic ectoderm and EEM are derived from the ICM and epiblast of the developing embryo and are not part of the fetus proper, while the cytotrophoblast is derived from the trophoblast (Bianchi *et al.* 1993; Figure 8). It should be noted that no test directly analyzes the chromosomes of the fetus proper during pregnancy.

Regardless of whether mosaicism is present in the entire individual or is confined, the mechanisms that mosaicism occurs are the same.

1.8 Mechanisms of Chromosome Malsegregation

There are three main mechanisms of chromosomal aneuploidy and mosaicism that can occur, leading to a gain and/or loss of chromosomes: non-disjunction, anaphase lag, and chromosome gain referred to as endoreplication. Anaphase lag accounts for the majority of chromosomal errors during embryonic development while non-disjunction and endoreplication occur to a lesser extent.

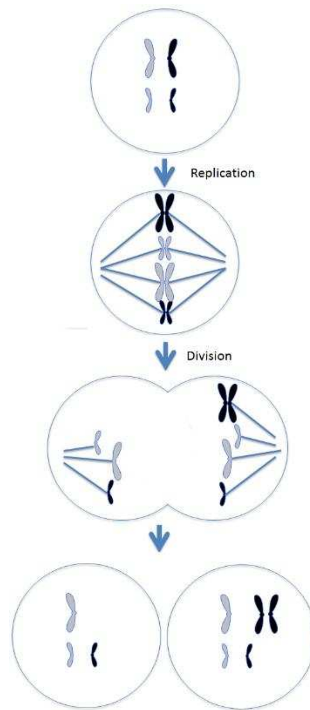
1.8.1 Non-disjunction

Non-disjunction is the failure of sister chromatids to separate during mitosis. Instead of separating, the entire chromosome (two chromatids) is pulled to one cell, creating a cell with a monosomy and another cell with a trisomy (Figure 10). If non-disjunction occurs prior to cell differentiation, for example in a preimplantation embryo, a general mosaic is created. Non-disjunction can also lead to a confined mosaicism. For example, if the non-disjunction event occurs in the trophoblast, after differentiation, then only the placenta will contain the mosaic cell lines, while the embryo proper could be euploid.

The incidence of non-disjunction during preimplantation development is subject to debate, and depends greatly on the stage of development and chromosome involved.

For example, non-disjunction has been shown to be the least prevalent mechanism associated with aneuploidy during meiosis I and II amongst the autosomes (Forman *et al.*, 2013a; Handyside *et al*., 2013a), but it is established as the main mechanism for sex chromosome malsegregation during the first cleavage stage divisions (Bean *et al*., 2001; Bean *et al*., 2002). It is evident that chromosomes may be more or less susceptible to non-disjunction depending on stage of development.

Figure 10: A non-disjunction event leading to a monosomy in one cell and a trisomy in another.



1.8.2 Anaphase Lag

Anaphase lag is the failure of a single chromatid to be incorporated into the daughter nucleus resulting in a monosomy of that chromosome in one cell and a disomy in the corresponding chromosome in the other cell (Figure 11). Anaphase lag occurs when the chromatid fails to attach to the spindle or when the chromatid attaches to the spindle but then fails to be incorporated in the nucleus. If this mechanism occurs prior to differentiation, then the organism will contain two distinct cell lines, thereby creating a general mosaic. If this event occurs after differentiation in the trophoblast, then the placenta will contain a normal and monosomic cell line, an example of CPM.

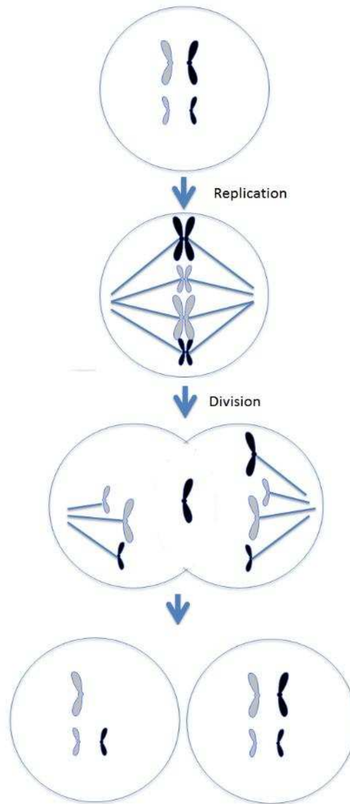
In a study using discarded day 5 embryos, Ioannou *et al*. (2012) demonstrated that monosomy can occur at a 7x greater rate than trisomy. This would implicate anaphase lag as the main source of mosaicism in human preimplantation development. This observation is supported by Coonen and colleagues (2004) and Capalbo and colleagues (2013a) who found anaphase lag at rates of 5x and 3x that of non-disjunction, respectively.

If a cell presents with a trisomy, then the process of anaphase lag can "correct" the trisomy and revert the reciprocal chromosome back to disomy, a process referred to as trisomic rescue (Figure 12). Although the frequency of this process is largely unknown, reports indicate that trisomy rescue of meiotic errors can occur during preimplantation development (Capalbo *et al*, 2013a; Barbash-Hazan *et al.*, 2009).

1.8.3 Endoreplication

Endoreplication is the replication of a chromosome without division. This results in a trisomic chromosome in one cell and a disomic chromosome in the other. Chromosome gain is believed to derive from two mechanisms, a cell cycle malfunction when a chromosome is replicated without subsequent cytokinesis or when mitosis is initiated and shortly thereafter shutdown, resulting in a replicated chromosome. Regardless of the mechanism, the result is the same: the gain of a single chromosome (Figure 13). Endoreplication can lead to polyploidy. Polyploidy has been shown to exist in blood, gut, skin, and brain (for review see Fox and Duronio, 2013).

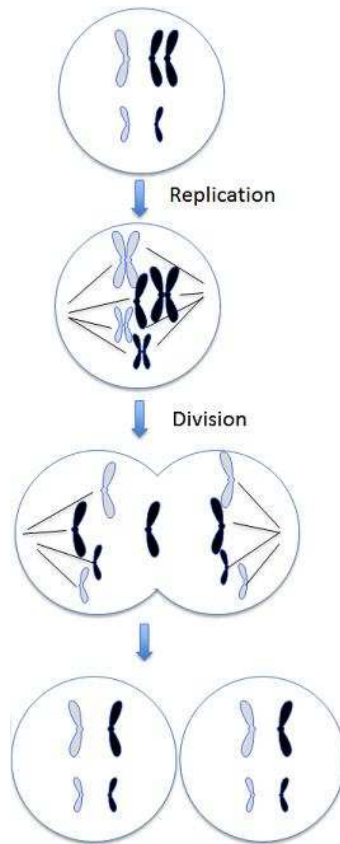
Figure 11: An anaphase lag event leading to a disomy in one cell and a monosomy in another.



1.8.4 Uniparental Disomy

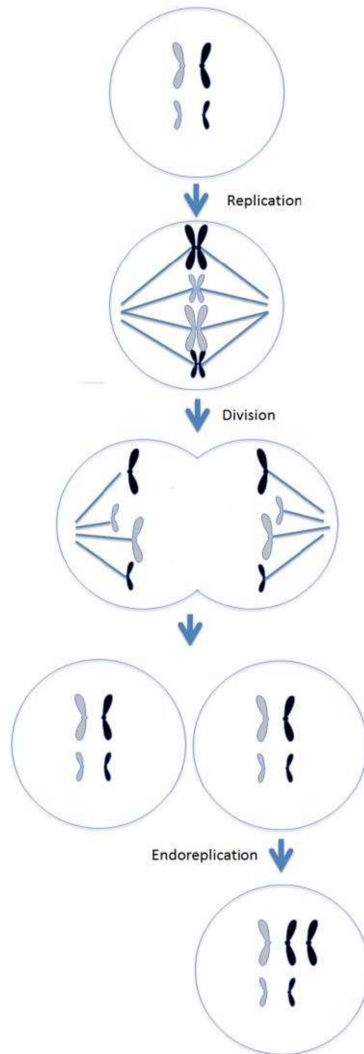
Non-disjunction, anaphase lag, and endoreplication lead to aneuploidy. However, there are mitotic events that lead to mosaicism, but still present a disomic cell line. Instead of chromosomes presenting in a gain or loss fashion, a phenomenon known as uniparental disomy (UPD) can occur. As UPD implies, there are two chromosomes present; however, instead of one maternal and paternal chromosome, there are two copies of either a maternal or paternal chromosomes. This may be the result of a trisomic rescue event after an error during meiosis (Figure 14).

Figure 12: A trisomic rescue event whereby a trisomic chromosome undergoes an anaphase lag event that corrects the cell line back to disomy.



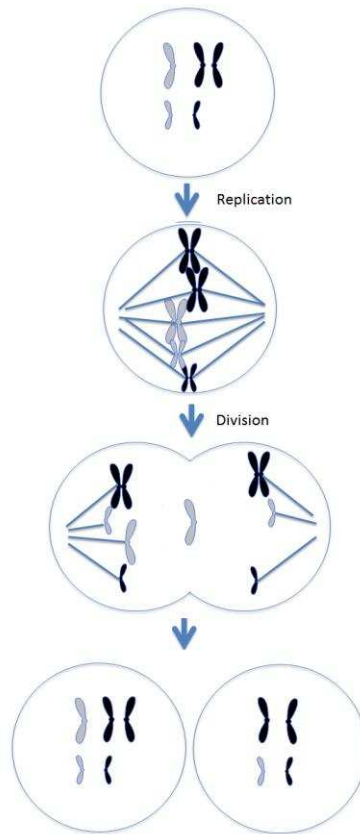
The most frequent chromosome that UPD occurs in is chromosome 15, where two paternal copies is referred to as Angelman syndrome or two maternal copies is known as Prader-Willi syndrome. Other chromosomes that can present with UPD are chromosomes 7, 11, and 16 (Kaluosek *et al*, 1992).

Figure 13: An endoreplication event resulting in a trisomy.



UPD and endoreplication occur to a lesser extent while anaphase lag and non-disjunction seem responsible for the majority of whole chromosomal abnormalities within the human embryo (Daphnis *et al.*, 2008). These mechanisms promote improper chromosome segregation, leading to multiple cell lines presenting with either chromosome gain and/or loss. Because of this, it is important to understand the origins of these mechanisms.

Figure 14: A uniparental disomy event resulting in a trisomy.



1.9 Origins of Aneuploidy

1.9.1 Paternal Origin

The centrosome is inherited from the sperm and is responsible for the first mitotic divisions within the human embryo (Palermo, 1994). The disruption of the sperm centrosome can produce mosaicism in the preimplantation embryo (Palermo *et al*, 1997). Furthermore, research has indicated that sperm aster formation could be delayed in infertile males when compared to fertile males (Yoshimoto-Kakoi *et al*, 2008; Terada *et al*., 2004). A delay in sperm aster formation could cause a delay in syngamy and

subsequent cleavages and possibly induce aneuploidy. Thus, studies have shown that chromosomal aneuploidies are more prevalent in patients with severe male factor infertility (Magli *et al*, 2009; Gianaroli *et al.*, 2000; Silber *et al.,* 2003). If that is the case, then it is possible that mosaicism may also be more prevalent during preimplantation development in this group of patients.

1.9.2 Maternal Origin

The increase in aneuploidy with maternal age is well documented (Munne *et al.*, 1995, 2002a). Although mosaicism is a mitotically derived phenomenon, the proper segregation of chromosomes can be hindered by maternal processes. As previously stated, the centrosome is paternally inherited but the mitochondria and mRNA stores necessary for proper chromosome division originate from the oocyte. Indeed, research has indicated that mitochondrial function is affected by maternal age, possibly influencing chromosome segregation (Wilding *et al.*, 2001; Schon *et al*., 2000).

The oocyte pool in a woman has been arrested at prophase since prenatal development. As the woman ages, so too does the length of exposure of the oocytes to reactive oxygen species and environmental factors that may have negative effects during embryological development. Furthermore, an increase in reproductive age correlates with a decrease in the cohesion molecule that is responsible for binding the sister chromatids together (Duncan *et al.*, 2012; Hodges *et al*., 2005). If this cohesion is reduced in older women, this could cause an unequal separation of chromosomes leading to aneuploidy.

Depending on when and where in development the error occurs, this could lead to mosaicism. Finally some of the genes necessary for mitosis have been shown to be down regulated in fibroblasts from older patients when compared to younger patients (Ly *et al.*, 2000), although unfortunately this study did not give an indication of the actual ages. Interestingly no correlation has been observed between the incidence of preimplantation chromosomal mosaicism and maternal age (Munne and Cohen, 1998; Munne *et al.*, 2002b).

Abnormalities in spindles are also more prevalent in older women. For example, meiotic spindles from older women can have an abnormal shape and produce more chromosome misalignment compared to spindles of younger women, which appear to have normal configurations (Battaglia *et al.*, 1996). Although the meiotic spindle does not directly influence mitotic chromosome segregation, it is possible that the presence of abnormal meiotic spindles could suggest that the process of chromosome segregation in older women is flawed from the onset, leading to mosaicism. An increase in mitotic spindle abnormalities of arrested day 3 and day 4 embryos compared to blastocysts has been reported (Chatzimeletiou *et al.* 2005). Furthermore, poor quality blastocysts have been shown to have more abnormal spindles when compared to good quality ones (Hashimoto *et al.*, 2013). If abnormal spindles are present within a human embryo, then one would expect a higher incidence of aneuploidies, which may indicate a direct relationship between maternal age, spindle abnormalities, and chromosomal mosaicism.

While there is a direct relationship between advanced maternal age and aneuploidy, no relationship between maternal age and mosaicism has been shown. Aneuploidy has also been shown to be prominent in young, fertile, oocyte donors and women <35 years old (Munne *et al*., 2006; Baart *et al*., 2006; Fragouli *et al.* 2009; Ata *et al*., 2012). It is possible that other cells within the embryos tested in these studies could present with different cell lines which would indicate the presence of mosaicism.

1.9.3 External Influences

External factors also contribute to mosaicism. For example, the production of oocytes for IVF requires controlled ovarian hyperstimulation of ovaries by exogenous follicle stimulating hormone. Hyperstimulation has been implicated in increased rates of cleavage stage aneuploidy. Munne and colleagues (1997) have demonstrated different mosaicism rates between IVF centers, implicating differences in stimulation protocols as a potential reason. However, other research has shown that even embryos derived from unstimulated ovaries produce similar rates of chromosomal aneuploidies (Verpoest *et al.,* 2008).

Munne and colleagues (1997) have suggested that embryo culture conditions may also cause differences in mosaicism. Improper culture conditions can compromise embryo quality. Furthermore, 5% oxygen versus atmospheric oxygen levels has been found to improve embryo quality and decrease sex chromosome mosaicism when compared to culture in atmospheric oxygen levels (De Los Santos *et al.* 2013; Bean *et al*., 2002). Proper

embryo culture is essential for embryo development and poorer quality embryos tend to have higher rates of chromosomal abnormalities (Munne *et al.*, 2007). Thus, it is plausible that embryo culture may increase aneuploidy and subsequent mosaicism in the human preimplantation embryo (Beyer *et al.*, 2009). However, in-vitro derived blastocysts have lower rates of aneuploidy when compared to cleavage stage embryos (Fragouli *et al*., 2014). The reason for the decrease in aneuploidies at the blastocyst stage is due to the selection against embryos carrying multiple aneuploidies, resulting in a large number of embryos becoming arrested at the cleavage or morula stage (Vega *et al.*, 2014). Embryos that develop to the blastocyst stage have progressed further than cleavage stage embryos and so the culture media may have less of an effect on chromosome segregation as development progresses.

1.10 Incidence of Mosaicism

1.10.1 Incidence in Cleavage Embryos

Mosaicism occurs in approximately 15% to 90% of all cleavage stage human embryos (Rubio *et al.*, 2007; Daphnis *et al.*, 2005; Harper *et al.*, 1995). It is believed that if the majority of the cells are diploid, then these embryos could be deemed viable, even though they are general mosaics. In a systematic review looking at all the cells from each embryo, van Echten-Arends and colleagues (2011) found that 73% of 815 cleavage embryos examined were mosaic and 59% of all embryos were diploid-aneuploid mosaic. The

authors noted that not all embryos were examined with the same technology, indicating that the mosaic rate may be higher when more chromosomes are analyzed. However, other research indicates similar rates of mosaicism at the cleavage stage (Mertzanidou *et al*, 2013; Vanneste *et al.*, 2009; Wells and Delhanty, 2000). A majority of research at the cleavage stage examining mosaicism was conducted utilizing FISH, a technique which has been shown to be inaccurate and difficult to perform due, possibly causing an overstatement in cleavage stage mosaicism (Northrop *et al.*, 2010). Nonetheless, it seems that mosaicism and aneuploidies are routine during the first cleavage divisions in human preimplantation development.

1.10.2 Incidence in Blastocysts

The blastocyst is composed of two distinct parts, the trophectoderm, which will become the placenta, and the ICM, which will become the fetus (Figure 8). Thus, the blastocyst represents the first stage of cellular differentiation in human embryonic development whereby totipotent cells become pluripotent cells. Compared to cleavage stage embryos, similar rates of mosaicism appear to exist in the human blastocyst. Liu *et al.* (2012) reported that 69% of abnormal blastocysts from women of advanced age are mosaic for both the ICM and trophectoderm. However research from younger women has demonstrated that 80% of blastocysts are euploid, with the majority of the abnormal blastocysts presenting with only one or two structural chromosome abnormalities. This suggested a lower level mosaicism at the blastocyst stage (Johnson *et al*, 2010). Fragouli

et al. (2011) demonstrated that roughly one-third of all blastocysts are mosaic, while Northrop and colleagues (2010) found that only 16% (8/50) were mosaic. Currently, research has not demonstrated an association between maternal age and mosaicism (McCoy *et al.*, 2015). Similar to cleavage stage mosaicism detection, one of pitfalls of mosaicism detection at the blastocyst stage is technical errors. Some researches argue that technical aspects of the test may be overcalling mosaicism and limiting our understanding of mosaicism (Capalbo *et al*. 2017a; 2017b). Regardless of the variability in results, mosaicism may still be prevalent at the blastocyst stage.

1.10.3 Incidence Post Implantation

CPM is detected via CVS samples typically at 10-12 weeks and is typically conducted on patients who are at an increased risk of genetic abnormalities; therefore, the true incidence of CPM in a general population is unknown. Research indicates that 1-2% of viable pregnancies present with a chromosomally abnormal placenta but a normal fetus (Ledbetter *et al.*, 1992). The diagnosis of CPM is determined from a limited number of cells from one particular biopsy site, and it is also possible that the cells sampled could accidently be from the mother (a euploid individual), an artifact known as maternal contamination. When maternal contamination is controlled for, CPM exists in roughly 6% of all pregnancies (Griffin *et al*, 1997). CVS sampling takes place at 10-12 weeks, when the placenta is not yet fully mature, therefore CPM quantified at CVS sampling has been shown to be different to that in term placenta (Schuring-Blom *et al.*, 1993). When term

placentas have been reanalyzed after a 10 week diagnosis of CPM, trisomic cells have been found within both the cytotrophoblast and the EEM (Figure 8; Schuring-Blom *et al*, 1993; Artan *et al*, 1995).

The majority CPM abnormalities are autosomal trisomies (Lestou and Kalousek, 1998). Unfortunately there is not always concordance between the EEM and cytotrophoblasts. For example, trisomies 2 and 17 are more prevalent in the EEM while trisomies 3, 6, 9, 12, 13, 14, 16, 18, 20, 21, and 22 are more prevalent in the cytotrophoblasts. The trisomies that present equally between the two tissues are chromosomes 4, 5, 7, 8, 11, and 15 (Hahnemann and Vejerslev, 1997; Lebedev, 2011). These errors could be attributed to either meiotic errors or mitotic errors during development. If meiotic in origin, the errors would also be present throughout the embryo including the EEM unless trisomic rescue occurred prior to differentiation. Likewise, it is possible that a non-disjunction event or endoreplication occurred at the onset of cellular divergence to either the EEM or cytotrophoblasts creating a mosaic within those lines compared to other embryonic and extraembryonic tissue. Regardless of how the error occurred, it is evident that chromosomes are differently affected during development.

1.11 Clinical Consequences

The clinical consequences of mosaicism are dependent on a variety of factors including when during development the error occurs and if the error can continue to propagate.

Because the cleavage stage embryo only contains 4 to 8 cells, the consequences of chromosomal mosaicism are much more pronounced during this stage then if mosaicism occurs when more cells are present. For example, it was previously mentioned that the rate of mosaicism at the cleavage stage varies greatly from 15-90%, however the rate of mosaicism seen in prenatal diagnosis ranges from 1-2% (Ledbetter *et al.*, 1992). This would indicate a selection mechanism against mosaicism in the later stages of development. Therefore, mosaicism during the preimplantation stage has a greater consequence than post implantation mosaicism.

It is believed that the actual fetus only derives from three cells of the ICM (Markert and Peters, 1978). Any cells not destined to become the fetus may not be a true representation of the fetus itself but rather of extraembryonic tissue (i.e., EEM, cytotrophoblasts, placenta, chorion, etc.). Studies have shown that abnormal cells can be forced away from the fetus lineage (James and West, 1994). Stetten and colleagues (2004) have found that 76% of CVS samples which are identified as mosaics have a normal amniocentesis result; however, they note that it is unknown if the fetus presents with low, undetectable levels of mosaicism. These tissues could be mosaic while the fetus itself is normal, at least phenotypically. In line with this, Staals and colleagues (2003) reported a mosaic female for trisomy 12 with normal development. Down syndrome patients with low levels of mosaicism tend to have less severe manifestations than typical Down syndrome patients, indicating that the clinical significance of mosaicism is directly associated with the ratio of abnormal to normal cells (Leon *et al*., 2010).

As previously mentioned, mosaic cell lines can arise from either mitotic or meiotic circumstances. If a meiotic error has occurred and the error is corrected at the cleavage stage, mosaicism will result. It is possible then that the abnormal cells are forced into the trophoblast while the normal (rescued) cells are destined to become the fetus (Figure 15). Conversely, it is also possible that the meiotic error is corrected at the cleavage stage, and the mosaic cell lines do not become isolated but rather persist throughout the trophectoderm and ICM (Figure 16). In either case, the meiotic errors are typically more devastating due to the initial onset of the abnormality, which allows the mosaic cell line to dominate. However mitotic errors may be as severe as meiotic errors depending on when they occur. If a mitotic error occurs in the first one or two divisions, then the mosaic cell line could be present through all embryonic tissues (Figure 17). However, if the mitotic error occurs further along in development, the mosaic cell line could be confined to that particular region (Figure 18; Figure 19).

Figure 15: A meiotic error that is corrected at the cleavage stage resulting in a mosaic cleavage stage embryo. When forming a blastocyst, the mosaic cell line is isolated to the trophectoderm while the euploid cell line is isolated to the ICM.

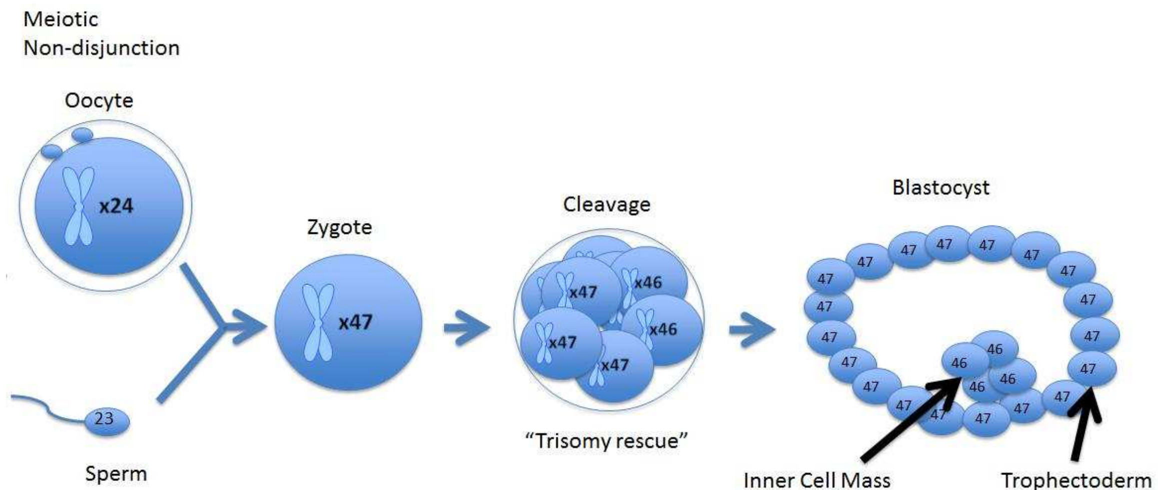


Figure 16: A meiotic error that is corrected at the cleavage stage resulting in a mosaic cleavage stage embryo. When forming a blastocyst, the mosaic cell line does not become isolated and persist throughout the trophectoderm and ICM.

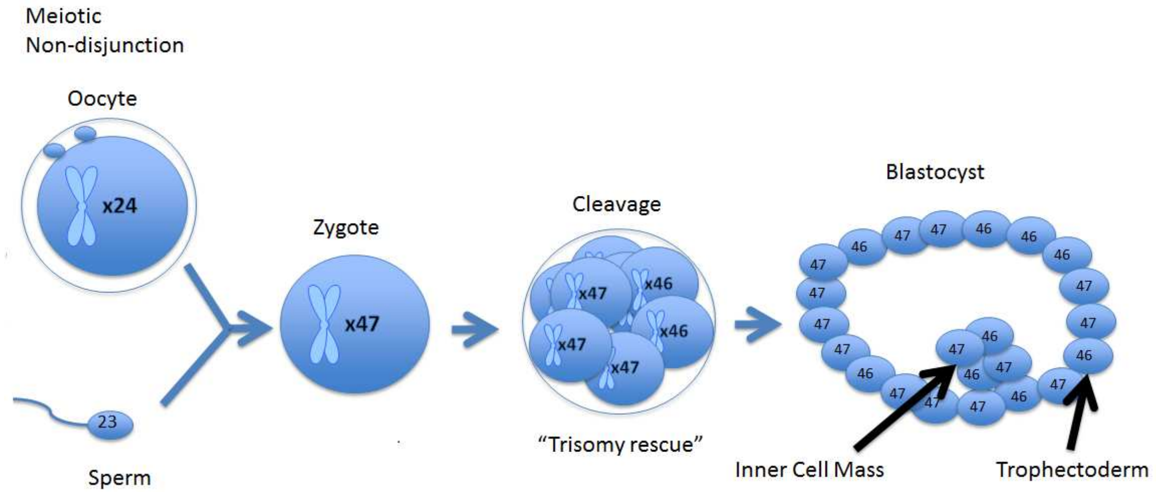


Figure 17: A mitotic error at the cleavage stage that resulted in a mosaic cleavage stage embryo. When forming a blastocyst, the mosaic cell line does not become isolated and persists throughout the trophectoderm and ICM.

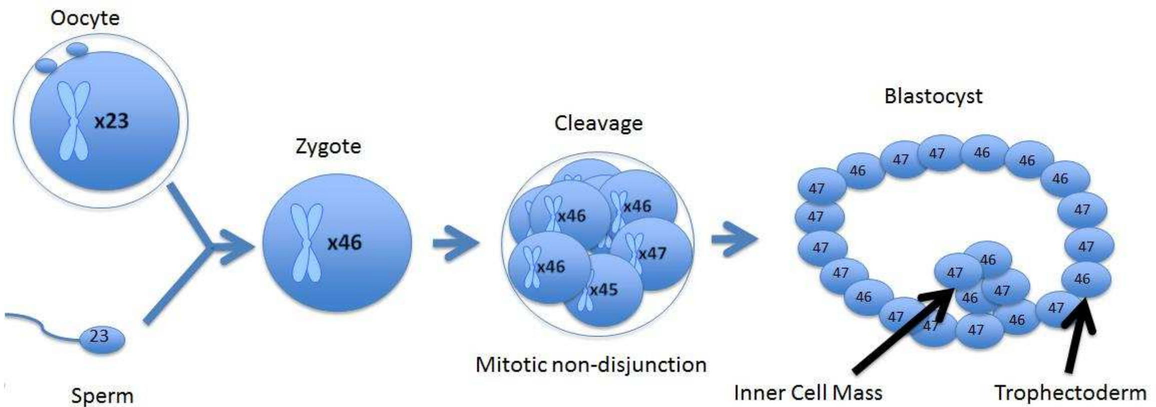


Figure 18: A mitotic error that occurred in the trophectoderm of the blastocyst. The blastocyst is a mosaic, however the error is isolated to the trophectoderm while the ICM remains euploid.

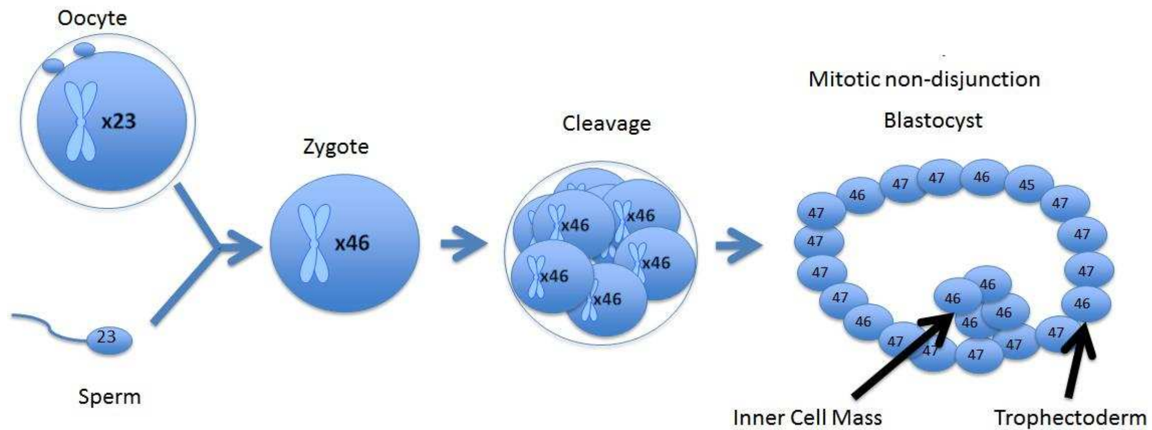
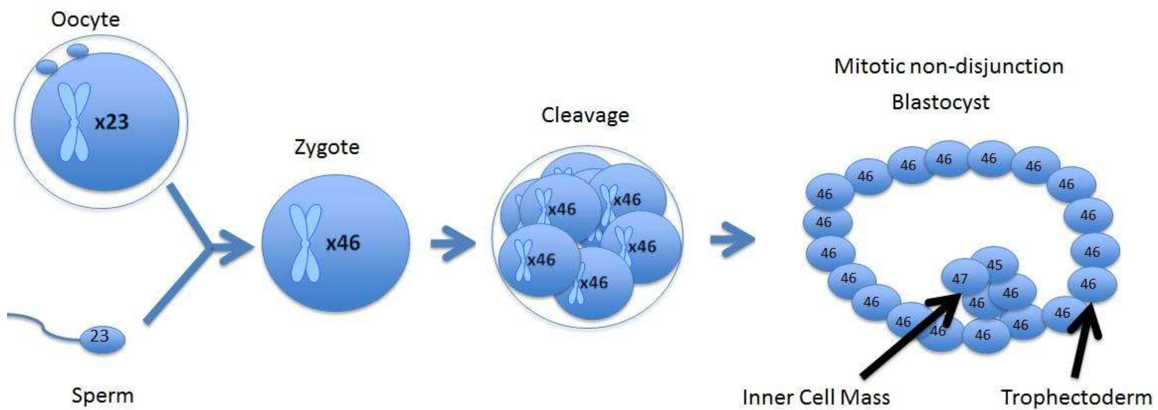


Figure 19: A mitotic error that occurred in the ICM of the blastocyst. The blastocyst is mosaic, however the error is isolated to the ICM while the trophectoderm remains euploid.



The clinical consequences of confined mosaicism are dependent upon the location of the mosaicism. For example, germ line mosaicism has been found to be associated with an increase in trisomic oocytes (Delhanty, 2011). In one report, CPM of trisomy 16 was discovered to also have propagated in the oocytes of the corresponding fetus (Stavropoulous *et al.*, 1998). Likewise, confined mosaicism for trisomy 21 was detected in the ovaries of eight fetuses that were phenotypically normal (Hulten *et al.*, 2008). It is

evident that the effects of the mosaicism depend greatly on the location of the mosaic cell line along with what chromosome is involved.

The clinical consequences of mosaicism are difficult to pinpoint and explain due to differences between individual chromosomes. For example, mosaicism of a particular chromosome may affect muscular development while mosaicism of another chromosome may affect organ development. It is not only the particular chromosome that matters but the prevalence or sheer numbers of the particular mosaic cell line. If an individual has a few muscle cells that are mosaic and the remaining are normal, then the effects of those abnormal cells is masked by the number of normal cells. The opposite also holds true, if all but a few muscle cells are mosaic, then the normal cells will be masked by the mosaic cells.

As previously mentioned, certain trisomies are more prevalent in certain tissues. This would suggest that there is a selection against some chromosomal abnormalities at certain stages of fetal development. This could be due to different growth rates of abnormal and normal cells. Likewise, each chromosome acts differently under different conditions and at different times during prenatal development. For example, if trisomy 13, 18, and sex chromosomes are detected in CVS, they typically will also present themselves within the fetal lineage (Hahnemann and Vejerslev, 1997), while CPM presenting with trisomy 2, 3, 7, and 8 are typically not associated with any adverse events and normally lead to a chromosomally normal fetus (Kalousek *et al*., 1996; Hahnemann

and Vejerslev, 1997; Sifakis *et al*, 2010; van Haelst *et al*, 2001). Taken together, the clinical relevance and consequences of mosaicism depends on a variety of factors.

1.12 Preimplantation Genetic Screening Biopsy Techniques

As previously alluded to, aneuploidy and mosaicism can have serious and disastrous consequences during pre-and post-implantation development. Thus, the screening of embryos and transferring of euploid embryos seems justified. In order to test embryos for their chromosomes, a piece of the oocyte or embryo must be removed and processed. Currently, there are three different stages that a biopsy can occur: the polar body, cleavage stage, and blastocyst (Harper *et al.*, 2012). Each of these represent a different stage of development and each present with their own set of advantages and disadvantages.

1.12.1 Polar Body Biopsy

As previously discussed during the course of oocyte maturation, two polar bodies are produced; therefore, there are two biopsy possibilities. First, the biopsy and analysis of the first polar body and second, the biopsy and analysis of the first and second polar body.

There are a multitude of ways to perform polar body biopsy. In general though, the mature oocyte must be placed in Hepes buffered media supplemented with 10% serum

and overlayed with oil. The oocyte, with the polar body at 12 o'clock is held in place by suction applied to a holding pipette. The ZP is either breached with a sharp biopsy needle, mechanically stripped by acid, or ablated by a laser, allowing the biopsy needle to aspirate the polar body. The first polar body is expelled before the completion of the first meiotic division; thus, further incubation may be required to allow completion of meiosis even though the polar body is visible (Wang et al. 2001; Verlinksy *et al*., 1990). If one is just removing the first polar body, then this procedure is performed on every mature oocyte prior on the day of retrieval. If this procedure is being performed on the first and second polar body, then the procedure is typically performed after fertilization when both polar bodies are present. One potential problem with waiting until after fertilization is that it would be unknown which polar body is the first or second polar body as there are no predictable markers to determine this (Forman *et al.*, 2013a; Salvaggio *et al.*, 2014).

In general there are many advantages to polar body biopsy. For example, polar bodies are considered extraembryonic tissue and are not involved in embryonic development; consequently, their removal should not impede embryological development. Because they are not part of the embryo, they have no legal status and ethical limitations are minimal or non-existent (Corveleyn *et al.*, 2008). Also, polar bodies are produced at the beginning of the IVF process and this allows for five to six days for analysis thereby facilitating fresh transfer. Biologically, the majority of aneuploidy is derived from the first meiotic division (the first polar body), so the biopsy of the first polar body could detect the majority of abnormalities present in the fetus. The problem with this approach is that sometimes the second meiotic division can correct the error in the first meiotic division

thus having a first aneuploidy polar body and euploid fetus (Forman et al. 2013; Scott *et al.*, 2012). Further research has also demonstrated that polar body biopsy is less predictive of implantation than blastocyst biopsy, 40% and 51% respectively (Salvaggio *et al.*, 2014).

From a technical standpoint, polar body biopsy is much more time consuming than either cleavage stage or blastocyst biopsy. With polar body biopsy it is possible that biopsied embryos may not have any reproductive potential. Moreover, after biopsy, embryos must be kept separate during culture, increasing the amount of time needed for culture preparation and grading of embryos under the microscope when compared to group culture. Lastly, the testing of two polar body can be an expensive add on to an already expensive IVF procedure and to pay hundreds of dollars for testing on embryos that may not develop is difficult to justify for the patient.

Because of the inability to detect post zygotic errors and technical difficulties surrounding polar body biopsy, the IVF field focused on cleavage stage (or blastomere) biopsy.

1.12.2 Cleavage Stage Biopsy

The first PGD cycles were performed at the cleavage stage for X-linked diseases and the process involves breaching of the ZP and removing either one or two cells from the embryo on day 3 (Handyside *et al*., 1990; Griffin et al. 1994). Previously, this was the most utilized procedure for PGS (Harper *et al.*, 2012). Over the years there have been additions

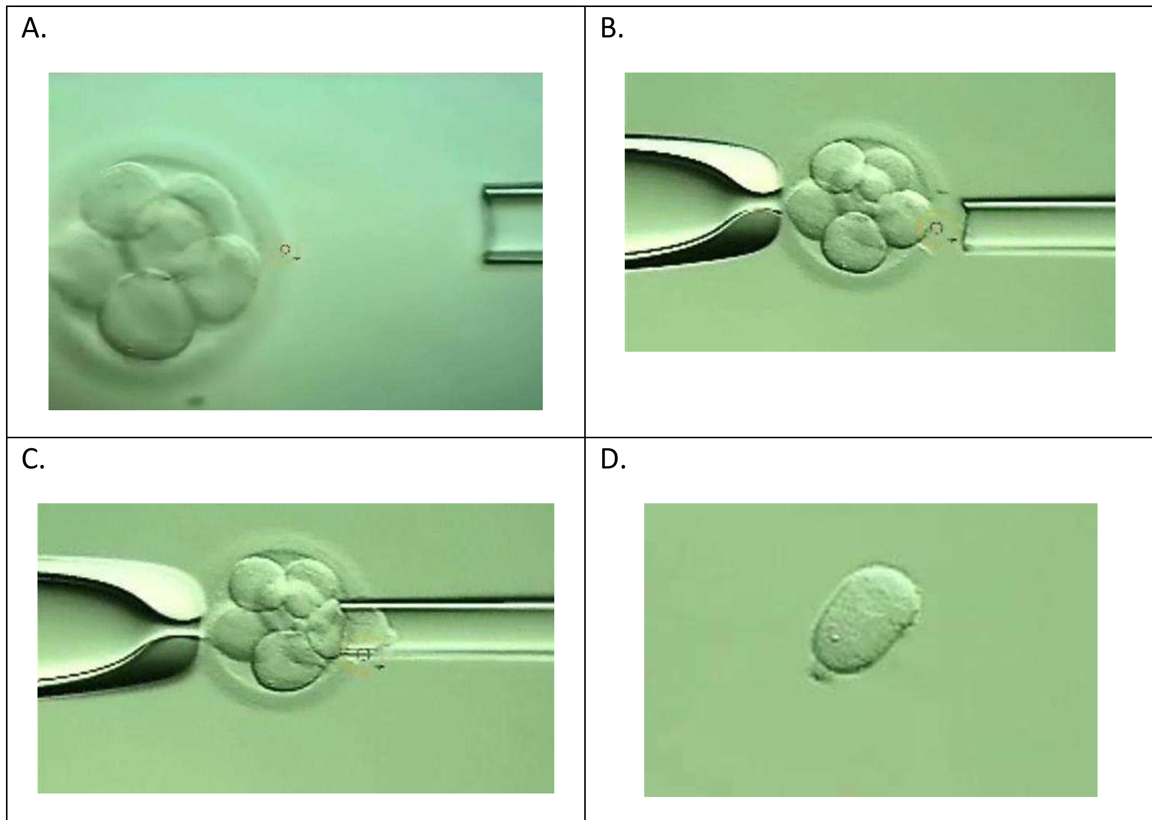
to the technique in hopes to optimize the procedure. Regardless of technique or modifications the principle is the same: remove a single cell from a day 3 embryo, perform testing on this cell and relate the results back to the embryo.

Cleavage biopsy is performed on good quality embryos presenting with six to eight blastomeres on day 3 of development (Figure 20A). Embryos selected to undergo the procedure are isolated and put into individual drops of calcium and magnesium (Ca/Mg) free media (Cooper Sage, Trumbull, Connecticut, USA) supplemented with 10% serum. Ca/Mg free media breaks down the gap junctions of the cleavage stage embryo and allows for easier separation of the individual blastomeres. With the aid of a biopsy pipette (Cook Medical, Bloomington, IL, USA) and either acid or laser (Zilos-tk, Hamilton Thorne, Beverly, Massachusetts, USA), a single cell is removed from the embryo (Figure 20B and 20C). The cell remains in the Ca/Mg free media while the embryo is washed in Hepes buffered media supplement with protein, before being placed back into culture media and the incubator. The individual cells are then prepped for PGS testing. After biopsy, embryos must be cultured individually to allow for identification post PGS results.

Cleavage stage biopsy was utilized during the course of IVF from the mid 1990's to late 2010's. This method may seem detrimental to embryo development but there are multiple reports that support the use of cleavage stage and PGS (Marquez et al. 2000; Munne et al. 2000; Hardy *et al.*, 1990; Grifo et al. 1994). Due to research that has shown there was no benefit to PGS at the cleavage stage, along with potential damage caused

by the biopsy procedure, many clinics have transitioned to blastocyst biopsy (Mastenbroek *et al.*, 2007; Scott *et al.*, 2013).

Figure 20: A) An 8 cell day 3 embryo being held in place by a holding pipette. The small red dot is where the laser will fire and the pipette on the right is the biopsy pipette. B) An opening is made in the ZP by firing the laser. The hole is just big enough for the biopsy pipette to grab the cell. C) The biopsy pipette removing the cell from the embryo, care if taken not to lyse the cell. D) An isolated cell with the nucleus clearly visible at the 2 o'clock position.



1.12.3 Blastocyst Stage Biopsy

Blastocyst biopsy refers to the removal of tissue from the trophectoderm layer of cells during preimplantation growth (Dokras *et al.*, 1990). Although initially a proof of principle study, it wasn't until Schoolcraft and colleagues (2010) published their work

demonstrating a pregnancy rate of 82.2% that blastocyst biopsy became more mainstream.

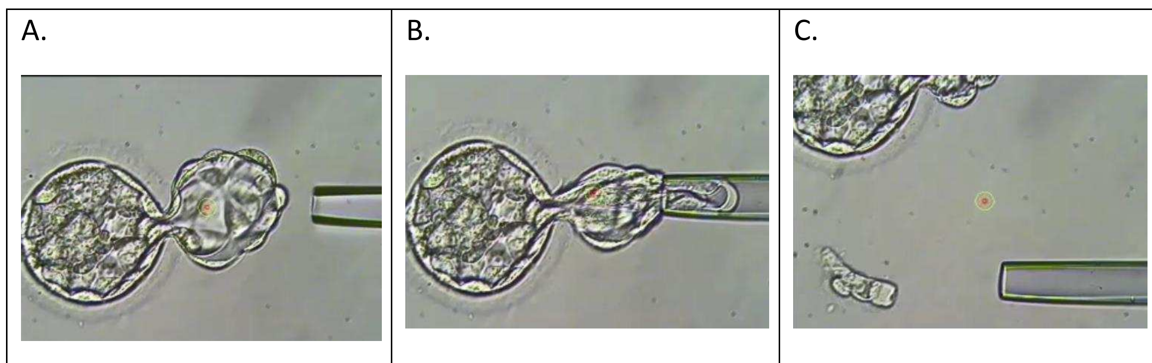
To facilitate hatching of the blastocyst out of the ZP, day 3 cleavage stage embryos are removed from the incubator and assisted hatching (AH) is performed with the aid of a laser (Zilos-tk, Hamilton Thorne, Beverly, Maine, USA). Using a pulse of 610 µs, the ZP is breached with 2-3 shots of the laser (Zilos-tk, Hamilton Thorne, Beverly, Maine, USA). After breaching the ZP with the laser, the embryos are placed back into the incubator until day 5.

On the morning of day 5 (112-115 hours post insemination), day 6 (136-139 hours post insemination), or day 7 (160-163 hours post insemination) embryos are removed from the incubator and those blastocysts that have a good or fair trophectoderm protruding from the ZP, along with good or fair quality ICM are biopsied (Figure 21A). If blastocysts are not suitable for biopsy on day 5, they are reevaluated on day 6. If embryos do not meet the criteria for biopsy on day 6, they are discarded.

Only blastocysts that present with a good or fair quality ICM and trophectoderm undergo the biopsy procedure. Briefly, blastocysts are placed in a drop of modified human tubal fluid (Irvine scientific, Santa Ana, California, USA) + 10% serum substitute supplement (SSS; Irvine scientific, Santa Ana, California, USA) and suction is applied to the blastocysts via a holding pipette (Humagen, Charlottesville, Virginia, USA). A biopsy pipette (Humagen, Charlottesville, Virginia, USA) gently aspirates the trophectoderm into the biopsy needle (Figure 21B). A laser (Zilos-tk, Hamilton Thorne, Beverly, Maine, USA), with

a pulse length of 610 µs, is used to "cut" the trophectoderm from the blastocyst, taking care not to expose the trophectoderm to unnecessary laser pulses (Figure 22C). The piece of trophectoderm is prepped according to the reference laboratories procedures for aCGH, next generation sequencing (NGS), single nucleotide polymorphism (SNP), or qPCR.

Figure 21: A) A blastocyst presenting with a good quality ICM and trophectoderm. The red circle is where the laser will fire and the biopsy pipette is shown at the 3 o'clock position. B) The trophectoderm being suctioned into the biopsy pipette. The red circle is where the laser will fire and "cut" the trophectoderm piece. C) The piece of the trophectoderm removed from the blastocyst.



1.13 PGS Platforms

As previously eluded to, PGS has been performed at multiple stages during the IVF process. Not only is the stage of biopsy important but also the technique that the genetic analysis occurs also can influence results. Previously, the most widely used method for analysis was fluorescence in-situ hybridization (FISH). More recently however, PGS has transitioned to testing at the blastocyst stage and using techniques that can detect aneuploidies across all chromosomes, referred to as comprehensive chromosome screening (CCS; Treff *et al.*, 2010a, 2012; Gutierrez-Mateo *et al.*, 2011).

1.13.1 Fluorescence In-Situ Hybridization

FISH allows for the detection of a limited number of chromosomes (Griffin *et al.*, 1994). Aneuploidy, as demonstrated by FISH, is well documented in scientific literature (Baart *et al.*, 2006; Li *et al.*, 2005; Rubio *et al.*, 2005; Munne *et al.*, 1995). FISH was widely utilized on individual blastomeres from cleavage stage biopsy. Initially, FISH was limited to three to twelve chromosomes; however, more recently, FISH technology has evolved to include all 24 chromosomes (Ioannou *et al.*, 2012). Regardless of how many chromosomes are examined, the principle remains the same.

As previously discussed, a cell is removed from the embryo on day 3. This cell is then exposed to hypotonic solution, allowed to swell, and placed on a glass slide (Munne *et al.*, 1993). Fixative is dropped onto the slide and breaks the cell open, dispersing the cytoplasm and fixing the nucleus to the slide.

Probes containing different fluorochromes are added to the slide and hybridized, allowing for the probe to bind to the chromosomes. These probes target certain loci within specific chromosomes and when attached emit a signal that is seen under a microscope. Thus, if two signals are seen under the microscope then two chromosomes are present in the nucleus. Similarly, if there are three signals or one signal present, then the nucleus is a trisomy or monosomy, respectively. To maximize the number of chromosomes one can examine with FISH, probes with different color fluorochromes are added, each color representing a different chromosome. This method was limited due to the number of colors available. Moreover, for more chromosomes to be viewed, new probes had to be

added after the slide is washed clean. It was very possible that washing and hybridization of probes degraded DNA causing inefficiencies in the technique. FISH as a technique was extremely difficult to perfect; moreover, FISH technology was also limited in that it only viewed whole chromosomes and could not detect segmental aneuploidy. Thus, limiting its clinical application as a screening tool for embryos during the course of IVF.

This technique was heavily utilized in the 90's and early turn of the century with success being demonstrated in retrospective and prospective studies (Gianaroli *et al.*, 1997; Gianaroli *et al.*, 1999; Pehlivan *et al.*, 2003; Rubio *et al.*, 2013a). However, multiple randomized control trails demonstrated a detrimental effect or no benefit on pregnancy rates leading to the reduction in the use of FISH in IVF (Mastenbroek *et al.*, 2007; Twisk *et al.*, 2008; Debrock *et al.*, 2010).

1.13.2 Whole Genome Amplification

Whole Genome Amplification (WGA) allows for multiple copies of DNA to be produced in an effective and efficient manner. One cell typically contains approximately 6pg of DNA; however, CCS using microarrays requires much more DNA, typically 0.2-1.0 µg, that's a 1000 fold increase (Dolezel *et al.* 2003). WGA is utilized to increase the amount of DNA that is needed for microarray analysis. WGA is performed by lysing the cell and then subjecting it to polymerase chain reaction based methods or multiple displacement amplification (Handyside *et al.*, 2004; Sher *et al.*, 2007; Fiegler *et al*., 2007). These two methods effectively increase the amount of DNA.

Increasing the DNA is not without its drawbacks. Current WGA have two distinct drawbacks. First, allele dropout is a phenomena when an allele fails to amplify; thus, the WGA product has an area of DNA that failed to amplify (although present in the original cell). In the final WGA product this would manifest as that specific allele not being present but in actuality that allele is present within the original cell. This can produce errors in the diagnosis. ADO is less of a problem with array based PGS due to the multiple alleles that are examined along the entire chromosome (Ling *et al.*, 2009). ADO is more of a problem with single gene disorders. For example, the array for CCS examines 50 alleles along a chromosome but determines that only 49 are present, it may call the loss of one allele an ADO event and thus all 50 alleles are present on the chromosome. However, if the ADO event occurs in an allele that is specific for a single gene disorder, than the final diagnosis will be a cell that is negative for the single gene disorder but in actuality the single gene disorder is present, it just failed to amplify. Another problem with WGA is that different protocols yield different results and depending upon what one needs to examine, should dictate the WGA product one uses (Treff *et al.*, 2011). It is entirely feasible that aneuploidy as determined by CCS platforms may be artifacts produced by the WGA procedure and not biological in nature (Capalbo *et al.*, 2015). Methods described below such as aCGH and SNP require WGA while qPCR does not.

1.13.3 Array Comparative Genomic Hybridization

aCGH has been adapted from comparative genomic hybridization (CGH) to be streamlined for the use of preimplantation embryos (Voullaire *et al*, 2000; Wells *et al.*, 2002). CGH will not be discussed in detail here; however, it should be noted that CGH was original developed for karyotyping tumor cells and is extremely labor and time consuming (Kallioniemi *et al.*, 1992; Kallioniemi *et al.*, 1993). These aspects make it not ideal for the use on preimplantation embryos.

Much like FISH, aCGH involves the hybridization of probes and fluorescence dyes to DNA. These probes and fluorescence dyes attach not to just a single reference point but to thousands of points of DNA across the entire genome. Initially, WGA DNA from the embryo is labeled with Cy5 (red) and the DNA from a known reference material is labeled with Cy3 (green). These two aliquots of DNA are mixed together in equal portions and placed on the microarray slide. The slide contains thousands of cloned DNA strands from known locations within the human genome. The slide with the DNA undergoes hybridization, which denatures the DNA and makes it single stranded. The single stranded DNA from both the reference and embryo compete for the same sites of cloned DNA on the slide. After hybridization the slide is visualized under a microscope using a computer program. If there is more embryo DNA (green) than reference DNA (red) then the green fluorescence will be visible. While if there is more reference DNA (red) than embryo DNA (green) then red fluorescence will be visible. If there are equal parts embryo DNA (green) and reference DNA (red), then yellow is visible. The computer program is able to

distinguish the different fluorescent probes and realign the colors with their corresponding cloned DNA location in the human genome and output chromosomal copy number changes of the embryo sample compared to the reference.

Although robust and well utilized within the IVF community for PGS testing, aCGH does have limitations. Namely that is not able to detect polyploidy within a sample and cannot deduce if the error is of mitotic or meiotic in nature. Regardless, due to the screening of all chromosomes and to studies demonstrating superior pregnancy and implantation rates compared to FISH, aCGH quickly became the gold standard for CCS (reviewed in Rubio *et al.*, 2013b).

1.13.4 Single Nucleotide Polymorphism Array

SNPs are biallelic genetic markers that are utilized by SNP arrays to detect chromosome copy number. SNP arrays are used to detect specific SNP's along the length of the chromosome. Because there are more than 600,000 SNP's in the human genome, SNP arrays provide much more information and sensitivity than aCGH and qPCR. SNP's are able to detect uniparental disomy (UPD), parental origin of aneuploidy, polyploidy, and the ability to "clean" data (Handyside *et al.*, 2010; Johnson *et al.*, 2010; Treff *et al.*, 2011).

UPD refers to the presence of two chromosomes from the same parent and can result in the manifestation of recessive disorders or imprinting disorders such as Angelman or

Prader-Willi syndrome. Although only present in <1% of blastocysts and newborns, it can only be detected with SNP array (Robinson *et al.*, 1997; Gueye *et al.*, 2013).

Parental origin of aneuploidy is the ability to determine which parents caused the aneuploidy within the embryo. Since all embryo chromosomes are a combination of maternal and paternal chromosomes, it is possible to compare the SNP's of the embryo to the SNP's of the parents to determine the SNP heritance (Johnson *et al.*, 2010; Handyside *et al.*, 2010). Using this same principle, it is possible to "clean" the SNP results by removing errors. For example, in the figure below, one of the SNP's in the embryo is not possible with the maternal and paternal SNP's. Thus, this SNP would be considered an error in the WGA or analysis and discarded, a process referred to as "cleaning".

Figure 22: An overview of the SNP algorithm and an example of how the SNP protocol can give parental origin of aneuploidy and "clean" data.

Maternal SNP		Paternal SNP		Embryo SNP	Inheritance
AA	+	AA	=	AA	Unknown
BA	+	BB	=	BA	Maternal
AB	+	BA	=	AB	Maternal
AA	+	BB	=	BB	Paternal
BB	+	BB	=	AA	Error

SNP's ability to provide whole chromosomal aneuploidy calls, along with high resolution, ability to clean data and provide linkage based analysis for parental origin of aneuploidy, makes SNP a truly powerful tool.

1.13.5 Quantitative Polymerase Chain Reaction

qPCR is one of the most extensively validated CCS platforms, being validated with positive and negative controls and against known cell lines (Scott *et al.*, 2012; Treff and Scott, 2013). In regards to cell lines, not all chromosomes are tested against known cell lines because most whole aneuploid cell lines are not commercially available. This prevents the complete validation of any platform, including qPCR, against all chromosomes. It is entirely possible that some chromosomes tested by qPCR are more accurately detected than others.

As previously mentioned aCGH and SNP look at thousands of loci along the chromosome, while qPCR only examines four loci on the chromosome. The reasoning behind the minimal number of sites is two-fold. First, WGA is known to have errors; thus, any platform that requires WGA will produce inaccurate results. qPCR does not utilize WGA and therefore is not confounded by this variable. Secondly, as previously discussed, the main reason for miscarriages and embryo wastage is whole chromosomal aneuploidy. So, perhaps a platform such as aCGH looks at too many loci and some of these errors may not be clinically significant and are actually throwing away viable embryos. Research by Capalbo and colleagues (2015) demonstrated that aCGH overcalls aneuploidy compared to SNP and qPCR. Although SNP uses WGA, its ability to "clean" the data minimizes WGA errors and provides a more accurate result.

Because of the limited amount of coverage, qPCR cannot detect segmental aneuploidy and large duplications/deletions; however, the clinical significance of these errors has yet

to be determined. Research has demonstrated an incidence of segmental aneuploidy in preimplantation embryos approximately 15% while the incidence in products of conception is approximately 5% (Rabinowitz *et al.*, 2012; Sahoo *et al.*, 2016). This suggests that some segmental aneuploidy may be clinically significant and is missed by qPCR. Moreover, due to the lack of coverage along the chromosome, if an error occurs it could more easily influence results due to the limited number of sights observed. This could cause an increase in false negatives (i.e., embryo deemed euploid but is actually aneuploid).

Although extensively validated, qPCR has minimal clinical data associated with it. One of the objectives of this thesis is to determine the clinical outcomes of blastocysts screened with qPCR or aCGH.

1.13.6 Next Generation Sequencing

NGS is another method that chromosomal aneuploidy is determined. Instead of binding DNA to a microscope slide like aCGH, NGS simply sequences the DNA. Similar to qPCR where one can adjust how many loci one views, one can adjust how much DNA one should sequence in order to determine aneuploidy. Thus, one can sequence the entire genome and gain a massive amount of information or one can sequence just enough to obtain the desired information.

NGS can be targeted, much like qPCR where WGA is not performed, and only a limited number of loci are examined (Goodrich *et al.*, 2016; Kinde *et al.*, 2012). Conversely, NGS utilize WGA and examine areas similar to other tests (Fiorentino *et al.*, 2014). Regardless, NGS involves sequencing.

Sequencing refers to the process that the sample DNA is denatured and spliced into sections and labeled with adaptors. The adaptors are complimentary to the DNA that is desired. The adaptors allow the sample DNA to bind to specific clusters of desired DNA on the flow cell. When the binding occurs, the sample DNA is then amplified multiple times. Thus, we have hundreds of thousands of small, single strands of sample DNA that is bound to the flow cell. Sequencing begins when unbound nucleotides are allowed to bind with the sample DNA attached to the flow cell. Each unpaired nucleotide is attached to a different color fluorescence probe and when it binds to the sample DNA, the fluorescent probe is excited and the color produced is recorded by a computer. The computer then analyzes the colors produced to determine the aneuploidy call.

The most common NGS on the market, Veriseq by Illumina, simply sequences the same sites that aCGH examines. Thus, the validation of Veriseq is based off of aCGH (Fiorentino *et al.*, 2014). As previously discussed, the validation of aCGH is based on FISH. Research by Treff and colleagues (2010b) demonstrates that FISH is inaccurate compared to SNP. By validating Veriseq off of aCGH then it is possible that Veriseq is also inaccurate. This will be discussed in more detail in my objectives when comparing qPCR to aCGH.

1.14 Perspective

The ultimate goal of IVF is a delivery of a single healthy baby. I have previously discussed developmental biology, aneuploidy, mosaicism, biopsy techniques, and PGS platforms. In order to provide patients with the highest chances of pregnancy, an understanding is needed between all five of these topics. Unfortunately, the biological implications and understanding of PGS is not well understood by IVF technicians while the limitations and implications of the biopsy techniques are not understood by geneticists. Thus, there is a gap between the two fields. This gap may be hindering IVF labs from adopting PGS while also limiting geneticists in understanding the limitations of PGS testing. One of the goals of the thesis to help bridge that gap between genetics and the IVF lab. As a geneticist it is important to understand that biologically, aneuploidy may be a natural occurrence and as a field, we may be discarding viable embryos. As an embryologist it is important to understand the limitations of biopsy and PGS so that patients can be more informed.

1.15 Aims of This Thesis

As stated above, chromosome malsegregation is the most common cause of miscarriages, failed IVF, and embryo wastage. However, the incidence of aneuploidy during preimplantation tells us that aneuploidy and mosaicism may be common during embryological development. What is more important is trying to identify the clinical consequences of aneuploidy and mosaicism during preimplantation development. In fact, some aneuploidies may be clinically insignificant while others may not. The purpose of the work described in this thesis is to improve our understanding on the limitations of PGS for a biological and technical standpoint, identify patients that may or may not benefit from PGS, and to gain a greater understanding of preimplantation development through the use of PGS. With these objectives in mind, the specific aims of this thesis were to ask the following technical and biological questions:

1. Can we develop a technique that reconstructs a virtual image of a blastocyst after CCS in order to "map" chromosomal mosaicism?
2. What is the reproductive potential of poor quality blastocysts that would be discarded?
3. Can PGS be successfully extended to previously frozen blastocysts by thawing, biopsing, refreezing and rethawing?
4. Are there clinical differences between euploid blastocysts that develop on day 5 as opposed to day 6?

5. Are there differences in PGS outcomes between fertile and infertile couples undergoing IVF?
6. Are there differences in chemical and clinical pregnancy rates between blastocysts screened with qPCR or aCGH?
7. What mechanisms can a diagnosed aneuploid cleavage stage embryo develop to euploid blastocyst?
8. Are aneuploidy rates different between the polar, mid, and mural trophectoderm?
9. Does a diagnosed aneuploid trophectoderm predict the chromosomal constitution of the entire blastocyst?

2.1 Specific aim 1. Can we develop a technique that reconstructs a virtual image of a blastocyst after CCS in order to "map" chromosomal mosaicism?

For this specific aim, the following published works are presented:

Taylor TH, Wing R, Griffin DK. 2015. A technique to "map" mosaicism at the blastocyst stage using comprehensive chromosome screening. Fertil Steril. 104;3:e274.

Taylor TH, Griffin DK, Katz SL, Crain JL, Johnson L, Gitlin SA. 2015. Technique to isolate individual cells of the human blastocyst and reconstruct a virtual image of their location. J Clin Embryo. 18;2:31-38.

Taylor TH, Griffin DK, Katz SL, Crain JL, Johnson L, Gitlin S. 2016. Technique to 'map" chromosomal mosaicism at the blastocyst stage. Cytogenet Genome Res. 149;262-66.

2.1.1 My Personal Contribution to the Work

For this study I did the majority of the embryology including setup, biopsies, and tubing. Dr. Nathan Treff helped with the idea of biopsing in sections; however, I developed the technique to isolate individual cells. Moreover, I analyzed the data, and wrote the manuscript.

2.1.2 Chapter Summary

The purpose of this proof of concept study is to identify a technique that allows for CCS of individual cells within the human blastocysts along with the approximation of their location in the trophectoderm relative to the ICM. Three blastocysts were held by a holding pipette and the ICM was removed. While still being held, the blastocyst was further biopsied into quadrants. To separate the individual cells from the biopsied

sections, the sections were placed in Ca^{2+}/Mg^{2+} free medium with serum for 20 minutes. A holding pipette was used to aspirate the sections until individual cells were isolated. Individual cells from each section were placed into PCR tubes and prepped for either aCGH or NGS. A total of three blastocysts underwent the segregation technique. Of those, the first blastocyst was analyzed by aCGH and the second and third were analyzed by NGS. From the first blastocyst, analyzed with aCGH, a total of 18 cells were sent for analysis, 15 (83.3%) amplified and provided a result and three (16.7%) did not. Fifteen cells were isolated from the trophectoderm, 13 (86.7%) provided an aCGH result while two (13.3%) did not amplify. Twelve cells were euploid (46, XX) while one was complex abnormal (44, XX) presenting with monosomy 7, 10, 11, 13, 19 and trisomy 14, 15, 21. A total of three cells were isolated from the ICM, two were euploid (46, XX) and one did not amplify. The second and third blastocysts underwent NGS. Twenty-eight and 23 individual cells were isolated from those blastocysts, respectively. From the second blastocyst, two trophectoderm pieces and the ICM were mosaic. Of the 22 cells isolated from the trophectoderm, 15 (68.2%) were euploid, three (13.6%) were aneuploid, and four (18.2%) returned no result. Of the six cells isolated from the ICM, five (83.3%) were euploid and one (16.7%) was aneuploid. From the third blastocyst, two trophectoderm pieces were mosaic, while the ICM was euploid. A total of 20 cells were analyzed from the trophectoderm, nine (45.0%) were euploid, three (15.0%) were aneuploid, and 7 (35.0%) returned no result. Of the three cells analyzed from the ICM, two (66.7%) were euploid and one (33.3%) returned no result. Since the blastocyst sections were biopsied

in regard to the position of the ICM, it was possible to reconstruct a virtual image of the blastocyst while presenting with each cell's individual CCS results.

2.1.3 Introduction

The presence of two or more distinct cell lines, commonly referred to as chromosomal mosaicism, is one of the potential pitfalls when analyzing embryos by CCS. The ability to detect mosaicism accurately is determined by the technology used, number of chromosomes examined and number of cells analyzed (Taylor *et al.*, 2014a). Even if mosaicism is present, the impact on subsequent development varies depending upon which chromosome is involved and at what stage the chromosomal abnormality occurs (Taylor *et al.*, 2014a).

CCS requires that the cells be pipetted into a PCR tube for analysis rather than fixed on a slide as previously performed with FISH studies (Magli *et al.*, 2000). To examine individual cells, each cell needs to be pipetted individually into a PCR tube, and each tube must undergo the CCS procedure. This makes the process labor intensive and expensive compared to FISH.

Although multiple studies have examined mosaicism at the blastocyst stage with CCS, these studies have all involved biopsied sections with multiple cells in each section, perhaps masking the true extent of mosaicism (Capalbo *et al.*, 2013b; Liu *et al.*, 2012; Fragouli and Wells, 2011). The examination of individual cells at the blastocyst stage is

particularly important to gain insight into possible origins and mechanisms of mosaicism, such as non-disjunction, endoreplication, anaphase lag, uniparental disomy, and their prevalence during preimplantation development (Taylor *et al.*, 2014a). Indeed, mosaicism could be responsible both for false negative and false positive PGS diagnoses (Haddad *et al.*, 2013; Werner *et al.*, 2014).

In this present study, I expand upon a novel technique, by which individual cells of a blastocyst could be isolated and a virtual image of the blastocyst with CCS results could be created (Taylor *et al.*, 2016a). Unfortunately, the previous study did not perform CCS. With this report, I have successfully isolated individual cells from the blastocyst, mapped their location in reference to the ICM, and successfully performed CCS on the individual cells. This proof of concept study could allow insights into the mechanism through which mosaicism arose in the blastocyst.

2.1.4 Methods

This study was approved by an institutional review board (WIRB #1138244) and utilized blastocysts deemed not viable and destined for discard. The University of Kent Research Ethics Advisory Group also approved this study.

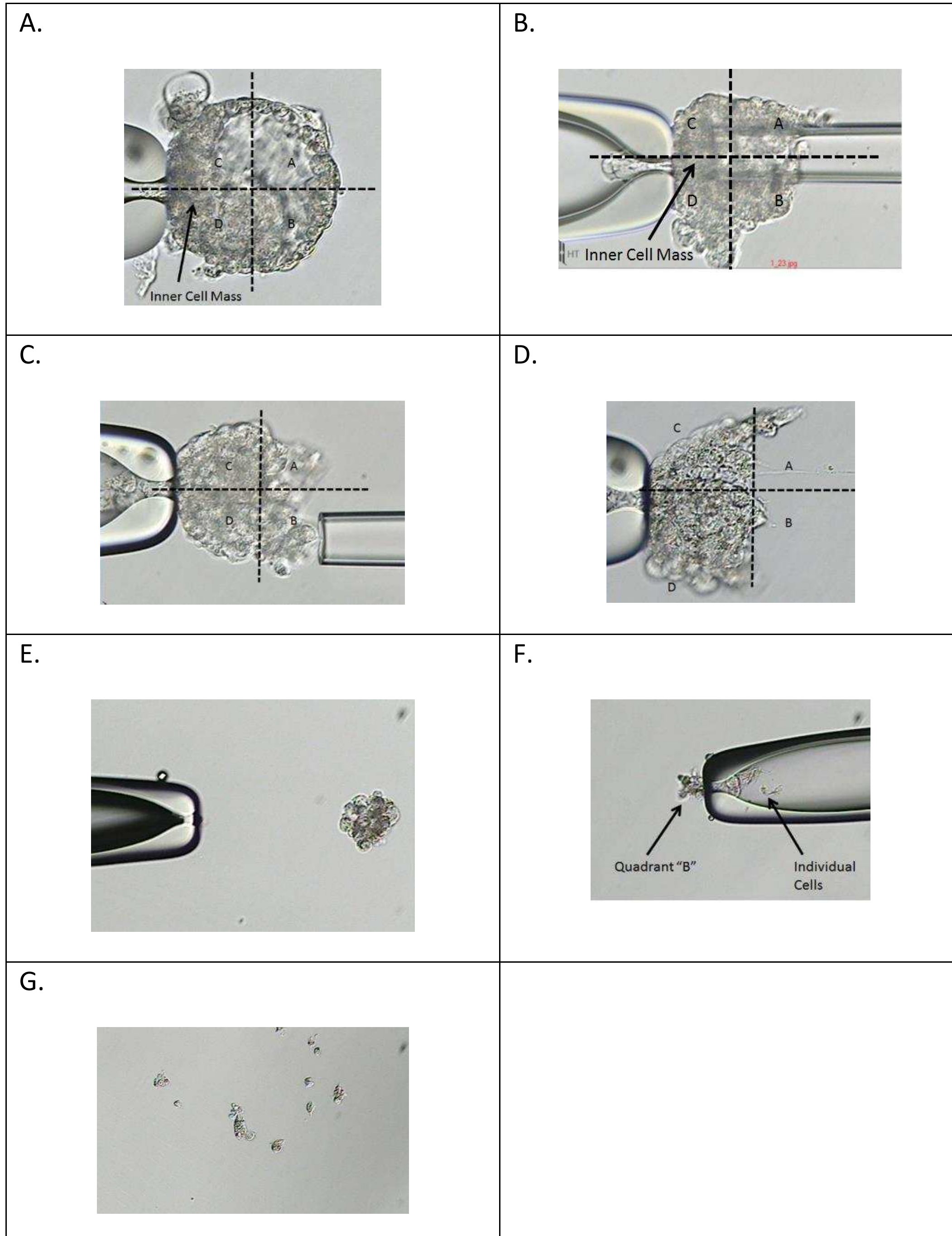
One blastocyst from a 33 year old patient and two poor quality blastocysts from a 37 year old, donated to research, that did not initially have AH, underwent the following procedure. The whole blastocyst was placed into a 20 µL drop of Ca^{2+}/Mg^{2+} free medium

(Cooper/Sage, Trumbull, CT, USA) with 10% SSS (Irvine Scientific, Santa Ana, California, USA) and overlayed by oil (Irvine Scientific, Santa Ana, California, USA). The blastocyst was held with a holding pipette (Origio, Denmark), positioning the ICM at the 9 o'clock position (Figure 23A). A laser was used to create a hole in the trophectoderm at the 3 o'clock position. A biopsy pipette was inserted into the blastocyst and the ICM was removed with gentle suction and isolated (Figure 23B). The ICM was removed from the drop and placed into another drop of Ca^{2+}/Mg^{2+} free with 10% SSS. Using a similar method, Capalbo and colleagues (2013b) demonstrated a 2% trophectoderm contamination rate when removing the ICM.

The blastocysts underwent four further biopsies, thereby separating the blastocyst into quadrants (Figure 23C and Figure 23D). After each biopsy, the biopsy needle was changed and the biopsied piece was pipetted out of the biopsy drop and into an individual drop of Ca^{2+}/Mg^{2+} free medium + 10% SSS for 20 minutes (Figure 23E). This process was repeated after each section so there was no cross contamination or mislabeling of sections during the procedure. After 20 minutes, a holding pipette was used to gently aspirate the sections of the blastocysts (Figure 23F). Doing so allowed the sections of the blastocyst to break apart into smaller pieces. Therefore, multiple, individual cells were obtained from each quadrant (Figure 23G).

The cells of the blastocyst were identified under a dissecting scope. Cells were rinsed in wash solution and prepped for aCGH or NGS.

Figure 23: (A) The whole blastocyst with the quadrants and ICM marked prior to biopsy. (B) Blastocyst undergoing ICM removal, the quadrants are marked. (C) The blastocyst during the biopsy of the "B" quadrant. The "A" quadrant has already been biopsied. (D) The blastocyst after the biopsy of ICM, quadrant "A", and quadrant "B". (E) Quadrant "B" of the blastocyst prior to separation into single cells. (F) Quadrant "B" being pipetted through the holding pipette. (G) Individual cells of Quadrant "B" prior to placement into the PCR tube.



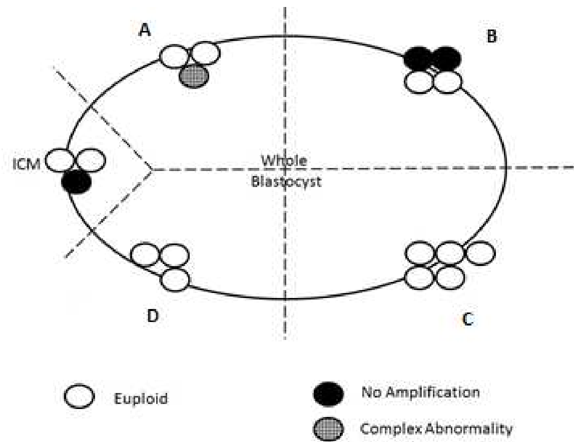
2.1.5 Results

From the first blastocysts, a total of 18 cells from one blastocyst were sent for aCGH. Of the 15 cells isolated from the trophectoderm, 13 (86.7%) provided a result while two (13.3%) did not amplify. Twelve were euploid (46, XX) and one was complex abnormal (44, XX) presenting with monosomy 7, 10, 11, 13, 19 and trisomy 14, 15, 21. The complex aneuploid cell was located in region "A", which is from the polar trophectoderm adjacent to the ICM (Figure 24). A total of three cells were isolated from the ICM, 2 (66.7%) were euploid and one did not amplify (Figure 24; Table 1).

Table 1: Chromosomal results of individual cells within the first blastocyst, tested by aCGH.

Section	Cell No.	Result
ICM	1	46, XX
	2	46, XX
	3	None
Troph A	1	46, XX
	2	46, XX
	3	Complex
Troph B	1	46, XX
	2	46, XX
	3	None
	4	None
Troph C	1	46, XX
	2	46, XX
	3	46, XX
	4	46, XX
	5	46, XX
Troph D	1	46, XX
	2	46, XX
	3	46, XX

Figure 24: Reconstructed trophectoderm and ICM of the first blastocyst analyzed with aCGH.



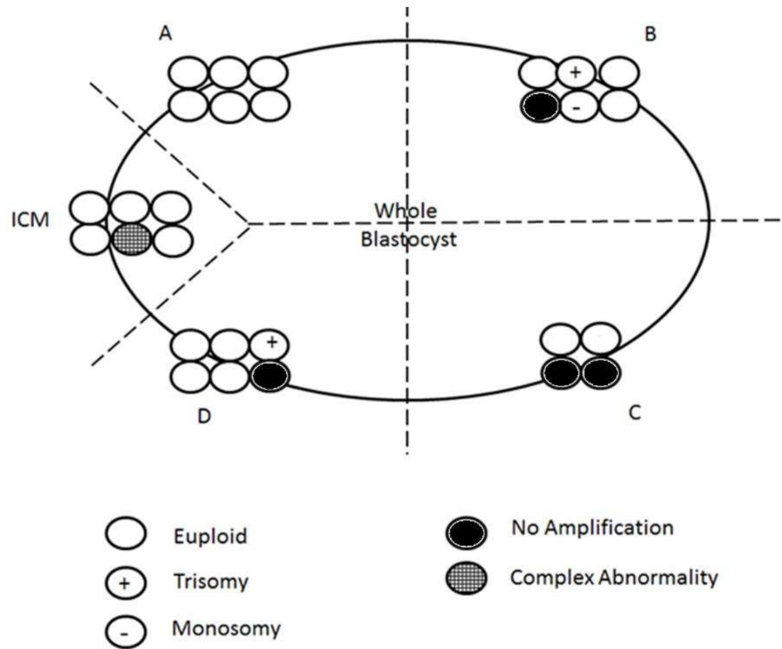
A second and third blastocyst underwent the same procedure but testing was performed with NGS. A total of 26 and 23 cells were analyzed from these blastocysts, respectively. Both blastocysts exhibited evidence of mosaicism. In the second blastocyst, 24 of 28 (92.3%) cells received a diagnosis resulting in four different cell lines. In the third blastocyst, 13 of 23 (56.5%) cells received a diagnosis, resulting in three different cell lines. Any cell presenting with 3 or more aneuploidies was classified as complex aneuploid.

From the second blastocyst (table 2) tested with NGS, 20/28 (71.3%) cells were euploid, four (14.3%) were aneuploid, and four (14.3%) returned no result. Of the four trophectoderm sections, two were mosaic. Moreover, the ICM was also mosaic (with a single cell displaying a complex pattern and the remainder euploid). Of the 22 cells isolated from the trophectoderm, 15 (68.2%) were euploid, three (13.6%) were aneuploid, and four (18.2%) returned no result. Of the six cells isolated from the ICM, five (83.3%) were euploid and one (16.7%) was aneuploid. A reconstructed image of this blastocyst is shown in Figure 25.

Table 2: Chromosomal results of individual cells within the second blastocyst, tested by NGS.

Section	Cell No.	Result
ICM	1	46, XX
	2	46, XX
	3	46, XX
	4	46, XX
	5	46, XX
	6	Complex
Troph A	1	46, XX
	2	46, XX
	3	46, XX
	4	46, XX
	5	46, XX
Troph B	1	46, XX
	2	47, XX, +6
	3	46, XX
	4	None
	5	45, XX, -22
	6	46, XX
Troph C	1	None
	2	None
	3	46, XX
	4	46, XX
Troph D	1	47, XX, +4
	2	None
	3	46, XX
	4	46, XX
	5	46, XX
	6	46, XX

Figure 25: Reconstructed trophectoderm and ICM of the second blastocyst, as analyzed by NGS.



From the third blastocyst (table 3), 12/23 (52.2%) of the cells were euploid, three (13.0%) cells contained a single aneuploidy, and 8 (34.8%) cells returned no result. Of the four sections of the trophectoderm, two were mosaic. The ICM was uniformly euploid. A total of 20 cells were analyzed from the trophectoderm, 10 (50.0%) were euploid, three (15.0%) were aneuploid, and 7 (35.0%) returned no result. Of the three cells sent from the ICM, two (66.7%) were euploid and one (33.3%) returned no result. A reconstructed image of this blastocyst is shown in Figure 26.

Table 3: Chromosomal results of individual cells within the third blastocyst as tested by NGS.

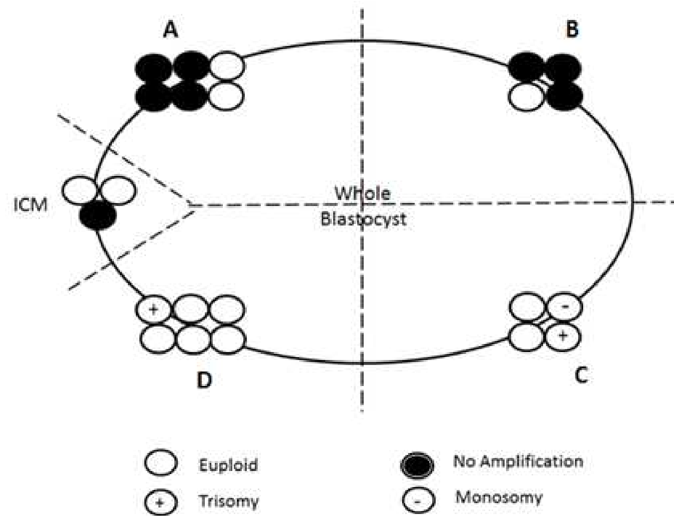
Section	Cell No.	Result
ICM	1	46, XX
	2	None
	3	46, XX
Troph A	1	None
	2	46,XX
	3	None
	4	46, XX
	5	None
	6	None
Troph B	1	46, XX
	2	None
	3	None
	4	None
Troph C	1	45, XX, -8
	2	47, XX, +6
	3	None
	4	None
Troph D	1	46, XX
	2	46, XX
	3	46, XX
	4	47, XX, +6
	5	46, XX
	6	46, XX

2.1.6 Discussion

This project describes a novel approach that is believed to be the first to combine isolation of individual blastocyst cells with the utilization of CCS. This powerful approach can be used to determine the extent of mosaicism in the human blastocyst. Moreover, by examining the CCS results of individual cells within the blastocyst, the mechanisms of mosaicism can be determined (e.g.; non-disjunction, uniparental disomy, endoreplication, or anaphase lag; Taylor *et al.*, 2014a). I must caution however, that this

study utilized poor quality blastocysts and it is difficult to ascertain the true nature of aneuploidy utilizing poor quality embryos.

Figure 26: Reconstructed trophectoderm and ICM of blastocyst number two tested with NGS.



Multiple studies have attempted to determine mosaicism at the blastocyst stage with mosaicism rates ranging from 16-70% (Liu *et al.*, 2012; Fragouli and Wells, 2011; Northrop *et al.*, 2010). All three of these studies examined mosaicism in two to three sections of the trophectoderm, each containing several cells. Examining these large of sections would not allow the chromosome constitution of individual cells within the blastocyst to be determined and thus, the true rate of mosaicism may be masked by the presence of multiple cells. In order to minimize the impact of multiple cells on the rate of mosaicism, the chromosome results for individual cells must be examined.

As previously mentioned, the detection of mosaicism is dependent upon on how many cells are analyzed. All of these aforementioned studies examined mosaicism in these

large sections that contained multiple cells. In this study, the blastocyst was mosaic but this mosaicism would not have been detected had individual cells been analyzed. Eight individual aneuploidies were detected in the trophectoderm. In a background of otherwise euploid cells I would infer that each was an individual post-zygotic error. In the absence of a reciprocal pattern for each (i.e. a corresponding trisomy and monosomy of the same chromosome) I would infer that the +14, +15, +21 aneuploidies arose via independent chromosome gain (perhaps some mechanism involving endoreplication) and the monosomies -7, -10, -11, -13, -19 by independent chromosome loss (anaphase lag). Using FISH, Delhanty and colleagues (1997) and Ioannou and colleagues (2012) demonstrated a lack of mitotic non-disjunction (3+1 pattern), suggesting that mitotic non-disjunction is rare as a mechanism for post-zygotic aneuploidy in human development. This project didn't test individual cells and it's possible that the corresponding reciprocal aneuploidies were "missed". Further studies are certainly warranted to improve upon this technique.

The way mosaicism presents itself during the blastocyst stage can influence PGS results. For example, if a population of aneuploid cells lines are clumped within the trophectoderm, it is likely that the biopsy portion will remove a section of the abnormal cell line, thereby giving a false positive aneuploid result as the majority of the blastocyst is truly euploid. Of the twelve pieces of trophectoderm biopsied, five (41.7%) contained an aneuploid cell line. Only one (20.0%) would have returned an aneuploidy result, while the other four (80.0%) would have returned a euploid result because the majority of the cells were euploid. Thus, results of CCS following blastocyst biopsy may mask the true

nature of mosaicism in the embryo. The opposite (false negative) scenario is also possible in that the blastocyst could be diagnosed euploid when the majority of the embryo (including the ICM) is in fact aneuploid.

Mosaicism in the developing fetus is well documented and can lead to a normal live birth with limited to no clinical consequences (Staals *et al.*, 2003; Leon *et al.*, 2010). Studies in the mouse have shown that the fetus does not develop from the entire ICM, rather only a certain number of cells are destined to become the fetus (Markert and Peters, 1978). Thus, the ICM is responsible for both the fetus and parts of the placenta and aneuploid cell lines in the ICM could give rise to CPM, as could an aneuploid trophoblast with a euploid ICM (Haddad *et al.*, 2013; Bianchi *et al.*, 1993). With this in mind, as well as simply determining the presence of chromosome copy number, the need to determine the origin of the error as well is paramount. Approaches that can achieve this have been described within the last five years using SNP chips and the approach could easily be adapted to analysis of NGS data (Handyside *et al.*, 2010; Gabriel *et al.*, 2011). Aneuploidy arising at meiosis tends to affect 50%-100% of embryo cells depending on whether subsequent post-zygotic events "rescue" the trisomy (Webb *et al.*, 1995). Even when trisomy rescue occurs, uniparental disomy can arise and the majority of mosaic trisomies with adverse obstetric outcomes are meiotic in origin. Conversely, many mosaic trisomies (e.g. those with CPM) that have arisen via post-zygotic errors proceed uneventfully to term. Indeed it is likely that the majority of these remain undetected for this reason (Webb *et al.*, 1995). In this study, even by analysis of only three embryos, the presence of 7 out of 32 aneuploid cells in the trophectoderm, representing seven independent post-zygotic

chromosome segregation errors, would support this notion. Importantly, these findings stress the need to perform a much larger similar study on a greater number of embryos with the ultimate aim of both improving diagnosis for PGS families and better understanding the nature of our own early development.

A meiotic error should be present in the entire, or at least the majority, of cells analyzed. In this proof of concept study, only one cell contained aneuploidies while the remaining cells were euploid. This would suggest that the error arose during mitosis and not meiosis. Previous research has demonstrated that approximately 25% of polar bodies are aneuploid while approximately 50% of blastocysts are aneuploid (Northrop *et al.*, 2010; Salvaggio *et al*, 2014; Taylor *et al.*, 2014b, Taylor *et al.*, 2014c). Interestingly, patients with advanced maternal age have demonstrated a polar body aneuploidy rate of 63% (Geraedts *et al.*, 2011). This would indicate that polar bodies from patients with advanced maternal age have a higher rate of meiotic aneuploidy than mitotic. A higher incidence of aneuploidy at the blastocyst stage would suggest that the majority of aneuploidy may be mitotic in origin. However, the incidence of meiotic and mitotic aneuploidies may be heavily influenced by maternal age. Indeed, previous research has indicated that mitotic errors are not associated with maternal age but meiotic errors are. Moreover, mitotic errors are the dominant mechanism for aneuploidy within the blastocyst (McCoy *et al.*, 2015). My approach described in this study will allow us to test the hypothesis that post-zygotic aneuploidy of individual cells is commonplace in the trophoblast during human development but less so in the ICM.

This research utilized aCGH and NGS, unfortunately, this particular protocol has not been verified against single cells. However, it is unknown if the tubes contained single cells or multiple cells. Moreover, the amplification failure seen in this proof of concept study was high. This is likely due to the fact that individual cells are incredibly small and visualization of cells into the tubes is extremely difficult. Thus, the failure of the amplification may be due to the lack of genetic material rather than the NGS procedure.

It cannot be overlooked that an aneuploid diagnosis could be due to an error in the CCS test. Capalbo and colleagues (2015) demonstrated that aCGH overcalls aneuploidy. However, Capalbo and colleagues (2015) also demonstrated that on a per chromosome basis the accuracy of aCGH is >98%. Another source of error could be due to "noise" within the plot of the CCS result. Some NGS protocols minimize "noise" and produce cleaner CCS plots, reducing the chance of misdiagnosis. The NGS used in this study had not been validated on single cells when this study occurred, whereas aCGH had (Gutierrez-Mateo *et al.*, 2011). Moreover, Fiorentino and colleagues (2014) reran 192 aCGH samples with NGS and found 191 (99.5%) were concordant. Nonetheless, future studies should utilize validated NGS platforms to reduce the chances of misdiagnosis.

Ozawa and Hansen (2011) were able to desegregate individual bovine blastocysts by exposure to trypsin and pipetting the blastocysts through a small glass pulled pipette. Similarly, this project utilized a holding pipette designed for holding the oocyte or embryo during micromanipulation procedures. This pipette had a very small bore size and assisted in the separation of cells from the trophectoderm. This technique could also

prove valuable for human embryonic stem cells (hESC). Often times these cells are in clumps and clusters and the isolation of single hESC may be desired for hESC culture. Prowse *et al*. (2009) performed a similar process, by which clumps of hESC were washed with Ca^{2+}/Mg^{2+}. After the wash, they added trypsin to help in the dissociation of cells. Similarly, Hasegawa and colleagues (2006) also disassociated clumps of hESC into individual cells using trypsin. Trypsin was not added to the blastocyst cells and it is unknown if this would have aided in separation. In these studies, trypsin was used on hESC whereas this study dealt with trophectoderm cells and trypsin may not separate trophectoderm cells as easily as hESC cells. Ca^{2+}/Mg^{2+} free media was utilized because it was readily available and has been used in conjunction with CCS tests and embryo biopsy for years and its influence on CCS results would be minimal (Orris *et al.*, 2010). Another problem is the difficulty in the visualization of the cells after isolation. One suggestion could be the addition of a hypotonic solution to the isolated cells, thereby allowing them to swell and become more easily distinguishable under a microscope (Drey *et al.*, 2013). Another technique referred to as optical tweezing allows for the control of small particles and possibly could be used to isolate individual cells (Grier, 2003; Prada *et al.*, 2016). However, this technique would require an expensive piece of equipment and training, neither that this technique requires.

Given the success with this proof of concept study, larger studies are certainly warranted, despite the cost of CCS. Even increasing the number of blastocysts to 10 would utilize approximately 200-250 CCS tests and patients may present with different rates of mosaicism thereby making a well-designed, high powered study difficult and costly.

These findings stress the need to perform a similar study on a greater number of embryos with the ultimate aim of both improving diagnosis for PGS families and better understanding the nature of our own early development.

2.2 Specific aim 2. What is the reproductive potential of poor quality blastocysts that would be discarded?

For this specific aim, the following published works are presented:

Taylor TH, Patrick JL, Das D, Gitlin SA, Katz SL, Griffin DK. Clinical experience transferring embryos deemed unbiopsiable and unfreezeable on day 6 of a fresh in-vitro fertilization cycle. Submitted 2017.

2.2.1 Personal Contribution to the Work

For this study I did the majority of the embryology including setup and transfers. I developed the idea, analyzed the data, and wrote the manuscript.

2.2.2 Chapter Summary

During course of extended culture, embryos must be of sufficient quality to frozen, transferred, or biopsied. The reproductive potential of these embryos that are of not sufficient quality to be frozen, transferred, or biopsied is not known. The purpose of this study is to determine the reproductive potential of poor quality embryos transferred ET on day 6 of a fresh IVF cycle. Patients undergoing routine IVF treatment with no preimplantation genetic screening, with a fresh day 6 ET where implantation and live birth was either 100% or 0%. Of the 256 IVF cycles that had a day 6 ET, 100 cycles had good quality blastocysts (group 1), 88 cycles had fair quality blastocysts (group 2), and 79 cycles had poor quality embryos transferred (group 3). The control group were all other cycles that had a day 6 ET during their fresh IVF cycle and the embryo that implanted could not

be determined (group 4). A total of 159, 132, 130, and 147 embryos were transferred in groups 1, 2, 3, and 4 respectively. Implantation rates were significant between group 1 (74/159, 42.8%), group 2 (40/132, 30.3%), group 3 (5/130, 3.8%), and group 4 (65/147, 44.2%; P<0.0001). Live births were significantly higher between group 1 (61/159, 38.4%), group 2 (29/132, 22.0%), group 3 (2/130, 1.5%), and group 4 (49/147, 33.3%; P<0.0001). When poor quality embryos are subdivided based on morphology, only full blastocysts exhibited the ability to result in a live birth. A total of 26 full blastocysts were transferred on day 6 of a fresh IVF cycle, and only 2 (7.7%) resulted in a live birth. This data suggest that poor quality day 6 embryos have a minimal chance of resulting in a live born. Therefore, the utilization of only good and fair quality embryos during extended culture is warranted.

2.2.3 Introduction

Extended culture to the blastocyst stage has become a routine practice in the field of IVF and has been credited with an increase in pregnancy rates while decreasing multiples (Gardner and Lane, 1997; Gardner *et al.*, 1998). Furthermore, extended culture allows for the ability to more accurately select embryos capable of a live born and more optimal synchronization between embryo and endometrium compared to cleavage stage transfer (Jones and Trounson, 1999; Tsirigotis, 1998).

Although research indicates that extended culture can lead to higher implantation rates, the correct patient population that this technology has been shown to be beneficial has been conflicting. For example, studies randomizing "good" prognosis patients to either cleavage stage or blastocyst stage transfer have shown a beneficial effect when the latter is utilized, while studies in a general or "poor" patient population have yielded conflicting results (Gardner *et al.*, 1998; Coskun *et al.*, 1000; Levitas *et al.*, 2004; Glujovsky *et al.,* 2012). One of the risks of extended culture in a "poor" patient population is the inability to make blastocysts in vitro. If these patients' embryos are cultured to the blastocyst stage they may not survive and therefore the patients' transfer will be canceled. Papanikolaou and colleges (Papanikolaou *et al.,* 2008) demonstrated an increase in canceled transfers when a general patient population utilized extended culture rather than "good" prognosis patients.

As previously mentioned, a benefit of extended culture is the ability to select those embryos that are most likely to result in a live birth (Gardner *et al.*, 1998). Studies have demonstrated that the transfer of a single blastocyst does not drastically lower live birth rate but does significantly reduce the risk of twins (Mullin *et al.*, 2010). Unfortunately, extended culture is also decreasing additional embryos available to the patient because only good or fair quality blastocysts are transferred or cryopreserved (Papanikolaou *et al.*, 2008; Papanikolaou *et al.*, 2005; Papanikolaou *et al.*, 2006).

More recently, extended culture is being utilized for blastocyst biopsy and freeze all cycles (Schoolcraft *et al.*, 2009; Shapiro *et al.*, 2011). During these processes is typical to only

utilize good or fair quality blastocysts whilst discarding the remaining poor quality embryos (Taylor *et al.*, 2014b, Taylor *et al.*, 2014c). Therefore, the reproductive potential of those discarded, poor quality embryos is unknown. It is the goal of this study to determine the reproductive potential of poor quality embryos that would have otherwise been discarded during extended culture. I hypothesize that poor quality embryos have minimal reproductive potential and that utilization of only good and fair quality, blastocysts during extended culture is warranted.

2.2.4 Methods

This study is retrospective in nature and was deemed exempt from institutional review board (Sterling IRB, #4445). Only cycles in which patients undergoing IVF at Reproductive Endocrinology Associates of Charlotte between 2008 and 2014 that had a fresh embryo transfer on day 6 were included in this study. No patients undergoing PGS were included in this study. Patients only had a transfer on day 6 during a fresh IVF cycle if the most advanced embryos on day 5 could not be selected. Due to this, embryos were given one more day in culture to correctly identify the best embryo. Only cycles in which embryo(s) transferred could be correctly identified as implanted (i.e., an implantation rate of 100% or 0%) were included in the data analysis. All cycles that had a day 6 transfer were subdivided into three groups. Group 1 were cycles in which only good quality blastocysts were implanted, group 2 were cycles in which only fair quality blastocysts were implanted, and group 3 were cycles in which only poor quality embryos were implanted.

The control group were those patients that had a day 6 ET during a fresh IVF cycle whereby the embryos that implanted could not be identified (Figure 27).

2.2.4.1 Oocyte and Embryo Culture

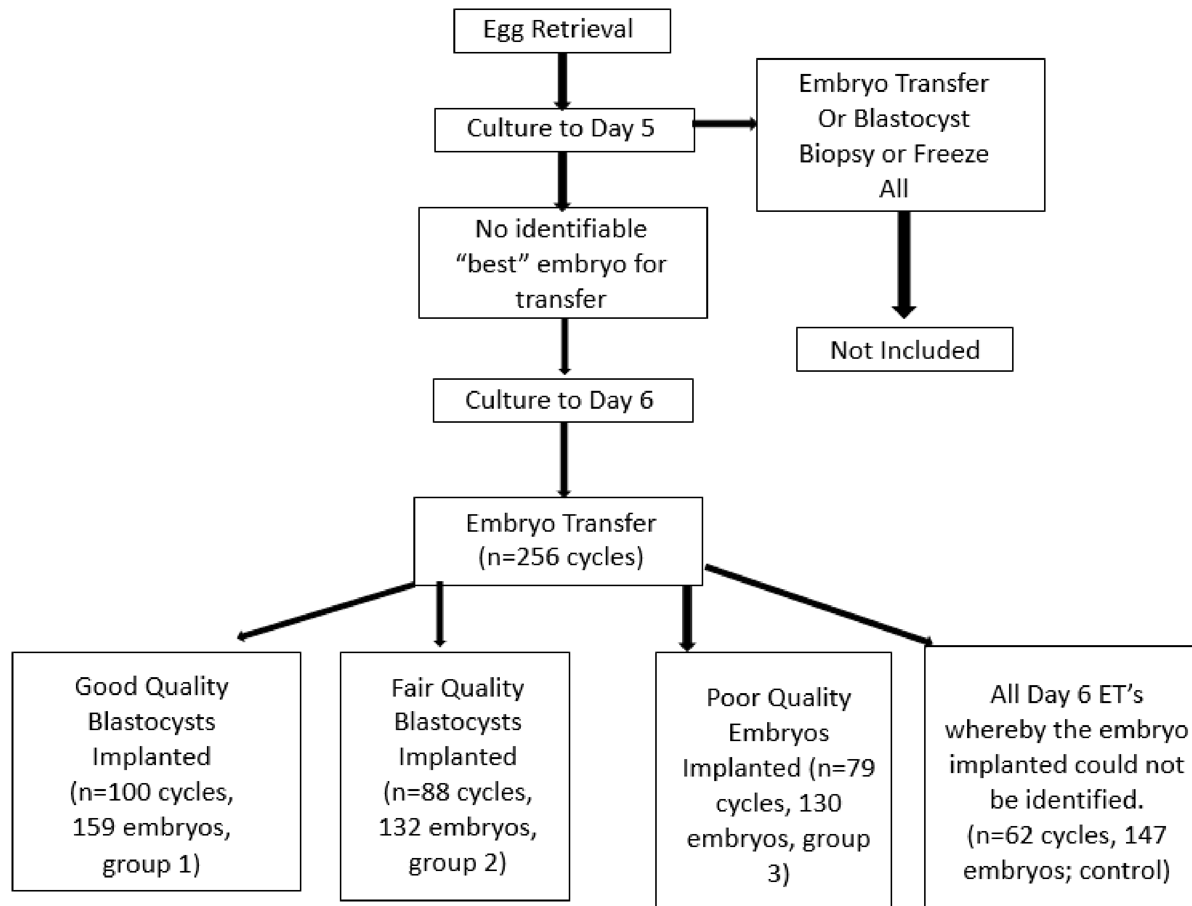
Oocytes were retrieved from follicular aspirations. All oocytes were designated for ICS); therefore, all oocytes were trimmed of excess cumulus cells and pipetted into 10 µL drops of Cumulase (Origio, Mt. Laurel, New Jersey, USA) covered in oil (Irvine Scientific, Santa Ana, California, USA) at 37C. While exposed to Cumulase, oocytes were stripped of cumulus cells with the use of increasing smaller diameter pipette tips until the oocytes were cleaned of all cumulus cells. Oocytes were then rinsed in Hepes (Cooper Surgical, Trumbull, Connecticut, USA) buffered media with 10% SSS (Irvine Scientific, Santa Ana, California, USA). Because this data analysis encompassed such a large time period; two different culture systems were implemented. From January 2008 to March 2011, a sequential culture system with sage protein plus (Cooper Surgical, Trumbull, Connecticut, USA**)** overlayed with oil Irvine (Scientific, Santa Ana, California, USA) and from March 2011-December 2014, CSC+10%SSS overlayed with oil was used. Oocytes were segregated based on maturity and pipetted into culture drops overlayed with oil and placed into the incubator at 37^{0}C with 95% N_2 and 5% CO_2 for one and half hours prior to ICSI.

ICSI was performed as previously described by Nagy and colleagues (1995). Briefly, a single sperm was injected into an oocyte while the polar body was located at the 12 o'clock position. After ICSI, oocytes were returned to the incubator until 16-18 hours.

Oocytes presenting with two pronuclei and two polar bodies were deemed successfully fertilized. Those that were fertilized were placed back into the incubator until day 3. Embryos were not visualized on day 2. On day 3, embryos were removed from the incubator and examined for cell number, fragmentation, and symmetry. After observation, all embryos were placed back into the incubator. On day 4, embryos were removed from the incubator and visualized, embryo quality recorded, and returned to the incubator. On day 5, embryos were removed from the incubator and observed. If the best embryos were a morula or less on day 5, no transfer was performed, and embryos were given to day 6 to demonstrate viability by continuing progression.

On day 6, those embryos that progressed to at least the morula stage were transferred into the patient. Pregnancy was detected with beta hCG levels at 10 days post ET. Those patients that presented with a positive hCG level were continually monitored with blood and ultrasound. After 6 weeks, those presenting with a fetal cardiac heartbeat were released from the IVF practice to be monitored by their physician.

Figure 27: Flow chart of inclusion criteria.



2.2.4.2 Blastocyst Grading

Embryos were graded identically on day 5 and day 6. Embryos were given a grade of "good", "fair", or "poor". Embryos that were an early blastocyst, morula, or early morula were considered "poor" quality; therefore, only poor quality embryos are embryos that would not be biopsied during a PGS cycle. Only expanded blastocysts, hatching blastocysts, or hatched blastocysts received a grade of "good" or "fair". Blastocysts were graded based on the number and symmetry of cells within the trophectoderm and ICM. Grades of the trophectoderm and ICM were given an "A" for good, "B" for fair, or "C" for poor quality. Any blastocysts that received a grade of "A" for either the ICM and/or the trophectoderm was considered "good" quality. Blastocysts with grades of "B" for both the ICM and trophectoderm were considered "fair" quality. Blastocysts that received a grade of "C" for either the ICM and/or trophectoderm were considered "poor" quality.

2.2.5 Results

Only patients in which the good, fair, or poor quality embryos could be determined to implant and result in live birth were included in this study. A total of 256 cycles had a day 6 fresh ET. Of those, 100 cycles had a day 6 transfer in which only good quality blastocysts (group 1) implanted, 88 cycles had a day 6 transfer which only fair quality blastocysts (group 2) implanted, and 79 cycles had only poor quality embryos (group 3) implanted. Out control group were those cycles which patients had a day 6 ET and it is unknown

which embryos implanted. A total of 159, 132, 130, and 147 embryos were transferred in group 1, group 2, group 3, and the control group, respectively. Maternal age at time of retrieval was significant between group 1 (32.6±3.9 years), group 2 (33.7±4.2 years), group 3 (35.0±4.6 years), and control (34.0±4.4; P=<0.001; Table 4).

Table 4: Cycle characteristics of patients undergoing a fresh embryo transfer on day 6 of an IVF cycle.

	Good quality blastocyst (Group 1)	Fair Quality Blastocysts (Group 2)	Poor Quality Embryos (Group 3)	Control (unknown implantation)	P value
No. Cycles	100	88	79	62	
Avg. Maternal Age (± SD)	32.6±3.9[a]	33.7±4.2	35.0±4.6[a]	34.0±4.4	<0.001[1]
No. Embryos ET'd	159	132	130	147	
Avg. No. Embryos Et'd (± SD)	2.0±0.4[b]	2.2±0.6	2.2±0.7	2.4±0.7[b]	<0.001[1]
No. Implantation (%)	74 (42.8%)	40 (30.3%)	5 (3.8%)	65 (44.2%)	<0.001[2]
No. Fetal Cardiac Activity (%)	68 (42.8%)	35 (26.5%)	2 (1.5%)	56 (38.1%)	<0.001[2]
No. Miscarriages (%)	13 (17.6%)	11 (27.5%)	3 (60.0%)	16 (24.6%)	0.1342[2]
No. Live Birth (%)	61 (38.4%)	29 (22.0%)	2 (1.5%)	49 (33.3%)	<0.001[2]

[1] = Kruskal-Wallis Test [2] = Chi-square test

Average number of embryos transferred was significant between group 1 (2.0±0.4), group 2 (2.2±0.6), group 3 (2.2±0.7), and control (2.4±0.7; P=0.0045; Table 4). Implantation rates, as defined by the detection of a gestational sac, were significant between group 1 (74/159, 42.8%), group 2 (40/132, 30.3%), group 3 (5/130, 3.8%), and control (65/147, 44.2%; P<0.001; Table 4). Likewise, fetal cardiac heartbeats were significant between group 1 (68/159, 42.8%), group 2 (35/132, 26.5%), group 3 (2/130, 1.5%) and control (56/147, 38.1%; P<0.001). Live born rates was also significant between group 1 (61/159, 38.4%), group 2 (29/132, 22.0%), group 3 (2/130, 1.5%), and control (49/147, 33.3% P<0.001; Table 4).

When poor quality embryos are subdivided based on morphology, only full blastocysts exhibited the ability to result in a live birth. No early morulas (n=3), morulas (n=9), early blastocysts (n=39), blastocysts (n=28), or expanded blastocysts with either a "C" grade trophectoderm or ICM (n=25), resulted in a live birth (Table 5). A total of 26 full blastocysts were transferred on day 6 of a fresh IVF cycle, and only 2 (7.7%) resulted in a live birth (Table 6).

2.2.6 Conclusion

This data supports the hypothesis that poor quality embryos have a very minimal chance of resulting in a live born. Only two poor quality embryos resulted in live births (1.5%), both were graded full blastocysts. The utilization of only expanded blastocysts and better during extended culture cycles is warranted.

Table 5: Description based on grade of the ICM or trophectoderm of the blastocyst.

ICM Grade	Description
A	ICM easily distinguishable with multiple cells that are tightly compacted
B	ICM distinguishable with fewer cells that are loosely joined together
C	ICM not distinguishable or very few cells that are not joined together or appear necrotic

Trophectoderm Grade	Description
A	Uniform layer of cells in a continuous layer around the blastocoel
B	Non-uniform layer of cells around the blastocoel, cells may be larger and not continuous
C	Very few, large cells formed around the blastocoel, non-uniform, possibly necrotic

Table 6: Live birth rates of embryos graded as "poor" quality on day 6.

	Early Morula	Morula	Early Blast.	Blast.	Full Blast.	Expand. Blast.	P value
No. Transferred	3	9	39	28	26	25	na
No. Live Birth (%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	2 (7.7%)	0 (0%)	0.1495

Multiple reports have demonstrated implantation and live birth rates of day 6 blastocysts, however these tend to be good quality blastocysts. For example, Elgindy and Elsedeek (2012) demonstrated that blastocysts that were expanded by day 6 had a significantly lower implantation rate than those blastocysts that were expanded by day 5. In this study, the majority of the embryos that were expanded were deemed to be fair quality

embryos and the implantation rate for fair quality blastocysts was 30.3%, higher than the 19% reported by Elgindy and Elsedeek (2012). Similarly, Elgindy and Elsedeek (2012) report that the later that blastocyst expands, the lower the implantation percentage. In their study, 40% of those expanded by day 5 implanted while only 19% of those that were expanded on day 6, implanted. Only poor quality blastocysts that resulted in a live birth were graded as a full blastocyst with a poor quality ICM and poor quality trophectoderm. This blastocyst was expanded at time of transfer, yet was graded “poor” because of the poor quality trophectoderm and ICM.

These data demonstrated that poor quality, unbiopsiable embryos result in live birth roughly 1.5% of the time. It could be potentially higher as only cycles with 100% or 0% implantation were included. Maternal age was higher in the poor quality group, possibly influencing the observed decrease in implantation. There were multiple other cycles that had a combination of good, fair, and poor quality embryos transferred where only one or two embryos implanted. It is unknown if the resultant live born derived from the good, fair, or poor quality embryo. Regardless, on those cycles that I could determine the live birth rate of poor quality embryos, the percentage remains significantly lower. This study only examined the outcomes of poor quality, unbiopsiable and unfreeze embryos at transfer on day 6. It is entirely possible that extended culture as a procedure may be causing some embryos that would be viable to perish. This is evident in research that indicates blastocyst culture and embryo transfer increases implantation rates in good prognosis patients but has shown no increase in implantation rates in poor quality patients over cleavage stage transfer (Blake *et al.*, 2007; Weissman *et al.*, 2008). The term

“unbiopsied” embryos is subjective. Research has now demonstrated that even morula stage embryos can be biopsied (Kort *et al.*, 2016). However, this research biopsied morulas on day 5 and it is possible that if cultured to day 6 that these morulas would be of sufficient quality to biopsy or transfer. In our study, morulas on day 5 were cultured to day 6 to allow for them to demonstrate development. Lastly, biopsy occurs when the blastocyst is properly developed on day 5 or day 6. There are no published cases of good quality embryos on day 5 that are cultured an extra day (day 6) in order to gain more cells. Research has suggested a direct relationship between the number of cells taken at biopsy and a higher incidence of miscarriage rates; thus, if a good quality blastocyst is given more time to develop then the biopsy should have less of an impact (Neal *et al.*, 2017). Further research is needed to determine if this particular IVF approach is warranted.

Along with discarding embryos during blastocyst biopsy, IVF may also be discarding embryos during freeze all cycles. Freeze all cycles, in which embryos are cryopreserved and used in a subsequent FET cycle, have become increasingly popular due to recent research suggesting that the transfer of a blastocysts during an unstimulated cycle yield a higher pregnancy rate than transfer during a fresh IVF cycle (Shapiro *et al.*, 2011; Coates *et al.*, 2017). The selection criteria for blastocysts cryopreserved during a freeze all cycle is similar, if not identical, to the selection criteria for blastocysts utilized during a blastocyst biopsy cycle. Thus, many centers only biopsy those embryos that have been deemed as good or fair quality (Taylor *et al.* 2014b; 2014c). Regardless, it seems that the discarding of viable embryos during the course of freeze all or blastocyst biopsy cycles seems to be incredibly low, approximately 1.5%.

In regards to using blastocyst culture in conjunction with PGS, this data suggest that poor quality embryos, that are otherwise unbiopsiable, although rare, can result in a live born. In order to fully maximize a patient's cycle, it may be best to keep patients on progesterone during their IVF cycle; thereby allowing them to transfer embryos that are not biopsiable but could potentially result in a live birth. Although good quality blastocysts are typically utilized in during a blastocyst biopsy cycle, they are not the only embryos that can result in a live birth, and patients should be made aware of this limitation concerning blastocyst biopsy.

2.3 Specific aim 3. Can PGS be successfully extended to previously frozen blastocysts by thawing, biopsing, refreezing and rethawing?

For this specific aim, the following published works are presented:

Taylor TH, Das D, Whitesides D, Crain JL, Wilson M, Patrick JL. 2013. Pregnancy, implantation, and live birth rates of blastocysts that were thawed, warmed, biopsied, vitrified, and rewarmed for euploid blastocysts transfer. Fertil Steril. 100;3:S134.

Taylor TH, Patrick JL, Gitlin SA, Wilson JM, Crain JL, Griffin DK. 2014. Outcomes of blastocyst biopsied and vitrified once versus those cryopreserved twice for euploid blastocyst transfer. Reprod Biomed Online. 29:59-64.

2.3.1 Personal Contribution to the Work

For this study I did the majority of the embryology including setup, biopsies, and tubing.

I developed the idea, analyzed the data, and wrote the manuscript.

2.3.2 Chapter Summary

Trophectoderm biopsy with CCS has been shown to increase implantation and pregnancy rates. Some patients desire CCS for previously cryopreserved blastocysts, resulting in blastocysts that are thawed/warmed, biopsied, vitrified, and warmed again. The effect of two cryopreservation procedures and two thawing/warming procedures on outcomes has not been effectively studied. Patients' cycles were divided into two groups, group 1 underwent a cryopreserved embryo transfer with euploid blastocysts that were vitrified and warmed once. Group 2 had a cryopreserved embryo transfer of a euploid blastocyst that was cryopreserved, thawed/warmed, biopsied, vitrified, and warmed. A total of 85 and 17 women aged 35.6±3.9 and 35.3±4.9 years (not significantly different) were

included in groups 1 and 2, respectively. The survival rate between group 1 (114 of 116, 98.3%) and the second warming in group 2 (21 of 24, 87.5%) was significantly different (P=0.0463). There was no difference between biochemical (68.2% and 62.5%) and clinical pregnancies (57.6% and 50.0%), implantation (58.4% and 52.4%), and live birth/ongoing pregnancy rates (53.9% and 47.6%) between groups 1 and 2, respectively. Although it is unconventional to thaw/warm, biopsy, revitrify, and rewarm blastocysts for cryopreserved embryo transfer, the results indicate that outcomes are not compromised.

2.3.3 Introduction

Typically, only good quality blastocysts derived from a fresh cycle of IVF are utilized in trophectoderm biopsy with CCS. This approach has yielded pregnancy outcomes higher than standard morphological assessment alone (Scott *et al.,* 2013a). Clinical pregnancy rates using this technology range from 60-75% which is comparable to anonymous oocyte donation (Grifo *et al*, 2013). However promising, the utilization of this technology is typically limited to blastocysts derived from fresh IVF cycles.

In order to benefit from trophectoderm biopsy and CCS, a patient has to undergo a fresh IVF procedure or have zygote or cleavage stage embryos previously cryopreserved thawed and cultured to the blastocyst stage. There are a large number of patients who have had IVF previously and have good quality, unbiopsied blastocysts cryopreserved (Zhu *et al*., 2013). These patients could simply want to utilize the current technology or have previous outcomes that may warrant utilization of CCS with trophectoderm biopsy.

For example, if a patient suffered a miscarriage or had failed attempts with fresh embryos, they may choose to utilize trophectoderm biopsy and CCS on previously cryopreserved blastocysts to allow for the transfer of a euploid embryo.

Blastocyst biopsy involves the removal of 3-10 cells from the trophectoderm of blastocysts at either day 5 or 6 of culture (Scott *et. al*, 2013b). If a blastocyst is biopsied on day 5, it is possible to get results by day 6 for a fresh transfer. Most clinics do not conduct onsite CCS, therefore the majority of the time, blastocysts are vitrified post biopsy. Current research indicates that the transfer of an embryo into an unstimulated uterus may yield higher pregnancy outcomes than a transfer during a fresh cycle (Shaprio *et. al,* 2011).

After biopsy, the sample is sent to the genetics laboratory while the blastocysts remain cryopreserved awaiting results. Even in the hands of the most experienced embryologist and geneticists, readings are not possible 100% of the time (Harton *et al*., 2011). Therefore, it is possible that the CCS report would reveal a "no result". In this particular instance, the patients are left with a cryopreserved blastocyst that has no genetic result.

Although biopsy, obtaining CCS results, and transfer without the need to vitrify can be achieved, particularly with methods such as four-hour quantitative real-time polymerase chain reaction, this approach cannot be utilized by every IVF clinic, due to logistics (Treff and Scott, 2013). Because of this, blastocysts with a "no result", as well as blastocysts that have been previously cryopreserved without undergoing trophectoderm biopsy during the fresh cycle would need to be thawed/warmed for biopsy or rebiopsy and

subsequently cryopreserved again while awaiting CCS results. Furthermore, if euploid, these blastocysts would undergo an additional warming procedure before being transferred into the uterus. Few studies have focused on patients that have previously cryopreserved blastocysts that undergo thawing/warming, biopsy, vitrifying, and a second warming prior to a cryopreserved embryo transfer. The purpose of this study was to test the hypothesis that blastocysts, which were previously cryopreserved can be successfully utilized for subsequent trophectoderm biopsy and CCS, and to determine the clinical efficiency of those blastocysts when used in a subsequent cryopreserved embryo transfer cycle.

2.3.4 Methods

This retrospective chart review was deemed exempt by Sterling Institutional Review Board. Patients attending Reproductive Endocrinology Associates of Charlotte from January 1, 2009 to April 31, 2013 were included in this study. Cycles were subdivided into two groups. Group 1 (n=85 cycles, 113 blastocysts) consisted of patients who underwent the traditional method of trophectoderm biopsy and CCS, by having their oocytes retrieved via IVF, embryos cultured to the blastocyst stage, and having all viable blastocysts biopsied and vitrified according to laboratory protocol. Group 2 consisted of cycles that had cryopreserved blastocysts and subsequently desired to have their blastocysts biopsied (n=19 cycles, 70 blastocysts) or those who desired a rebiopsy due to a “no result” (n=2 cycles, 3 blastocysts; Figure 27; Table 7). Outcomes consisted of

biochemical pregnancy (positive beta-hCG test), clinical pregnancy (visualization of gestational sac on ultrasound), fetal cardiac activity (FCA), and ongoing/live birth rates.

Figure 28: Flow chart of study design.

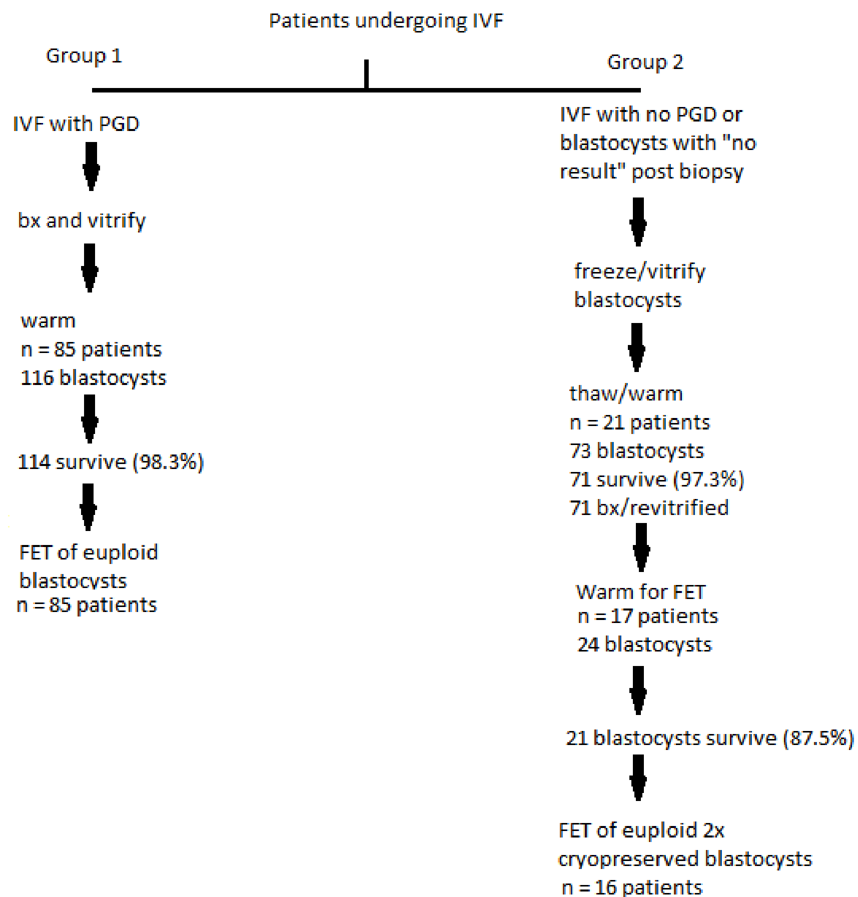


Table 7: Cycle characteristics between patients undergoing a euploid blastocyst transfer with a single vitrified and warm blastocyst (group 1) compared to patients that underwent euploid blastocysts transfer with twice cryopreserved and rewarmed blastocysts (group 2). Group 2 is subdivided into the possible interventions. Bx=biopsy, vit=vitrification, FET=frozen embryo transfer, slow=slow cryopreservation, rebx=rebiopsy

	Group 1	Group 2				P value
Intervention	Bx/vit/warm /FET	Vit/warm/bx/revit /rewarm/FET	Slow/thaw/bx/vit /warm/FET	Bx/vit/warm/rebx/ revit/rewarm/FET	combined	
No. patients (thaws)	85	9	6	2	17	
Age at retrieval (years)	35.6±3.9	35.3±4.8	34.1±5.5	39.0±2.8	35.3±4.9	0.5314[a]
No. transfers	85	9	5	2	16	
No. blastocysts cryopreserved	116	12	9	3	24	
No. blastocysts survived thaw/warm (%)	114 (98.3%)	12 (100.0%)	7 (77.8%)	2 (66.7%)	21 (87.5%)	<0.0001[b]
Avg. No. transferred during FET	1.3±0.6	1.3±0.5	1.2±0.8	1.0±0.0	1.2±0.6	0.7801[a]
No. embryos transferred during FET	113	12	7	2	21	
+hCG (%) per FET	58 (68.2%)	6 (66.7%)	4 (80.0%)	0 (0%)	10 (62.5%)	0.2105[b]
+sac (%) per FET	52 (61.2%)	5 (55.6%)	4 (80.0%)	0 (0%)	9 (56.3%)	0.2655[b]
+fca (%) per FET	49 (57.6%)	5 (55.6%)	3 (60.0%)	0 (0%)	8 (50.0%)	0.4453[b]
Implantation (%)	66 (58.4%)	6 (46.2%)	5 (71.4%)	0 (0%)	11 (52.4%)	0.3112[b]
No. clinical miscarriages (%)	3 (5.8%)	0 (0.0%)	1 (25.0%)	0 (0.0%)	1 (11.1%)	0.4455[b]
Live birth/ongoing (%)	61 (53.9%)	6 (46.2%)	4 (57.1%)	0 (0%)	10 (47.6%)	0.4954[b]

a = one-way ANOVA [b] = chi-square test for independence

2.3.4.1 In-Vitro Fertilization and Embryo Culture

Oocytes were retrieved under ultrasound guidance and placed in Hepes buffered solution (Cooper Surgical, Trumbull, Connecticut, USA) +10% serum protein substitute (SPS; Cooper Surgical, Trumbull, Connecticut, USA) overlayed with oil (Irvine Scientific, Santa Ana, California, USA). All oocytes were designated for intracytoplasmic sperm injection (ICSI) and trimmed and stripped of excess cumulus cells as described by Taylor and colleagues (2006). Oocytes were separated based on maturity and placed back into the incubator. After 2 hours, all mature oocytes underwent ICSI (Nagy *et* al., 1995).

Because this study occurred over a long period of time, two different culture systems were utilized. From January 2009 to August 2012 sequential media (Cooper Surgical, Trumbull, Connecticut, USA) + 10% SPS overlayed with oil was used. From September 2012 to present, a continuous single culture media (CSC; Irvine Scientific, Santa Ana, California, USA) + 10% SSS (Irvine Scientific, Santa Ana, California, USA) overlayed with oil was utilized. Regardless of what culture system was utilized, all oocytes and embryos were cultured in 95% N_2, 5% $C0_2$, and 98% humidity.

2.3.4.2 Trophectoderm Biopsy and aCGH

With the aid of a laser (Zilos-tk, Hamilton Thorne, Beverly, Maine, USA), all embryos from group 1 underwent AH on day 3. Group 2 included blastocysts previously cryopreserved, these were thawed or warmed and AH was performed during the thawing/warming

procedure while the blastocyst was compacted. Only blastocysts that presented with a good quality ICM and trophectoderm were biopsied. Blastocysts were placed in a drop of modified human tubal fluid (Irvine Scientific, Santa Ana, California, USA) + 10% SSS. Gentle suction was applied to the blastocyst via a holding pipette (Cook Medical, Bloomington, Illinois, USA). The hatching trophectoderm was located at the 3 o'clock position. A biopsy pipette (Cook Medical, Bloomington, Illinois, USA) was used to gently aspirate the trophectoderm into the bore of the needle. Laser pulses were used to "cut" the trophectoderm away from the blastocyst. Care was taken to minimize the number of laser pulses needed to remove a piece of the trophectoderm. The piece of trophectoderm was prepared for aCGH (Genesis Genetics, Detroit, MI, USA).

Briefly, the tube with the sample of the trophectoderm was amplified according to manufacturer's instructions (Sureplex, Rubicon Genomics/Bluegnome;CPC4, Capital Park). Those samples that produced an amplification result were labeled with Cy3 dye for sample DNA and Cy5 dye for reference male DNA (Bluegnome) according to manufacturer's instructions. Separately, the sample DNA and reference DNA were denatured at 74°C. After hybridization, the sample and test DNA were mixed together and added to the microarray (Bluegnome). The microarrays were hybridized overnight at 47^{0}C. After hybridization, microarrays were washed at room temperature for 10 minutes in a 2x SSC with 0.05% Tween-20, 10 minutes in 1 x SSC, 5 minutes in 0.1x SSC at 60^{0}C, and finally 2 minutes in 0.1x SSC. Microarray slides were scanned and analyzed by Bluefuse (Bluegnome; Harton *et al.*, 2013).

2.3.4.3 Vitrification

Blastocysts were individually vitrified immediately following trophectoderm biopsy. Blastocysts were placed in equilibration solution (Irvine Scientific, Santa Ana, California, USA) for 15 min and then rinsed in vitrification solution (Irvine Scientific, Santa Ana, California, USA) for <1 min and placed on a Cryolock® (Biodiseno, Atlanta, Georgia, USA) and plunged into liquid nitrogen.

2.3.4.4 Warming

The cryolock was uncapped under liquid nitrogen and plunged into 37^0C warming solution (Irvine Scientific, Santa Ana, California, USA). The blastocyst was “knocked” off the cryolock and left in the warming solution for one minute. The blastocyst was transferred to dilute solution (Irvine Scientific, Santa Ana, California, USA) for three minutes, and finally washing solution (Irvine Scientific, Santa Ana, California, USA) for 10 minutes. For group 2, where the blastocyst had not been subjected to AH in the fresh cycle, AH was performed in the washing solution. After warming, blastocysts were placed into CSC+20% SSS overlaid with oil. Culture media was changed in October of 2013, therefore prior to that date, blastocysts were placed in sequential blastocyst media (Cooper Surgical, Trumbull, CT, USA) + 20% SPS (Cooper Surgical, Trumbull, CT, USA).

Twenty blastocysts were initially cryopreserved using a slow freeze protocol (Cooper Surgical, Trumbull, CT, USA) prior to the introduction of vitrification. Therefore, those

blastocysts were thawed according to manufacturer's instructions (Cooper Surgical, Trumbull, CT, USA).

Regardless of how the embryos were initially cryopreserved, vitrification was used for the second cryopreservation of all blastocysts after trophectoderm biopsy.

One-way ANOVA tests were utilized for the continuous variables and chi-squared test for independence was utilized for categorical variables. When comparing two categorical variables with n≤5, a Fisher's exact t-test was used. Logistical regression was conducted to adjust for female age and asses the relationship between twice cryopreserved blastocysts and implantation. Regardless of test, significance was set at P<0.05.

2.3.5 Results

Only patients who underwent a cryopreserved embryo transfer with euploid blastocysts were included in this study. Group 1 included 85 cycles, in women aged 35.6±3.9 years, who underwent a cryopreserved embryo transfer with euploid blastocysts derived from a fresh cycle (once vitrified). Group 2 included 17 cycles, in women aged 35.3±4.9 years, who underwent a cryopreserved embryo transfer with euploid blastocysts derived from previously cryopreserved, thawed/warmed, biopsied (or rebiopsied), vitrified, and rewarmed blastocysts (twice cryopreserved). There are multiple scenarios that may require this treatment approach: 1) vitrification, warming, biopsy, revitrification, rewarming, and cryopreserved embryo transfer, 2) slow freezing, thawing, biopsy, vitrification, warming, and cryopreserved embryo transfer, 3) biopsy, vitrification,

warming, rebiopsy, revitrification, rewarm, and cryopreserved embryo transfer. Therefore, group 2 was further subdivided into these three scenarios (Table 7).

Of the 73 total blastocysts from group 2 that were thawed/warmed for trophectoderm biopsy and revitrified, 20 of the blastocysts were initially frozen using a slow freeze protocol. Of those 20 blastocysts, 18 (90.0%) survived the initial thaw, were biopsied, and vitrified. From those, only 9 blastocysts have been warmed for a cryopreserved embryo transfer (Table 7). A total of 53 vitrified blastocysts were warmed from group 2 and all 53 survived (100%). Blastocyst survival rate following warming of blastocysts from group 1 (114 of 116, 98.3%) compared with initial thawing/warming from group 2 (71 of 73, 97.3%) was not significantly different. However, the survival rate for blastocysts undergoing second warming in group 2 was significantly lower (21 of 24, 87.5%) compared with the survival rate for blastocysts warmed in group 1 (114 of 116 blastocysts, 98.3%; P=0.0463; Table 7). From group 2, those blastocysts that underwent two vitrification and warming events showed the highest rate of survival post second warming (12 of 12 blastocysts, 100%), compared to those that were initially slow frozen (7 of 9 blastocysts, 77.8%), and those that underwent two biopsies (2 of 3, 66.7%), however the differences failed to reach statistical significance (Table 7).

From group 1, a total 113 euploid blastocysts were transferred in 85 cycles, with an average of 1.3±0.6 blastocysts per transfer (Table 7). From group 2 a total of 21 euploid blastocysts were transferred in 16 cryopreserved embryo transfer cycles with an average of 1.2±0.6 blastocysts per transfer (Table 7). For the cycle in which no blastocysts survived

on the second warming, the two blastocysts were initially slow frozen. The blastocysts survived the initial thaw and were biopsied; however they did not survive the second warming.

Biochemical (58 of 85, 68.2% versus 10 of 16, 62.5%) and clinical pregnancy (49 of 85, 57.6% versus 8 of 16, 50.0%), implantation rates (66 of 113, 58.4% versus 11 of 21, 52.4%) were not significantly different between group 1 and group 2 respectively (Table 7).

Logistical regression analysis adjusted for maternal age revealed that when blastocysts are exposed to two cryopreservation/thawing or warming events and biopsy, implantation rate was not impacted (P=0.5391; OR 0.7013, 95% CI 0.2444-2.0123).

2.3.6 Discussion

These data demonstrate that previously cryopreserved blastocysts can be successfully thawed/warmed, biopsied, revitrified, rewarmed and utilized for CCS. Trophectoderm biopsy with CCS has been implicated in higher pregnancy rates and lower spontaneous abortions (Forman *et. al.*, 2012). However, research has focused on the biopsy, vitrification, and subsequent cryopreserved embryo transfer of euploid blastocysts. A previous case report by Peng and colleagues (2011) demonstrated that a twice vitrified and warmed blastocyst in conjunction with trophectoderm biopsy can achieve live birth. However, to my knowledge this is the first published cohort study that demonstrates the

effectiveness of using previously cryopreserved blastocysts for use with trophectoderm biopsy and CCS.

In this study, the thawing/warming, biopsy, and revitrification, were all performed on the same day, which may have caused the significantly lower warming survival rates in group 2. Therefore, an extra day may allow the blastocysts to recover from the warming and biopsy procedure. This treatment approach may prove advantageous with day 5 blastocysts as it has been shown that there is no difference in pregnancy rates if a blastocyst is transferred on day 5 or day 6 (Elgindy and Elsedeek, 2012). However, in the case of day 6 blastocysts, an extra day would make that embryo a day 7 blastocyst; and day 7 blastocysts have been shown to result in lower pregnancy rates compared to day 5/6 blastocysts (Kovalevsky *et al*., 2013). Interestingly, some reports indicate similar pregnancy and implantation rates between vitrified day 5, 6, and 7 blastocysts (Hiraoka *et al*, 2009a; Hiraoka *et al*, 2008).

In the two cryopreserved embryo transfer cycles of twice biopsied (due to a "no result") and twice cryopreserved blastocysts, neither resulted in a pregnancy (Table 7). Of the three blastocysts that were twice biopsied and twice cryopreserved, two (66.7%) survived the second warming (Table 7). The lower survival rate could be attributed to the low numbers or possible removal of too many cells from the trophectoderm. However, research has shown that the trophectoderm biopsy procedure is not detrimental to implantation and live birth rates (Scott *et al*., 2013b). Conversely, any additional intervention has the potential to damage an embryo; therefore, it is plausible that twice

biopsied blastocysts are severely impacted by the biopsy procedure due to the removal of too many trophoblast cells. Furthermore, blastocysts that have been biopsied once prior to vitrification already have a hole in the ZP. This hole allows for the direct exposure of cells to the cryoprotectants, which may affect warming survival rates. The artificial collapse of blastocysts with the laser results in a similar size hole in the ZP and improves vitrification outcomes (Iwayama *et al.*, 2011; Mukaida *et al.*, 2006). Likewise, the vitrification of blastocysts that are hatching or hatched from the ZP is a common practice with IVF. Thus, the poor results in the twice biopsy group seem to be due to the biopsy procedure itself rather than direct exposure of the cells to the cryoprotectant.

There are multiple reports showing that cryopreservation of embryos twice or cryopreservation of oocytes followed by cryopreservation of the resultant embryos does not affect outcomes. However, these reports focus on vitrification at two different stages of development. Cobo and colleagues (2013) showed no differences in live births between fresh embryos and vitrified oocytes that were later vitrified at either the cleavage or blastocyst stage. Likewise, pregnancies have been achieved from vitrified cleaving embryos that were warmed and revitrified at the blastocyst stage (Hiraoka *et. al,* 2009b). Montag and colleagues (2006) demonstrated that previously frozen oocytes that were thawed and cryopreserved again at the pronuclear stage, can result in a live birth.

Interestingly, there is little literature describing the outcomes of embryos twice cryopreserved on the same day using a slow freeze protocol. This may be due to the lack of sufficient data concerning the subject or the inability of the slow freeze protocols to

offer this type of procedure successfully. In this study, 20 blastocysts were initially cryopreserved using slow freeze protocols. Of those, 18 survived the initial thaw (90.0%), while all of the previously vitrified blastocysts survived the initial warming. Furthermore, of the 24 twice warmed blastocysts, three (12.5%) did not survive the second warming. Of those three, two were previously cryopreserved with a slow protocol. This may indicate that blastocysts cryopreserved with slow freeze protocols are not as able to survive two cryopreservation procedures compared with vitrified blastocysts, larger studies are needed to confirm to support this conclusion.

Lastly, the majority of patients desiring CCS on previously frozen blastocyst utilize this treatment approach for family balancing or because a miscarriage occurred during their initial fresh transfer. Patients that utilize CCS initially are typically those that present with advanced maternal age or diminished ovarian reserve; therefore, controlling for these two patient populations is difficult. Further studies, using larger subsets of patients are needed to confirm these findings.

Although cryopreserved embryo transfer cycles using euploid blastocysts that have been subjected to two cryopreservation and two thawing/warming events are slightly compromised, the overall viability of this procedure is encouraging. Even though survival appears to be lower, the overall data suggests similar outcomes. Current blastocyst vitrification protocols support the ability to vitrify, warm, biopsy, revitrify, and rewarm blastocysts for subsequent cryopreserved embryo transfer.

2.4 Specific aim 4. To determine the clinical differences between euploid blastocysts that develop on day 5 as opposed to day 6?

For this specific aim, the following published works are presented:

Taylor TH, Welch L, Katz S, Crain JL, Wilson M, Patrick JL. 2013. Comparison of aneuploidy, pregnancy, and implantation rates between day 5 and day 6 blastocysts, a sibling oocyte study. Fertil Steril 100;3:S84.

Taylor TH, Patrick JL, Gitlin SA, Wilson JM, Crain JL, Griffin DK. 2014. Comparison of aneuploidy, pregnancy and live birth rates between day 5 and day 6 blastocysts. Reprod Biomed Online. 29;3:305-10.

2.4.1 My Personal Contribution to the Work

For this study I did the majority of the embryology including setup, biopsies, and tubing.

I developed the idea, analyzed the data, and wrote the manuscript.

2.4.2 Chapter Summary

CCS is typically utilized for aneuploidy analysis of blastocysts. It is believed that either day of blastocyst development is acceptable. This projected examined euploidy rates and outcomes between day 5 and day 6 blastocysts in two studies. First, euploidy rates were examined between day 5 and day 6 blastocysts on a per embryo and per patient basis. Second, IVF outcomes were examined when only euploid day 5 or day 6 blastocysts were transferred in a cryopreserved embryo transfer cycles. With cycles that had blastocysts biopsied on both day 5 and day 6, day 5 blastocysts had a higher chance of being euploid than day 6 blastocysts, 54.6% (125/229) and 42.8% (77/180), respectively (P=0.0231). When euploid rates are calculated on a per patient basis, 235/421 (55.8%) day 5 and

184/413 (44.6%) day 6 blastocysts were euploid (P=0.0014). In the second study, 50 patients (36.1±4.3 years) and 39 patients (35.1±3.8 years) had only euploid day 5 or euploid day 6 blastocysts transferred during a cryopreserved embryo transfer. Although underpowered, these data suggests that euploid day 6 blastocysts are as capable of positive outcomes as their euploid day 5 counterparts.

2.4.3 Introduction

The relationship between chromosomal abnormalities and embryo development has been previously described (Kroener *et al*., 2012). Often those embryos that are slower to progress present with chromosomal abnormalities compared with embryos that progress normally (Kroener *et al.*, 2012; Rubio *et al.*, 2007). However, those data were from chromosome analysis of cleavage stage embryos. With the advent of trophectoderm biopsy, the relationship between chromosomal abnormalities and those embryos that develop to blastocysts can be more completely examined.

Trophectoderm biopsy is performed when the embryo has become a blastocyst, either on day 5 or 6 of development. IVF clinics typically utilize day 5 blastocysts similarly to day 6 blastocysts, allowing them to be transferred, cryopreserved, or biopsied for preimplantation genetic screening. The transfer of cryopreserved day 6 blastocysts tends to result in slightly lower pregnancy rates when compared with the transfer of cryopreserved day 5 blastocysts (Muthukumar *et al.*, 2013). Likewise, those embryos that blastulate on day 6 have been shown to give a lower pregnancy rate than those that

blastulate on day 5 during a fresh cycle embryo transfer (Barrenetxea *et al.*, 2005). Using a time-lapse culture system, Campbell and colleagues (2013a; 2013b) demonstrated that aneuploid blastocysts were slower to blastulate compared with euploid blastocysts. The lower pregnancy and implantation rates associated with day 6 blastocysts could be attributed to spindle abnormalities, mitochondrial deficiencies, or gene expression (Shapiro *et al.*, 2013; Hashimoto *et al.*, 2013; Hsieh *et al.*, 2004; Wood *et al.*, 2007). These deficiencies could impact the blastocysts ability to implant and develop in-utero.

Since research has shown that embryos with poor progression tend to be chromosomally abnormal, then it would stand to reason that late developed blastocysts (i.e., those that develop on day 6 as opposed to day 5) should have higher rates of chromosomal abnormalities. The purpose of this study is to test the hypothesis that blastocysts derived on day 6 have higher rates of chromosomal abnormalities than those derived on day 5. Regardless of chromosomal ploidy, a day 6 blastocyst is still one day behind in development, possibly indicating that an abnormality other than chromosomes is influencing growth. In order to control for endometrial and embryonic synchrony, only cryopreserved embryo transfers into an unstimulated uterus should be considered when examining outcomes. Therefore, this study compared pregnancy and implantation rates of blastocysts biopsied on day 5 versus those biopsied on day 6 in cryopreserved embryo transfer cycles.

2.4.4 Methods

This study was deemed exempt from Institutional Review Board approval by Sterling IRB on 26th August 2013. This project examined the differences in aneuploidy rates and outcomes between day 5 and day 6 blastocysts in two studies. Only patients undergoing IVF, trophectoderm biopsy, and aCGH between January 2011 and April 2013 at Reproductive Endocrinology Associates of Charlotte (Charlotte, North Carolina, USA) were included in this study. Aneuploidy rates between day 5 and day 6 blastocysts were compared by two different means. First, aneuploidy rates were compared between patients that had trophectoderm biopsy on both day 5 and day 6 blastocysts in the same cycle (n=70). If patients had trophectoderm biopsy on day 5 and not day 6, and vice-versa, they were not included in the first calculation. Second, the overall aneuploidy rates of day 5 and day 6 blastocysts were compared from all patients whom had a biopsy at the blastocyst stage (n=193). The second study compared pregnancy, implantation, and live birth rates when only euploid day 5 (group 1, n=50) or only euploid day 6 (group 2, n=39) blastocysts were transferred in a subsequent cryopreserved embryo transfer. All patients that had a euploid blastocyst transferred were included in this analysis.

Because these data is not normally distributed, non-parametric tests were used. For continuous variables, Wilcoxon matched pairs test and Mann-Whitney tests were utilized, while categorical variables utilized a chi-squared test. Significance was set at $P<0.05$ for all tests.

2.4.4.1 Embryo Culture

All oocytes were designated for ICSI. Oocytes were retrieved, trimmed of blood, and stripped of cumulus cells as described by Taylor and colleagues (2006). Oocytes were graded for maturity, separated, placed into a 60 mm dish (Thermo scientific, Rochester, New York, USA) containing 250 µL drops of CSC (Irvine Scientific, Santa Ana, California, USA) supplemented with 10% SSS (Irvine Scientific, Santa Ana, California, USA) and overlayed with 8 mL's of oil (Irvine Scientific, Santa Ana, California, USA). The dish containing the oocytes was placed into an incubator at 37°C, 6% CO_2 and 5% O_2 for 2-3 hours. After 2 hours, all oocytes presenting with a polar body underwent ICSI as described by Nagy and colleagues (1995), placed back into the same dish, and put back into the incubator.

The next morning, 16-18 hours post ICSI, oocytes were evaluated for fertilization by the presence of two pronuclei. Embryos that exhibited two pronuclei were group cultured in a fresh dish of CSC+10%SSS overlayed with oil and placed back into the incubator. Embryos were not viewed on day 2.

On day 3, the embryos were removed from the incubator, graded, and AH was performed on all cleaving embryos with the aid of a laser (Zilos-tk, Hamilton Thorne, Beverly, Maine, USA). AH facilitated the protrusion of the trophectoderm from the ZP. Using a pulse of 610 µs, the ZP was breached with 2-3 shots of the laser (Zilos-tk, Hamilton Thorne, Beverly, Maine, USA) and was breached where there were no blastomeres that could be

directly affected by the laser pulse. After breaching the ZP with the laser, the embryos were left in the same drop and placed back into the incubator.

On the morning of day 5 (112-115 hours post insemination) and day 6 (136-139 hours post insemination), embryos were removed from the incubator, blastocysts were graded based on Schoolcraft and colleagues (1999), and those blastocysts that had a good or fair trophectoderm, which was protruding from the ZP, along with good or fair quality ICM were biopsied. Blastocysts were only viewed once in the morning and at no other times. If the blastocysts were not suitable for biopsy in the morning of day 5, they were reevaluated on the morning of day 6. Blastocysts were biopsied on day 5 or day 6, which ever day they met the biopsy criteria. If embryos did not meet the criteria for biopsy on day 6, they were discarded. There was no difference between blastocysts that were biopsied on day 5 or day 6 other than the embryos needed an extra day to reach the appropriate stage for biopsy.

2.4.4.2 Trophectoderm Biopsy

Blastocysts that presented with a good or fair quality ICM and trophectoderm were placed in a drop of modified human tubal fluid (Irvine scientific, Santa Ana, California, USA) + 10% SSS (Irvine scientific, Santa Ana, California, USA). Suction was applied to the blastocysts via a holding pipette (Humagen, Charlottesville, Virginia, USA). A biopsy pipette (Humagen, Charlottesville, Virginia, USA) gently aspirated the trophectoderm into the biopsy needle. A laser (Zilos-tk, Hamilton Thorne, Beverly, Maine, USA), with a pulse

length of 610μs, was used to "cut" the trophectoderm from the blastocyst, taking care not to expose the trophectoderm to unnecessary laser pulses. The piece of trophectoderm was prepped according to the reference laboratories procedures for aCGH (Genesis Genetics, Detroit, Michigan, USA). aCGH slides were supplied by Bluegnome (Cambridge, United Kingdom) and were validated through FISH reanalysis of embryos (Fragouli *et al*, 2011; Capalbo *et al.*, 2013b).

2.4.4.3 Vitrification

Blastocysts were individually vitrified immediately following biopsy. Blastocysts were placed in equilibration solution (Irvine Scientific, Santa Ana, California, USA) for 15 min and then rinsed in vitrification solution (Irvine Scientific, Santa Ana, California, USA) for <1 min and placed on a Cryolock® (Biodiseno, Atlanta, Georgia, USA), plunged into liquid nitrogen, and capped.

2.4.4.4 Warming and Cryopreserved Embryo Transfer

The Cryolock was uncapped under liquid nitrogen and plunged into 37^0C warming solution (Irvine Scientific, Santa Ana, California, USA). The blastocyst was visualized while in the thawing solution, "knocked" off the Cryolock, and remained in the warming solution for one minute. The blastocyst was transferred to dilute solution (Irvine Scientific, Santa Ana,

California, USA) for three minutes, and a washing solution (Irvine Scientific, Santa Ana, California, USA) for 10 minutes before being placed into CSC+20% SSS overlaid with oil.

After warming, blastocysts were transferred to the uterus with a Wallace catheter (Smiths Medical, Dublin, Ohio, USA) under ultrasound guidance. Regardless of if the blastocysts were day 5 or day 6, all cryopreserved embryo transfers occurred on the sixth day of progesterone administration (50 mg/daily; Actavis, Parsippany, New Jersey, USA) but prior to that day's dose.

2.4.5 Results

In the first study I calculated differences in aneuploidy rates between day 5 and day 6 blastocysts by two different means. First, in order to minimize patient differences, only patients (n=70; 35.9±3.7 years) who had blastocysts to biopsy on both day 5 and day 6 during the same IVF cycle were included in this study. The mean number of blastocysts biopsied on day 5 was 3.3±2.2 while on day 6, a mean of 2.6±1.5 blastocysts were biopsied (P=0.0472; Table 8). Blastocysts biopsied on day 5 had a significantly higher chance of being euploid than those biopsied on day 6, 54.6% (125/229) and 42.8% (77/180), respectively (Table 8; P=0.0231). When this set of data are examined on a per patient basis, day 5 blastocysts had a significantly higher euploid rate compared to day 6 blastocysts, 56.0 ± 35.7% and 43.9 ± 37.2%, respectively (Table 8; P=0.0351). With an alpha of 0.05 and type II error of 0.20, a power analysis reveals that 824 embryos are

required per group to detect a 10% difference in aneuploidy rates between day 5 and day 6 blastocysts. Regardless, statistical significance is achieved.

Table 8: Differences between day 5 and day 6 blastocysts euploid rates from the same IVF cycle, diagnosed by aCGH (n=70; maternal age, 35.9±3.7 years).

	Day 5 Blastocyst	Day 6 Blastocyst	P value
Average No. Biopsied (±SD)	3.3 ± 2.2	2.6 ± 1.5	0.0472[a]
Total Biopsied	229	180	
No. Euploid (%)	125 (54.6%)	77 (42.8%)	0.0231[b]
% Euploid mean ± SD	56.0% ± 35.7%	43.9% ± 37.2%	0.0351[a]

[a] = Wilcoxon matched pairs test [b] = chi-square test

The overall aneuploidy rates were also calculated on a per embryo and patient basis. A total of 193 patients (35.7 ± 4.2 years) had blastocysts biopsied on day 5 and/or day 6. Of the 421 and 413 blastocysts biopsied on day 5 and day 6, 235 (55.8%) and 184 (44.6%) were euploid, respectively (Table 9; P=0.0014). The average percent euploid per patient for day 5 and day 6 blastocysts was 55.0 ± 37.5% and 45.4 ± 36.6%, respectively (Table 9; P=0.0286). A power analysis with an alpha of 0.05 and type II error of 0.20, reveals that 822 embryos are required to detect a 10% difference in aneuploidy rates.

Table 9: Aneuploidy rates of day 5 and day 6 blastocysts. (n=193 patients; 35.7 ± 4.2 years)

	Day 5	Day 6	P value
Total No. Biopsied	421	413	0.0014[a]
Total No. Euploid (%)	235 (55.8%)	184 (44.6%)	
% Euploid (mean ± SD)	55.0% ± 37.5%	45.4% ± 36.6%	0.0286[b]

[a] = chi-squared test [b] = mann-whitney test

In the second study I examined the difference in implantation and pregnancy rates in patients who had only euploid day 5 versus euploid day 6 blastocysts transferred in an FET cycle. Fifty patients (36.1±4.3 years) had only euploid day 5 blastocysts transferred during a cryopreserved embryo transfer, while 39 patients (35.1±3.8 years) had only euploid day 6 blastocysts transferred during a cryopreserved embryo transfer (Table 10). An average of 1.3±0.5 and 1.2±0.6 blastocysts were transferred from day 5 and day 6 cryopreserved embryo transfer groups, respectively (Table 10). Biochemical pregnancy rates were not significantly different when only euploid day 5 or day 6 blastocysts were transferred, 34/50 (68.0%) and 25/39 (64.1%), respectively (Table 10). Clinical pregnancy rates were not significant when only euploid day 5 or day 6 blastocysts were transferred, 30/50 (60.0%) and 24/39 (61.5%), respectively (Table 10). There was no significant difference in implantation rates when only day 5 euploid blastocysts were transferred (38/65, 58.5%) compared with day 6 euploid blastocysts (26/48, 54.2%; Table 10).

There were no statistically significant differences between the two groups according to Mann-Whitney Test or chi-squared test.

With an alpha of 0.05 and in order to detect a 10% difference in implantation rates between euploid day 5 and day 6 blastocysts, a power analysis reveals that 678 embryos are required.

Table 10: Outcomes of cryopreserved embryo transfer cycles with euploid day 5 or day 6 blastocysts, diagnosed by aCGH.

	Day 5 blastocysts	Day 6 blastocysts	P Value
No. Patients	50	39	
Maternal Age at Retrieval (mean±SD)	36.1±4.3	35.1±3.8	NS[a]
No. transferred (mean ± SD)	1.3±0.5	1.2±0.6	NS[a]
Total no. transferred	65	48	
Positive HCG test (%)	34 (68.0%)	25 (64.1%)	NS[b]
Gestational sac (%)	30 (60.0%)	24 (61.5%)	NS[b]
Fetal cardiac activity (%)	27 (54.0%)	23 (59.0%)	NS[b]
Implantation (%)	38 (58.5%)	26 (54.2%)	NS[b]
No. clinical miscarriages (%)	4 (13.3%)	1 (4.2%)	NS[c]
Live birth/ongoing (%)	26 (52.0%)	23 (59.0%)	NS[b]

[a] = Mann-Whitney test   [b] = Chi-square test   [c] = Fisher's exact test

2.4.6 Discussion

The hypothesis that day 6 blastocysts present with higher rates of chromosomal aneuploidy when compared with day 5 blastocysts has been validated with these data. This is the first study that compares blastocyst aneuploidy rates between day 5 and day 6 blastocysts using a sibling embryo model. This model effectively minimizes any patient differences that could cause bias. Kroener and colleagues (2012) demonstrated no difference in aneuploidy rates between day 5 and day 6 blastocysts. However this research did not utilize sibling embryos and different patient populations have widely varying aneuploidy rates (Thum *et al*., 2008; Voullaire *et al*., 2007). This research utilized

day 3 biopsy and aneuploidy screening; the high incidence of chromosomal mosaicism in cleavage embryos suggests that even those that are diagnosed as aneuploid may be euploid (Wells *et al.*, 2000; Northrop *et al*., 2010). Mosaicism is not just an artifact of cleavage stage embryos but also persists at the blastocyst stage. For example, Northrop and colleagues (2010) biopsied multiple pieces of trophectoderm from the same blastocysts and found a mosaic rate of 24%, while Fragouli and colleagues (2011) demonstrated a mosaic rate of 32%. Regardless of stage, mosaicism is present during preimplantation development.

This study found that the risk of aneuploidy was 10% higher in the embryos that did not blastulate until day 6. This would suggest that not all blastocysts are created equal and that delayed development to the blastocyst stage may be caused by chromosomal errors. Alfarawati and colleagues (2011) found a correlation between aneuploidy and blastocyst morphology whereby the most advanced blastocysts had lower rates of aneuploidy compared with those blastocysts of slower progression. Similarly, studies have suggested that euploid embryos tend to blastulate before those that are aneuploid (Campbell 2013a; Campbell 2013b). These data parallels these reports; however, these data also suggest that even those embryos that are diagnosed as euploid can develop to the blastocyst stage at varying rates, suggesting that other factors aside from chromosomes influence growth. For example, mitochondrial, spindle abnormalities, and specific gene expression have all been shown to influence embryo development (Hashimoto *et al*., 2013; Hsieh *et al*., 2004; Wood *et al.*, 2007).

The use of AH may confound these data, causing embryos to artificially blastulate prematurely. However, AH on day 3 and the biopsy of protruding trophectoderm at the blastocyst stage is a common technique and has been shown not to affect implantation rates (Scott *et al.*, 2013a). This study utilized daily observations of embryos, a technique that may be proven obsolete with the development of time-lapse equipment (Kirkegaard *et al.*, 2013; Montag *et al.*, 2013; Sundvall *et al.*, 2013). However, daily observations are routine procedures for the majority of IVF clinics as many clinics cannot afford the cost of time-lapse equipment.

Research on blastocyst morphology typically fails to include the day of blastulation as a parameter even though research has indicated that day 6 blastocysts have lower implantation rates than day 5 blastocysts (Shapiro *et al.*, 2001). Conversely, current research that transferred patients on day 5 and on day 6, regardless of day of blastulation found no difference in pregnancy and implantation rates (Elgindy *et al*., 2012).

Because slower progression may affect implantation, outcomes of those patients that had a cryopreserved embryo transfer of only euploid day 5 or day 6 blastocysts were examined. The numbers are small and it is difficult to draw conclusive results; however, these data suggest that there is no difference in pregnancy, implantation, or ongoing/live birth rates between day 5 and day 6 euploid blastocysts when they are utilized in FET cycles. Larger studies are needed to support or rebut these findings, however even with larger studies there are numerous factors that need to be examined. For example, the laboratory protocol is to transfer day 5 blastocysts first; thus, it is expected that a higher

implantation rate with day 5 cryopreserved embryo transfers is observed. On the other hand, day 6 euploid blastocysts selected for transfer are typically patients that have utilized all their euploid day 5 embryos or only had day 6 blastocysts available for biopsy during their fresh cycle, possibly indicating slower developing embryos. Secondly, it is possible that day 6 blastocysts were only slightly behind biopsy on day 5, therefore they had more cells and could be less damaged by the biopsy procedure. This is unlikely as the transfer of day 6 euploid blastocysts yielded similar pregnancy rates as euploid day 5 blastocysts. Furthermore, blastocyst biopsy does not significantly impact implantation (Scott *et al*., 2013a). Lastly, culture conditions, stimulation protocols, and other specific patient parameters could affect day of blastulation, while warming protocols and progesterone supplementation protocols during cryopreserved embryo transfer cycles, could influence implantation rates.

Aneuploidy is directly linked to slow developing cleavage stage embryos (Kroener *et al.*, 2012). Using a sibling embryo model, this study determined that embryos that did not blastulate until day 6 had a 10% increase in aneuploidy than those that blastulated on day 5. Although the data set is small, it demonstrated that day 6 euploid blastocysts, although a day behind, are just as capable of positive outcomes as their day 5 counterparts.

2.5 Specific aim 5. To determine if there are differences in PGS outcomes between fertile and infertile couples undergoing IVF?

For this specific aim, the following published works are presented:

Taylor TH, Welch L, Wing R, Crain JL, Wilson M, Patrick JL. 2013. Pregnancy, implantation, and live birth rates of fertile and infertile patients following transfer of euploid blastocysts. Fertil Steril. 100;3:S195.

Taylor TH, Patrick JL, Gitlin SA, Crain JL, Wilson JM, Griffin DK. 2014. Blastocyst euploidy and implantation rates in a young (<35 years) and old (≥35 years) presumed fertile and infertile patient population. Fertil Steril. 102;1318-23.

2.5.1 My Personal Contribution to the Work

For this study I did the majority of the embryology including setup, biopsies, and tubing. I developed the idea, analyzed the data, and wrote the manuscript.

2.5.2 Chapter Summary

The purpose of this study is to test the hypothesis that infertile patients exhibit a higher rate of aneuploidy and lower implantation potential of euploid blastocysts compared to a presumed fertile population. This study compares blastocyst euploid rates in presumed fertile and infertile patients, both from a young (<35 years) and old (≥35 years) female patient population. Furthermore, this study will compare pregnancy and implantation rates of the same presumed fertile and infertile patients in a corresponding FET cycle. Patients were divided into two categories, presumed fertile and infertile. Presumed fertile patients were those undergoing IVF either with anonymous oocyte donor, social

sex selection, or single gene defect (excluding fragile X syndrome). Infertile patients included all other diagnosis. In order to gain further insight into the effect of age on aneuploidy, patients were subdivided based on maternal age, <35 and ≥35 years old. All patients underwent blastocyst biopsy with CCS and a transfer in a corresponding FET. There was no significant difference in number of euploid blastocysts between presumed fertile (68/118, 57.6%) and infertile (75/132, 56.8%) patients <35 years old. Likewise, there was no significant difference in number of euploid blastocysts between presumed fertile (42/86, 48.8%) and infertile (97/206, 47.1%) patients ≥35 years old. When those same patients underwent a corresponding frozen embryo transfer (FET) cycle, presumed fertile patients demonstrated a significantly higher chemical pregnancy rate when compared to infertile patients, 28/33 (84.8%) and 50/81 (61.7%), respectively. Moreover, presumed fertile patients exhibited significantly higher implantation rates compared to infertile patients, 36/42 (85.7%) and 54/109 (66.7%), respectively. When subdivided by maternal age, no significant difference was seen in blastocyst euploidy rates between presumed fertile and infertile patients; however, chemical pregnancy and implantation rates were significantly higher in a presumed fertile patient population even when transferring only euploid blastocysts. This would indicate infertility, as a disease, may encompass other aspects such as uterine or other unknown embryological factors that can influence outcomes.

2.5.3 Introduction

One of the factors contributing to the lack of success in IVF is the high incidence of whole chromosomal abnormalities, or aneuploidies in the developing embryo (Ledbetter, 2009). PGS was adopted to test for aneuploidies in preimplantation embryos in order to decrease miscarriages and increase live birth rates. This concept was first applied to specific patient groups believed to be more prone to aneuploidies, such as patients diagnosed with recurrent pregnancy loss, multiple IVF failures, previous aneuploid conceptions, male factor, or patients of advanced maternal age (Platteau *et al.*, 2005; Wilton *et al.*, 2003; Munne *et al.*, 2004; Silber *et al.*, 2003; Staessen *et al.*, 2004; Gianaroli *et al.*, 1997). Studies have indicated that the majority of embryos, regardless of patient diagnosis, contain aneuploidies (Van Echten-Arends *et al.*, 2011; Wells and Delhanty, 2000; Mertzanidou *et al.*, 2013; Vanneste *et al.*, 2009). Because of the high incidence of aneuploidies during human preimplantation development, screening the embryos prior to transfer makes sense, as the majority of miscarriages and embryo wastage is derived from chromosomal aneuploidies (Hassold and Hunt, 2001).

Since the majority of embryos analyzed during the course of IVF are derived from infertile couples or from discarded embryos, the true nature of preimplantation aneuploidy may be overrepresented (Baart *et al.*, 2006). The only embryos that may reflect the true incidence of preimplantation aneuploidy would be those derived from fertile, not infertile, patients. Unfortunately, IVF is not heavily utilized by fertile patients; however, there are specific patient groups that are presumed to be fertile. For example,

anonymous oocyte donors offer insights into aneuploidy rates in young and presumed fertile patients but fail to yield aneuploidy rates in an older (≥35 years), fertile patient population. Therefore, patients that are ≥35 years old, undergoing IVF for single gene disorder in combination with aneuploidy screening or social sex selection, offer a glimpse into the preimplantation aneuploidy in an older, presumed fertile population. Given that the incidence of aneuploidy is patient dependent, it is possible that fertile patients may not exhibit the same rate of aneuploidy seen in infertile women of the same age (Thum *et al.*, 2008; Voullaire *et al.*, 2007).

The purpose of this study is to test the hypothesis that infertile patients exhibit a higher rate of aneuploidy and lower implantation potential of euploid blastocysts compared to a presumed fertile population. This study compares blastocyst euploid rates in presumed fertile and infertile patients, both from a young (<35 years) and old (≥35 years) female patient population. Furthermore, this study will compare pregnancy and implantation rates of the same presumed fertile and infertile patients in a corresponding FET cycle.

2.5.4 Methods

This study was deemed exempt by Sterling Institutional Review Board (Atlanta, Georgia, USA). Only patients attending Reproductive Endocrinology Associates of Charlotte for IVF and CCS at the blastocyst stage, using aCGH, SNP microarray, or qPCR between January 2010 and January 2014 were included in this study. Patients were divided into two categories, presumed fertile and infertile. Presumed fertile patients were those

undergoing IVF either with anonymous oocyte donor, social sex selection, or single gene defect (excluding fragile X syndrome). Infertile patients included all other diagnosis. In order to gain further insight into the effect of age on aneuploidy, patients were subdivided based on maternal age, <35 and ≥35 years old.

Kruskal-Wallis, Mann-Whitney U test, chi-square, and Fisher's exact test were utilized and significance was set at $P<0.05$.

2.5.4.1 Embryo Culture

Oocytes were retrieved, stripped of cumulus cells, separated by maturity, and placed into individual 250 µL drops of (Irvine Scientific, Santa Ana, CA, USA) media supplemented with 10% SSS (Irvine Scientific, Santa Ana, CA, USA) overlayed with oil (Irvine Scientific, Santa Ana, USA). The dish with the oocytes was placed into an incubator at 37^0C with 5% CO_2, 95% N_2, and 95% humidity for two to three hours until ICSI.

ICSI was performed on all mature oocytes as described by Nagy and colleagues (1995). Those with two pronuclei at 16-18 hours after ICSI were separated into a separate dish of CSC+10%SSS, overlayed with oil, and placed back into the incubator. On day 3, all embryos underwent AH using a laser (Zilos-tk, Hamilton Thorne, Beverly, Maine, USA). Two to three laser shots at a pulse of 610 µs were utilized to breach the ZP. Embryos were placed back into the incubator and allowed to culture until day 5 or day 6.

Those blastocysts that were observed to have a good or fair quality ICM and trophectoderm that was protruding from the ZP were biopsied and vitrified the same day. When desired by the patients, those embryos with a poor quality ICM or trophectoderm were biopsied and vitrified. Embryos were given until day 6 to reach the blastocyst stage; those that did not blastulate by day 6 were discarded.

2.5.4.2 Trophectoderm biopsy

Blastocysts were placed in a drop of modified human tubal fluid (Irvine scientific, Santa Ana, California, USA) + 10% SSS. The protruding trophectoderm was aspirated into the biopsy pipette (Humagen, Charlottesville, Virginia, USA). Laser pulses of 610 µs were used to "cut" the trophectoderm, taking care to minimize the number of laser shots necessary. Reference labs were used for all CCS procedures; therefore the piece of trophectoderm was prepared according to their protocols.

2.5.4.3 Vitrification

Immediately following biopsy, blastocysts were individually vitrified. Blastocysts were placed in equilibration solution (Irvine Scientific, Santa Ana, California, USA) for 15 min and then transferred to vitrification solution (Irvine Scientific, Santa Ana, California, USA) for <1 min. Blastocysts were pipetted onto a Cryolock® (Biodiseno, Atlanta, Georgia, USA), plunged into liquid nitrogen, and capped.

2.5.4.4 Warming and transfer

Only blastocysts with euploid results were warmed in a subsequent FET cycle. The Cryolock® containing the blastocyst was uncapped under liquid nitrogen and plunged into 37^0C thawing solution (Irvine Scientific, Santa Ana, California, USA) for one minute. The blastocyst was transferred to dilute solution (Irvine Scientific, Santa Ana, California, USA) for three minutes, and finally washing solution (Irvine Scientific, Santa Ana, California, USA) for 10 minutes before being placed into CSC+20% SSS overlaid with oil.

For FET cycles, the endometrial lining was prepped with estrogen patches for approximately 12 days. Progesterone in oil was administered (Day 0) when the endometrial lining was ≥8 mm. The blastocysts were warmed on the 6th day of progesterone administration. After warming, blastocysts were transferred to the uterus with a Wallace catheter (Smiths Medical, Dublin, Ohio, USA) under ultrasound guidance. Patients continued daily progesterone in oil shots until a negative pregnancy test or 8 weeks gestation.

2.5.5 Results

Only patients that underwent a FET cycle of a euploid blastocyst were included in the aneuploidy analysis.

2.5.5.1 Aneuploidy in Presumed Fertile and Infertile Patients, <35 Years Old

A total of 48 CCS cycles from 42 patients were performed on women <35 years old. Eighteen (31.5±2.2 years; range, 21-34 years) presumed fertile patients underwent 21 IVF cycles and 24 infertile (32.1±2.1 years; range, 25-34 years) patients underwent 29 IVF cycles. Twenty-one presumed fertile patients underwent CCS for the following reasons: single gene (n=6), gender selection (n=8), and anonymous oocyte donor (n=7). Of those embryos derived from patients with single gene disorders, embryos may be diagnosed euploid but may be affected with the single gene in question; therefore, those embryos were not available for transfer. The primary diagnoses' for the 29 infertile cycles were as follows: polycystic ovarian syndrome (PCOS; n=4), severe male factor (n=6), recurrent pregnancy loss (RPL; n=9), diminished ovarian reserve (n=1), unexplained (n=5), secondary infertility (n=2), primary infertility (n=1), and endometriosis (n=1). There was no significant difference between estradiol level at hCG, day of hCG, or total IU's of gonadotropins used between presumed fertile and infertile patients <35 years old (Table 11). There was no significant difference between maternal age, average number of oocytes, average number of embryos, and number of euploid blastocysts between presumed fertile and infertile patients <35 years old (Table 11). However, a significantly higher percentage of blastocysts were biopsied from the presumed fertile group (118/244, 48.4%) than from the infertile group (132/340, 38.8%; P<0.0001; Table 11).

Table 11: Cycle characteristics between presumed fertile and infertile patients <35 years old.

	Presumed Fertile	Infertile	P value
No. patients	18	24	
No. cycles	21	29	
Maternal age at retrieval ± SD	31.5±2.2	32.1±2.1	0.3470[a]
E2 at hCG ± SD	3865.5±1413.6	4526.8±1708.6	0.2306[a]
Total IU Gonadotropins ± SD	2787.3±1188.4	2782.2±1261.3	0.8983[a]
Day of hCG ± SD	9.6±1.2	9.8±1.1	0.4818[a]
Avg. No. eggs	17.2±6.8	18.1±7.3	0.7377[a]
Avg. No. embryos	11.6±5.6	11.7±5.4	0.9057[a]
No. eggs	361	525	--
No. embryos	244	340	--
No. blasts bx (%)	118 (48.4%)	132 (38.8%)	<0.0001[b]
Total euploid (%)	68 (59.3%)	75 (56.8%)	0.9992[b]
Avg. No. Euploid (%)	3.2±2.0	2.4±1.9	0.1702[a]

[a] = Mann-Whitney U test [b] = Chi-square test for independence

2.5.5.2 Aneuploidy in a Presumed Fertile and Infertile Patients, ≥35 Years Old

A total of 66 CCS cycles were performed on embryos derived from women ≥35 years. Seventeen cycles from 13 presumed fertile (37.6±1.9 years; range, 35-42) patients and 49 cycles from 38 infertile (38.3±2.2 years; range, 35-44) patients underwent IVF with blastocyst biopsy. Seventeen presumed fertile cycles underwent CCS for the following reasons: gender selection (n=12) and single gene (n=5). The primary diagnoses' for 49 infertile cycles were; unexplained (n=13), diminished ovarian reserve (n=8), endometriosis (n=2), severe male factor (n=4), PCOS (n=4), RPL (n=8), secondary infertility (n=2), uterine factor (n=2), and advanced maternal age (n=6). Estradiol levels at day of hCG were not significantly higher in the presumed fertile group when compared to the

infertile group (Table 12). Total gonadotropins used during the stimulation cycle were significantly lower in the presumed fertile group when compared to the infertile group, 2660.4±1039.8 IU's and 3749.3±1362.6 IU's, respectively (P=0.0061). There was no difference in number of stimulation days, number of oocytes produced, and number of embryos generated, and number of blastocysts biopsied between presumed fertile and infertile patients (Table 11). There was no difference in number of euploid blastocysts between presumed fertile patients (42/86, 48.8%) and infertile patients (97/206, 47.1%; P=0.8852; Table 12). The average number of euploid blastocysts was also not significantly different between the presumed fertile group and the infertile group (Table 12).

Table 12: Characteristics between presumed fertile and infertile patients' ≥35 years old.

	Presumed Fertile	Infertile	P value
No. patients	13	38	
No. cycles	17	49	
Maternal age at retrieval ± SD	37.6±1.9	38.3±2.2	0.4643[a]
E2 at hCG ± SD	4455.1±2252.9	3852.0±2081.5	0.2979[a]
Total IU Gonadotropins ± SD	2660.4±1039.8	3749.3±1362.6	0.0061[a]
Day of hCG ± SD	9.7±1.4	10.5±1.3	0.0620[a]
Avg. No. eggs	17.3±7.3	16.7±6.9	0.8086[a]
Avg. No. embryos	11.5±4.7	9.8±4.7	0.3140[a]
No. eggs	294	819	--
No. embryos	195	482	--
No. blasts bx (%)	86 (44.1%)	206 (42.7%)	0.8112[b]
Total euploid (%)	42 (48.8%)	97 (47.1%)	0.8852[b]
Avg. No. Euploid (%)	2.5±1.4	2.0±1.4	0.3478[a]

[a] = Mann-Whitney U test [b] = Chi-square for independence

2.5.5.3 Implantation

Presumed fertile and infertile patients were grouped together when comparing pregnancy and implantation rates in a corresponding FET cycle. A total of 114 FET's of euploid blastocysts were analyzed. Thirty-one presumed fertile patients underwent 33 FET's, while 62 infertile patients underwent 81 FET's. The primary diagnoses of all the cycles undergoing an FET are presented in table 13. Maternal age at time of retrieval was not statistically significant between presumed fertile and infertile patients, 34.2±3.8 years and 35.5±3.6 years, respectively (P=0.1168; Table 14). All patients were urged to only transfer 1 euploid blastocyst; however, some patients decided to transfer two euploid blastocysts based on their medical history. Regardless, the average number of euploid blastocysts transferred in an FET cycle was not significantly different between presumed fertile (1.3±0.5 blastocysts) and infertile (1.4±0.5 blastocysts) patients (Table 14; P=0.5695). There was also no significant difference in embryo quality transferred between the two groups. Chemical pregnancy rates, as defined by a positive beta hCG level, were significantly higher in the presumed fertile patients when compared to infertile patients, 28/33 (84.8%) and 50/81 (61.7%), respectively (Table 14; P=0.0251). Clinical pregnancies, as defined by the presence of a gestational sac, were significantly higher in the presumed fertile (28/33, 84.8%) patients compared to infertile patients (44/81, 54.3%; Table 14; P=0.0025). Presumed fertile patients exhibited a significantly higher implantation rate of euploid blastocysts in a corresponding FET cycle when compared to infertile patients, 36/42 (85.7%) and 54/109 (66.7%), respectively (Table 14;

P=0.0001). There was no difference between number of multiples or spontaneous abortions between presumed fertile and infertile patients (Table 14).

2.5.6 Discussion

These data do not support the hypothesis that infertile patients exhibit a higher rate of aneuploidy than age matched presumed fertile patients (Table 11; Table 12). However, these data do support the hypothesis that euploid blastocysts derived from infertile couples have significantly lower implantation potential when compared to euploid blastocysts from presumed fertile couples (Table 14).

Table 13: Primary diagnosis of patients undergoing an FET cycle in presumed fertile and infertile patients.

	Presumed Fertile	Infertile
No. patients	31	62
No. frozen embryo transfer cycles	33	81
No. recurrent pregnancy loss (%)	-	17 (21.0%)
No. primary infertility (%)	-	1 (12.4%)
No. secondary infertility (%)	-	2 (2.5%)
No. polycystic ovarian syndrome (%)	-	9 (11.1%)
No. diminished ovarian reserve (%)	-	8 (9.9%)
No. uterine (%)	-	2 (2.5%)
No. severe male factor (%)	-	13 (16.1%)
No. unexplained (%)	-	14 (17.3%)
No. advanced maternal age (%)	-	7 (8.6%)
No. endometriosis (%)	-	5 (6.2%)
No. single gene (%)	10 (30.3%)	-
No. sex selection (%)	8 (24.2%)	-
No. anonymous egg donor (%)	15 (45.5%)	-

Table 14: Outcomes in frozen embryo transfer cycles of euploid blastocysts in presumed fertile and infertile patients.

	Presumed Fertile	Infertile	P value
No. patients	31	62	
No. frozen embryo transfer cycles	33	81	
Maternal age at retrieval ± SD	34.2±3.8	35.5±3.6	0.1168[a]
No. blastocyst thawed	43	109	
No. blastocyst survived (%)	42 (97.8%)	109 (100%)	0.2829[b]
Avg. No. transferred ± SD	1.3±0.5	1.4±0.5	0.5695[a]
No. transferred	42	109	
Good quality blastocysts (%)	12 (28.6%)	20 (18.4%)	0.3790[c]
Fair quality blastocysts (%)	28 (66.7%)	82 (75.2%)	
Poor quality blastocysts (%)	2 (4.8%)	7 (6.4%)	
+hCG (%) per transfer	28 (84.8%)	50 (61.7%)	0.0251[b]
+sac (%) per transfer	28 (84.8%)	44 (54.3%)	0.0025[b]
No. of sacs (% implantation)	36 (85.7%)	54 (66.7%)	0.0001[c]
No. of multiples per pregnancy (%)	8 (24.2%)	10 (20.0%)	0.5607[c]
No. clinical miscarriages (%)	3 (8.3%)	2 (4.0%)	0.3436[b]
Avg. No. Euploid available	3.4±1.7	2.6±1.6	0.0154[a]

[a] = Mann-Whitney U test [b] = Fisher's exact test [c] = Chi-square for independence test

In both age groups, no statistical difference in aneuploidy rates was seen between presumed fertile and infertile patients. The literature is limited concerning the rate of aneuploidy in fertile women ≥35 years as this patient group typically does not resort to IVF to achieve pregnancy. Ata and colleagues (2012) described a direct relationship between aneuploidy and maternal age, indicating that approximately 30% of blastocysts from oocyte donors are aneuploid while approximately 80% of blastocysts from women ≥43 years old are aneuploid. This study found approximately 40% of blastocysts produced by presumed fertile patients (which included oocyte donors) <35 years old, were aneuploid, which is similar to their rate of aneuploidy described in donor oocyte cycles.

Fragouli and colleagues (2009) utilized comparative genomic hybridization and examined the polar bodies from young donors. They found a low aneuploidy rate of 3%. In contrast, some reports indicate aneuploidy rates in polar bodies derived from donors as high as 65% (Sher *et al.*, 2007). It is important to examine aneuploidy rates in an older, presumed fertile patient population because individuals achieve pregnancy in their late 30's and early 40's without the aid of IVF. When subdivided by age, these data demonstrated that regardless if a patient is fertile or infertile, the incidence of preimplantation aneuploidy is similar.

Fragouli and colleagues (2009) also hypothesize that during a natural cycle in a normal, proper functioning ovary, aneuploid oocytes may be selected against. However, controlled ovarian hyperstimulation or aging may circumvent this mechanism. Presumably, fertile patients have proper functioning ovaries that are exposed to hyperstimulation drugs. Therefore, regardless if a patient is fertile or not, the hyperstimulation of ovaries produces similar aneuploidy rates. In this study, presumed fertile patients <35 years old had significantly higher blastocysts euploid rates (59.3%; Table 11) when compared to presumed fertile women ≥35 years old (42.9%; Table 12). The same trend was seen when blastocysts euploid rates were compared between infertile patients <35 years old (56.3%; Table 11) and ≥35 years old (42.2%; Table 12). Therefore, these data suggest that age and not hyperstimulation undermines this mechanism. Further evidence of this is the fact aneuploidy is present in embryos derived from non-stimulated ovaries (Verpoest *et al.*, 2008).

Other studies have reported similar pregnancy rates between fertile and infertile women when only euploid blastocysts were transferred during FET cycles (Harton *et al.*, 2013). Pregnancy rates from euploid blastocysts have been reported to range 65-70% (Grifo *et al.*, 2013). Overall pregnancy rates are similar to this report (68.4%); however, this study also experienced a significantly higher pregnancy and implantation rate in the presumed fertile patients compared to infertile patients. This would indicate that a factor other than chromosomes is hindering pregnancy in infertile patients such as an increase amount of mitochondrial activity, altered gene expression, or possible uterine factors (Hsieh *et al.*, 2004; Wood *et al.*, 2007). One possible explanation for the increase in implantation rates seen with presumed fertile patients is the fact that they had more blastocysts to biopsy when compared to infertile patients (Table 11). With more blastocyst to biopsy and vitrify, then these patients would have more euploid blastocysts to choose from in the corresponding FET cycle. This assumption is correct, however, when embryo quality prior to warming is examined, there is no difference between presumed fertile and infertile patients (Table 14). Lastly, the majority of presumed fertile patients underwent PGS for either sex selection or single gene disorders. Therefore, the sex of the embryo or the single gene diagnosis, not embryo quality, was used as the first line of selection in this group of patients.

Lastly, these data are retrospective and requires an understanding of presumed fertile and infertile. There are many diagnoses that may include or exclude patients from these categories and possibly influence the results. Presumed fertile patients were only those that desired IVF for either gender selection or single gene disorder. All these patients,

theoretically, do not require IVF to get pregnant and therefore they were presumed fertile. Infertile patients encompass a much larger spectrum of diagnosis; however, all these patients underwent IVF to achieve pregnancy. First, only patients with severe male factor (<1 million per mL) were included in the infertile group. Second, secondary infertility are patients that have exhibited previous fertility but have not been successful after a full year of unprotected intercourse. Possibly reasons for secondary infertility included increased maternal age and weight gain. In this study, all of the secondary infertility patients had a secondary diagnosis as well (advanced maternal age, ovulation disorder, or unexplained infertility). Lastly, maternal age is the largest single factor affecting chromosomal aneuploidy and IVF success rates (Franasiak *et al.*, 2014). Patients with AMA were included in this study. By definition, those patients that were presumed fertile and ≥35 years old could be diagnosed with AMA, however they did not need IVF to achieve pregnancy. Infertile patients diagnosed with AMA were undergoing IVF specifically because they could not conceive naturally. The presumed fertile group was not undergoing IVF to conceive, but rather to select a child with certain characteristics. The infertile group was undergoing IVF specifically because they could not achieve a successful pregnancy.

In conclusion, these data suggest that blastocyst euploidy rates are similar, regardless if a patient is presumed fertile or infertile. Although the numbers are small, presumed fertile patients show a significantly higher pregnancy and implantation rate compared to infertile patients when a euploid blastocyst is transferred during an FET cycle. This would indicate infertility as a disease may encompass other aspects such as uterine or other

unknown embryological factors that can influence outcomes. Further studies are needed to determine these factors.

2.6 Specific aim 6. To determine if blastocyst screened by qPCR have similar aneuploidy rates and implantation rates compared to blastocysts screened with aCGH?

For this specific aim, the following published works are presented:

Taylor TH, Griffin DK, Wilson JM, Wing RL, Johnson L, Katz SL. A comparison of aneuploidy rates and ongoing pregnancy rates between blastocysts screened using quantitative polymerase chain reaction or array comparative genomic hybridization, results from a single center. Submitted.

2.6.1 My Personal Contribution to the Work

For this study I did the majority of the embryology including setup, biopsies, and tubing. I developed the idea, analyzed the data, and wrote the manuscript.

2.6.2 Chapter summary

The use of CCS has become widespread in IVF. Data comparing pregnancy and implantation rates utilizing different CCS platforms is lacking. The goal of this study is to examine clinical outcomes of blastocysts tested with qPCR or aCGH. Our control group consisted of all patients undergoing an IVF cycle at the same time as the study patients where untested blastocysts were frozen and utilized in a FET. Chemical clinical pregnancy rates did not differ between qPCR, aCGH, or untested blastocysts. Loss of implantation, as defined as a positive beta pregnancy that was lost pre or post heartbeat was significantly different between euploid diagnosed aCGH blastocysts (9/68, 13.2%) and untested blastocysts (21/71, 29.6%; Table 2; P=0.0328). The loss rate between qPCR

diagnosed euploid blastocysts (8/25, 32.0%) was similar to the loss rate of untested blastocysts (21/71, 29.6%; Table 2; P=0.8205). Our study demonstrated that patients that have a euploid qPCR blastocysts transfer have a 32% chance of a miscarriage once a positive beta is achieved. This rate more closely approaches patients that had untested blastocysts transferred (29%) than the aCGH euploid blastocysts (13.2%: P=0.0374).

2.6.3 Introduction

The use of technologies such as CCS, which allows for the simultaneous testing of all 24 chromosomes, has allowed the field of in-vitro fertilization (IVF) to make radical advances towards the goal of a single healthy baby. The four most common technologies utilized during CCS are aCGH, SNP array, NGS, and qPCR.

A validation process has been described that all CCS platforms should undergo. The validation process provides the patient and clinician the rate at which a positive diagnosis (euploid blastocyst) leads to a positive result (euploid live birth) and the rate at which a negative diagnosis (aneuploid blastocyst) leads to a positive result (euploid live birth; Scott *et al.*, 2012). Moreover, the comparison of CCS tests to known cell lines should be undertaken. Only qPCR and some NGS have been validated in this manner (Scott *et al.*, 2012; Treff *et al*., 2013; Werner *et al.*, 2015). aCGH was validated against fluorescence in-situ hybridization (FISH) which has been demonstrated to have higher rates of aneuploidy compared to SNP and have a detrimental effect on live birth rates, indicating

that aCGH and some NGS could be as problematic as FISH (Mastenbroek *et al.*, 2007; Treff *et al.*, 2010; Gutierrez-Mateo *et al.*, 2011; Fiorentino *et al.*, 2014).

Pregnancy and implantation rates also need to be examined to determine the clinical efficiency of each platform. Data comparing pregnancy rates utilizing different platforms is lacking. The goal of this study is to examine the pregnancy rates of blastocysts tested with qPCR or aCGH. Given the current research, we believe that qPCR will increase the likelihood of a euploid embryo, while maintaining high pregnancy and implantation rates associated with CCS testing.

2.6.4 Methods

This study was determined to be exempt from Institutional Review Board by Sterling IRB. Only patients attending Reproductive Endocrinology Associates of Charlotte and undergoing a fresh cycle from June 2013 to December 2014 were included in this retrospective study. Starting in June 2013, doctors were given the option to send their patients desiring PGS to either The Foundation for Embryonic Competence (FEC; Morristown, New Jersey, USA) for qPCR or Genesis Genetics (Detroit, Michigan, USA) for aCGH (Figure 29). Thus, some doctors decided to send their patients to FEC while others continued to send to Genesis. This study was not randomized. From the patients desiring PGS, two groups were created: those that had their blastocysts screened with qPCR at FEC and those patients that had their blastocysts screened with aCGH at Genesis Genetics.

To examine clinical outcomes between platforms a control group was determined. Our control group consisted of all patients undergoing an IVF cycle at the same time as the study patients where untested blastocysts were frozen and later utilized in a FET. Patients whom had a fresh blastocyst transfer and then subsequent FET's were not included in the control group. The control group were not aged matched and did not have PGS performed during their fresh cycle (Figure 29).

All patients, regardless of where they were sent for PGS or if they were in the control arm, underwent a retrieval and FET between June 2013 and January 2015.

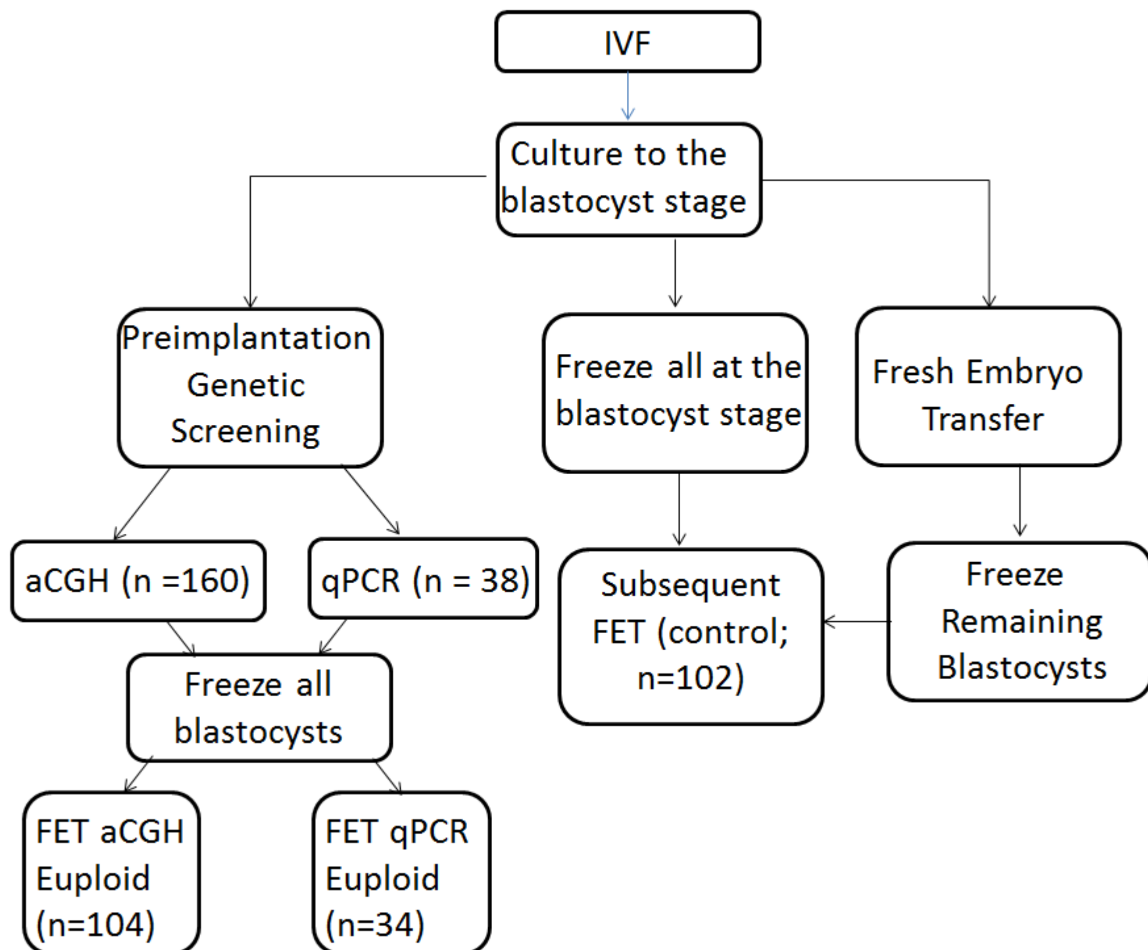
All patients underwent IVF with intracytoplasmic sperm injection, embryo culture, assisted hatching, and blastocyst biopsy, vitrification, and FET protocols as reviewed by Taylor and colleagues (2014b; 2014c). Briefly, oocytes were retrieved, stripped of cumulus cells, separated by maturity, and placed into individual 250 µL drops of CSC (Irvine Scientific, Santa Ana, CA, USA) media supplemented with 10% SSS (Irvine Scientific, Santa Ana, CA, USA) overlayed with oil (Irvine Scientific, Santa Ana, USA). The dish with the oocytes was placed into an incubator at 37^0C with 5% CO_2, 95% N_2, and 95% humidity for two to three hours until ICSI.

ICSI was performed on all mature oocytes. Those that properly fertilized were separated into a separate dish of CSC+10% SSS, overlayed with oil, and placed back into the incubator. On day 3, all embryos underwent assisted hatching utilizing a laser (Zilos-tk, Hamilton Thorne, Beverly, Maine, USA). Two to three laser shots at a pulse of 610 µs

were utilized to breach the zona pellucida. Embryos were placed back into the incubator and allowed to culture until day 5 or day 6.

Those blastocysts that were observed to have a good or fair quality inner cell mass and trophectoderm which was protruding from the zona pellucida were biopsied and vitrified on the same day. Embryos were given until day 6 to reach the blastocyst stage; those that did not blastulate by day 6 were discarded.

Figure 29: Flow chart of study design for qPCR vs. aCGH.



2.6.5 Results

The primary diagnoses of all the cycles undergoing an FET are presented in table 15. To determine if there were differences in aneuploidy rates between qPCR and aCGH screened blastocysts, we examined all cycles undergoing PGS by either technique. Between June 2013 and January 2014, a total of 198 cycles underwent PGS, 38 for qPCR and 160 for aCGH (Table 16). The maternal age at time of retrieval was not significant between qPCR (35.5 ± 6.6 years) and aCGH (36.2 ± 4.4 years; P=NS). The number of blastocysts biopsied, number of euploid blastocysts, number of cycles with euploid blastocysts, and number of blastocysts that had a "no read" signal were not significant between qPCR and aCGH (Table 16).

We attempted to determine the reproductive outcome when transferring blastocysts diagnosed as euploid by qPCR (n=34 cycles) or aCGH (n=60 cycles) in a subsequent FET. Our control group consisted of patients who had an untested blastocyst transferred during a subsequent FET (n=102).

For the corresponding FET cycles, maternal age at time of retrieval, biochemical pregnancy rates, and clinical pregnancy rates was not significant between groups (Table 17). More blastocysts were transferred in the untested group (1.7±0.5) as compared to the qPCR (1.2±0.4) and aCGH (1.2±0.4) groups (Table 17; <0.0001). The total number of fetal heartbeats was significantly higher with aCGH euploid blastocysts (68/129, 52.7%) compared to untested blastocysts (64/168, 38.1%; Table 17; P=0.0166), however there was no significant difference between the number of heartbeats of qPCR euploid

blastocysts (20/41, 48.8%) compared to untested blastocysts (64/168, 38.1%; Table 17). Loss of implantation, as defined as a positive beta pregnancy that was lost pre or post heartbeat, was significantly different between euploid diagnosed aCGH blastocysts (9/68, 13.2%) and untested blastocysts (21/71, 29.6%; Table 17; P=0.0328) and similar between qPCR diagnosed euploid blastocysts (8/25, 32.0%) and untested blastocysts (21/71, 29.6%; Table 17; P=0.8205).

Table 15: Cycle characteristics of the patient populations undergoing frozen embryo transfers.

	qPCR	aCGH	Untested
Total No. of patients	32	96	96
No. frozen embryo transfers	34	104	102
No. recurrent pregnancy loss (%)	5 (14.7%)	12 (11.5%)	2 (2.0%)
No. secondary infertility (%)	1 (2.9%)	3 (2.8%)	4 (3.9%)
No. polycystic ovarian syndrome (%)	2 (5.9%)	5 (4.8%)	5 (4.9%)
No. tubal (%)	0	2 (1.9%)	9 (8.8%)
No. diminished ovarian reserve (%)	0	2 (1.9%)	9 (8.8%)
No. uterine (%)	0	1 (1.7%)	1 (1.0%)
No. male factor (%)	5 (14.7%)	14 (13.5%)	15 (14.7%)
No. unexplained (%)	1 (2.9%)	12 (11.5%)	21 (20.6%)
No. advanced maternal age (%)	5 (14.7%)	6 (5.8%)	0
No. endometriosis (%)	0	12 (11.5%)	21 (20.6%)
No. sex selection (%)	6 (17.6%)	6 (5.8%)	0
No. anonymous egg donor (%)	4 (11.8%)	6 (5.8%)	1 (1.0%)
No. ovulation (%)	2 (5.9%)	8 (7.7%)	8 (7.8%)
No. previous failed IVF (%)	2 (5.9%)	3 (2.9%)	2 (2.0%)
No. other (%)	1 (2.9%)	5 (4.8%)	4 (3.9%)
No. single gene (%)	0	7 (6.7%)	0

Table 16: IVF cycle characteristics and aneuploidy rates between qPCR and aCGH tested blastocysts.

	qPCR	aCGH	P Value
No. Cycles	38	160	
Avg. Age	35.5 ± 6.6	36.2±4.4	0.8808[1]
Avg. No. Eggs	15.5 ± 6.7	16.5 ± 7.1	0.6066[1]
Avg. No. Embryos	7.9 ± 3.9	9.8 ± 5.0	0.0507[1]
Avg. No. Biopsied	3.7 ± 2.4	4.0 ± 2.6	0.6150[1]
Total Biopsied	141	634	0.1110[2]
Total Euploid (%)	76 (53.9%)	292 (46.1%)	
No. Cycles with no Euploid Blastocysts (%)	7 (18.4%)	38 (23.8%)	0.3651[2]
No. Blastocysts with no read (%)	4 (2.8%)	37 (5.8%)	0.2101[3]

[1] Mann-Whitney [2] Chi-Square test [3] Fisher's exact

Table 17: Pregnancy and implantation rates per cycle when euploid blastocysts diagnosed by qPCR, aCGH, and untested embryos are transferred during a frozen embryo cycle.

		qPCR	aCGH	Untested	P value
Per FET	No. Cycles	34	104	102	
	Avg. Age	36.2 ± 5.1	36.2 ± 5.1	34.2 ± 4.5	0.1425[1]
	Avg. No. ET'd	1.2 ± 0.4[a]	1.2 ± 0.4[b]	1.7 ± 0.5[a,b]	<0.0001[1]
	No. cycles w/+hCG (%)	25 (73.5%)	68 (65.4%)	71 (69.6%)	0.6317[2]
	No. cycles w/+sac (%)	20 (58.8%)	63 (60.6%)	58 (56.9%)	0.8636[2]
	No. cycles w/+FCA (%)	17 (50.0%)	59 (56.7%)	50 (49.0%)	0.5150[2]
	No. cycles w/a loss (%) after +hCG	8 (32.0%)	9 (13.2%)[c]	21 (29.6%)[c]	0.0387[2]
	No. clinical miscarriages (%)	3 (15.0)	4 (6.3%)	8 (13.8%)	0.3285
Per Embryo	Total No. ET'd	41	129	168	
	Total No. +sac (%)	24 (58.5%)	73 (56.6%)	77 (45.8%)	0.1159[2]
	Total No. FCA (%)	20 (48.8%)	68 (52.7%)[d]	64 (38.1%)[d]	0.0374[2]

[1] Man-Whitney [2] Chi-square test

[a,b,c,d] = indicates statistical significance between the two groups

[a,b] = <0.0001 [c] = 0.0328 [d] = 0.0166

2.6.6 Discussion

Our data demonstrates similar aneuploidy rates between blastocysts tested with qPCR and aCGH. qPCR diagnosed euploid blastocysts had a higher rate of miscarriage compared to aCGH diagnosed euploid blastocysts. The rate of loss described with qPCR tested blastocysts was similar to the loss observed in untested blastocysts, suggesting that qPCR diagnosed euploid blastocysts are not the same as euploid diagnosed blastocysts by aCGH. We believe this discrepancy can be explained by qPCR's inability to detect segmental aneuploidy and deletions/duplications (Treff *et al.*, 2013; Treff *et al.* 2012).

Research has indicated that the main cause of miscarriages and IVF failure are whole chromosomal abnormalities (Hassold and Hunt, 2001). To test for whole chromosomal abnormalities, the question becomes "how many loci should be analyzed to determine whole chromosomal errors?" qPCR only examines four loci (Treff *et al.*, 2013; Treff *et al.* 2012). The exact location of these loci is unknown however there are two on the p and q arm. If those loci are present in the PCR reaction, then that dictates the diagnosis given. With only four loci examined, compared to the thousands loci examined with aCGH, the confidence in the copy number calls is greatly reduced. For example, if one of the PCR reactions fails due to technical error then an embryo can be misdiagnosed as aneuploidy. Alternatively, if a technical error occurs with aCGH, multiple other loci are present to "disqualify" the error and thus increase confidence in the diagnosis. Moreover, the lack of loci examined prevents qPCR from detecting segmental aneuploidies or duplications/deletions which can cause miscarriages.

In terms of segmental aneuploidy, Rabonowitz *et al.* (2012) demonstrated the presence of segmental aneuploidy in approximately 15% of preimplantation embryos. In spontaneous aborted tissue, the incidence decreases to approximately 5%, suggesting a selection against segmental aneuploidy slightly before or after implantation (Sahoo *et al.*, 2016). Large deletions/duplications may cause a miscarriage as well. Shen and colleagues (2016) demonstrated 5.3% of all spontaneous miscarriages contain a large duplication or deletion. The incidence of large deletions/duplications within the blastocyst is currently unknown however preimplantation embryos are of particularly high risk for chromosome instability, resulting in chromosome breakage and fusion (Voet *et al*., 2011; Vanneste *et al*., 2012). If these errors were present in the blastocyst, their diagnosis would be missed if qPCR were utilized.

Utilizing the same technology, Forman and colleagues (2013) conducted a single embryo transfer of 140 euploid blastocysts and had 19/140 (13.6%) loss of implantation rate, which more closely resembles those of aCGH in our study. In our dataset with qPCR, our clinical pregnancy rate was 58.8% and ongoing pregnancy rate was 50.0%. The differences in loss of implantation could be attributed to different definitions for chemical pregnancy, different cutoffs for biochemical pregnancy, different patient populations, or different treatment protocols.

Ours is not the first report to demonstrate a high miscarriage rate with qPCR. Anderson and colleagues (2015) reported a similar pregnancy, implantation, and miscarriage rate between qPCR blastocysts and untested blastocysts. However, these results could be

attributed to damage caused by the blastocyst biopsy procedure as they did not compare their pregnancy rates with another CCS platform. In our study, patients underwent IVF for both qPCR and aCGH at the same time with the same embryologists, eliminating the effect of the blastocyst biopsy procedure. Moreover, research has indicated that the blastocyst biopsy procedure does not affect implantation and that the blastocyst biopsy procedure seems consistent across multiple IVF centers and embryologists (Scott *et al.*, 2013; Capalbo *et al*., 2016).

We believe that culture conditions, laboratory techniques, and FET protocols did not play a role in our high miscarriage rate with qPCR. During this study, no media was changed, embryo biopsy was performed by the same personnel for both groups, and only proven vitrification and transfer methods were utilized (Taylor *et al.*, 2014b; Taylor *et al*., 2014c). In terms of FET protocols, progesterone was administered on the same day for all 3 groups. Larger studies are needed to confirm our findings.

Overall, those embryos diagnosed as euploid by qPCR may have up to a 20% chance of having either a segmental aneuploidy (15%) or large duplications/deletions (5%) that may cause a miscarriage. These undiagnosed errors could have attributed to our high miscarriage rate. Our data demonstrate that both technologies provide excellent outcomes; however, the miscarriage rate of qPCR blastocysts is similar to untested blastocysts.

2.7 Specific aim 7. To determine the mechanisms by which diagnosed aneuploid cleavage stage embryos can produce euploid blastocysts?

For this specific aim, the following published works are presented:

Taylor TH, Patrick JP, Das D, Crain JL, Wilson JM, Griffin DK. Euploid live births from aneuploid cleavage stage embryos. Submitted 2017.

Taylor TH, Patrick JP, Das D, Crain JL, Wilson JM, Griffin DK. Euploid live births from aneuploidy cleavage stage embryos. PGDIS 2014.

2.7.1 My Personal Contribution to the Work

For this study I did the majority of the embryology including setup, biopsies, and tubing. I developed the idea, analyzed the data, and wrote the manuscript.

2.7.2 Chapter summary

From a single cell, normal human development requires one chromosome from each parent to replicate and evenly segregate into two daughter cells with identical diploid number. When a malsegregation event occur, aneuploidy results. Thus, the assumption is that an aneuploid cleavage stage embryo will give rise to an aneuploid pregnancy. This research challenges the dogma for human development. A total of 34 human embryos from 10 patients were biopsied at the cleavage and blastocyst stage. Each blastomere was assessed for aneuploidy by single nucleotide polymorphism microarray. Only cleavage stage embryos (Day 3) that were diagnosed as aneuploid were rebiopsied at the blastocyst stage (day 5 or 6. Of the 34 cleavage stage embryos that were diagnosed as aneuploid, 16 (47.1%) were diagnosed as euploid. On the basis of a euploid diagnosis at

blastocyst stage, three patients underwent four embryo transfers. Two have resulted in chromosomally and phenotypically normal live births while two others have resulted in biochemical pregnancies. The results suggest that the human embryo is prone to chromosomal abnormalities at the cleavage stage but the dogma of a uniformly euploid or aneuploid embryo should be reconsidered. Chromosomal diagnosis at the cleavage stage may be an unreliable indicator of chromosome copy number in the child for biological or technical reasons.

2.7.3 Introduction

In human development (akin to virtually all diploid eukaryotic organisms), normal mitosis usually ensures faithful segregation of chromosomes so that one cell divides into two identical daughter cells in all subsequent divisions. The received wisdom therefore is that euploid preimplantation embryo usually remains euploid in all later divisions and leads to a euploid individual. Alternatively, an aneuploid embryo remains uniformly aneuploid, leading to implantation failure, miscarriages, and birth defects**.**

In reality, it has been widely demonstrated that the human embryo is in fact commonly mosaic, containing two or more distinct cell lines (Delhanty *et al.*, 1993; Fragouli *et al.*, 2011; Wells and Delhanty, 2000; Daphnis *et al*., 2008; Taylor *et al.*, 2014a). Proposed mechanisms of post-zygotic chromosome segregation error, which typically leads to chromosome mosaicism include: mitotic non-disjunction (where one daughter cell inherits three copies of a chromosomes pair and the other daughter cell inherits the remaining one copy); chromosome gain (presumably by some mechanism of

endoreplication) and chromosome loss (e.g. by anaphase lag) (Taylor *et al*., 2014a). A variant of the latter is so-called "trisomy rescue", where a cell with three chromosomes purportedly expels one of the chromosomes present. These phenomena can lead to UPD and thence such disorders as Prader-Willi or Angelman syndromes.

The clinical consequences of aneuploidy are well reported and were one of the reasons why PGS was developed (i.e. to selectively transfer embryos diagnosed as euploid). Ultimately this stemmed from the desire to improve IVF success rates, particularly in high-risk groups (e.g. advanced maternal age, repeated implantation failure). Previously, PGS required the removal of a piece of the embryo at the cleavage stage, followed by multi-color fluorescence in-situ hybridization; FISH. However, this was largely discredited following the results of randomized clinical trials (Staessen *et al.*, 2004; Mastenbroek *et al.*, 2007). The practice of PGS with FISH at the cleavage stage largely under-estimated the phenomenon of chromosome mosaicism in the cleavage stage embryo, or at least thought it of sufficiently low incidence not to be clinically significant (reviewed in Taylor *et al.*, 2014a).

Recently, FISH technology has been superseded by aCGH or other CCS techniques for the diagnosis of preimplantation aneuploidy; however the contemporary CCS technology has a logistical drawback, namely its expense. For this reason, current research points to a greater incidence of aneuploidy at the cleavage stage compared to the blastocyst stage (Adler *et al.*, 2014). This then raises the question of whether an embryo biopsied and diagnosed as aneuploid at day 3 could develop as a chromosomally normal fetus. Of

course it would be unethical to knowingly transfer an embryo diagnosed as aneuploid in the absence of independent evidence that it was indeed chromosomally normal. However, the aforementioned evidence certainly lends credence to this hypothesis.

Several mechanisms have been described that might account for different patterns of ploidy in day 3 vs day 5 human embryos. These mechanisms include preferential proliferation of euploid cell lines (Ruangvutilert *et al.*, 2000) and preferential allocation of the aneuploid cells to the trophectoderm (James and West, 1994). These proposed mechanisms, in addition to evidence that the cleavage stage mammalian embryo is particularly prone to chromosome segregation errors (Bean *et* al., 2001; Bean *et al.*, 2002; Wells and Delhanty, 2000), suggest that the blastocyst stage is the most appropriate stage to perform embryo biopsy for PGS. Moreover, recent randomized trial data provide indirect evidence to support this, by demonstrating the efficacy of trophectoderm biopsy followed by CCS (Scott *et al.*, 2012 and 2013b). To the best of my knowledge however, direct evidence showing that an embryo diagnosed as aneuploid at the cleavage stage can subsequently develop as a chromosomally normal fetus has yet to be established. In this study I was fortunate enough to be able to analyze data from cycles that both cleavage stage and blastocyst biopsy was performed. Retrospective data mining allowed us to test the hypothesis that chromosomal aneuploidy during the cleavage stage may not be clinically relevant to future development and can result in chromosomally normal live born offspring.

2.7.4 Materials and Methods

Patients included in the study underwent IVF at Reproductive Associates of Charlotte between 2010 and 2012 and additionally received PGS using single nucleotide polymorphism (SNP) with cleavage stage biopsy due to either advanced maternal age (n=5), recurrent pregnancy loss (n=1), or previous IVF failures (n=4). During this time, there was some evidence to suggest that blastocyst stage is a more ideal stage to perform PGS testing (Schoolcraft *et al*., 2010). Therefore, if cleavage stage embryos diagnosed as aneuploid developed to a morphologically normal blastocyst, then these embryos were subject to a second biopsy to either confirm or refute the cleavage stage findings. Moreover, the re-biopsy at the blastocyst stage would confirm to the patient and to the clinicians that potentially viable embryos would not be discarded. To this end, consent was sought from patients in order to allow us to rebiopsy and vitrify diagnosed aneuploid cleavage embryos if they developed to morphologically normal blastocysts. This study was approved by Independent Review Consult (protocol #10040-01A) and by the University of Kent local research and ethics committee.

All IVF procedures were conducted as routine standard of care. Procedures for IVF, sperm preparation and intracytoplasmic sperm injection are extensively referenced elsewhere (Nagy *et al*, 1995; Taylor *et al.*, 2014b; Taylor *et al.*, 2014c). A total of 34 human embryos from 10 patients were biopsied at both the cleavage and blastocyst stage as follows:

2.7.4.1 Cleavage Stage Biopsy

Embryos were group cultured using cleavage media (Cooper Sage, Trumbull, Connecticut, USA) + 10% SPS (Cooper Sage, Trumbull, Connecticut, USA) and overlayed with oil (Irvine Scientific, Santa Ana, California, USA) until day 3. On day 3, embryos with greater than four blastomeres were placed in individual drops of Ca^{2+}/Mg^{2+} free media (Cooper Sage, Trumbull, Connecticut, USA) supplemented with 10% SPS. With the aid of a biopsy pipette (Cook Medical, Bloomington, IL, USA) and laser (Zilos-tk, Hamilton Thorne, Beverly, Massachusetts, USA) , a single cell was removed from a cleavage embryo and prepared for SNP microarray according to a reference lab protocol (Natera, San Carlos, California, USA). After biopsy, embryos were cultured individually in blastocyst media (Cooper Sage, Trumbull, Connecticut, USA) supplemented with 10% SPS. Those embryos that were diagnosed as aneuploid on day 3 and that developed to a morphologically normal blastocyst by day 6 were re-biopsied and vitrified.

2.7.4.2 Blastocyst Biopsy

Only blastocysts that presented with good quality ICM and trophectoderm were re-biopsied. Each individual blastocyst was placed in 20 µL of HEPES (Cooper Sage, Trumbull, Connecticut, USA) supplemented with 10% SPS overlayed with oil. The blastocyst was held in place by a holding pipette (Cook Medical, Bloomington, Illinois, USA). A biopsy pipette was then used to aspirate a piece of the trophectoderm into the bore of the

pipette. Using a pulse of 610 µs and minimizing the number of pulses, a piece of the trophectoderm was "cut" from the blastocyst. This piece of trophectoderm was then prepared for SNP microarray according to reference lab protocol (Natera, San Carlos, California, USA).

2.7.4.3 Blastocyst Vitrification

After biopsy, blastocysts were individually vitrified. Blastocysts were pipetted into equilibration solution (Irvine Scientific, Santa Ana, California, USA) for 15 minutes and then pipetted into vitrification (Irvine Scientific, Santa Ana, California, USA) solution for <1 minute. From the vitrification solution, blastocysts were transferred to a Cryolock® (Biodiseno, Atlanta, Georgia, USA). Care was taken to remove as much vitrification media as possible from the cryolock before plunging into liquid nitrogen.

2.7.4.4 Frozen Embryo Transfer Cycles

If a patient had no euploid embryos at the cleavage stage or failed to achieve pregnancy after the transfer of euploid diagnosed cleavage stage embryos, the patient was consulted on the prospect of using those blastocysts that were diagnosed as aneuploid at the cleavage stage yet euploid at the blastocyst stage. Patients could utilize their euploid diagnosed blastocysts in a subsequent FET cycle. Prior to their decision, all patients were

extensively counseled on the prospect of chromosomal mosaicism and possible clinical consequences, including live birth defects and miscarriages.

2.7.5 Results

SNP microarray results for the cleavage and blastocyst stage biopsy are represented in Table 18. Only embryos that were biopsied at the cleavage stage and diagnosed as aneuploid were included in this study. A total of 34 embryos from 10 patients were biopsied. Embryo were only included if results were received from both a single cell from the cleavage stage and the trophectoderm at the blastocyst stage (Table 18).

Of the 34 embryos diagnosed as aneuploid by SNP microarray at the cleavage stage, 16 (47.1%) were found to be euploid when re-biopsied at the blastocyst stage.

A total of 95 individual errors occurred at the cleavage stage and of those, 71/95 (74.7%) were not present in the blastocyst biopsy. Both trisomic and monosomic errors at the cleavage stage were present at the same rate in the blastocyst biopsy, 34/95 (35.8%) and 34/95 (35.8%), respectively (P=1.0000; chi-square test). Overall, the autosomes presented with three nullisomies (3/95, 3.2%) at the cleavage stage, all that are not present in the blastocyst biopsy. Seven non-disjunction events were observed with errors occurring at the cleavage stage, and the reciprocal error being detected at the blastocyst stage. A total of 16/95 (16.8%) individual errors occurred at the cleavage stage that were also present in the blastocyst.

Data from the sex chromosomes were analyzed independently of the autosomes. A total of 9 embryos presented with sex chromosome aneuploidies at the cleavage stage, six had an additional X, while 3 presented with XO. Of the 6 embryos that presented with an additional X, all 6 (100%) were XX at the blastocyst stage. All embryos that presented with XO on day 3 were XO at the blastocyst stage.

Three patients underwent four transfers of seven abnormally diagnosed cleavage stage embryos that were identified as euploid at the blastocyst stage. Of those four transfers, two have resulted in a live birth (patient #2, embryo #4 and patient #5, embryo #14; table 18) and the other two resulted in a biochemical pregnancies (patient #10, embryos #29 and 32; patient #10, embryos #31 and 34; table 18).

2.7.6 Discussion

Multiple studies have re-biopsied cleavage stage embryos diagnosed as aneuploid at the blastocyst stage (Li *et al.*, 2005; Magli *et al.*, 2000; Daphnis *et al.*, 2008, Barbash-Hazan *et al.*, 2009, Northrop *et al.*, 2010; Capalbo *et al.*, 2013a), however, only one study by Capalbo and colleagues (2013a) has utilized CCS at all stages. Northrop and colleagues (2010) re-biopsied aneuploid cleavage stage embryos diagnosed by FISH and subsequently performed CCS at the blastocyst stage. Unfortunately, FISH has been shown to be insufficient in detecting chromosomal abnormalities; therefore, it is unknown if those diagnosed as aneuploid at the cleavage stage were truly aneuploid or if the abnormalities were a product of the FISH procedure (Treff *et al.*, 2010). Consequently,

ours is the second study to utilize CCS at both the cleavage and blastocyst stage and the first to report on live births from previously diagnosed aneuploid cleavage stage embryos that developed to euploid blastocysts.

There are a number of mechanisms that would allow an aneuploid cleavage stage embryo to develop to a euploid blastocyst: preferential allocation of aneuploid cell lines to the trophectoderm (Figure 30), trisomic rescue (Figure 31A), endoreplication leading to UPD (Figure 31B), and advantageous growth of euploid cell lines during preimplantation development. In this study, only the latter seems to be prevalent during preimplantation development.

Figure 30: An aneuploidy cleavage stage embryo demonstrating preferential allocation of aneuploidy cell lines to the trophectoderm.

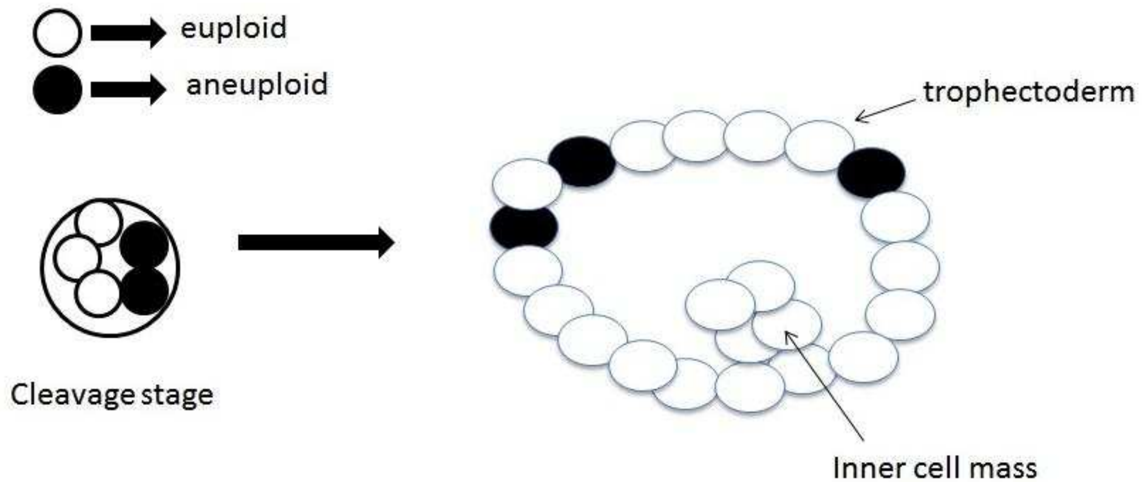
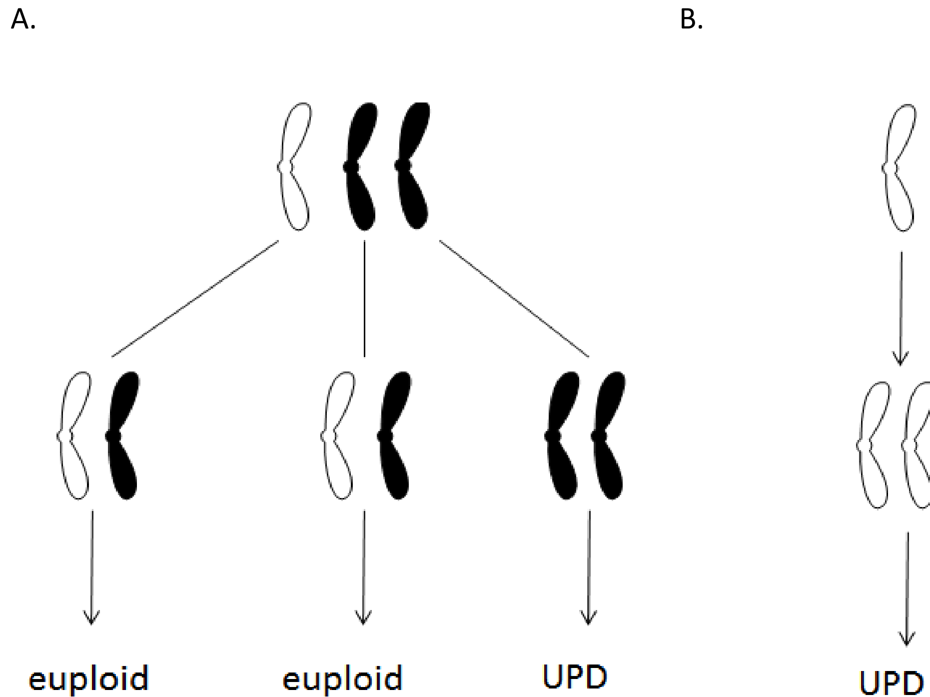


Table 18: SNP results of the same embryo at the cleavage and blastocysts stage. (0) denotes no chromosomes present (i.e., (0)3, indicates no chromosome 3). Sex chromosomes are disomic unless noted. ^ denotes <85% confidence in diagnosis (dx).

Pt	Emb.	Day 3 Dx	Day 5 dx	Destiny	Outcome
1	1	+X	Euploid	Cryo	NA
2	2	+19	+19	Discarded	NA
2	3	-1,-2,-5,-16	Euploid	Cryo	NA
2	4	+X	Euploid	FET	Liveborn
2	5	+21,+22	Euploid	Cryo	NA
2	6	+X,-15	-15	Discarded	NA
2	7	+10	Euploid	Cryo	NA
3	8	XO,-14,-15,-17,-20	XO	Discarded	NA
3	9	+X,+3,+4,+5,+6,+9,+11,+15,+18	+15	Discarded	NA
3	10	69XXX	69XXX	Discarded	NA
4	11	Euploid	Euploid	Cryo	NA
4	12	Euploid (^chromo 22, sex)	-7	Discarded	NA
4	13	Euploid (^chromo 22)	Euploid	Cryo	NA
4	14	(0)1,-2,(0)3,-5,-7,-8,-9,-10,-11,-13,-16,-17,-20,-21,+22	+22	Discarded	NA
4	15	-17	Euploid	Cryo	NA
4	16	+X,-13	Euploid	Cryo	NA
4	17	Euploid (^chromo 22, sex)	Euploid	Cryo	NA
5	18	Euploid (^chromo 21)	Euploid	Cryo	NA
6	19	+20	Euploid	FET	Liveborn
7	20	-5,+22	Euploid	Cryo	NA
7	21	-16	-16	Discarded	NA
7	22	Euploid	Euploid	Cryo	NA
8	23	+19	+19	Discarded	NA
8	24	-19	-19	Discarded	NA
8	25	+5	+5	Discarded	NA
8	26	Euploid (^chromo 22)	Euploid	Cryo	NA
9	27	69XXX	Euploid	Cryo	NA
9	28	-1,-2,+13,+20	+1,+2,-20	Discarded	NA
10	29	-2	-2	Discarded	NA
10	30	Euploid (^chromo 22)	Euploid	Cryo	NA
10	31	-15	-15	Discarded	NA
10	32	-9,-22	-22	Discarded	NA
10	33	-10	Euploid	Cryo	NA
10	34	Euploid (^chromo 21, sex)	Euploid	Cryo	NA
10	35	-20	-20	Discarded	NA
11	36	XY, -2,0(4),-8,-9,+10,+13,-17	OY,+2,+8,+9,+17	Discarded	NA
11	37	Euploid*	Euploid	Cryo	NA
11	38	XO,-1,-3,-9,-13,-14,-16,-17,-20	XO	Discarded	NA
11	39	Euploid*	+20	Discarded	NA
11	40	+X,+1,+5,+8,+10,+11,+12,+13,+14,+17,+0,+22	Euploid	FET#1	Biochem
11	41	+11	Euploid	Discarded	NA
11	42	Euploid (^chromo 21)	Euploid	FET#1	NA
11	43	-22	Euploid	FET#2	Biochem
11	44	+15	Euploid	FET#1	NA
11	45	XO	XO	Discarded	NA
11	46	-2	Euploid	FET#2	NA

Figure 31: Proposed chromosomal mechanisms of self-correction that can lead to uniparental disomy (UPD). A) Correction of a trisomic cell line by trisomic rescue, leading to a proper disomic cell line 2/3 of the time and UPD 1/3 of the time. B) Correction of a monosomic cell line by endoreplication, leading to UPD 100% of the time.



Preferential allocation to the trophectoderm describes a process that the aneuploid cell line is forced to the trophectoderm of the preimplantation embryo. If aneuploid cell lines were forced to the trophectoderm and euploid cell lines to the ICM, one would expect CPM. This has been shown to exist in 1-2% of all pregnancies (Ledbetter *et al.*, 1992), and there are data to suggest that it may be meiotic in origin (Robinson *et al.*, 1997) or mitotically derived later in development when the cytotrophoblast invades the uterine wall (Weier *et al.*, 2005). However, current research has indicated that there is a high degree of concordance between the chromosomal status of the trophectoderm and ICM indicating no preferential allocation of aneuploid cell lines to the trophectoderm (Capalbo *et al.*, 2013b; Johnson *et al.*, 2010). In order to demonstrate preferential allocation of

aneuploid cells lines to the trophectoderm, the ICM would have to be biopsied. Unfortunately this was not conducted during this particular study. Nonetheless, some valuable observations have been made by Northrop and colleagues (2010). In this study 50 aneuploid cleavage stage embryos were biopsied at the blastocyst stage, and only 2 (4%) of these presented with a euploid ICM and the same error detected at the cleavage stage present in the trophectoderm. However, both of these embryos also presented with euploid sections of trophectoderm. If allocation of aneuploidies to the trophectoderm was active, one would expect more abnormalities to be present at the blastocyst stage. However, this is not the case, and aneuploidies are more common at the cleavage stage then at the blastocyst stage (Adler *et al.*, 2014).

This study had two live births and two biochemical pregnancies from diagnosed aneuploid cleavage stage embryos. These embryos had euploid cell lines present in the trophectoderm as evident by re-biopsy at the blastocyst stage. It is possible that the aneuploid cell line from the cleavage stage embryo was incorporated into the trophectoderm, however it did not propagate and the biopsy at the blastocyst stage did not detect the aneuploidies. Everett and West (1998) demonstrated that mosaicism within the blastocyst can have either a checkerboard or scattered pattern. However, this observation was made from a 4N-->2N mouse chimera, and has not been demonstrated in human blastocysts or with trisomies. Nonetheless, it is plausible that the blastocyst stage biopsy did not detect the aneuploidies because of the location of the biopsy. Alternatively, it is also possible that the aneuploid cell lines along with euploid cell lines are present within both the ICM and the trophectoderm. In the two embryos that

resulted in a live born, the euploid cells could have been destined to become the fetus while the aneuploid cell lines became extraembryonic tissue. Likewise, in the two biochemical pregnancies, the aneuploid cells could have been destined to become the fetus while the euploid cell lines became the extraembryonic tissue. The two biochemical pregnancies occurred in the same patient, at two different transfers; therefore, it cannot be ruled out that these biochemical pregnancies were patient related as opposed to embryonic.

A second mechanism that an aneuploid cleavage stage embryo might develop to a euploid blastocyst is trisomic rescue. In this instance, anaphase lag can alter a trisomy cell line by reducing the number of chromosomes from 47 to 46 (reviewed in Taylor *et al.,* 2014a). This occurs when a chromatid fails to become incorporated during the final stages of mitosis. In this study, a total of 34 trisomies detected at the cleavage stage were not present at the blastocyst stage. If a lag event occurred in a trisomic cell line, it is possible that the trisomy would be rectified to a disomy state. Since a trisomic chromosome contains 2 chromatids from one parent and a single chromatid from the other, 2/3 of the time the process of reducing a trisomic chromosome should result in a proper disomic cell line, while 1/3 of the time the process should result in UPD (Figure 31A). Similarly, 34 monosomic events occurred at the cleavage stage and corrected themselves by the blastocyst stage. In order to correct this error, an endoreplication event, the duplication of an entire chromosome, resulting in UPD must occur (Figure 31B). Given these errors, one would have expected UPD to occur in approximately 45 of the errors that corrected; however, this was not the case, as UPD was not detected in any sample within the

trophectoderm. This finding parallels research demonstrating that UPD occurs in approximately 0.06% of blastocysts, indicating that UPD is an extremely rare event (Gueye *et al.*, 2014). The overall suggestion therefore, is that the mechanisms of endoreplication and trisomy rescue do not explain the embryos ability to compensate for cleavage stage aneuploidy.

Research has indicated that the cleavage stage is highly prone to chromosomal malsegregation, particularly in ambient air as opposed to lower oxygen culture conditions (Bean *et al.* 2001; Bean *et al.*, 2002). However, more recent studies have shown an increase in euploid cells as the embryo progresses (Ruangutilert *et al.*, 2008; Adler *et al.*, 2014). For example, Santos and colleagues (2010) demonstrated an increase in euploid rates from day 4 (6%), day 5 (37%), and day 8 (58%) while Munne and colleagues (2005) cultured aneuploid embryos with fibroblasts and found approximately 50% of the cells were normal by day 12. In this study, 16 embryos that were aneuploid at the cleavage stage were euploid at the blastocyst stage. Moreover, a total of 95 errors occurred at the cleavage stage, while only 26 errors occurred at the blastocyst stage. This supports other research showing that blastocyst development favors euploid cell lines (Ruangutilert *et al.*, 2008; Adler *et al*., 2014).

Of the embryos that resulted in a successful child, one was diagnosed on day 3 as trisomy 20 and the other as XXX. Trisomy 20 is one of the more common abnormalities and typically leads to miscarriage in the first trimester (Robinson *et al*., 2005). Triple XXX syndrome is a rare disease that often goes undiagnosed because it presents with little to

no clinical manifestations (Ben Hamouda *et al.*, 2009). It is of course possible that these liveborns may be mosaic, however in order to fully eliminate mosaicism as a possibility one would have had to examine the placenta (as mosaicism can become isolated to the placenta), which was not carried out in this study (Kalousek and Dill, 1983). Moreover, mosaicism in the blastocyst seems to be incredibly low, (approximately 5%; Capalbo *et al.*, 2013b) and so even if the blastocysts were mosaic, it may not have been clinically significant. Nonetheless, both babies are currently healthy and present with no abnormalities.

Lastly, one must be careful in interpreting these results, as no test is 100% accurate. SNP microarray has been validated to have a false-positive rate of 3.9% and a false-negative rate of 2.1% (Johnson *et al.*, 2010). However, research suggests that analysis of single cells by SNP array is flawed and can lead to the over stating of errors (Handyside *et al.*, 2010; Bisignano *et al.*, 2011). Scott and colleagues (2012) demonstrated that both cleavage stage and blastocyst stage euploidy is predictive of live birth rate. In their study, only 1/53 (1.9%) embryo that was diagnosed as aneuploid at the cleavage stage developed to a live birth, while 3/46 (6.5%) blastocysts diagnosed as aneuploid developed to a liveborn. In this respect, this study contradicts theirs by demonstrating that the chromosomal status of the cleavage stage embryo is a poor predictor of the chromosome copy number in the child.

Due to the lack of evidence in support of preferential allocation to the trophectoderm and the low incidence of UPD during preimplantation development, I propose that the

only active corrective mechanism during preimplantation development is the advantageous growth of euploid cell lines. In this study, the majority of errors at the cleavage stage were corrected by the blastocyst stage (71/95. 74.7%), indicating that this mechanism is fairly efficient. Lastly, the dogma that the chromosomal status of the cleavage stage embryo is predictive of the chromosome copy number in the blastocyst or child should be reconsidered.

2.8 Specific Aim 8. To assess aneuploidy rates between the polar, mid, and mural trophectoderm?

For this specific aim, the following published works are presented:

Taylor TH, Crain JL, Katz SL, Griffin DK. Preliminary assessment of aneuploidy rates between the polar, mid and mural trophectoderm. Submitted JARG 2017.

Stankewicz-McKinney TL, **Taylor TH**, Glassner MJ, Orris JJ, Basile DR, Griffin DK. 2015. Preliminary assessment of aneuploidy rates between the polar, mid, and mural trophectoderm. PGDIS, Chicago.

2.8.1 My Personal Contribution to the Work

For this study I did the majority of the embryology including setup, biopsies, and tubing. I developed the idea, analyzed the data, and wrote the manuscript.

2.8.2 Chapter Summary

It has been suggested that ploidy is consistent throughout the trophectoderm. Thus, cells removed from the mural trophectoderm should mirror the chromosome content of the remaining cells. To test this hypothesis, this projected aimed to compare aneuploidy rates between three distinct areas of trophectoderm: mural, polar, and a region in between these two locations termed the "mid" trophectoderm. All fertilized oocytes were cultured to day 3 and AH was performed. Embryos were placed back into incubator and cultured to the blastocyst stage. Embryos whose trophectoderm was hatching out of the ZP underwent the biopsy procedure. Biopsied blastocysts were divided into three groups

depending on which area (polar, mid, or mural) of the trophectoderm was protruding from the ZP and was biopsied. Aneuploidy rates were significantly higher with cells from the polar region of the trophectoderm (56.2%) compared to cells removed from the mural region of the trophectoderm (30.0%). Although not significant, this data does show a strong trend in decreasing aneuploidy from the polar (56.2%), mid (47.4%), and mural trophectoderm (30.0%; Figure 32). The non-concordance demonstrated between polar and mural trophectoderm can be attributed to biological occurrences, differences between embryologists in the biopsy procedure, and chromosomal mosaicism.

2.8.3 Introduction

Aneuploidy refers to the presence of absence of whole chromosomal abnormalities. In order for a euploid live birth to occur, chromosomes must divide equally in the developing fetus. Any abnormal division during development can have disastrous downstream effects, leading to poor embryo development, failed implantation, obstetric complications, pregnancy loss, stillbirth, neonatal congenital abnormality, and infertility. Thus, PGS has been created to tests for aneuploidy prior to implantation thereby allowing the transfer of only diagnosed euploid embryos. The transferring of euploid embryos has demonstrated a higher pregnancy rate, lower miscarriage rate, and higher live birth rate than the transfer of untested embryos (Scott *et al.*, 2013b).

Biopsy for PGS occurs the majority of the time at either the cleavage (day 3) or blastocyst stage (day 5/6). For cleavage stage biopsy, a single cell is removed from a 5-8 cell embryo.

In contrast, at the blastocyst stage, approximately 5-10 cells are removed from a blastocyst that can contain hundreds of cells. Thus, the removal of one cell from a cleavage stage embryo represents a larger proportion of the embryo and has been shown to cause a decrease in implantation potential when compared to blastocyst stage biopsy (Scott *et al.*, 2013a). Not only is blastocyst biopsy less detrimental but more cells removed for analysis can improve accuracy and decrease “no reads”. It is believed that all cells within the trophectoderm have the same karyotype and aneuploidy or euploidy is consistent throughout the blastocyst.

The blastocyst represents the first stage of differentiation in preimplantation development. The blastocyst differentiates into the ICM, which will become the fetus, and the trophectoderm that will become the placenta. The trophectoderm itself is subdivided into two areas based on the location of the ICM: the mural trophectoderm, the area furthest away from the ICM, and the polar trophectoderm, the area adjacent to the ICM. Typically during PGS, cells are removed from the mural trophectoderm as not to expose the ICM to the damage caused by the laser (Taylor *et al.*, 2014b; Taylor *et al.*, 2014c). However, blastocyst biopsy is not standardized, which can lead to inter and intra differences with embryologists in terms of the area of biopsied.

It has been suggested that ploidy is consistent throughout the trophectoderm i.e. that all cells have the same karyotype. Thus, cells removed from the mural trophectoderm should mirror the chromosome content of the remaining cells. To test this hypothesis, this projected aimed to compare aneuploidy rates between three distinct areas of

trophectoderm: mural, polar, and a region in between these two locations termed the "mid" trophectoderm.

2.8.4 Methods

This study was deemed exempt by Sterling IRB because it only incorporated routine IVF procedures. Only patients undergoing IVF with PGS between January 2012 and April 2013 at Reproductive Endocrinology Associates of Charlotte (Charlotte, North Carolina, USA) were included in this study. All biopsy specimens were sent to Genesis Genetics (Detroit, Michigan, USA) where samples underwent NGS by the Veriseq kit (Illumina, San Diego, USA).

Briefly, all fertilized oocytes were cultured to day 3 and AH was performed. Embryos were placed back into incubator and cultured to the blastocyst stage. Embryos whose trophectoderm was hatching out of the ZP underwent the biopsy procedure. Biopsied blastocysts were divided into three groups depending on which area (polar, mid, or mural) of the trophectoderm was protruding from the ZP and was biopsied.

2.8.4.1 Egg Retrieval and Embryo Culture

All retrieved oocytes were designated for intracytoplasmic sperm injection (ICSI). Oocytes were retrieved, trimmed of blood, and stripped of cumulus cells as described by Taylor

and colleagues (2006). Oocytes were separated based on maturity and placed into a 60 mm dish (Thermo scientific, Rochester, New York, USA) with approximately 100 µL drops of CSC (Irvine Scientific, Santa Ana, California, USA) supplemented with 10% SSS (Irvine Scientific, Santa Ana, California, USA) and overlayed with oil (Irvine Scientific, Santa Ana, California, USA). After grading, the dish containing the oocytes was placed into an incubator at 37°C, 6% CO_2 and 5% O_2 for 2-3 hours. After 2 hours, all oocytes presenting with a polar body were ICSI'd as described by Nagy and colleagues (1995), placed back into the same dish, and put back into the incubator.

The next day, 16-18 hours post ICSI, oocytes were evaluated for proper fertilization. Embryos that exhibited two pronuclei were group cultured in a fresh dish of CSC+10%SSS overlayed with oil and placed back into the incubator. Embryos were not viewed on day 2.

On day 3, the embryos were removed from the incubator, graded, and AH was performed on all cleaving embryos with the aid of a laser (Zilos-tk, Hamilton Thorne, Beverly, Maine, USA). Using a pulse of 610 µs, the ZP was breached with 2-3 shots of the laser (Zilos-tk, Hamilton Thorne, Beverly, Maine, USA). The ZP was breached where there were no blastomeres that could be directly affected by the laser pulse. After breaching the ZP with the laser, the embryos were left in the same drop and placed back into the incubator.

On the morning of day 5 (112-115 hours post insemination) and day 6 (136-139 hours post insemination), embryos were removed from the incubator, blastocysts were graded based on Schoolcraft and colleagues (1999), and those blastocysts that had a good or fair

trophectoderm protruding from the ZP along with good or fair quality ICM were biopsied. Blastocysts were only viewed once in the morning and at no other times. If the blastocysts were not suitable for biopsy in the morning of day 5, they were reevaluated on the morning of day 6. Blastocysts were biopsied on day 5 or day 6, which ever day they met the biopsy criteria. If embryos did not meet the criteria for biopsy on day 6, they were discarded. There was no difference between blastocysts that were biopsied on day 5 or day 6 other than the embryos needed an extra day to reach the proper stage for biopsy.

2.8.4.2 Trophectoderm biopsy

Blastocysts that presented with a good or fair quality ICM and trophectoderm were placed in a drop of modified human tubal fluid (Irvine scientific, Santa Ana, California, USA) + 10% SSS (Irvine scientific, Santa Ana, California, USA). Suction was applied to the blastocysts via a holding pipette (Humagen, Charlottesville, Virginia, USA). A biopsy pipette (Humagen, Charlottesville, Virginia, USA) gently aspirated the trophectoderm into the biopsy needle. A laser (Zilos-tk, Hamilton Thorne, Beverly, Maine, USA), with a pulse length of 610 µs, was used to “cut” the trophectoderm from the blastocyst, taking care not to expose the trophectoderm to unnecessary laser pulses. The piece of trophectoderm was prepped according to the reference labs’ procedures for aCGH (Genesis Genetics, Detroit, Michigan, USA). aCGH slides were supplied by Bluegnome (Cambridge, United Kingdom) and were validated through FISH reanalysis of embryos (Fragouli *et al*, 2011).

2.8.5 Results

In total, 166 blastocysts were biopsied, 48 from the plural trophectoderm, 78 from the mid trophectoderm, and 40 from the mural trophectoderm. There was no significant difference in maternal age between the three groups, i.e. 35.8±4.9 years, 34.9±4.4 years, and 35.2±5.1 years, for the plural, mid, and mural trophectoderm, biopsied groups respectively (Table 19; P=0.8024). Aneuploidy rates were 27/48 in polar trophectoderm group (56.2%), 37/78 in the mid trophectoderm group (47.4%), and 12/40 in the mural trophectoderm group (30.0%; Table 19; P=0.1859). Interestingly, in a direct comparison between mural and polar trophectoderm, aneuploidy rates were significantly higher (Table 20; P=0.0243).

2.8.6 Discussion

The hypothesis that aneuploidy is evenly distributed throughout the trophectoderm cannot be supported by this study. Aneuploidy rates were significantly higher when cells were taken from the polar region of the trophectoderm (56.2%) compared to cells removed from the mural region of the trophectoderm (30.0%).

Table 19: A comparison of aneuploidy rates between the polar, mid, and mural trophectoderm.

	Polar	Mid	Mural	P value
Avg. Age (years)	35.8±4.9	34.9±4.4	35.2±5.1	0.80241[1]
No. Blastocyst	48	78	40	0.18592[2]
No. Aneuploid	27 (56.2%)	37 (47.4%)	12 (30.0%)	

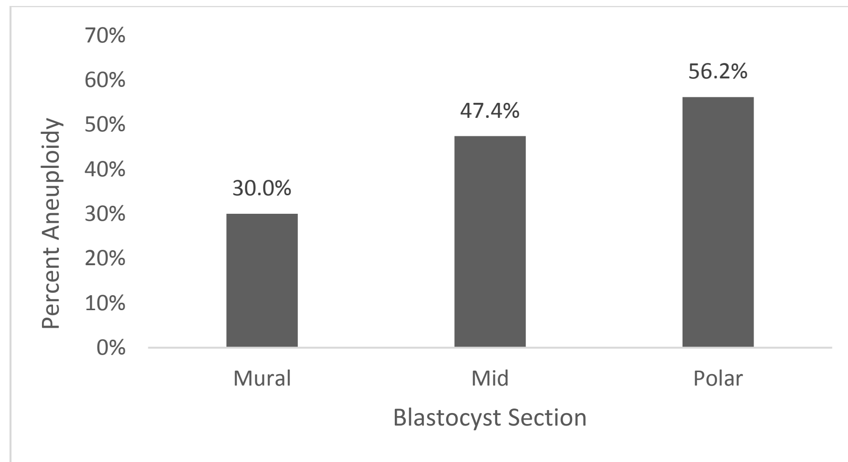
[1] Kruskal-Wallis test [2] Chi-square test

Table 20: A comparison of aneuploidy rates between polar and mural trophectoderm.

	Polar	Mural	P Value
Avg. Age (years)	35.8±4.9	35.2±5.1	0.8417[1]
No. Blastocyst	48	40	0.02432[2]
No. Aneuploidy	27 (56.2%)	12 (30.0%)	

[1] Kruskal-Wallis test [2] Chi-square test

Figure 32: Aneuploidy rates between polar, mid, and mural trophectoderm.



Although not significant, this data does show a strong trend in decreasing aneuploidy from the polar (56.2%), mid (47.4%), and mural trophectoderm (30.0%; Figure 32). The non-concordance demonstrated between polar and mural trophectoderm can be attributed to biological occurrences, differences between embryologists in the biopsy procedure, and chromosomal mosaicism.

Biologically speaking, Hogan and Tilly (1978) dissected mouse ICM from the trophectoderm and left the ICM in culture. Within 5 days, some of the individual ICM's had the appearance of a blastocyst. Moreover, the individual ICM's derived trophoblast giant cells. These studies suggest that cells from the ICM feed the trophectoderm. It is unknown if this mechanism is present in human embryos. However, if it were, it could

explain these data. For example, if the ICM were mosaic and contained equal proportions of aneuploid and euploid cells, then aneuploid cells would feed into the trophectoderm at the same rate as euploid cells. However, once in the trophectoderm, the euploid cells would proliferate at a faster rate than aneuploid cells (Ruangvutilert *et al.*, 2000). Thus, the blastocyst could have a higher proportion of aneuploid cells in the polar compared to the mural trophectoderm, which these data supports. Conversely, this theory would suggest that the blastocyst may be able to allocate aneuploid cells to the trophectoderm thereby correcting its chromosome state by the elimination of aneuploid cells from the ICM. Research using FISH and array based techniques have found no evidence of this correction mechanism in place for human blastocysts (Johnson *et al.*, 2010; Northrop *et al.*, 2010; Evsikov and Verlinsky, 1998; Magli *et al.*, 2000; Derhaag *et al.*, 2003; Fragouli *et al.*, 2008).

Another biological reason for the discrepancy between regions of the trophectoderm could be the process of implantation. During implantation, the blastocyst embeds itself with the ICM (polar trophectoderm) against the uterine wall. In order to invade into the uterine wall, the cytotrophoblasts, which are located in the polar region, have been shown to induce aneuploidy (Weier *et al.*, 2005). Aneuploidy in the polar region may be a completely normal pathway of cytotrophoblast invasion. These data suggests that aneuploidy is higher in the polar region, possibly because the embryo is undergoing chromosomal changes to prepare for implantation. Unfortunately this study did not examine implantation rates between the three different categories, so it is unknown if aneuploidy in the polar region is detrimental. However, transfers of “aneuploid” or

mosaic blastocysts have resulted in euploid live births suggesting that some aneuploidy and mosaicism may not be clinically significant (Scott *et al.*, 2013a; Florinto *et al.*, 2015; Taylor *et al.*, 2014a).

Literature is currently lacking in terms of the effects of the biopsy procedure on the outcomes of CCS cycles. For example, in this study, the embryologist has to biopsy from the mural trophectoderm. Because of its proximity to the ICM, it is possible that some ICM cells were removed with the trophectoderm during the biopsy. Unfortunately, the level of contamination between the ICM and trophectoderm during the biopsy is unknown. However, this may not affect the CCS result as research has indicated a high concordance between the two regions (Rabonowitz *et al.*, 2010; Capalbo *et al.*, 2012). Moreover, recent research has suggested a limited effect of the embryologist on the CCS results (Capalbo *et al.*, 2015). Interestingly, with the advent of NGS and its increase in the detection of mosaicism, the biopsy procedure has become a variable. For example, mosaicism is dependent on the number of cells analyzed. If embryologist “A” biopsies 2 cells from the blastocyst, mosaicism will not be detected. However, if embryologist “B” biopsies 10 cells from the blastocysts, the probability that mosaicism will be detected increases due to the increase number of cells biopsied. Research has also suggested that the majority of abnormalities at the blastocyst stage are mitotic in origin, suggesting that with enough cells present, CCS results could be altered (Rabonowitz *et al.*, 2015).

These data also suggests other occurrences during preimplantation development. Of the 166 blastocysts, there was no difference between which area (polar, mid, or mural)

hatched out of the blastocyst, 37.8%, 30.7%, 31.5%, respectively (P=NS). Schimmel and colleagues (2014) demonstrated a similar trend in mouse embryos that underwent laser AH whereby 32%, 33%, and 35% hatched from the polar, mid, and mural trophectoderm, respectively.

One argument to the current data is the phenomena of mosaicism (Taylor *et al.*, 2014a). One could biopsy from the polar, mid, and mural trophectoderm from a single blastocyst; however, this was not possible because these were patients undergoing IVF and not blastocysts donated to research. Northrop and colleagues (2010) examined three separate sections of the trophectoderm and demonstrated a concordance rate of 80% (40/50 blastocysts). Unfortunately this study did not record the location of the trophectoderm samples in relation to the ICM. Most of the research with mosaicism at the blastocyst stage deals with the reanalysis of aCGH samples or the mixing of known cell lines to determine the percent mosaicism present in the entire blastocyst (Ruttanajit *et al.*, 2016). Using a technique developed by Taylor and colleagues (2016b), it would be possible to effectively "map" aneuploidies in single cells from the blastocyst, which may help in understanding aneuploidy and blastocyst morphology.

In conclusion, these data does not support the hypothesis that aneuploidy is evenly distributed throughout the trophectoderm. This study adds to the pool of data that may help patients and clinicians understand why some embryos diagnosed as "euploid" fail to implant. Further research is needed to better understand aneuploidy at the blastocyst stage and its clinical consequences.

2.9 Specific aim 9. To determine if a previously diagnosed aneuploid trophectoderm relates to the embryo proper.

For this specific aim, the following published works are presented:

Taylor TH, Divic N, Katz SL, Gitlin SA, Griffin DK. Reanalysis of blastocysts previously diagnosed as aneuploidy demonstrates that one in four are rediagnosed as euploid. Submitted 2017.

2.9.1 My Personal Contribution to the Work

For this study I did the majority of the embryology including setup, biopsies, and tubing. I developed the idea, analyzed the data, and wrote the manuscript.

2.9.2 Chapter Summary

Preimplantation genetic screening is typically performed on a piece of the trophectoderm. The biopsied sample should reflect the whole embryo, however data showing the relationship between the chromosome content of the trophectoderm and its relationship to the chromosome content of the whole embryo is limited. Due to mosaicism, I hypothesize that the trophectoderm biopsy is not concordant to the whole embryo. Blastocysts with a previously diagnosed aneuploidy trophectoderm sample were assessed for chromosomes using aCGH. A total of 43 blastocysts from 34 patients (35.3±2.4 years) were analyzed. Of these, 24 (55.8%) exhibited full concordance between the trophectoderm sample and the blastocyst, six (11.6%) had at least one aneuploid chromosome that was concordant between the trophectoderm and blastocyst, two

(4.7%) were non concordant but both the trophectoderm and blastocyst were aneuploidy. Remarkably, 11 (25.6%) displayed a euploid result. Of the 65 aneuploidies detected in the trophectoderm, 37 (56.9%) were detected in the whole blastocyst. In approximately one quarter of the blastocysts analyzed, a diagnosis of aneuploidy did not reflect the chromosome constitution of the whole embryo. Possible explanations for this are mosaicism, mitotic non-disjunction, post-zygotic errors with sequestering of the aneuploid cells to the trophectoderm, and/or technical errors.

2.9.3 Introduction

Chromosome malsegregation, or aneuploidy, is the leading cause of miscarriages and IVF failure (Hassold and Hunt, 2001). In order to screen for aneuploidy during the course of IVF, PGS has been developed. Previously, PGS was performed on cleavage stage embryos using a technique known as FISH that allows for the detection of a limited number of chromosomes (Griffin *et al.*, 1994). Aneuploidy, as demonstrated by FISH, is well documented in scientific literature (Baart *et al.*, 2007; Li *et al.*, 2005; Rubio *et al.*, 2005; Munne *et al.*, 1995). More recently however, PGS has transitioned to testing at the blastocyst stage and using techniques that can detect aneuploidies across all chromosomes, referred to as CCS (Treff *et al.*, 2010, 2012; Gutierrez-Mateo *et al.*, 2011).

When PGS utilizes trophectoderm biopsy and an aneuploidy is diagnosed, the assumption is that this aneuploidy is present throughout the embryo as well. One study demonstrated a high concordance between the trophectoderm and ICM (Capalbo *et al.*,

2012). However this study was conducted using FISH which does not detect all errors (Treff *et al.*, 2010). Using CCS, Johnson and colleagues (2010) examined all the chromosomes from the ICM and trophectoderm and found a 96% concordance rate between the two sections. Subtle differences may be reflective of biological errors or technical errors. Mosaicism, or the presence of two distinct cell lines, is common at the blastocyst stage; therefore, a small piece of the trophectoderm may not be representative of the entire embryo (Taylor *et al.*, 2014a). Chromosomal mosaicism within the trophectoderm has been demonstrated in approximately 20% of embryos (Northrop *et al.*, 2010). However, the chromosome constitution of the whole embryo in regards to the biopsied trophectoderm is sparse.

Theoretically, the biopsied sample should reflect the embryo as a whole; however karyotype data showing the relationship between the chromosome content of the trophectoderm and its relationship to the average chromosome content of the blastocyst is sparse. Moreover, mosaicism may cause a non-concordant result between the trophectoderm and the remaining embryo. The objective of this study is to examine the chromosome constitution of trophectoderm biopsies and test the hypothesis that the corresponding blastocyst yields a contradictory result.

2.9.4 Methods

This is an observational study and was approved by an institutional review board (WIRB #1138244). The University of Kent Research Ethics Advisory Group also approved this

study. Only previously diagnosed aneuploid blastocysts that were donated to research were used in this study. Ovarian hyperstimulation, IVF, ICSI, extended culture, and vitrification are discussed in detail elsewhere (Taylor *et al.*, 2014b, 2014c).

Briefly, embryos from patients requesting PGS were cultured to the blastocyst stage. Only blastocysts presenting with a good or fair quality ICM and trophectoderm were biopsied on day 5 or day 6. Blastocysts chosen for biopsy were placed in modified human tubal fluid (Irvine Scientific, Santa Ana, California, USA) + 10% SSS (Irvine Scientific, Santa Ana, California, USA) overlayed with oil (Irvine Scientific, Santa Ana, California, USA). The blastocyst was held in place with a holding pipette (Humagen, Charlottesville, Virginia, USA). Using a laser (Zilos-tk, Hamilton Thorne, Beverly, Maine, USA) and a biopsy pipette (Humagen, Charlottesville, Virginia, USA), care was taken to remove approximately 5-10 cells of the trophectoderm. Immediately after biopsy, the piece of the trophectoderm was prepped for aCGH while the biopsied blastocysts were individually vitrified on a Cryolock ® (Biodiseno, Atlanta, Georgia, USA) as previously described (Taylor *et al.*, 2014b).

Donated aneuploid blastocysts were “knocked off” the Cryolock into 37^0C thawing solution (Irvine Scientific, Santa Ana, California, USA). The protocol for aCGH requires that the cells be lysed for analysis. For this reason, it was decided that the blastocysts did not have to undergo the complete warming procedure. Upon visualization of the blastocyst in the thawing solution, a 150 um stripper tip was used to pipette the blastocyst up and down until the ZP was separated from the blastocyst. The entire blastocyst was prepped

for aCGH by sending it through a series of rinses with wash buffer and placed into the PCR tube for analysis. The reference laboratory was blinded to the previous analysis.

2.9.4.1 Array Comparative Genomic Hybridization

The cells in the PCR tubes underwent whole genome amplification (WGA; Sureplex, Rubicon Genomics/Bluegnome; CPC4, Capital Park). Cy3 dye and Cy5 dye was used for the sample and reference DNA, respectively. The sample DNA and reference DNA were denatured at 74^0C. Both sets of DNA were mixed, placed on the microarray slide, and hybridized overnight at 47^0C. Afterwards, the microarray slides were washed at room temperature for 10 minutes with 2x saline sodium citrate (SSC) and 0.05% Tween-20, 10 minutes with 1x SSC, 5 minutes with 0.5 SSC at 60^0C, and lastly in 0.1x SSC. Microarray slides with DNA were scanned and analyzed by Bluefuse software (Harton *et al.*, 2013).

2.9.5 Results

A total of 43 whole blastocysts from 34 patients (35.3±2.4 years) were analyzed by aCGH. Of these, 24 (55.8%; Table 21) exhibited full concordance between the trophectoderm sample and the whole blastocyst, six (11.6%; Table 21) had at least one aneuploid chromosome that was concordant between the trophectoderm and blastocyst, two (4.7%; Table 21) were non concordant but both the trophectoderm and blastocyst were aneuploidy. Remarkably, 11 (25.6%; Table 21) displayed a euploid result.

In the original biopsied trophectoderm samples, a total of 65 whole aneuploidies were diagnosed and two segmental aneuploidies. Of the 65 aneuploidies, 37 (56.9%) were detected in the whole embryo consisting of 19 (51.4%) gains and 18 (48.6%) losses. There were 28 aneuploidies detected in the trophectoderm sample that were not in the blastocyst, composing of 16 (57.1%) gains and 12 (42.9%) loses.

Two trophectoderm samples presented with segmental aneuploidies (#22 and #31). Trophectoderm sample #22 presented with a deletion on the p arm on chromosome 6, the same deletion was seen throughout the entire embryo. Trophectoderm sample #31 presented with a deletion on the q arm on chromosome 1. This deletion was not present in the entire blastocyst. Although this blastocyst did not present with the segmental aneuploidy seen in the original trophectoderm sample, the blastocyst still presented with a monosomy 19 error.

From the 31 whole aneuploid blastocysts, a total of 42 aneuploid chromosomes were determined. Of these, 37 (88.1%) had been initially detected in the trophectoderm, composing of 18 (48.7%) gains and 19 (51.3%) losses. Five (11.6%) aneuploidies in the whole blastocyst were not detected by the trophectoderm biopsy, composing of three (60.0%) gains and two (40.0%) loses.

2.9.6 Discussion

These results indicate that the trophectoderm yielded full concordance with the whole blastocyst approximately 56% of the time. Interestingly, at the chromosomal level, 88.1%

of aneuploidies detected in the trophectoderm were detected in the whole embryo. Biological reasons for discrepancies include chromosomal mosaicism (Figure 33A), mitotic non-disjunction with equivalent number of trisomy and monosomy cells (Figure 33B), preferential sequestering of the aneuploid cells to the trophectoderm (Figure 33C) and technical errors in the CCS test.

As previously discussed, chromosomal mosaicism refers to the presence of two or more distinct cell lines and is a well-documented phenomenon during human preimplantation development. In these data, 19 of 43 (44.2%) could be classified as mosaics. Reports have demonstrated a mosaicism rate of 69%, 33%, and 16% in the blastocyst (Liu *et al.*, 2012; Fragouli *et al.*, 2011; Northrop *et al.*, 2010). Conflicting results on the rate of mosaicism could be due to the tests utilized, number of cells examined, and patient populations. Regardless, mosaicism is present in the blastocyst; what is unknown is when mosaicism becomes clinical significant. Approximately 59% of all mosaic embryos are diploid-aneuploid mosaics, composed of both diploid and aneuploid cell lines (Echten-Arends *et al.*, 2011). In this study, 11 of 43 (25.6%) of the blastocysts could be classified as a diploid-aneuploid mosaic.

Mitotic non-disjunction occurs when sister chromatids fail to separate during mitosis. Instead of separating equally, one cell receives 3 copies of a chromosome (trisomy) and the other cell receives a single copy of the reciprocal chromosome (monosomy; Taylor *et al.*, 2014a).

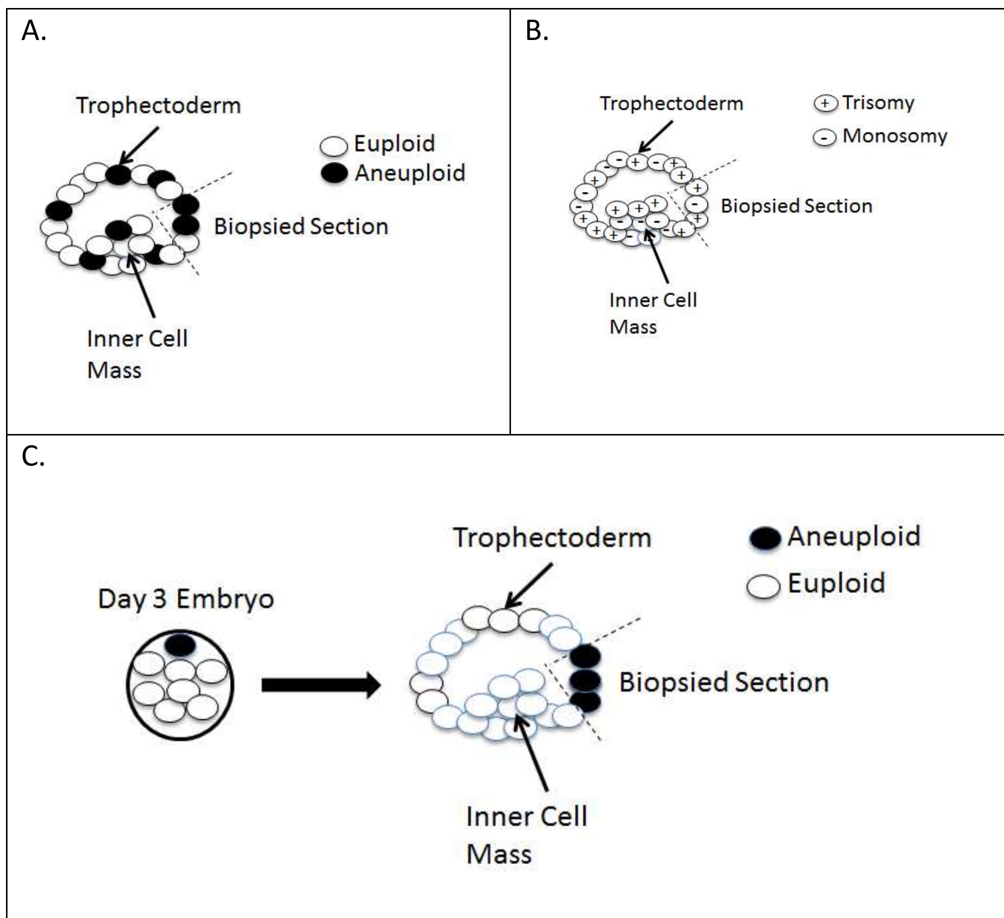
Table 21: Trophectoderm samples and their corresponding whole embryo aCGH analysis.

Full concordance		
Sample	**Trophectoderm**	**Whole Embryo**
1	-8, XX	-8, XX
2	+21, XY	+21, XY
3	+22, XY	+22, XY
4	-21, XY	-21, XY
5	-21, XY	-21, XY
6	+22, XY	+22, XY
7	-7, XX	-7, XX
8	-16, XX	-16, XX
9	-16, XX	-16, XX
10	+13, XX	+13, XX
11	+16, XX	+16, XX
12	+9, +22, XX	+9, +22, XX
13	-13, XX	-13, XX
14	XYY	XYY
15	+19, XX	+19, XX
16	-14, XY	-14, XY
17	+22, XX	+22, XX
18	+16, XX	+16, XX
19	+15, XX	+15, XX
20	+19, -22, XY	+19, -22, XY
21	+20, XX	+20, XX
22	-6p, XY	-6p, XY
23	+16, XY	+16, XY
24	-11,-18, XX	-11,-18, XX
Nonconcordant but aneuploid whole embryo		
25	XO, -15	XO; -5
26	-10, -13, XY	-10, -13, +15, XY
27	-6, -16, +20, XY	-6, -16, XY
28	+5, -6, +7, +9, -10, +15, -16, +18, XX	-16, XX
29	-9, +15, +19, XX	-9, +15, +19, +22, XX
30	-22, XY	+19, XY
31	+1q, XY	-19, XY
32	-5, +14, +21, XY	+14, +21, XY
Nonconcordant but euploid whole embryo		
33	+4, XY	Euploid, XY
34	+22, XX	Euploid, XX
35	+2, XX	Euploid, XX
36	+11, +17, +20, XX	Euploid, XX
37	-7, -8, XY	Euploid, XY
38	+8, +14, XY	Euploid, XY
39	+14, XX	Euploid, XX
40	-6, -10, -18, XY	Euploid, XY
41	+21, XY	Euploid, XY
42	-14, XX	Euploid, XX
43	-14, XX	Euploid, XX

Although this is an error, it might not be identified by aCGH because the cells are pooled together, creating the same amount of DNA that is present in euploid cells (Harton *et al.*, 2013). However, it depends on when and where the non-disjunction occurs as to how it will influence the aCGH results. For example, if the non-disjunction occurs in the zygote, then the two cell lines can propagate throughout the embryo. Depending on the number of cells biopsied from the trophectoderm, this particular scenario could yield an aneuploid trophectoderm and euploid whole embryo result. If the non-disjunction event occurred in the trophectoderm, then the number of aneuploid cells may be outnumbered by the euploid cells in the ICM, causing a euploid result. However, research has shown as high as a 96% concordance between the two tissues (Johnson *et al*., 2010).

Another possibility for the euploid result is that the majority of the embryo is in fact euploid while the trophectoderm piece was aneuploid. Research has indicated that mouse 4N->2N chimeras have the ability to exclude 4N's from the epiblast (Tarkowski *et al.*, 1977; Nagy *et al.*, 1990; James *et al.*, 1995). This demonstrates that a mechanism is in place to force abnormal cell lines out of the ICM and into the trophectoderm. This mechanism has not been demonstrated in humans or trisomies, although CPM has been documented (Kalousek and Dill, 1983). Research in the mouse has shown that as little as three cells from the ICM are destined to be the fetus (Markert and Peters, 1978). This suggests that even if aneuploidy exists in the ICM, a euploid fetus is possible. The only way to fully detect preferential allocation of aneuploidy cell lines is to individually screen each cell of the blastocyst and be able to reconstruct a virtual image of the blastocyst (Taylor *et al.*, 2016a).

Figure 33: Possible biological explanations for discrepancies between the aCGH results of the trophectoderm and whole embryo. (A) Two or more aneuploid cell lines are presented throughout the embryo resulting in an aneuploid trophectoderm biopsy and possible euploid whole embryo diagnosis. (B) The biopsied trophectoderm results in a trisomy diagnosis; however the whole embryo is diagnosed as euploid due to equal numbers of trisomy and monosomy cells. (C) An aneuploid cell in the day 3 embryo is forced to the trophectoderm, resulting in an aneuploid diagnosis whilst the ICM is still euploid.



Scott and colleagues (2010) biopsied a piece of trophectoderm but transferred the blastocyst without testing. After a live birth was obtained they retrospectively analyzed the piece of trophectoderm. They found that of the 46 blastocysts that were biopsied and transferred, 3 (6.5%) live births derived from a subsequently diagnosed aneuploidy

in the trophectoderm. It is unknown however if this aneuploid result in the trophectoderm was due to an error in the test. Given that the test performed by Scott and colleagues (2010) has shown an extremely low error rate against known cell lines (97.6%), it seems as if their results, and in combination with ours, supports the theory that human preimplantation embryos may be able allocate aneuploid cell lines to the trophectoderm (Treff and Scott, 2013). To fully identify this mechanism, isolation and testing of the ICM is needed, which was not performed in this study. Further research is needed to determine if this mechanism is purposeful, accidental, or just a product of mosaicism.

Segmental aneuploidies, or aneuploidies that only involve a piece of the embryo, are detectable by aCGH or SNP. Although detectable, the clinical significance of segmental aneuploidies during preimplantation development has yet to be established. In this study, two of the blastocysts contained segmental aneuploidies in the trophectoderm. One of these segmental aneuploidies was present when analyzing the whole embryo (#40) and one was not (#42). Therefore, some segmental aneuploidies may not have any clinical significance and may result in a euploid live birth, while others may result in miscarriages and affected live births. Segmental aneuploidy can be biological or technical in nature. For example, it is possible that the WGA procedure does not cover the area with the segmental error. Thus, the result will return a euploid result, even though there is segmental error. Further studies in regards to segmental aneuploidies are warranted.

This study does have limitations. Research has described conflicting results against known cell lines using different WGA protocols (Treff *et al.*, 2010). In a recent study, Capalbo and colleagues (2014) demonstrated a 7% error rate with aCGH, which requires WGA, compared to qPCR (quantitative polymerase chain reaction), which doesn't require WGA, and SNP, which does require WGA. In this study, of the 43 whole aneuploidies in the entire blastocyst, 37 (88.1%) were detected in the trophectoderm. Therefore, 11.9% of the aneuploidies in the whole blastocysts were not detected by trophectoderm biopsy, which is similar to the 7% error rate as determined by Capalbo and colleagues (2014). Another limitation is the number of cells removed or the quality of these cells removed for testing. However, the initial blastocyst biopsy was performed by a knowledgeable technician and were biopsied according to laboratory procedures that have resulted in high pregnancy rates. Thus, the biopsy or laboratory procedures could not have attributed to these errors and these errors are either biological or a failure in the test as previously mentioned (Taylor *et al.*, 2014a; Taylor *et al.*, 2014b)

In conclusion, these data support the theory that the biopsied piece of the trophectoderm does not always coincide with the remaining chromosome content of the embryo; however, 88.1% of single aneuploidies in the trophectoderm were detected in the whole embryo. These discrepancies could be due to mosaicism, the inability of aCGH to distinguish between mitotic non-disjunction and euploidy, preferential allocation of aneuploid cell lines to the trophectoderm, or an error in the test. Further studies are needed to distinguish between these scenarios.

3.0 General Discussion

This work has identified some of the limitations surrounding PGS testing. Given the shortcomings of PGS, certain questions still need to be answered in order to determine who may benefit from this technology. Thus, PGS at the blastocysts stage requires the understanding of a few questions.

1) Can an embryo diagnosed as aneuploidy lead to a euploid individual?
2) Is the chromosomal status of preimplantation embryos stagnant?
3) What is the chromosomal relationship between the trophectoderm and ICM?
4) Are all preimplantation aneuploidies equal?
5) What is the success rate of poor quality embryos?

3.1 Can an embryo diagnosed as aneuploidy lead to a euploid individual?

Scott and colleagues (2012) addressed this question in a well-designed study that determined the reproductive potential of CCS diagnosed cleavage stage embryos and blastocysts. Scott and colleagues biopsied both cleavage stage and blastocyst stage embryos prior to transfer. CCS results were not available until after the transfer was conducted and a fetus obtained. They found that of the 53 cleavage stage embryos that resulted in a live birth, 52 were diagnosed as euploid while only 1 (1.9%) was diagnosed as aneuploid. Of the 46 blastocysts that resulted in a live birth, 43 were diagnosed as euploid and 3 (6.5%) were diagnosed as aneuploid. This leads the authors to correctly state that those embryos diagnosed as euploid have an incredibly high reproductive

potential. Unfortunately, DNA was not obtainable in those embryos that failed to implant or miscarried; therefore, it is unknown if the diagnosis was correct. Thus, the diagnostic value of the CCS can only loosely be determined from this study.

In my study, approximately a quarter of the embryos that were diagnosed as aneuploid were, in fact, euploid. The reason for an aneuploid diagnosis leading to a euploid individual is either due to an error in the test (as no test is accurate 100% of the time), modification of the embryo by the removal of aneuploid cell(s), or mosaicism that is the presence of two or more cell lines within an individual. In my study, it is possible that the day 3 analysis was incorrect due to the complications of WGA on an individual cell. When the rebiopsy occurred at the blastocyst stage, the multiple cells present in the blastocyst biopsy allowed for a more accurate WGA procedure and the embryo was diagnosed correctly as euploid.

The phenomenon of mosaicism would lead one to believe that more aneuploid cleavage and blastocyst stage embryos could result in a euploid individual. What is unknown is that if any of those embryos that were diagnosed as euploid were mosaic, and if the researchers had biopsied different cell(s), would they have had a different CCS result. Obviously this type of approach cannot be undertaken with current technology; however, one can estimate using the current understanding of diploid-aneuploid mosaicism. Diploid-aneuploidy mosaicism is the presence of both aneuploid and diploid cell lines within the same individual and is believed to occur in approximately 59% of all cleavage stage embryos and 14% of blastocysts (van Ecten-Arends *et al.*, 2011; Northrop *et al.*,

2010). If those rates of diploid-aneuploid mosaicism are applied to the Scott *et al.* (2012) study, then of the 52 cleavage stage embryos that were diagnosed as euploid that lead to a live birth, approximately 30 (57.7%) contained aneuploid cells. Of the 43 euploid blastocysts that lead to a live birth, approximately 6 (14.0%) blastocysts diagnosed as euploid could have also been diagnosed as aneuploid had a different piece of the trophectoderm been biopsied. Therefore, those diagnosed as euploid may not be entirely euploid and those diagnosed as aneuploidy, may not be entirely aneuploid. This analysis is purely hypothetical and generalized. The rate of diploid-aneuploid mosaicism may differ upon test utilized, embryo quality, number of cells analyzed, and patient characteristics.

Mosaicism at the blastocyst stage can present in many different forms. According to research in the mouse, mosaicism in the trophectoderm may be either a checkerboard pattern or in isolated clumps (Everett and West, 1996). Whether these patterns are true of human preimplantation development is currently unknown, however the way mosaicism presents itself during the blastocyst stage can influence CCS results. For example, if aneuploid cells lines are clumped within the trophectoderm, it is entirely possible that the biopsy portion will remove a section of the abnormal cell line, thereby giving an aneuploid result while the blastocyst is truly euploid (Figure 34). The opposite also holds true in that the blastocyst could be diagnosed euploid but actually contain a large portion of aneuploid cells (Figure 35). If mosaicism presents in a checkerboard pattern, the test utilized could yield both a euploid (Figure 36) or aneuploid (Figure 37) results, depending on the number of aneuploid cells within the biopsied sample. Lastly,

it is also possible that the trophectoderm is completely euploid but the ICM is mosaic (Figure 38).

Figure 34: Mosaic blastocyst with an aneuploid cell line isolated to the trophectoderm. The section biopsied is represented with broken, black lines. If these cells were biopsied, the results would be aneuploid but the embryo would be euploid.

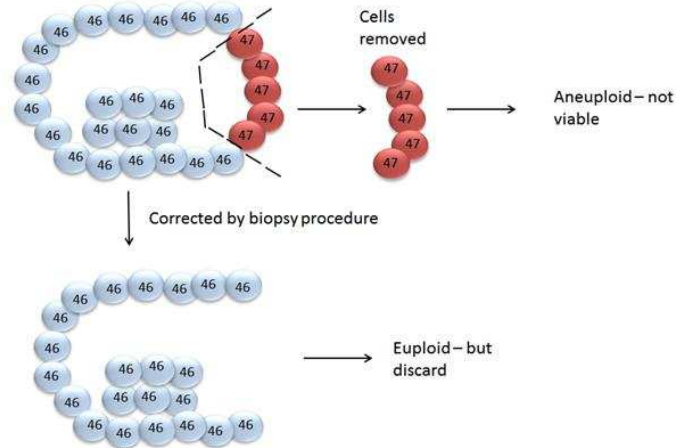


Figure 35: Mosaic blastocyst with an aneuploid cell line isolated in the trophectoderm. The section biopsied is represented with broken, black lines. If these cells were biopsied the result would be euploid but the embryo would be mosaic.

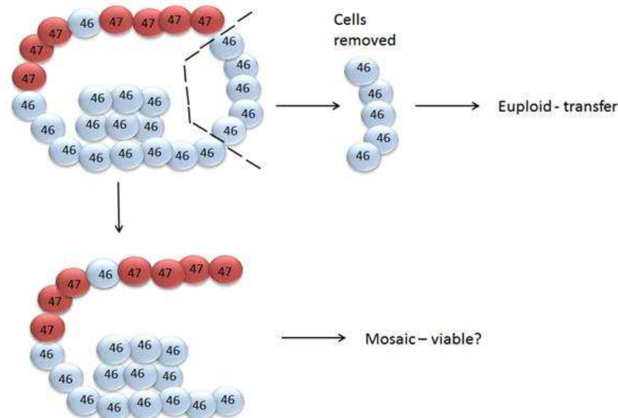


Figure 36: A mosaic blastocyst with an aneuploid cell line isolated to the trophectoderm and presenting in a checkerboard fashion. The section biopsied is represented with broken, black lines. If these cells were biopsied, the result would be euploid but the embryo would be mosaic.

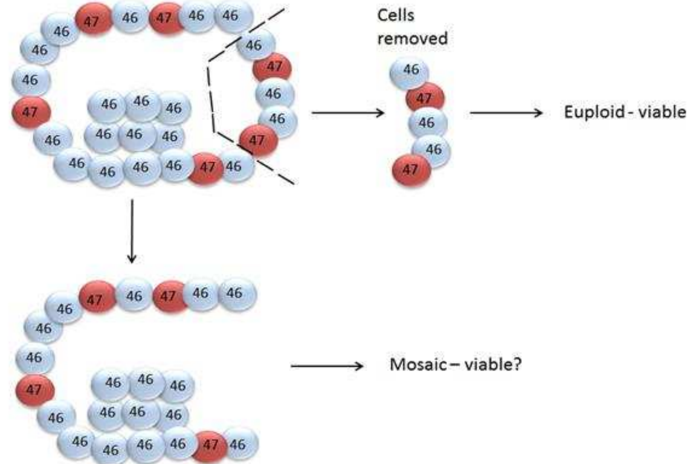


Figure 37: A mosaic blastocyst with an aneuploidy cell line isolated to the trophectoderm and presenting in a checkerboard fashion. The section biopsied is represented with broken, black lines. If these cells were biopsied, the result would be aneuploidy but the embryo would be mosaic.

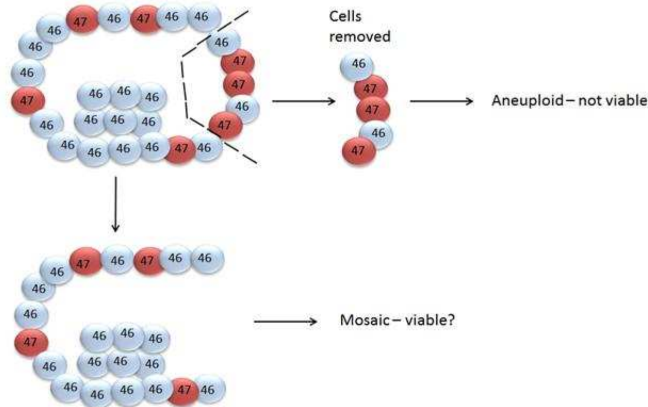
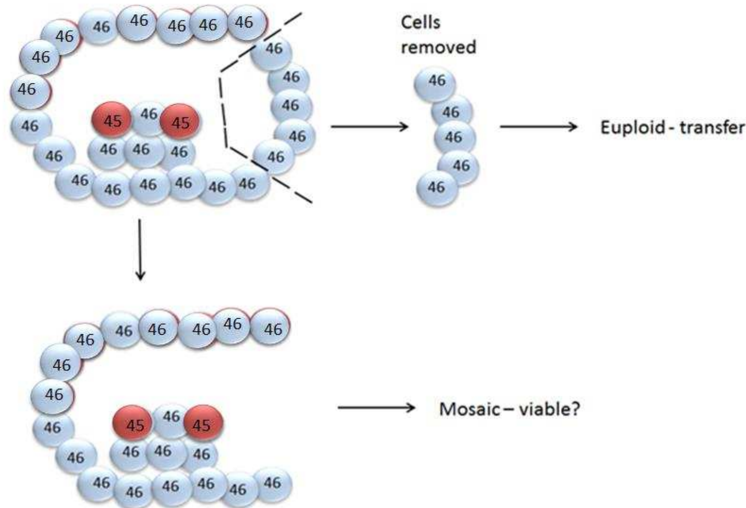


Figure 38: A mosaic blastocyst with a euploid cell line in the trophectoderm but mosaic inner cell mass. The section biopsied is represented with broken, black lines. If these cells were biopsied, the result would be euploid but the embryo would be mosaic.



Mosaicism is common and routine during both pre and post implantation development. In essence all of us are mosaic, as we all contain at least one cell that is aneuploid. For example, it is entirely possible that during cleavage stage biopsy the cell removed and diagnosed is the only cell that is aneuploid. If only one cell is aneuploid while the others are euploid, it may be possible for the embryo to develop to a euploid individual. Likewise, at the blastocyst stage, if 10 cells are removed from the trophectoderm and two of the cells are aneuploid and eight are euploid, the diagnosis is euploid; however, the blastocyst is truly an undiagnosed mosaic because two distinct cell lines are present. Although mosaic, the clinical relevance of this mosaicism at these stages is unknown and the number of cells affected, along with those aneuploidies involved, may have different influences during development.

3.2 Is the chromosomal status of preimplantation embryos stagnant?

Not only can aneuploidy occur at a single point during preimplantation and/or post implantation development but aneuploidy is also fluid, occurring at many different points and under many different conditions. An error earlier during development gives the aneuploid cell line more time to propagate and outnumber the euploid cells. Conversely, confined mosaicism involves an aneuploidy that typically occurs post implantation, thereby containing that aneuploidy within a particular area. Confined mosaicism has been reported in the brain, skin, and gonads, amongst other organs (Yurov *et al.*, 2007; Happle, 1993). For these particular cases, the fetus (pre and post implantation) could be euploid; however, an error occurs post implantation: an error that would not be diagnosed by PGS (Kalousek and Dill, 1983).

Numerous reports have re-examined blastocysts from diagnosed aneuploid cleavage stage embryos, providing evidence that cleavage stage embryos with at least one aneuploid cell can be diagnosed as a euploid blastocyst (Norhtrop *et al.*, 2010; Magli *et al.*, 2000; Fragouli *et al.*, 2008). My study contained in this thesis demonstrated that approx. 50% of diagnosed aneuploid cleavage stage embryos can develop to euploid blastocysts. Although interesting, there are multiple problems with these studies. First, these studies only examined a limited number of chromosomes at the cleavage stage or blastocyst stage, or both. Secondly, the data can be interpreted a multitude of ways. For example, Magli and colleagues (2000), examined multiple cells from the ICM of blastocysts derived from aneuploid cleavage stage embryos, six ICM's had at least one

euploid cell while only one had ≥50% euploid cells present in the ICM. This leads the authors to conclude that because ≥50% of the ICM was euploid, this embryo had successfully corrected itself. Lastly, none of these studies ascertain the reproductive potential of the aneuploid cleavage stage embryos that are diagnosed as euploid at the blastocyst stage. The study presented in this thesis using SNP on day 3 and day 5 blastocysts demonstrated that diagnosed day 3 aneuploid embryos can develop into a euploid blastocyst and can achieve a live birth. These data, along with others indicates that the chromosomal constitution of the embryo is not stagnant.

Aneuploidies can occur on every chromosome and at any time. It is possible that one aneuploidy may not propagate whilst another may become established. Moreover, the clinical significance of each aneuploidy can be examined at the individual chromosome level or preimplantation embryo as a whole. Capalbo and colleagues (2013a) examined correction at a chromosomal level. Of 62 meiotic derived aneuploidies, 12 (19.4%) were not present at the blastocyst stage, while only 5 (20.0%) of 25 mitotic errors at the cleavage stage were not present at the blastocyst stage. Lastly, Northrop and colleagues (2010) examined multiple pieces of the trophectoderm and the whole ICM after embryos were diagnosed as aneuploid at the cleavage stage. If one includes any blastocysts that had a euploid section of the trophectoderm or ICM, then 37 (74.0%) of the aneuploid cleavage stage embryos contained euploid cell lines at the blastocyst stage. However, only 29 (58.0%) embryos were completely euploid by the blastocyst stage. Using FISH, Munne and colleagues (2005) examined 24 embryos on day 12 that were diagnosed as aneuploid on day 3. They demonstrated that 7 (29.2%) were completely euploid and 11

(45.8%) were partially euploid. All of these studies suggest that aneuploidy can occur at any given moment and cleavage stage errors may not be predictive of further chromosomal status in the embryo.

It has also been shown that euploid cells proliferate at a higher rate than aneuploid cells, at least during preimplantation development (Ruangvutilert *et al.*, 2000). Moreover, it is possible that the higher rate of proliferation of euploid cell lines may be a product of natural selection, i.e., a mechanism developed by nature to combat the faulty chromosome segregation during preimplantation development. In yeast, aneuploidy has been shown to be induced under stressful conditions. The aneuploidy allows for the survival of the yeast by increasing the rate of cellular division, thereby allowing the yeast to propagate (Chen *et al.*, 2012). In mouse embryos, aneuploidy rates differ between low oxygen and atmospheric oxygen culture conditions, which supports the idea that aneuploidy is a mechanism induced by stress (Adler *et al,* 2014). If this is the case, then perhaps the presence of aneuploidy in a human embryo is a mechanism to compensate for stressful conditions. The aneuploidy is induced to overcome a conditional stress, and then once that stress dissipates, the aneuploidy is weaned out by the more aggressive growth of euploid cell lines. For example, in humans, the cleavage divisions are known to have more aneuploidies than the blastocyst stage (Braude *et al.*, 1988). It may be that the first cellular divisions are extremely stressful and aneuploidy results. Once the embryonic genome activates at the six to eight cell stage, stress is reduced and the growth of euploid cells lines is favored over aneuploid cell lines (Lejeune *et al.*, 1959). Therefore, aneuploidy maybe a correction mechanism, and although the embryo may be under

stress, the embryo could potentially be viable. Nonetheless, chromosomes are not stagnant, and each stage of development may be more or less prone to aneuploidy.

3.3 Are all preimplantation aneuploidies equal?

This is perhaps one of the largest misconceptions within IVF. For example, it is known that trisomy 21, or Downs Syndrome, can result in a live born, whereas a monosomy (except 45 XO) is lethal shortly after implantation, and triple X syndrome or 47, XXX is fairly benign and often goes undiagnosed (Lejeune *et al.*, 1959; Ford *et al.*, 1959; Gustavson, 1999). There are others, but these examples demonstrate that individual aneuploidies have different outcomes. It may be that certain preimplantation aneuploidies are benign and impact little during the course of development, while other aneuploidies have extreme clinical consequences. However, when PGS is conducted, all aneuploid embryos are diagnosed as abnormal and discarded; thereby treating all aneuploidies equally, even those that may present with little to no clinical significance.

Every chromosome acts differently and is influenced by different factors at each stage of development (Griffin, 1996). Precocious separation of sister chromatids is the most common error during meiosis while non-disjunction is more prominent in the sex chromosomes during the first cleavage divisions (Bean *et al.*, 2002, 2001; Handyside *et al.*, 2012; Forman *et al.*, 2013a). Alternatively, the autosomes are more prone to anaphase lag during preimplantation development (Capalbo *et al.*, 2013a; Ioannou *et al.*,

2012; Coonen *et al.*, 2004). Lastly, rates of aneuploidy differ between each chromosome during preimplantation development.

Blastocyst biopsy in conjunction with PGS determines the chromosomal constitution of the trophectoderm, which is destined to become the placenta, along with other extraembryonic tissue. The placenta is biopsied during CVS to determine the chromosomal constitution of the fetus. It is known that certain aneuploidies that present within the placenta are not present in the fetus. For example, trisomy 13, 18, or sex chromosome aneuploidies are present in CVS and will typically present in the fetus (Cytogenetic, 1994). On the other hand, trisomies 2, 3, 7, and 8 typically do not present with adverse effects and the fetus is typically euploid (Wolstenholme, 1996; Kalousek *et al.*, 1996). It is unknown if these relationships are merely a product of post-implantation development or are also relevant during preimplantation development.

Similarly, there are differences between aneuploidies seen in the extraembryonic mesoderm (derived from the ICM) and the cytotrophoblast (derived from the trophectoderm). For example, trisomy 2 and 17 typically present themselves within the EEM while trisomy 3, 6, 7, 12, 13, 14, 16, 18, 20, 21, and 22 typically are associated with the cytotrophoblast (Ledbetter *et al.*, 1992). It is evident that aneuploidies act and are influenced by different factors at every stage of development. Thus, treating all aneuploidies as equal during preimplantation development should be reconsidered.

3.4 What is the Chromosomal Relationship between the Trophectoderm and ICM?

CPM occurs in roughly 1-2% of all pregnancies indicating that discrepancies between the ICM and trophectoderm exist (Ledbetter *et al.*, 1992). However, when IVF utilizes blastocyst biopsy and an aneuploidy is diagnosed, it is assumed that this aneuploidy is present throughout the embryo as well. Research has shown a high concordance between the trophectoderm and ICM (Capalbo *et al.*, 2013b). However this research was conducted using FISH, which may not be accurate (Treff *et al.*, 2010). Johnson and colleagues (2010) examined all the chromosomes from the ICM and trophectoderm and found a 96% concordance rate between the two tissues. These differences in mosaicism could be attributed to patient characteristics, as patients with different diagnosis tend to have different rates of aneuploidy. Research has also shown that mosaicism is common at the blastocyst stage; therefore, a small piece of the trophectoderm may not be representative of the entire embryo (Liu *et al.*, 2012). Indeed, mosaic trophectoderm has been shown in approximately 20% of embryos (Northrop *et al.*, 2010; Fragouli *et al.*, 2011).

This study examined individual cells from the blastocyst and determined that mitotic errors are common. In fact, all of the blastocysts presented with at least two different cell lines meaning that every blastocyst was mosaic.

As previously discussed, the fetus is derived from the ICM. However, not all of the cells from ICM become the fetus. In fact, it is believed as little as three cells are needed to

become the fetus, while the reminder of the ICM is destined to become extraembryonic tissue (Markert and Peters, 1978). This poses a potential problem, as one is unable to determine, which cells within the ICM will become the fetus and those that are destined for extraembryonic tissue. It may very well be possible that only three euploid cells are needed to be present in the ICM to develop into a euploid individual. The chromosomal status of the remaining ICM cells may be irrelevant.

The goal of genetic testing is to determine the chromosome composition of the fetus. This is done via CVS, amniocentesis, or PGS. Ironically, the majority of genetic sampling is not conducted on the fetus; therefore, understanding the relationship between tested material and the fetus is essential. For example, CVS sampling is known to have roughly a 6% rate of maternal contamination (Grifo *et al.*, 2013). Consequently, if CVS sampling demonstrates an aneuploid result, amniocentesis is typically recommend as a follow up. This demonstrates that aneuploidies can present within extraembryonic material that do not present within the fetus proper. In a way, amniocentesis acts as a double check for CVS results, no such double check exists for PGS. It may be plausible to suggest that embryologist's rebiopsy all embryos that have been diagnosed as aneuploid to confirm their aneuploidy status.

3.5 What is the Success Rate of Poor Quality Embryos?

CCS at the blastocyst stage provides pregnancy rates comparable to anonymous oocyte donation (Handyside, 2013b). Unfortunately, studies have been conducted on patients that generate a fair amount of blastocysts; therefore, these patients are typically not poor responders. An increase in pregnancy rates with blastocyst biopsy theoretically makes sense considering there are two selection mechanisms, morphology and chromosomal constitution, as opposed to just standard morphology (Handyside, 2013b). With blastocyst biopsy, embryos are given until day 6 to develop. If they fail to progress to a blastocyst by day 6, they are discarded. Research has shown that live births have occurred from day 7 blastocysts; therefore, the discarding of non-biopsiable blastocysts on day 6 may be disposing of viable embryos (Kovalevsky *et al*, 2013).

Blastocysts that undergo PGS would have eventually been transferred (i.e., if they didn't undergo genetic testing they would be transferred or cryopreserved without biopsy because they were of optimal quality). Therefore, PGS at the blastocyst stage does not increase pregnancy rates, it decreases time to pregnancy. One could even argue that if there is only one or two blastocyst available, the time to pregnancy is actually increased. For example, results are typically not available same day, so if there are only one or two blastocysts (which would have been transferred anyway), then delaying the transfer until a FET would only delay the time to pregnancy. Not to mention the added cost and potential harm caused by the biopsy and vitrification/warming procedure. Therefore, one could make an argument that PGS on poor responders actually lengthens time to

pregnancy and increases the risk of embryo death, thereby having no beneficial effect to the patient. So, what should we do with patients that are not producing any blastocysts in culture? As a field, do we discard embryos because they are not blastocysts? The data generated in this thesis concludes that the reproductive potential of these poor quality embryos is minimal, around 1.5% and thus I believe that the utilization of only good and fair quality blastocysts is justifiable.

4.0 Conclusion

It is not just blastocyst biopsy and PGS that may be discarding viable embryos. For example, zygotes displaying one pronuclei (as opposed to two) may contain a Y chromosome, indicating that these embryos may be viable (Staessen and Van Steirteghem, 1997). Furthermore, the vitrifying and warming (even if they are optimal quality), could potentially result in the loss of viable embryos. Lastly and as previously discussed, live births have occurred from day 7 blastocysts (Kovalevsky *et al.*, 2013). Although a blastocyst has to form in order for implantation to occur and given that day 7 blastocysts have been shown to implant and produce liveborns, perhaps we should rethink day 6 as the final day of culture.

One of the benefits of blastocyst biopsy with CCS is the effectiveness of this technique at limiting multiple births. However, this is simply because it adds another selection criteria in addition to extended culture (Forman *et al.*, 2013b). However, the application of this

technology may also be preventing patients from achieving pregnancy and potential live born. The more blastocysts that are tested, the more likely the patient will meet the assumptions described and could benefit from blastocyst biopsy with CCS. Some patients, especially poor responders, who produce a single blastocyst or no blastocysts during IVF treatment, may not benefit from blastocyst biopsy and CCS. However, it should be noted that female age is the main determinant of aneuploidy and thus, aneuploidy screening may be warranted in an older patient population. Moreover, there are a multitude of factors that are not discussed that could influence these data, including embryo quality, sperm quality, maternal age, paternal age, PGS methodology, site of biopsy, etc. It is imperative that we continue to research these areas in an attempt to perfect IVF and PGS.

Given the inability of this field to determine which embryo is viable and which is not, it is important to discuss the limitations of CCS with anyone considering PGS. The goal of IVF is to give every patient that undergoes retrieval a chance at pregnancy, not just those that produce blastocysts in culture.

4.1 Future Work

Although the technologies of PGS have advanced radically in the previous years, the application of this technology still remains as controversial as ever. The attractiveness of a test that can predict those embryos which will implant and those that cannot is the

"silver bullet" for all of IVF. Future work needs to focus on technical limitations of PGS testing.

Technical limitations of PGS testing include the biopsy site and technique. As described in this work, perhaps the location of the biopsy influences results; thus, embryologists should be more aware of what piece of the trophectoderm they are removing. Moreover, it seems logical to suggest that during the biopsy procedure, chromosomes may be loss in the media due to the excessive laser heat or from the ripping and tearing that is required to remove a piece of the trophectoderm. If a chromosome is damaged during the biopsy procedure or lost in the culture media, then false aneuploidy readings are to be expected. This begs the question, how much of our understanding of preimplantation genetics is wrong simply due to technical error? It is entirely possible that we are not as mosaic as we believe and that the presence of mosaicism is merely a technical artifact created during the biopsy procedure. Non-invasive biopsy procedures should also be pursued. Currently, removing a piece of the trophectoderm has not demonstrated a reduction in implantation potential; however, the process is still invasive. The ability to correctly determine the chromosome content of the embryo without biopsy could be extremely beneficial. Small studies have been performed which have demonstrated the ability to obtain the chromosomal content of embryo by testing the surrounding culture media and should be pursued further (Shamonki *et al.*, 2016; Feichtinger *et al.*, 2017). Further studies are warranted to understand the contributions of the biopsy procedure to the detection of aneuploidy.

Biologically, aneuploidy is induced in the cytotrophoblast during implantation; thus, the aneuploidy detected may be a biologically normal event (Weier *et al.*, 2005). Also, CPM occurs in approximately 6% of all pregnancies (Kalousek *et al.*, 1991; Griffin *et al.*, 1997). Both of these signify a difference between the fetus and placenta. To overcome these biological pitfalls perhaps a different biopsy technique needs to be developed. In the mouse, biopsies of the ICM have resulted in live births. Perhaps future research needs to develop a technique that can determine the chromosomal constitution of the actual fetus as opposed to the placenta (Dittrich *et al.*, 2011). In fact, I have already started to conduct these studies. Using a small gauge ICSI needle, a small portion of the ICM is aspirated into the needle, expelled, and placed in a PCR tube for PGS testing. Pilot studies have yielded PGS results that are similar to the trophectoderm. Many more samples need to be taken and results analyzed before any conclusions can be drawn, but the direct sampling of cell lines that will become the actual fetus is an important aspect that should be explored.

Finally, future research needs to focus on the clinical significance of aneuploidy. For example, some aneuploidies may present with clinical consequences and some may not. What if the presence of trisomy 10 or monosomy 12 in the trophectoderm has no clinical implications? Obviously, there are differences between a blastocyst diagnosed with trisomy 21 as opposed to monosomy 4, for example. Instead of discarding embryos because they are aneuploid, what if we ranked them based on the severity of the clinical consequences of that corresponding aneuploidy? This way, a patient can still opt for transfer knowing the limitations and consequences of those aneuploidies. With this

viewpoint, almost every embryo can be deemed viable and the discarding of potential babies can be limited.

4.2 Personal Perspective

I have found that this field is divided into two camps. The first is the geneticists who feels that everything must be perfect and euploid to deliver a healthy baby. The second camp, and likely the one I fall into, is the embryologist who is on the front lines, biopsing and discarding embryos, and perhaps most importantly seeing and feeling the emotions patients have when you tell them that we have to discard their embryos because they are abnormal. I have seen numerous patients consulted to pursue egg donation because they produced all aneuploid embryos. What if my biopsy technique contributed to that decision? What if something I did caused this patient not to get pregnant? What if the PGS testing was inaccurate and those embryos could have resulted in a live birth? We will never know.

Due to the lack of studies surrounding good quality/euploid embryos and the plethora of retrospective data, I feel larger well controlled, randomized controlled trial would benefit not only me but the field, in better understanding preimplantation aneuploidy and its clinical consequences.

I feel I have a better grasp on preimplantation genetics but I'm no closer to an answer than I was before this research started. Understanding the limitations of PGS testing

makes me more of a critic than a believer. The deeper I dig, the more confused I become. How is it possible that an embryo produces this much aneuploidy? Are we really that horrible at reproducing, or is our knowledge flawed because we are examining preimplantation outside of its natural environment? There is research to suggest that aneuploidy is induced to overcome stress and once that stress is overcome, the aneuploidy is phased out (Chen *et al.*, 2009). So is aneuploidy a phenomena induced by our culture system used during IVF? Are the techniques employed in the IVF lab influencing aneuploidy (Swain *et al.*, 2016)? Is aneuploidy a corrective measure by the embryo to overcome stress? I guess it boils down to one question, is aneuploidy all that significant during preimplantation development? I still don't know.

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