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## Fluorescence in Situ Hybridization Analysis of Human Embryos Derived From in Vitro and in Vivo Matured Oocytes

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**FLUORESCENCE IN SITU HYBRIDIZATION ANALYSIS OF  
HUMAN EMBRYOS DERIVED FROM  
IN VITRO AND IN VIVO MATURED OOCYTES**

by

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B.S. May 1988, Siena College**

**A Dissertation submitted to the Faculty of  
Old Dominion University  
and  
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**DOCTOR OF PHILOSOPHY**

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**OLD DOMINION UNIVERSITY  
EASTERN VIRGINIA MEDICAL SCHOOL  
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## **ABSTRACT**

### **FLUORESCENCE IN SITU HYBRIDIZATION ANALYSIS OF HUMAN EMBRYOS DERIVED FROM IN VITRO AND IN VIVO MATURED OOCYTES**

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August 1997  
Director: Dr. Susan Lanzendorf**

Despite adequate hormonal stimulation, oocytes collected for the purpose of in vitro fertilization and embryo transfer display several levels of nuclear maturity. Preovulatory or mature oocytes, technically those that are Metaphase I or II, are inseminated shortly after aspiration and assessed for fertilization the following day. Prophase I oocytes, also called germinal vesicle-bearing or immature oocytes, require a 24-36 hour period in culture before being exposed to spermatozoa. During this time, the majority of Prophase I oocytes complete nuclear maturation in vitro, progressing from germinal vesicle breakdown through first polar body extrusion. If inseminated, many in vitro matured oocytes fertilize and appear to develop normally. However, compared to embryos derived from mature oocytes, embryos derived from Prophase I oocytes produce significantly fewer pregnancies following intrauterine transfer. To determine if the reduced developmental potential of embryos derived from Prophase I oocytes can be explained in part by an increase in nuclear and/or genetic abnormalities, this study used Fluorescence In Situ Hybridization analysis to compare 65 embryos derived from oocytes that were Metaphase I or II at aspiration to 61 embryos derived from oocytes that were Prophase I at aspiration. Although there was no difference in the incidence of multinucleated blastomeres in the two groups, embryos derived from Prophase I oocytes had a

significantly higher incidence of both anuclear blastomeres and blastomeres with a fragmented nucleus compared to their counterparts derived from mature oocytes. Because nuclear fragmentation is a hallmark of programmed cell death and subsequent apoptosis, which has been implicated in the processes of follicular atresia in vivo and cleavage arrest in vitro, we speculate that Prophase I oocytes obtained following controlled ovarian hyperstimulation originate from follicles in early stages of atresia. This study found no difference in the rate of aneuploidy for chromosomes X, Y, and 18, or in the incidence of mosaicism involving these chromosomes in the two groups of embryos. However, according to our classification system, 23% of embryos derived from Metaphase I or II oocytes were normal compared to only 3% of embryos derived from Prophase I oocytes. Our findings suggest that few embryos derived from Prophase I oocytes are normal, perhaps explaining in part why they rarely establish pregnancies in our IVF program.

I wish to dedicate this work to my parents,  
Orlando and Christine Montalto,  
My husband,  
Dominic,  
And our children,  
Cristina and Michael

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## **I. INTRODUCTION**

At or shortly after birth, the human ovary consists of millions of follicles, each containing an oocyte that has begun meiosis only to become arrested early in the first meiotic division at a stage referred to as the dictyate stage. The nucleus of the oocyte, defined as the germinal vesicle, has become arrested in Prophase I of meiosis, and will remain arrested throughout childhood. Following the maturation of the hypothalamic-pituitary-ovarian axis that occurs at puberty, a 28-35 day reproductive cycle is established. During each cycle, under appropriate hormonal stimulation, a group of follicles is "recruited" to begin growth and development. Approximately halfway through the cycle, a gonadotropin (follicle stimulating hormone and luteinizing hormone, called FSH and LH respectively) surge occurs, inducing the resumption of meiosis in a single oocyte, that within the "dominant follicle". The remaining follicles, and the oocytes within, will have succumbed to atresia at various stages of development. Progression of this oocyte through germinal vesicle breakdown (GVBD) to first polar body extrusion follows, and ovulation ensues. The nucleus of the ovulated oocyte is once again arrested, this time in metaphase of the second meiotic division (Metaphase II), and it will remain arrested until fertilization occurs. This description of oogenesis and the more detailed one that follows, represent decades of work by many investigators as reviewed by Byskov (1982), Baker (1982), and Wassarman and Albertini (1994).

Reproductive endocrinologists have developed controlled ovarian hyperstimulation (COH) protocols which utilize the exogenous administration of gonadotropic hormones to increase the number of mature oocytes available for assisted reproductive techniques. By artificially increasing the serum level of

FSH, the cohort of developing follicles can avoid the usual "selection" phase in which all but the dominant follicle undergo atresia, allowing the oocytes in all of the recruited follicles to achieve growth and maturation. Prior to ovulation, the oocytes are collected in a transvaginal, ultrasound-guided procedure, and used for in vitro fertilization and embryo transfer (IVF-ET). Despite adequate hormonal stimulation, these fully-grown oocytes demonstrate various levels of nuclear maturity. Preovulatory, or "mature" oocytes, technically those in Metaphase I or Metaphase II of meiosis, are inseminated shortly after collection and assessed for fertilization the following day. Prophase I oocytes, also called germinal vesicle bearing or "immature" oocytes, must be cultured for 24-36 hours to permit germinal vesicle breakdown and first polar body extrusion before being exposed to spermatozoa. Despite the fact that the majority of such in vitro matured oocytes do fertilize and appear to cleave normally, the resulting embryos demonstrate an extremely low incidence of pregnancy following intrauterine transfer when compared to embryos derived from oocytes that were mature at collection (Reviewed by Edwards and Brody, 1995).

The reduced developmental potential of embryos derived from in vitro matured oocytes is well-documented but poorly understood. The purpose of this study was to compare the rate of aneuploidy and the incidence of mosaicism in embryos derived from in vitro matured oocytes to that observed in embryos derived from in vivo matured oocytes using Fluorescence In Situ Hybridization (FISH), a technique with proven application for the genetic analysis of human embryos.

#### A. Oogenesis

Oogenesis, the formation, growth, and maturation of the oocyte, begins

early in fetal life when, under the influence of two X chromosomes, (and in the absence of a Y chromosome), the primitive bipotential gonads begin to differentiate into ovaries. Primordial germ cells, identifiable as early as 24 days after conception, migrate from extra-embryonic sites to a region of tissue called the genital ridge located on the ventral surface of the primitive kidney, the mesonephros. Upon arrival at the genital ridge, which will form an ovary in the female, the germ cells are called oogonia. As a result of vigorous mitotic activity, their number rapidly increases from 600,000 at eight weeks of gestation to nearly 7,000,000 by twenty weeks of gestation. Upon entering meiosis, a process which begins around 12 weeks of gestation immediately following sexual differentiation, the oogonia are called oocytes. During the second half of fetal life, the number of oocytes falls to approximately 2,000,000 at birth. This dramatic decline is the result of chromosomal breaks and other errors leading to oocyte degeneration that occur early in the first meiotic division, as described below.

Meiosis, a series of two cell divisions in which the number of chromosomes is reduced from diploid to haploid, begins before birth and is not truly completed until, or unless, sperm penetration occurs following ovulation, many years later. The first meiotic division begins shortly after the last mitotic division of the oogonium is completed. Following interphase, in which the DNA is replicated in preparation for meiosis, the primary oocyte enters Prophase I. This phase is exceptional for two reasons. First, it is during Prophase I that crossing over takes place and second, Prophase I is characterized by a prolonged resting phase in females during which development is arrested.

Prophase I can be divided into five stages called leptotene, zygotene, pachytene, diplotene, and diakinesis. Leptotene begins when, in cytological

preparations, the diploid number of chromosomes can be identified, each chromosome comprised of two identical chromatids. During zygotene, homologous chromosomes of maternal and paternal origin associate and eventually attach forming pairs called “tetrads” in a manner so precise that homologous genes associate with one another. “Crossing over”, in which the exchange of genetic material between pairs of homologous chromosomes occurs, happens during pachytene. Diplotene is characterized by the separation of homologous chromosomes except at those places in which crossing over has occurred, termed chiasmata. Crossing over is critical in that this reassortment of genes ensures that the genetic material of the oocyte is unique.

In humans, Prophase I is completed through diplotene in all oocytes at or shortly after birth. The oocytes then enter a prolonged resting stage termed dictyate or the dictyotene stage. Although meiosis is arrested during dictyate, (which lasts throughout childhood until puberty, when the maturation of the hypothalamic-pituitary-ovarian axis occurs), dictyate is not truly a “resting stage”. Cytoplasmic organelles such as Golgi apparatus, endoplasmic reticulum, and ribosomes are present in oocytes at dictyate, and both transcription and translation actively occur (Telford et al., 1990; Wassarman and Kinloch, 1992). The “lampbrush” chromosomes present, characteristic of those found in the oocytes of many vertebrate and invertebrate species, bear lateral projections which replicate ribonucleic acid (RNA). Since transcription ceases at the time of ovulation (Telford et al., 1990), this RNA guides protein synthesis in the oocyte and organizes the early development of the embryo following fertilization until activation of the embryonic genome occurs, believed to be between the 4- 8 cell stage in the human (Braude et al., 1988).

The term "primordial follicle" is given to the oocyte at dictyate when it becomes surrounded by a single layer of cuboidal "theca" cells. These layers increase in number by mitosis, until the oocyte, in its arrested meiosis, is surrounded by several layers of cells called granulosa cells. These cells, along with the oocyte itself, participate in the formation of the zona pellucida, a mucoid material which ultimately surrounds the oocyte. Granulosa cell processes penetrate the zona pellucida, contacting the oocyte in various regions, permitting exchange of both substrates and waste materials. Eventually, follicular fluid accumulates in the spaces between the granulosa cells, and the follicle is said to be vesicular. These spaces coalesce to form a single antrum, transforming the follicle into a preovulatory or Graafian follicle.

The process of follicular growth described above, resulting in the development of vesicular and finally Graafian follicles from that of the primordial type, begins shortly before birth and continues throughout childhood in a small fraction of the follicle pool at all times. Before the onset of puberty, all such growing follicles undergo degeneration at some point in their development, a process called follicular atresia, due to a low serum FSH level. Thus, of the 2,000,000 oocytes present at birth, about 250,000 remain at the age of seven years. At the onset of puberty, approximately 150,000 are viable. Following the maturation of the hypothalamic-pituitary-ovarian axis that occurs at puberty, a 28-35 day reproductive cycle is established. Only at this time, with the correct hormonal support (i.e. FSH), is a growing follicle allowed to proceed to the point of ovulation. During each cycle, although many follicles begin to grow, only one oocyte is induced to resume meiosis and proceed to ovulation. The remaining follicles undergo atresia. During a woman's reproductive lifetime, no more than 400 oocytes are normally ovulated.

Shortly before ovulation, approximately 36 hours in the human female, there is a dramatic increase in the release of gonadotropins from the pituitary gland, termed the "LH surge". This surge results in the final maturation of the Graafian follicle, inducing a wave of mitosis in the granulosa cells. Those cells immediately adjacent to the oocyte, the cumulus oophorus, become columnar in shape and eventually separate from the remaining membrana granulosa, rendering the oocyte "free-floating" within the follicle or loosely attached to the follicle wall.

The LH surge also induces the resumption of meiosis in the oocyte to be ovulated. Meiosis progresses to diakinesis, also called the germinal vesicle stage since the nucleus of the oocyte at this time is defined as the germinal vesicle. Thus, the prophase of the first meiotic division begun so long ago is finally completed. Metaphase I is rapidly followed by Anaphase I and then Telophase I. Homologous chromosomes separate, and an unequal division of cytoplasm occurs, resulting in a rather large secondary oocyte and a small first polar body. Meiosis continues to Metaphase II, which in the human female represents another stage of arrested development similar to dictyate. It is at this time that ovulation generally occurs. Completion of the second meiotic division is dependent upon penetration of the oocyte by a spermatozoon at fertilization, and an unequal division of cytoplasm occurs once again, resulting in the extrusion of a small second polar body.

Great strides have been made in recent years toward understanding gene expression during oogenesis. Maturation-promoting factor (MPF), a protein dimer consisting of a catalytic subunit composed of a serine threonine kinase, and a regulatory subunit composed of cyclin-B (Pines and Hunter, 1989), is a key regulatory component of the cell cycle in both meiotic and mitotic

cells (Murray et al., 1991). During oogenesis, two peaks of MPF activity have been demonstrated (Mattioli et al., 1991). The first one occurs at the resumption of meiosis following dictyate, and the second during meiotic arrest at Metaphase II. The proto-oncogene *c-mos* has been implicated in the up-regulation of MPF activity that occurs at both these times (Sagata et al., 1989). The protein product of *c-mos* is a kinase which enhances MPF activity directly by phosphorylating the cyclin-B subunit (Roy et al., 1990), and indirectly by interfering with its proteolytic degradation (O'Keefe et al., 1991). A study performed at the Jones Institute (Heikinheimo et al., 1995) supports the theory that *c-mos* messenger RNA is a stored maternal message that is translated in a temporally-specific manner during oogenesis, allowing *c-mos* kinase to play a vital role in meiotic maturation.

#### **B. In Vitro Maturation of Oocytes**

Despite vast improvements in the success of assisted reproduction for the treatment of infertility, from the cryopreservation of human embryos to the use of intracytoplasmic sperm injection (ICSI) for the achievement of fertilization, there has been little success with the maturation of human oocytes in the laboratory. As mentioned earlier, oocytes collected following COH for the purpose of IVF-ET display varying levels of nuclear maturity. Preovulatory, or mature oocytes, those which have been induced to resume meiosis in vivo, are inseminated shortly after collection. These oocytes demonstrate high fertilization rates in vitro and, upon transfer to the uterus, the resulting embryos initiate significantly more pregnancies than embryos derived from oocytes that were Prophase I at collection.

Prophase I oocytes are cultured for 24-36 hours before being reassessed

for maturity. It has been the Norfolk experience that by this time, the majority (80%) have achieved meiotic competency, progressing from germinal vesicle breakdown through first polar body extrusion (Veeck, 1984). Upon insemination with freshly prepared spermatozoa, approximately 80% of such "in vitro matured" oocytes fertilize and appear to undergo normal development. However, when the resulting embryos are transferred to the uterus, an extremely low incidence of pregnancy (4%) is realized (unpublished data from the Jones Institute data base).

Despite the fact that COH protocols producing the greatest number of mature oocytes are utilized, approximately 22% of the oocytes collected for the purpose of IVF-ET are Prophase I (unpublished data from the Jones Institute data base). Several investigators have modified in vitro culture conditions, generally believed to be suboptimal, in an attempt to improve in vitro maturation rates, and enhance the development and pregnancy potential of embryos derived from oocytes matured in vitro.

Prins and coinvestigators (1987) reported that immature oocytes cultured for 24-34 hours in medium supplemented with LH and FSH demonstrate higher maturation rates (73.5% versus 35.6%) and fertilization rates (64.0% versus 36.4%) than control oocytes cultured under standard conditions. The authors were unable to determine if gonadotropin supplementation of culture medium increased the ability of these oocytes to initiate pregnancy, as the resulting embryos were transferred with embryos derived from oocytes matured in vivo.

Other attempts to improve the in vitro maturation and development of immature oocytes collected following COH have utilized coculture. This technique, which involves culturing oocytes and embryos along with various somatic cells such as epithelial cells and fibroblasts from numerous sites, or

granulosa cells, is an effort to mimic the environment within the ovarian follicle.

Dandekar et al. (1991) reported that “immature oocytes” cultured with granulosa cells obtained from preovulatory follicles from the same patient demonstrated higher maturation and fertilization rates compared to “immature oocytes” cultured without granulosa cells (59% versus 35% and 54% versus 20% respectively ). Unfortunately, nuclear maturation was not assessed in this study and oocytes were classified as “immature” based solely on cumulus and corona morphology rather than on the presence of a germinal vesicle. In addition, because the embryos were transferred along with embryos derived from in vivo matured oocytes, the authors were unable to determine if there was a difference in the pregnancy potential between the two groups.

Janssenswillen and coworkers (1995) reported that human cumulus-free immature oocytes cultured with green monkey kidney epithelial cells (Vero cells) demonstrate a higher maturation rate than similar oocytes cultured in medium alone. Thirty hours after collection, 82% of the immature oocytes in the coculture group progressed to first polar body extrusion compared to 38% of those cultured in medium alone. Because all in vitro matured oocytes were used for other studies, the authors were unable to assess their fertilization and further development .

Using the cynomolgus monkey model, Lanzendorf and coinvestigators (1996) have shown that the developmental potential of embryos derived from immature oocytes is significantly improved following coculture with Vero cells. In this study, 40% of embryos derived from in vitro matured oocytes reached the expanded blastocyst stage when cultured with Vero cells compared to 0% when cultured in medium alone. Interestingly, embryos derived from in vivo matured oocytes did not benefit from coculture, reaching the expanded blastocyst stage

at a rate of 33% in both treatment groups. The authors concluded that embryos derived from in vitro matured oocytes differ in culture requirements when compared to embryos derived from oocytes matured in vivo.

The studies described above demonstrate that it is possible to improve the developmental potential of Prophase I oocytes on two levels: at the level of the oocyte, by improving in vitro maturation and fertilization rates; and, at the level of the embryo, by improving development to the expanded blastocyst stage. However, because embryos derived from Prophase I oocytes are rarely transferred alone, it is unclear if these improvements translate into improvements in pregnancy rates.

The fact that some Prophase I oocytes undergo maturation, fertilization, and normal development in vitro and, upon transfer to the uterus, are capable of implanting and progressing to live births, is of great interest to infertility specialists. It suggests that, in the future, IVF-ET may be possible with little or no exogenous hormonal stimulation, particularly if the developmental potential of in vitro matured oocytes improves such that it approximates that of oocytes matured in vivo during COH. This is extremely important given the fact that the long-term effects of COH on the patient remain largely unknown. In addition to the possibility that there is an association between ovarian stimulation and an increased risk of ovarian cancer, the severe form of ovarian hyperstimulation syndrome, although rare, represents a critical illness (Edwards and Brody, 1995).

The developmental potential of Prophase I oocytes is also of great interest for another reason. Although the cryopreservation of mature human oocytes has met with little success, Toth and coworkers (1994) have reported that Prophase I human oocytes, obtained from both stimulated and unstimulated

ovaries, are able to survive cryopreservation and undergo nuclear maturation in vitro following thaw. The oocytes obtained from unstimulated ovaries were not inseminated, but those obtained from stimulated ovaries, upon insemination, demonstrated fertilization and cleavage rates similar to that of control oocytes. Although the cryopreservation of Prophase I oocytes may be an option for women, particularly those anticipating loss of ovarian function following extirpative therapy, radiation, or chemotherapy, the low pregnancy rate realized after intrauterine transfer of embryos derived from these oocytes remains a serious concern.

Cytogenetic analyses of Metaphase II human oocytes obtained after ovarian hyperstimulation have been limited not only by the scarcity of material, but also by the inefficiency of the karyotyping procedure. These studies have consistently found, however, that many such oocytes are aneuploid, containing a chromosomal constitution different from the normal haploid constitution by loss or duplication of one or more chromosomes or chromosome segments. Van Blerkom and Henry (1992) reported that 25-40% of mature oocytes collected after COH are aneuploid, and suggested that this may result from exposure to abnormal follicular conditions during a critical point in maturation. This is in sharp contrast to a 1-3% aneuploidy rate in Prophase I oocytes allowed to undergo maturation in vitro (VanBlerkom, 1989). It must be noted, however, that the immature oocytes in this study were obtained from unstimulated ovaries, and whether or not this low aneuploidy rate applies to immature oocytes collected following COH remains unknown. Indeed the fact that the follicles containing Prophase I oocytes were able to be identified and punctured during the retrieval, suggests at least a limited exposure and response to the gonadotropins used for stimulation. This suggests that the

Prophase I oocytes collected after COH may be different from those obtained from unstimulated ovaries, but this remains to be seen.

Mammalian oocytes released from follicles and placed in culture undergo meiotic maturation spontaneously (Pincus, 1935; Edwards, 1965; Eppig, 1985), provided they have reached a species-specific minimum size (Iwamatsu, 1975; Sorensen, 1976; Lanzendorf, 1992; Durinzi, 1995). Investigators have shown that increased levels of intracellular cAMP maintain meiotic arrest in vitro (Schultz, 1983), and that a significant decrease in the intracellular cAMP level precedes GVBD in the oocyte, both in vitro and in vivo (Dekel, 1980). It has been suggested that a cAMP-dependent protein kinase modulates the phosphorylation/dephosphorylation of various proteins in the oocyte which in turn regulate meiotic maturation (Bornslaeger, 1986; Schultz, 1983). In addition, there is evidence suggesting a role for intracellular calcium levels (Bornslaeger, 1984), steroid hormones (Eppig, 1983), and gonadotropins (Freter, 1984), as well as oocyte maturation inhibitor (OMI), a product of granulosa cells (Tsafriri, 1982). It is likely the regulation of meiosis in the mammalian oocyte is a complex system involving many or all of the factors mentioned above, but a detailed regulatory pathway remains elusive.

Despite success in the cattle industry, in which oocytes are routinely obtained from unstimulated ovaries and used for IVF-ET (Goto, 1988; Fukui, 1989), attempts to mature human oocytes obtained from unstimulated ovaries in the laboratory have met with limited success. However, three pregnancies resulting from in vitro-matured oocytes have been reported. In the first report, healthy triplet girls were delivered following the in vitro maturation and fertilization of oocytes recovered from ovariectomy specimens and used for donation (Cha et al., 1991). In the other two pregnancies, immature oocytes

were aspirated from the follicles of infertile patients without prior ovarian stimulation. Following in vitro maturation of the recovered oocytes, two pregnancies resulting in normal, live births were established (Trounson et al., 1994; Barnes et. al., 1995). Although these reports are both encouraging and exciting, much research is needed before immature oocyte recovery and use for the establishment of pregnancy becomes routine in the treatment of human infertility.

It has been suggested that although oocytes matured in the laboratory undergo normal nuclear maturation, perhaps their cytoplasmic maturation is impaired. Using the cynomolgus monkey model, investigators transferred ooplasm, a technique they called ooplasmic transfusion, from in vivo matured Metaphase II oocytes into Prophase I oocytes. A delivery rate of 13% was realized when, following nuclear maturation, the transfused oocytes were returned to the fallopian tube for fertilization. The authors suggest that in vitro matured oocytes lack a cytoplasmic factor found in oocytes matured in vivo, which, if replaced, improves their developmental potential (Flood, 1990).

Despite impressive strides made in recent years, there remains considerable mystery surrounding the female gamete, the oocyte. Folliculogenesis, described briefly above, in which several follicles are "recruited" to begin growth and development in a given reproductive cycle, is poorly understood. Investigators are at a loss to explain why some follicles become part of this developing "cohort" while neighboring follicles, seemingly identical, remain unaffected. It has been suggested that when meiosis is "asynchronised", as it is in humans, the first oocytes to enter meiosis during fetal life will be among the first recruited for further development later on, but this remains to be proven. In addition, scientists don't fully understand how one

oocyte, that within the “dominant follicle”, is selected to resume meiosis while those within the remaining follicles in the cohort succumb to atresia at various stages of their development. A working theory, however, has gained wide acceptance (Hodgen, 1982).

Considering the complex nature of all that mammalian oogenesis achieves, from bestowing genetic uniqueness on the female gamete, to synthesizing the macromolecules and organelles necessary for normal fertilization and early embryogenesis, it is not surprising that attaining the ability to carry out and investigate this entire process in the laboratory has proven a great challenge.

### **C. Fluorescence In Situ Hybridization For The Analysis Of Human Embryos**

Fluorescence in situ hybridization (FISH) is a very powerful tool for the genetic analysis of blastomeres from human embryos. Used in conjunction with COH and IVF for the production of numerous embryos, and embryo biopsy, for the removal of one or two blastomeres from cleavage stage embryos, FISH has successfully been used for the preimplantation diagnosis of genetic sex and aneuploidy in human embryos prior to transfer. In addition, several investigators have used FISH to detect chromosome abnormalities in cleavage-arrested and morphologically abnormal human embryos, as well as in excess and “deselected” embryos, those not transferred or cryopreserved following a stimulated cycle.

FISH analysis involves hybridizing fluorochrome-labelled DNA probes specific for regions of selected chromosomes to the fixed interphase nuclei of blastomeres. Fluorescence microscopy then allows direct visualization of the selected chromosomes, permitting numerical chromosome analysis. Because

several probes labeled with different fluorochromes can be hybridized simultaneously, FISH allows assessment of ploidy as well.

The preimplantation genetic diagnosis of sex allows the selective transfer of female embryos following IVF in couples at risk of transmitting X-linked disorders. FISH is currently the preferred method for embryo sexing with clear advantages over the previous method, DNA amplification via the polymerase chain reaction (PCR), in that it provides information on sex chromosome number, and is less susceptible to contamination by foreign DNA (Grifo et al., 1994; Griffin et al., 1994; Harper et al., 1994).

Following evaluation of 20 cleavage-arrested or abnormally developing monospermic embryos using FISH with DNA probes specific for chromosomes X, Y, 18, 13, and 21, Munne and coworkers (1993) reported that 70% contained numerical aberrations of these chromosomes, including errors in ploidy, mosaicism (the presence of two or more different cell lines within a single embryo), and aneuploidy. Similar evaluation of 10 normally developing monospermic embryos from patients with a mean age of 40 years found that 70% of these embryos were abnormal as well, with sex chromosome aneuploidy being the most common abnormality.

A similar study evaluating 131 cleavage-arrested or morphologically abnormal embryos using FISH with DNA probes specific for chromosomes X, Y, and 18 found numerical aberrations in 56.5% of the embryos (Munne et al., 1994). In this study, most of the abnormal embryos were polyploid or mosaic. The authors suggest that, if it were possible to evaluate all chromosomes simultaneously, the vast majority of cleavage-arrested and abnormally developing embryos would likely be found to carry numerical chromosome abnormalities.

In April 1995, Munne and coworkers published a study using FISH to evaluate 31 normally-developing human embryos, those that had reached the 6-8 cell stage by Day 3 of development with <15% fragmentation, even cells, and few or no vacuoles or multinucleated blastomeres. These embryos came from two sources: embryos determined to be male following preimplantation diagnosis of sex in couples at risk of transmitting X-linked disorders, and therefore desiring the selective transfer of female embryos; and, embryos donated for research by patients over 40 years of age, since cryopreservation rarely increases the chance of pregnancy in these patients. Using probes specific for chromosomes X,Y,18, and 16, the investigators determined that 23% of normally-developing embryos carried numerical abnormalities involving these chromosomes. And, if diploid embryos containing one or more tetraploid cells are considered abnormal, then this percentage increased to 49%. Although the aneuploidy rate tended to increase with maternal age, the differences did not reach statistical significance possibly due to a small sample size.

In a larger study evaluating 524 monospermic embryos with FISH using either three (X,Y, and 18) or five (X,Y,18,13, and 21) DNA probes simultaneously, Munne and coinvestigators (1995) correlated embryo morphology, developmental rate, and maternal age with numerical chromosome abnormalities. They reported that while polyploidy and multinucleation are the main chromosome abnormalities found in cleavage-arrested embryos, aneuploidy is the main chromosome abnormality in normally-developing embryos obtained after COH/IVF. In addition, the incidence of aneuploidy increases significantly with maternal age, reaching 37.2% in normally-developing embryos from patients 40 years or older.

The application of FISH for the genetic analysis of human embryos in recent years has had a large impact on the field of assisted reproduction. We have learned that a large proportion of embryos obtained following COH/IVF are genetically abnormal, including many with normal morphology, perhaps explaining in part, why so few embryos implant and progress to live births upon transfer to the uterus. Also, the occurrence of genetic abnormalities in human embryos increases significantly with maternal age, a fact of great importance considering that the average age of patients seeking treatment for infertility has increased over the past several years. In addition, the application of FISH in the area of preimplantation genetic diagnosis has led to the exciting possibility that, in the future, routine screening of human embryos after COH/IVF will allow the selective transfer of fewer, genetically normal embryos in all patients, thereby increasing the efficiency of IVF/ET while, at the same time, decreasing the incidence of multiple births. This possibility will soon be explored at The Jones Institute for Reproductive Medicine.

## **II. STATEMENT OF PURPOSE**

Despite adequate hormonal stimulation, oocytes collected following controlled ovarian hyperstimulation for the purpose of IVF-ET display several levels of nuclear maturity. Mature (Metaphase I or II) oocytes are inseminated shortly after retrieval and assessed for fertilization the following day. Prophase I (immature) oocytes are cultured for 24-36 hours before being exposed to spermatozoa. Of those that complete nuclear maturation in vitro during this time, progressing from germinal vesicle breakdown through first polar body extrusion, approximately 50% fertilize and appear to undergo normal development. However, an extremely low incidence of pregnancy is realized following intrauterine transfer of the resulting embryos. From January of 1986 through March of 1997, there were 4098 embryo transfers performed at the Jones Institute in which all embryos were derived from mature oocytes. Of these, 1177 (29%) resulted in clinical pregnancies. In contrast, of 96 embryo transfers performed during the same time period in which all of the embryos were derived from Prophase I oocytes, only 4 (4%) resulted in clinical pregnancies (unpublished data, Jones Institute database).

The reduced developmental potential of embryos derived from Prophase I oocytes is well documented and, as a result, ovarian hyperstimulation protocols producing the greatest number of mature oocytes are utilized in IVF programs. Embryos derived from Prophase I oocytes are transferred only when a sufficient number of embryos derived from mature oocytes is unavailable. In many cases, Prophase I oocytes are discarded or used in research protocols. Given the fact that last year, 22% of all oocytes aspirated at the Jones Institute were Prophase I (unpublished data, Jones Institute database), it is obvious that

in order improve the success rate and increase the efficiency of IVF-ET for our patients, we need to improve the developmental potential of Prophase I oocytes. Increasing our understanding of their reduced developmental potential, the goal of this study, is a first step toward this end.

The purpose of this study was to test our hypothesis that the reduced developmental potential of embryos derived from Prophase I oocytes can be explained in part by an increase in nuclear and/or genetic abnormalities in these embryos. We used Fluorescence In Situ Hybridization analysis with DNA probes specific for chromosomes X, Y, and 18 to compare the rate of aneuploidy and the incidence of mosaicism in embryos derived from oocytes that were Prophase I at aspiration to that observed in their counterparts derived from oocytes that were Metaphase I or II at aspiration.

### **III. EXPERIMENTAL TECHNIQUES**

#### **A. Materials**

The hormones, human menopausal gonadotropin (Pergonal) and human follicle stimulating hormone (Metrodin) used for ovarian hyperstimulation of patients at The Jones Institute for Reproductive Medicine were obtained from Serono Laboratories, Inc. (Norwell, MA). Leuprolide acetate (Lupron) was obtained from TAP Pharmaceuticals (Deerfield, IL). Falcon plasticware was used for tissue culture and sperm preparation (Becton Dickinson, Franklin Lakes, NJ). Hams F-10 culture medium and Dulbecco's phosphate buffered saline were supplied by GIBCO Laboratories (Grand Island, NY). Human serum albumin and synthetic serum substitute were obtained from Irvine Scientific (Santa Ana, CA). Percoll, sodium citrate, bovine serum albumin, methanol, and glacial acetic acid were purchased from Sigma Chemical Company (St. Louis, MO). The following materials, used in the preparation of Acid Tyrodes solution, were also obtained from Sigma Chemical Company: sodium chloride, potassium chloride, calcium chloride, magnesium chloride, glucose, and polyvinylpyrrolidone (PVP-40). Propanediol was supplied by Fisher Scientific (Pittsburgh, PA). Formamide was obtained from United States Biochemical (Cleveland, OH). The DNA probe mixtures, saline sodium citrate (SSC), NP-40, and DAPI (4',6-diamino-2-phenyl-indole) were purchased from Vysis (Downers Grove, IL).

#### **B. Equipment**

Oocytes and embryos were evaluated using a Nikon Diaphot inverted microscope (Nikon, Garden City, NY) equipped with Hoffman optics (Hoffman

Modulation Contrast, Greenvale, NY). Embryos were cryopreserved and later thawed in a Planer Kryo 10, Series II programmable biological freezer obtained from T.S. Scientific (Perkasie, PA). Blastomere isolation and fixation was performed under a Nikon SMZ-10 dissecting microscope. Fixed nuclei were located using a Nikon Labophot-2 phase contrast microscope, and their locations recorded with a Field Finder microscope slide obtained from Fisher Scientific. Fluorescence microscopy was performed on a Nikon Microphot-FX epifluorescent microscope, and the single bandpass filter sets for viewing both green and red fluorochromes were supplied by Nikon. The single bandpass aqua filter set and the triple bandpass filter set were obtained from Vysis.

### C. Human Embryos

Embryos for this study were obtained from two sources. The first source was cryopreserved embryos donated by IVF patients of the Jones Institute for Reproductive Medicine. Patients no longer wishing to keep their embryos cryopreserved are sent a form entitled "Authorization for Utilization or Disposition of Cryopreserved Pre-zygote(s)/Preembryo(s) at the Jones Institute" (Appendix B). One option they may choose is to donate their embryos for use in approved research. The use of such embryos for this study is covered by a protocol reviewed and approved by the Institutional Review Board of Eastern Virginia Medical School (IRB Approval #07-08-93-0043; Appendix A1).

A second source of embryos was obtained as follows: Prophase I oocytes were donated by non-male-factor IVF patients and oocyte donors at The Jones Institute for Reproductive Medicine. After overnight incubation in culture medium, the oocytes were inseminated with sperm samples obtained from the spouse in the case of IVF patients, or from consenting sperm donors in

the case of oocyte donors. The use of these embryos is covered by the protocol mentioned above (IRB Approval #07-08-93-0043; Appendix A1). The consent forms for oocyte and sperm donors are found in Appendices A2 and A3.

#### **D. Methods**

##### **1. In Vitro Fertilization and Embryo Culture**

Because this study utilized human embryos donated for research by former IVF patients which were in cryostorage for varying numbers of years, laboratory protocols differed. In addition, procedures such as stimulation protocol, sperm preparation, and oocyte insemination are routinely individualized and tend to vary slightly according to each patient's specific needs.

Ovarian hyperstimulation was accomplished as previously described for the Norfolk program (Muasher, 1992). Briefly, hMG (Pergonal) alone, FSH (Metrodin) alone, or a combination of hMG/FSH with or without pituitary suppression using a GnRH analog (Lupron) under long or short protocols were utilized. Transvaginal oocyte retrieval was performed 34-36 hours after human chorionic gonadotropin (hCG) administration.

Culture conditions for oocytes and embryos, as well as in vitro fertilization procedures, were as previously described for the Norfolk program (Veeck, 1991). Culture of oocyte and embryos occurred in organ culture dishes kept at 5% CO<sub>2</sub> in air under humidified conditions. Incubation medium consisted of modified Ham's F-10 supplemented with human fetal cord serum, human serum albumin, or synthetic serum substitute. Sperm was prepared by either standard swim-up methods or after Percoll gradient centrifugation. Insemination concentration was dependent upon sperm morphology. Oocytes

were classified according to nuclear maturity at aspiration, and inseminated as follows: Metaphase II oocytes, 3-5 hours after aspiration; Metaphase I oocytes, 3-5 hours after first polar body extrusion; Prophase I oocytes, 24-29 hours after aspiration. Oocytes were evaluated for the presence of pronuclei 12 to 19 hours after insemination.

Immediately before transfer, which occurred on the morning of Day 2 or 3 following fertilization, embryos were evaluated for cleavage status and their morphology graded using criteria previously published (Veeck, 1991). Grade 1 represented a perfect morphological condition and Grade 5 an embryo with severe or complete fragmentation. The grade of the embryo with the best morphology was recorded as the grade of the transfer.

## **2. Cryopreservation and Thawing Protocols**

Cryopreservation of pronuclear stage embryos was performed before 20 hours post-insemination using a slow freezing protocol in a programmable Planer Kryo 10, Series II biological freezer as previously described (Veeck et al., 1993). Freezing medium consisted of 1.5 M propanediol in modified Dulbecco's phosphate buffered saline. Embryos were placed into cryovials containing 0.3 mL of freezing medium, and allowed to equilibrate at room temperature for 30 minutes before being loaded into the freezer. The temperature within the freezer was cooled to  $-60^{\circ}\text{C}$  at a rate of  $10^{\circ}\text{C}/\text{minute}$ . After a 5 minute hold, each cryovial was manually seeded. Following an additional 5 minute hold, the temperature within the freezer was cooled to  $-800^{\circ}\text{C}$  at a rate of  $0.50^{\circ}\text{C}/\text{minute}$ . Each cryovial was then plunged directly into liquid nitrogen for storage.

The thawing procedure was performed in the Planer biological freezer in a similar manner. After cooling the freezer to  $-1000^{\circ}\text{C}$ , the cryovials containing

the embryos to be thawed were loaded. The temperature within the freezer was warmed to room temperature at a rate of 80 C/ minute. After a 5 minute hold, the embryos were removed from the cryovials, and taken through a series of decreasing concentrations of 1,2 propanediol for 5 minutes in each dilution (1.0 M, 0.5 M, and dPBS without propanediol). The embryos were washed and placed in equilibrated culture medium.

### **3. Blastomere Isolation and Fixation**

Following evidence of normal fertilization (i.e. the presence of two pronuclei 12-20 hours post-insemination), or, in the case of cryopreserved embryos, survival of the thawing procedure, embryos were cultured in Hams F-10 medium supplemented with 15% Synthetic Serum Substitute for 48 hours. Each embryo was placed briefly (2-3 seconds) in acidified Tyrodes solution (Hogan et. al., 1986) to effect zona pellucida removal, and then washed and transferred to a culture dish containing calcium and magnesium-free Dulbecco's phosphate buffered saline for 10-15 minutes to decrease cell-to-cell contacts. Blastomere separation was facilitated by gentle pipeting through a fine-drawn glass pipet. Individual blastomeres were placed in a hypotonic solution consisting of 1% sodium citrate and 6 mg/ml bovine serum albumin for 5 minutes, during which time they were evaluated for nuclear status under an inverted microscope. Figure 1 shows a representative blastomere with a single nucleus (A), one with multiple nuclei (B), and one found to be anuclear (C). Although the nuclear status before fixation was recorded, this was sometimes found to be incorrect, especially in cases where the cytoplasm demonstrated excessive granularity. For this reason, all blastomeres were fixed, even if a nucleus could not be identified under the inverted microscope. The absence of a nucleus in blastomeres was not a failure of the fixation procedure, since



**Fig. 1** Individual blastomeres from human embryos examined under the inverted microscope with Hoffman modulation contrast. (A) Blastomere with a single nucleus; (B) Multinucleated blastomere with 2 nuclei; (C) Anuclear blastomere. Original magnification x 400.

cytoplasm was present and could be readily identified on the slide. Also, some blastomeres lacked a distinct, membrane-bound nucleus but a clear, lightened area was present within the cytoplasm. Some of these blastomeres were found to contain metaphase chromosomes (Figure 2B).

Following the 5-minute incubation in hypotonic solution, individual blastomeres were transferred to a glass microscope slide. All slides were previously cleaned with methanol and marked with a small circle on the bottom using a diamond tip pen indicating the approximate location of the nucleus. Immediately before the microdroplet dried, 8.5 uL of fixative (methanol/acetic acid 3:1) was dropped on top of the blastomere, lysing the cell membrane and fixing the nucleus. The fixative was prepared fresh and stored on ice during the procedure. Gentle blowing across the slide was used to free the nucleus of remaining cytoplasm. The fixed nucleus was immediately located using a phase contrast microscope. Figure 2 shows a single nucleus (A), two nuclei (B), and a metaphase spread (C) viewed under the phase contrast microscope. These were obtained following the fixation of three individual blastomeres. The first was a mononuclear blastomere (A); the second was a multinucleated blastomere with 2 nuclei (B); and the third was a blastomere in which a distinct, membrane-bound nucleus was not visible under the inverted microscope. The location of the nucleus (or nuclei) was recorded with a Field Finder microscope slide. All slides were stored at 80 C until the FISH procedure was performed.

#### **4. Fluorescence In Situ Hybridization**

FISH was performed using a mixture of DNA probes specific for chromosomes X,Y, and 18 directly labeled with Spectrum Green, Spectrum Orange and Spectrum Aqua fluorochromes respectively. The hybridization target for chromosome X was DXZ1. The hybridization target for chromosome Y



**Fig. 2** Photographs taken with a phase contrast microscope following the fixation of three individual blastomeres. (A) A single nucleus from a mononucleated blastomere; (B) Nuclei from a multinucleated blastomere with two nuclei; (C) Metaphase chromosomes obtained from the fixation of a blastomere in which a distinct, membrane-bound nucleus was not visible under the inverted microscope. Original magnification x 600.

was DYZ1. The hybridization target for chromosome 18 was D18Z1. The probes were purchased pre-denatured and pre-mixed with appropriate hybridization buffer. Each nucleus, fixed onto an individual glass microscope slide, was denatured in the following way: 100 uL of denaturing solution consisting of 70 uL formamide, 10 uL 20X SSC, and 20 uL water, was placed on top of the nucleus and covered with a 22 mm x 40 mm coverslip. The microscope slide was then placed on a glass plate in an oven at 80° C for 5 minutes. After removing the coverslip, the denatured nucleus was dehydrated in an ethanol series (70%, 85%, 100%) for 1 minute in each dilution. Each slide was allowed to air dry before being placed on a slide warmer at 50° C for 2-3 minutes. Two uL of the probe mixture was applied to the target area and covered with a small coverslip prepared by quartering a 22 mm x 22 mm coverslip using a diamond tip pen. After excluding air bubbles, the coverslip was sealed with rubber cement. Hybridization was allowed to proceed overnight in a moist chamber at 37° C. The post-hybridization washes used to remove excess probe consisted of a 2 minute wash in 0.4X SSC/0.3% NP-40 at 73° C followed by a 1 minute wash in 2X SSC/0.1% NP-40 at room temperature. After allowing the slides to air dry protected from direct light, 10 uL DAPI I counterstain was applied to the target area and covered with a 22 mm x 22 mm coverslip.

Fluorescence microscopy for evaluation of the nuclei was performed on a Nikon epifluorescent microscope equipped with four single bandpass filter sets: a filter set for viewing ultraviolet light was used to locate the DAPI-counterstained nuclei; a filter set for viewing green fluorochromes was used to identify chromosome X; a filter set for viewing red fluorochromes was used to

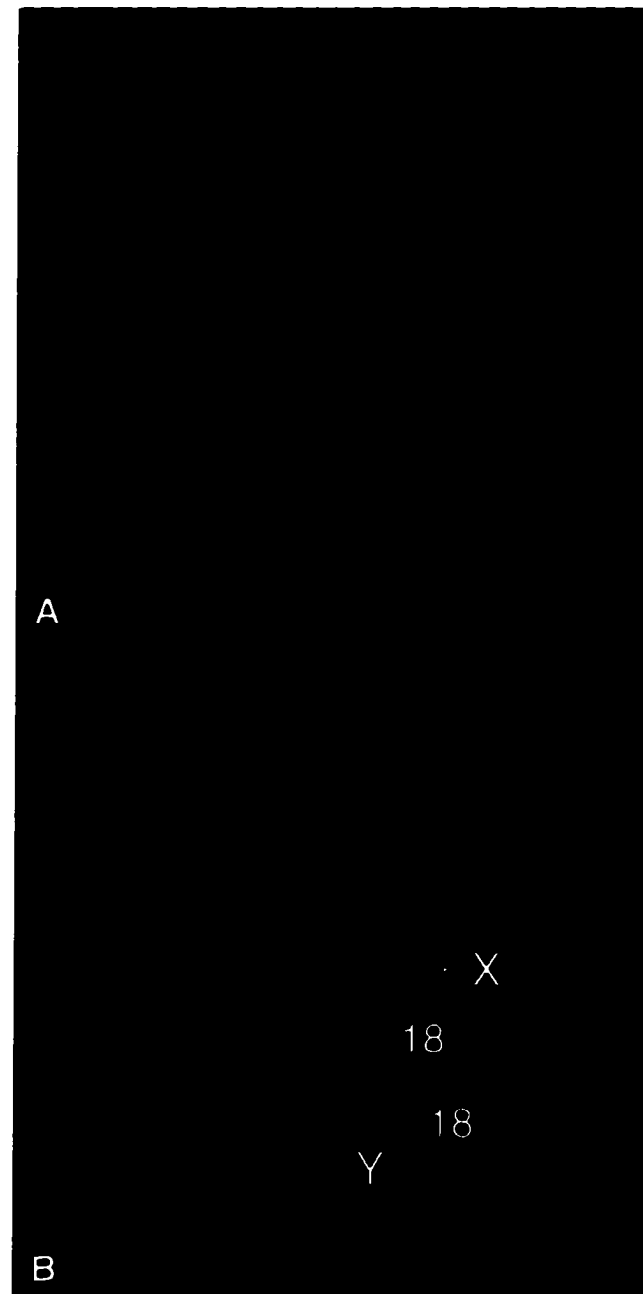
visualize chromosome Y; and a filter set for viewing the aqua fluorochrome was used to identify chromosome 18. In addition, a triple bandpass filter set which allows simultaneous viewing of all three fluorochromes was used to photograph the nuclei. Figure 3 shows the nucleus of a blastomere obtained from a human embryo viewed with the ultraviolet filter (A), and the triple bandpass filter (B) showing a normal male complement (XX1818). A computerized image analysis system was not used at any time. Initial location of the nucleus was performed using the Field Finder microscope slide and the coordinates recorded during the fixation procedure.

a. Scoring Criteria

The scoring criteria described by Hopman and coworkers (1988) were followed. Signals of low intensity, most likely the result of cross-hybridization to non-target DNA, were not scored. Double signals, two signals found close together or interconnected which may represent sister chromatids or split signals due to the nature of the target alphoid sequences, were scored as one signal.

In addition, since all or most of the blastomeres from each embryo were analyzed, the criteria described by Munne et al. (1995) were used to distinguish FISH failure from true mosaicism:

- 1) Blastomeres with one signal per chromosome analyzed were considered haploid cells.
- 2) Blastomeres with three or more signals per chromosome analyzed were considered polyploid cells.
- 3) Embryos in which all of the blastomeres analyzed contained the same abnormality, whether aneuploidy, haploidy, or polyploidy, were considered genetically abnormal.



**Fig. 3** FISH analysis using probes for chromosomes X (green), Y (red), and 18 (blue) of a nucleus from a blastomere obtained from a human embryo. (A) DAPI-counterstained nucleus viewed with an ultraviolet filter set; (B) The same nucleus viewed with a triple bandpass filter set showing a normal male complement (XY1818). Original magnification x 600.

4) Embryos in which sibling blastomeres had extra or missing signals compensating for those missing or extra in another blastomere were considered “compensated mosaic” embryos.

5) All other blastomeres with more or less than two signals for each chromosome analyzed were considered FISH failures.

In addition, blastomeres with a nucleus observed under phase microscopy following fixation, but either not found or covered by debris following FISH analysis, were considered FISH failures.

**b. Lymphocyte Controls**

Lymphocyte control slides, prepared using standard cytogenetic technique, were used to determine the efficiency of the FISH procedure. A total of 300 nuclei obtained from a healthy male were scored.

**c. Embryo Classification**

The following definitions were used to classify the embryos:

**Normal:** An embryo in which all blastomeres analyzed contain a single nucleus with the same normal diploid complement.

**Mosaic:** An embryo containing blastomeres with two or more different genetic complements, indicating the presence of two or more different cell lines. In a diploid mosaic, one of the cell lines is a normal diploid complement. Also included in this group are all embryos with one or more multinucleated blastomeres and/or blastomere(s) with a fragmented nucleus.

**Aneuploid:** An embryo in which all of the blastomeres analyzed contain the same genetic abnormality.

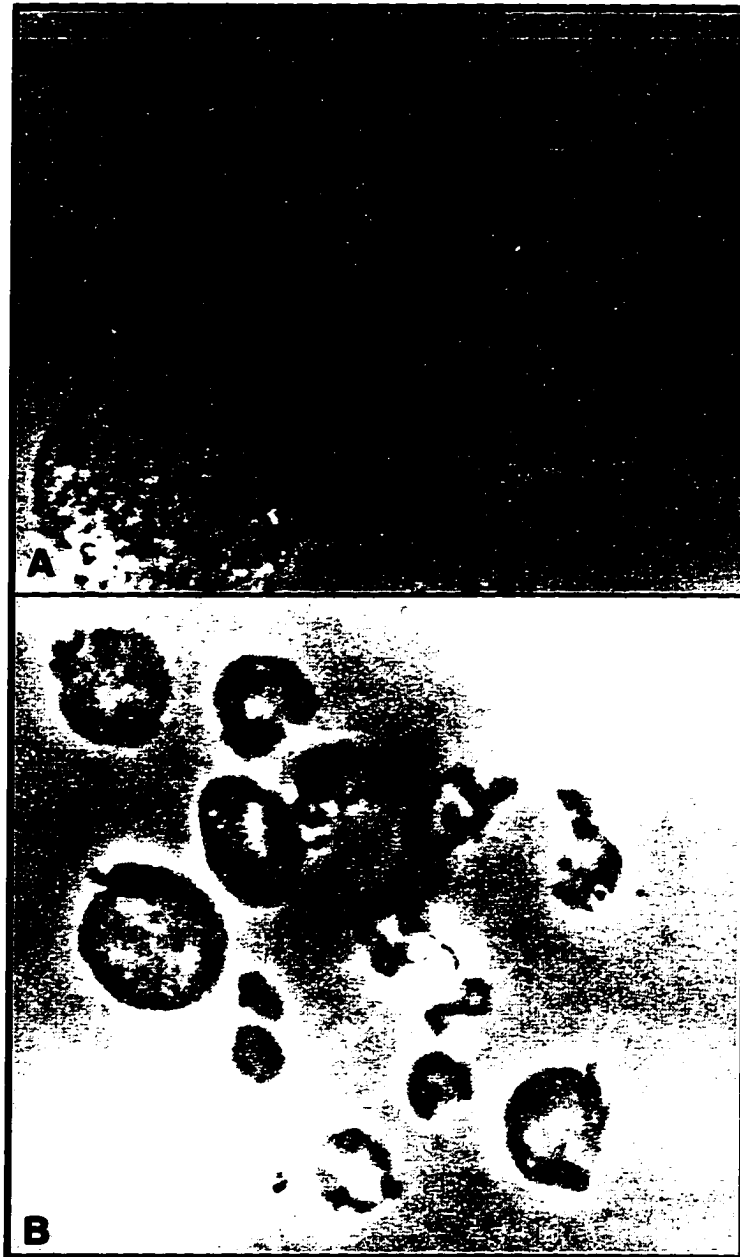
**Abnormal Nuclear Morphology:** An embryo in which 50% or more of the blastomeres demonstrated abnormal nuclear morphology. i.e. they were either multinucleated or contained a fragmented nucleus. Figure 4 illustrates the

difference between a multinucleated blastomere (A) and one containing nuclear fragments (B). In a multinucleated blastomere, the nuclei, which usually number two but occasionally number 3 or more, are roughly the same size and round in shape. In a blastomere with a fragmented nucleus, many (>5) smaller, irregularly-shaped pieces of nuclear material are present.

### **5. Statistical Analysis**

Data was collected on two groups of embryos. One group (**Immatures**) consisted of 61 embryos derived from oocytes that were Prophase I at collection, and the other group (**Matures**) consisted of 65 embryos derived from oocytes that were either Metaphase I or II at collection. Patient information was compared between the two groups using unpaired t-tests for the following continuous variables: age at retrieval, # amps of hMG administered, # amps FSH administered, # mature oocytes aspirated, # mature oocytes that fertilized normally, and the # of embryos transferred. Comparisons between the two groups were made with contingency table analysis (Chi-square) for categorical variables: stimulation protocol, highest transfer grade, establishment of a fresh pregnancy, and establishment of a cryo pregnancy.

Comparisons between the two groups with respect to embryo information were made as follows. An unpaired t-test was used to compare the mean number of blastomeres per embryo. A Chi-square test was used to compare the number of embryos in each group classified as Normal, Mosaic, Aneuploid, and containing Abnormal Nuclear Morphology according to the classification system outlined above. Chi-square tests were used to compare embryo classification with the following variables: patient age, type of infertility, stimulation protocol, highest grade of embryo(s) transferred, and fresh and cryo pregnancy status. In addition, a Chi-square test was used to compare the number of blastomeres in



**Fig. 4** Phase contrast photographs illustrating the difference between a multinucleated blastomere (A) and a blastomere containing a fragmented nucleus (B). The blastomeres were obtained from two different human embryos. Original magnification x 600.

each group that were lost during fixation, were anuclear, contained a single nucleus, contained multiple nuclei, contained a fragmented nucleus or nuclei, and contained metaphase spreads, independent of the embryos from which they came. Non-parametric rank tests were used to compare the number of blastomeres in each group that were lost during the FISH procedure, were lost during fixation, were anuclear, contained a single nucleus, contained multiple nuclei, contained a fragmented nucleus or nuclei, and contained metaphase spreads.

#### 6. Photography

Embryos and individual blastomeres were evaluated and photographed using a Nikon inverted microscope equipped with Hoffman optics and Kodak Technical Pan film (ASA 100). The fixed nuclei were evaluated and photographed using an Olympus phase-contrast microscope and Kodak Technical Pan film (ASA 100). Fluorescent signals were evaluated and photographed using a Nikon epifluorescent microscope and Kodak Gold film (ASA 400).

## **IV. RESULTS**

### **A. Patient Information, Transfer Status, and Pregnancy Outcome**

A total of 130 cryopreserved pronuclear stage embryos derived from oocytes that were mature (Metaphase I or II) at collection were donated by 27 patients in 28 stimulated cycles. Sixty-nine embryos survived the thaw for an overall survival rate of 53%. Two of these embryos were found to contain one pronucleus at thaw, and both arrested at this stage of development. Three embryos were lost before fixation. In addition, one of these patients also donated 2 cleavage-stage embryos, and one of these embryos survived the thaw and was included in the study. A final count of 65 embryos derived from mature oocytes were analyzed. These embryos were donated by 17 patients in 17 stimulated cycles. One patient was an oocyte recipient, and the remainder were infertility patients using their own oocytes. The types of infertility were as follows. There were 4 patients with tubal factor infertility, 3 with luteal phase defects, 3 with male factor infertility, 2 with immunologic factors, 2 with idiopathic infertility, 1 with endometriosis, and 1 with an ovulatory defect. Twelve (71%) of the patients received luteal lupron. Two patients (12%) received follicular lupron. Three patients (18%) received no lupron.

There were 64 embryos derived from oocytes that were Prophase I at aspiration donated by 23 patients in 27 stimulated cycles. Ten of these embryos were cryopreserved at the pronuclear stage. Three embryos did not survive the thaw, leaving 61 embryos from 23 patients for analysis. Twenty-one of these patients were oocyte donors. The remaining 2 were infertility patients, one diagnosed with polycystic ovaries and the other with tubal infertility. All patients received luteal lupron.

Comparisons between the two groups, designated as **Mature** and **Immature** based on the nuclear status of the oocyte at aspiration, were as follows. The average age of patients in the **Mature** group was 35.5 +/- 2.7 years compared to 28.0 +/- 3.8 years for patients in the **Immature** group. This difference was statistically significant ( $p < .0001$ ). Table 1 presents information on various IVF parameters collected in an attempt to compare the patient response to stimulation between the two groups. Note that the fertilization rate, the number of embryos transferred, embryo grade, and pregnancy outcomes for oocyte donors in the **Immature** group actually pertain to their respective recipient(s). In other words, these values were achieved with those oocytes, mature at aspiration, received by the recipients. Twenty-two oocyte donors had one recipient and 3 had two recipients. The fertilization rate was significantly higher in the **Mature** group than in the **Immature** group (88% vs. 67%,  $p = 0.00001$ ). No other differences were statistically significant (Table 1).

#### B. Maturation and Fertilization of Prophase I Oocytes

Of 176 Prophase I oocytes aspirated, 159 were inseminated. Of those inseminated, 71 fertilized normally, for a fertilization rate of 44.7%. Results on 7 embryos were lost to technical difficulties early in the study. As mentioned above, 3 of 10 embryos that were cryopreserved did not survive the thaw, leaving 61 embryos derived from Prophase I oocytes for analysis. The mean number of Prophase I oocytes aspirated per cycle was 6.5 +/- 4.1 and ranged from 2 to 16. The mean number of Prophase I oocytes inseminated per cycle was 5.9 +/- 3.7 and ranged from 1 to 15. The mean number of Prophase I oocytes that fertilized normally per cycle was 2.6 +/- 1.7 and ranged from 1 to 6.

**TABLE 1. IVF Parameters For Mature Oocytes In the Mature and Immature Groups**

Group	No. Mature Oocytes Aspirated	Fertilization Rate (%)	No. Embryos Transferred	Embryo Grade 1 or 2 (%)	Fresh Pregnancy Rate (%)	Cryo Pregnancy Rate (%)
Mature	15.5 +/- 10.9	231/264 (88) <sup>a</sup>	3.4 +/- 1.4	24/27 (89)	6/15 (40)	5/8 (63)
Immature *	16.1 +/- 6.8	291/436 (67) <sup>a</sup>	3.7 +/- 1.3	13/15 (89)	14/27 (53)	4/5 (80)

<sup>a</sup> p = .00001

\* Information on fertilization, transfer, and pregnancy outcome refer to mature oocytes received by recipient(s).

### C. Nuclear Status of Individual Blastomeres

A total of 331 blastomeres from 65 embryos were analyzed in the **Mature** group. A total of 342 blastomeres from 61 embryos were analyzed in the **Immature** group. The mean number of blastomeres per embryo was  $5.1 \pm 2.3$  and  $5.6 \pm 2.6$  for the **Mature** and **Immature** groups respectively (NS). Table 2 presents the nuclear status of the individual blastomeres independent of the embryos from which they came. Also included are those blastomeres lost during the fixation procedure. The **Mature** group contained a significantly greater percentage of blastomeres with a single nucleus than the **Immature** group (56% vs. 33%,  $p < .0001$ ). The **Immature** group had a significantly greater percentage of blastomeres with a fragmented nucleus or nuclei than the **Mature** group (19% vs. 3%,  $p < .0001$ ). The **Immature** group also had a significantly greater percentage of anuclear blastomeres than the **Mature** group (33% vs. 25%,  $p = 0.0181$ ). No other differences were statistically significant (Table 2).

### D. Oocyte Maturity and Embryo Classification

Table 3 presents the classification of each of the embryos analyzed according to the system outlined in the Methods section. A significantly greater percentage of embryos in the **Mature** group were classified as Normal compared to embryos in the **Immature** group (23% vs. 3%,  $p = 0.0012$ ). A significantly greater percentage of **Immatures** were classified as Abnormal compared to **Matures** (64% vs. 35%,  $p = 0.0014$ ). No other differences were significant (Table 3). In addition, if the mosaic embryos in each group are reevaluated disregarding multinucleated blastomeres and blastomeres containing a fragmented nucleus (i.e. considering mononucleated blastomeres

**TABLE 2. Nuclear Status of Individual Blastomeres in the Mature and Immature Groups**

Group	Single Nucleus (%)	Multiple Nuclei (%)	Fragmented Nucleus (%)	Anuclear (%)	Metaphase Spreads (%)	Lost During Fixation (%)	Total
Mature	185 (56) <sup>a</sup>	44 (13)	11 (3) <sup>b</sup>	82 (25) <sup>c</sup>	2 (1)	7 (2)	331
Immature	114 (33) <sup>a</sup>	37 (11)	65 (19) <sup>b</sup>	113 (33) <sup>c</sup>	9 (3)	4 (1)	342

<sup>a</sup>  $p < .0001$

<sup>b</sup>  $p < .0001$

<sup>c</sup>  $p = .0181$

**TABLE 3. Oocyte Maturity and Embryo Classification**

Group	Normal (%)	Aneuploid (%)	Mosaic (%)	Abnormal Nuclear Morphology (%)	Didn't Cleave (%)	Technical Failure (%)
Mature	15/65 (23) <sup>a</sup>	0 (0)	22/65 (34)	23/65 (35) <sup>b</sup>	3/65 (5)	2/65 (3)
Immature	2/61 (3) <sup>a</sup>	2/61 (3)	14/61 (23)	39/61 (64) <sup>b</sup>	3/61 (5)	1/61 (2)

<sup>a</sup>  $p = .0012$

<sup>b</sup>  $p = .0014$

only), 15/65 (23.1%) of embryos in the **Mature** group are mosaic compared to 13/61 (21.3%) of embryos in the **Immature** group. This difference is not statistically significant. Results of the FISH analysis of each individual blastomere of all embryos in both groups are presented in Appendix C.

Tables 4 and 5 present oocyte maturity and embryo classification for embryos with less than 4 blastomeres and embryos with 4 or more blastomeres respectively. There were no significant differences between the **Mature** and **Immature** groups when only those embryos with less than 4 blastomeres were considered (Table 4). However, when only those embryos with 4 or more blastomeres were considered, a significantly greater percentage of **Matures** were normal compared to **Immatures** (30% vs. 4%,  $p = 0.0009$ ). A significantly greater percentage of **Immatures** were classified as having abnormal nuclear morphology compared to **Matures** (64% vs. 28%,  $p = 0.0006$ ) (Table 5).

#### E. Other Patient Variables and Embryo Classification

There were no significant relationships between the following patient variables and embryo classification for either **Matures** or **Immatures**: patient age ( $p = 0.1509$  and  $p = 0.6385$  respectively); highest embryo grade at transfer ( $p = 0.3983$  and  $p = 0.3831$  respectively); and establishment of a pregnancy ( $p = 0.0971$  and  $p = 0.6075$  respectively). In the **Mature** group, there was no significant relationship between the following types of infertility and embryo classification ( $p = 0.1328$ ): idiopathic, immunologic, luteal phase defect, male factor, and tubal factor infertility. There were too few embryos in the remaining groups (endometriosis, ovulatory, polycystic ovarian disease) for statistical analysis. Also in the **Mature** group, there was no significant relationship

**TABLE 4. Oocyte Maturity and Embryo Classification for Embryos With Less Than 4 Blastomeres**

Group	Normal (%)	Mosaic (%)	Abnormal Nuclear Morphology (%)	Other (%)	Total No. of Embryos
Mature	0 (0)	3/15 (20)	9/15 (60)	3/15 (20)	15
Immature	0 (0)	0 (0)	7/11 (64)	4/11 (36)	11

**TABLE 5. Oocyte Maturity and Embryo Classification For Embryos With 4 or More Blastomeres**

Group	Normal (%)	Mosaic (%)	Abnormal Nuclear Morphology (%)	Other (%)	Total No. of Embryos
Mature	15/50 (30) <sup>a</sup>	19/50 (38)	14/50 (28) <sup>b</sup>	2/50 (4)	50
Immature	2/50 (4) <sup>a</sup>	14/50 (28)	32/50 (64) <sup>b</sup>	2/50 (4)	50

<sup>a</sup> p = .0009

<sup>b</sup> p = .0006

between stimulation protocol and embryo classification ( $p = 0.0057$ ).

#### F. Lymphocyte Controls

Of the 300 lymphocyte nuclei scored, 297 contained one signal for chromosome X (99%), and the remaining 3 nuclei contained no signal for X (1%); 293 nuclei contained one signal for chromosome Y (98%), 4 contained no signal for Y (1%) and 3 contained 2 signals for Y (1%); 286 nuclei contained two signals for chromosome 18 (95%), 9 contained only one signal for 18 (3%), and 5 contained 3 signals for 18 (2%).

#### G. Efficiency of the FISH Procedure

In the **Mature** group, 7/331 (2%) blastomeres were lost during the fixation procedure, and 2/324 (0.6%) nuclei were either covered with cytoplasm or without clear signals following the FISH procedure. In the **Immature** group, 4/342 (1.2%) were lost during the fixation procedure, and 5/338 (1.5%) nuclei were either covered with cytoplasm or without clear signals following FISH.

## V. DISCUSSION

In the present study, FISH analysis using DNA probes specific for chromosomes X,Y, and 18 was used to compare 61 embryos derived from Prophase I oocytes to 65 embryos derived from Metaphase I or II oocytes. We report that only 2 (3%) of the embryos in the **Immature** group were normal. This was significantly lower ( $p = 0.0012$ ) than the percentage of normal embryos in the **Mature** group (23%, Table 3). An embryo was considered normal when all of the analyzed blastomeres contained a single nucleus with the same normal diploid complement.

The percentage of embryos classified as having abnormal nuclear morphology was significantly higher ( $p = 0.0014$ ) in the **Immature** group (62%) than in the **Mature** group (34%). As defined in the Methods section, embryos in which 50% or more of the blastomeres were either multinucleated or contained a fragmented nucleus were classified as having abnormal nuclear morphology. Since the incidence of multinucleated blastomeres was the same in the two groups (13% vs 11% in the **Matures** and **Immatures** respectively), a difference in the incidence of blastomeres with a fragmented nucleus was responsible for this observed difference between the groups. Indeed a greater percentage of blastomeres in the **Immature** group (19%) contained a fragmented nucleus compared to their counterparts in the **Mature** group (3%,  $p < 0.0001$ , Table 2).

In a study of 1145 blastomeres from 147 normally fertilized human preimplantation embryos, Hardy et al. (1993) reported that 28 (2.4%) contained a fragmented nucleus. Although the nuclear status at aspiration of the oocytes from which these embryos were derived was not specifically mentioned, it can

be assumed that they were mature since insemination occurred the day of aspiration and fertilization assessment the following day. The investigators speculated that fragmented nuclei represent a form of cell death similar to apoptosis, with further degradation of the DNA fragments resulting in anuclear blastomeres. Interestingly, we report that the percentage of anuclear blastomeres was also significantly higher in the **Immature** group than in the **Mature** group (33% vs. 25% respectively).

Juriscova and coinvestigators (1996) published a study in which they used combined nuclear and terminal transferase-mediated DNA end labeling (TUNEL) to detect signs of DNA fragmentation compatible with programmed cell death (PCD) via apoptosis in arrested human preimplantation embryos. One of the characteristics of cell death via apoptosis, as opposed to necrosis, is DNA degradation into oligonucleosomal fragments which can be demonstrated by the appearance of DNA "laddering" on agarose gel electrophoresis. The small number of blastomeres in preimplantation embryos renders gel electrophoresis impractical. However, an in situ technique utilizing TUNEL (Gavrieli et al., 1991), allowed the authors to demonstrate extensive DNA fragmentation in the nuclei of blastomeres from arrested human embryos. They concluded that PCD with subsequent apoptosis may be responsible for the extensive fragmentation observed in human embryos cultured in vitro, ultimately leading to cleavage arrest. True PCD is genetically programmed, requiring activation of specific genes for execution. The genes responsible, as well as the "trigger" which activates them in human embryos, remain to be identified. In the present study, nuclear fragmentation was much more prevalent among disaggregated blastomeres in the **Immature** group than in the **Mature** group. Sixty-four percent of the embryos in the **Immature** group had one or more blastomeres

with a fragmented nucleus compared to 11% of embryos in the **Mature** group. Of 11 blastomeres in the **Mature** group in which a fragmented nucleus was observed, 4 were from the same embryo, and represented all of the cells from that embryo. The remaining 7 cells were from 6 embryos, all of which demonstrated abnormal nuclear morphology.

Apoptosis has also been implicated in the process of follicular atresia in avian as well as mammalian species (Tilly et al., 1991). Using DNA analysis by either ethidium bromide staining or 3'-end labeling followed by autoradiography, the investigators demonstrated the presence of DNA fragments characteristic of apoptosis in atretic but not normal, healthy follicles obtained from chicken and porcine ovaries. We speculate that Prophase I oocytes obtained following COH may come from follicles that have already begun the process of atresia, the effect of which is profound on the oocyte within. Indeed the fact that these follicles are large enough to be identified and punctured at aspiration suggests at least a limited exposure and response to the gonadotropins used for stimulation. Although these follicles were "selected" by the ovary for inclusion into the "cohort" of developing follicles, their oocytes failed to respond completely with a resumption of meiosis. Despite the fact that some of these oocytes are able to mature upon removal from the follicle, and to fertilize and sustain apparently normal development following insemination, perhaps their fate, to become one of several million oocytes destined for atresia, has already been sealed.

It is well known that early embryogenesis is dependent upon and directed by macromolecules and organelles synthesized during the final stages of oocyte growth and maturation (Telford et al., 1990; Wassarman and Kinloch, 1992). In particular, maternally-derived messenger RNA transcripts (mRNA)

vital in supporting early cleavage divisions of the embryo are detectable through the 4-6 cell stage in the human embryo (Heikenheimo et al., 1995). Activation of the embryonic genome, the timing of which varies among species, is thought to occur at the 4-6 cell stage in the human embryo as well (Braude et al., 1988), thus marking the commencement of embryonic transcription. In a study performed at the Jones Institute (Heikenheimo et al., 1996), levels of maternally-inherited mRNA transcripts for c-mos kinase and cyclin -B1, two proteins involved in the up-regulation of maturation promoting factor (MPF) activity, were not significantly different in Prophase I oocytes and Metaphase II oocytes before or after in vitro culture. The authors concluded that the reduced developmental potential of embryos derived from Prophase I oocytes may not be attributable to extensive degradation of these transcripts during culture or to reduced levels of their respective gene products early in development. These data can be used to further the theory outlined above, namely that Prophase I oocytes from stimulated ovaries are aspirated from follicles that have begun the process of atresia. Perhaps a cytoplasmic factor synthesized at or shortly after the onset of follicular atresia remains stable through oocyte maturation, fertilization and early development, eventually activating genes responsible for the execution of cell death via apoptosis, observed as DNA fragmentation in the present study.

We acknowledge that the method used in the present study is not the most sensitive available for the detection of DNA degradation compatible with PCD and subsequent apoptosis. The in situ technique utilizing TUNEL described by Gavrieli and coworkers (1991) would have been preferable. However, the finding that blastomeres containing a fragmented nucleus are prevalent among embryos derived from Prophase I oocytes was not

anticipated. Indeed, we expected to find a higher incidence of aneuploidy and/or mosaicism in embryos derived from Prophase I oocytes compared to their counterparts derived from Metaphase I or II oocytes. Although it would have been best if the nuclear fragmentation could have been documented in the intact blastomere before fixation, helping to rule out the possibility that the observed fragmentation was a procedural artifact, it is notable that in the vast majority of blastomeres found to contain a fragmented nucleus, a distinct, membrane-bound nucleus was not visible under the inverted microscope before the fixation procedure. Rather, a “suspicious”, lightened area was present within the cytoplasm. We did not recognize the nuclear fragments as such until the FISH procedure was performed. Perhaps improved microscope optics would have allowed us to observe the fragmentation in the intact blastomere. We acknowledge that additional investigation is required to clarify this issue.

In the study mentioned briefly above, Hardy et al. (1993) used Hoechst 33342, a polynucleotide-specific fluorochrome, to label the nuclei of disaggregated blastomeres from human preimplantation embryos. In addition to the blastomeres with fragmented nuclei, the investigators noted the presence of blastomeres with other nuclear abnormalities. The most common of these abnormalities, binucleate blastomeres, occurred in 17% of normally fertilized embryos at the 2-4 cell stage and in 65% of those at the 9-16 cell stage. Although various mechanisms have been implicated in the formation of binucleate blastomeres, including cell fusion, nuclear amitotic splitting, and acytokinesis, estimates of blastomere volume based on cell diameter and measurements of nuclear size in their study led the authors to speculate that binucleate blastomeres in human embryos most often arise through a failure of

cytokinesis between the second and fourth cleavage divisions.

In the present study, there was no difference in the incidence of multinucleated blastomeres between the groups. Forty-nine percent and 44% of embryos in the **Mature** and **Immature** groups respectively contained one or more multinucleated blastomeres. In addition, 13% and 11% of the disaggregated blastomeres in the **Mature** and **Immature** groups respectively were multinucleated. In the **Mature** group, 80% of these were binucleate compared to 63% in the **Immature** group. Our analysis of binucleate blastomeres suggests that at least two of the mechanisms mentioned above, acytokinesis and nuclear amitotic splitting, contribute to the formation of binucleate blastomeres in human embryos. We observed binucleated blastomeres in which each nucleus contained the same number of signals for chromosomes X, Y, and 18 as sibling mononucleated cells, and others in which the sum of the signals in the two nuclei was the same as that observed in sibling mononucleated cells. However, the number and distribution of chromosomes in the nuclei of binucleate blastomeres varied greatly, suggesting that other mechanisms may be involved as well. Our results are comparable to that reported by Munne and coworkers (1993) who found that 11.5% of blastomeres from arrested human embryos and 27.6% of blastomeres from normally developing human embryos were multinucleated. They also reported that a vast majority (79.6%) of the multinucleated blastomeres contained two nuclei.

Another abnormality observed in preimplantation embryos is the presence of anuclear blastomeres. Munne et al. (1993) reported the incidence of anuclear blastomeres in human embryos as follows: 24.8% of blastomeres in arrested embryos; 11% of blastomeres in slow and/or fragmented embryos; and 4.4% of blastomeres in normally developing embryos. In the present study,

25% of the disaggregated blastomeres in the **Mature** group were anuclear and 33% of their counterparts in the **Immature** group were anuclear. One possible explanation for the higher incidence of anuclear blastomeres in the study reported here is that all blastomeres, even those without a visible nucleus under the inverted microscope, were fixed and recorded. Some of these may have been cytoplasmic fragments. In later stages of development (8-12 cells), it is difficult to distinguish an anuclear blastomere from a cytoplasmic fragment.

Nuclear abnormalities in blastomeres of human embryos obtained by IVF contribute to developmental arrest, and may explain, in part, the low implantation and pregnancy rates realized following embryo transfer. Clearly, anuclear blastomeres and those with a fragmented nucleus lack the potential for further development. Although the exact mechanisms responsible for the formation of blastomeres with nuclear abnormalities in IVF-generated embryos remain uncertain, it has been suggested that the culture conditions routinely used, likely to be sub-optimal, contribute (Hardy et al., 1993).

The incidence of mosaicism in human embryos may also be related to in vitro culture conditions. Mosaicism is defined as the presence of two or more different cell lines within a single embryo. In a study in which normally developing human embryos obtained from four IVF centers were evaluated using FISH with DNA probes specific for chromosomes X, Y, 13, 18, and 21, Munne and coinvestigators (1997) found that the rate of mosaicism differed greatly between the centers, ranging from 11% in one center to 52% in another. They concluded that in vitro culture conditions and/or hormonal stimulation protocols may affect the incidence of mosaicism in the resulting embryos, perhaps explaining in part, the differences in success rates between IVF centers. In the study reported here, 34% of embryos in the **Mature** group were

mosaic compared to 23% in the **Immature** group. This difference was not statistically significant, perhaps due to the fact that the embryos in both groups were cultured under identical conditions. As outlined in the Methods section, embryos with one or more multinucleated blastomeres and/or blastomeres with a fragmented nucleus were automatically classified as mosaic. We acknowledge that this classification system may result in an overestimation of the incidence of chromosomal mosaicism in preimplantation embryos. In an attempt to address this issue, we reevaluated all of the mosaic embryos in each group considering only those blastomeres with a single nucleus (i.e. disregarding multinucleated blastomeres and blastomeres with a fragmented nucleus). Considering mononucleated blastomeres only, 7 of the mosaic embryos in the **Mature** group and one of the mosaic embryos in the **Immature** group would be classified as normal, changing the incidence of mosaicism in the **Mature** group from 34% to 23%, and in the **Immature** group, from 23% to 21%. Although this difference is still not statistically significant, it did make the difference in the incidence of normal embryos in the two groups more apparent (34% in the **Mature** group vs. 5% in the **Immature** group).

The occurrence of mosaicism in human embryos is of great interest because it has profound implications on the relatively new field of preimplantation genetic diagnosis. Embryo biopsy, the removal of one or two blastomeres from a preimplantation embryo for the purpose of genetic analysis, could lead to misdiagnosis in the case of a mosaic embryo. It has been suggested that there is a mechanism which diverts abnormal cells to the trophectoderm, thereby selecting against them during embryonic development (James and West, 1994). In this way, chromosomally abnormal cells are prevented from participation in the formation of the embryo proper. Whether or

not mosaicism occurs in normally conceived human embryos remains uncertain.

We also investigated the incidence of aneuploidy for chromosomes X,Y, and 18 in the two groups of embryos. Aneuploidy, defined as a chromosomal constitution different from the normal diploid constitution by loss or duplication of one or more chromosomes or chromosome segments, was found to be low in both the **Mature** (0%) and **Immature** (3%) groups. This is in contrast to a rate of 5.3% reported by Munne et al. (1995). There are several possible explanations for this discrepancy between the two studies. First, the average age of patients in the study presented here was quite low, 35.5 +/- 2.7 years and 28.0 +/- 3.8 years in the **Mature** and **Immature** groups respectively. While our data includes only one embryo from a patient over 38 years of age, more than a third of the embryos in Munne's study were from patients 40 years of age or older. In addition, although Munne reported a significant increase in aneuploidy with maternal age, this increase was mostly due to an increase in aneuploidy for chromosomes 13 and 21. Indeed the trend toward increasing aneuploidy for gonosomes and chromosome 18 in his study, which began only after 40 years of age, did not reach statistical significance. Lastly, according to Munne's classification system, embryos are both mosaic and aneuploid when the average of their cells, corrected for errors in ploidy, are aneuploid for a specific chromosome. In the present study, such embryos were classified as mosaic only.

Although an ideal study designed to evaluate the effect of oocyte maturity at aspiration on embryo classification would evaluate embryos derived from the different types of oocytes obtained from the same patients, the realities of human reproduction make this impractical. We feel justified in comparing

embryos in the **Mature** and **Immature** groups, because evaluation of various IVF parameters in the patients from which they came suggests a similar response to stimulation (Table I). With the exception of the fertilization rate, which was significantly lower in the **Immature** group, there were no differences between the groups. Note that information on fertilization, transfer, and pregnancy outcome for oocyte donors in the **Immature** group pertain to the mature oocytes received by their recipient(s). With this in mind, one possible explanation for the lower fertilization rate in the **Immature** group is an unsuspected male factor infertility in the oocyte recipient couple. Indeed there were several cases in which fertilization was poor, including one in which of 23 mature oocytes donated to a single recipient, only 5 fertilized normally.

A total of 176 Prophase I oocytes were donated for this study, and following insemination, 45% fertilized as evidenced by the presence of two pronuclei. This fertilization rate was low compared to the 80% previously published (Veeck, 1984), but may may be due in part to the fact that some of the inseminated oocytes were arrested at the germinal vesicle stage. In order to reduce the amount of extra work required for this study, many Prophase I oocytes were inseminated the day after aspiration without assessing their nuclear status, including some that had failed to undergo germinal vesicle breakdown. In addition, we evaluated more recent data from the clinical laboratory on the fate of Prophase I oocytes collected from January of 1995 to March of 1996 (Series 58-62) and found that 46% of the Prophase I oocytes aspirated matured in vitro, and of those that were inseminated, 60% fertilized (unpublished data, Jones Institute database).

In the study reported here, we were unable to detect a correlation between patient age and embryo classification in either the **Mature** or

**Immature** group. This may be due to the fact that the embryos we analyzed were from relatively young patients. This is especially true of the oocyte donors in the **Immature** group. Such a correlation is likely to become more apparent in embryos from older patients. In the **Mature** group, there was no significant relationship between stimulation protocol and embryo classification or type of infertility and embryo classification. If these correlations exist, detection may require analysis of a larger group of embryos.

We acknowledge several differences in the two groups of embryos in our study which may have contributed to the results we observed. For example, all of the embryos in the **Mature** group were cryopreserved and subsequently thawed before analysis. In contrast, all but 7 of the embryos in the **Immature** group were fresh. In addition, embryos in the **Immature** group, by virtue of the fact that they were derived from Prophase I oocytes which required a 24-36 hour incubation period to complete nuclear maturation before insemination, were in culture one day longer than embryos in the **Mature** group. It is possible that this extra day in culture conferred instability to the nuclear membrane in blastomeres from these embryos, observed as nuclear fragmentation in our study. If so, perhaps a change in culture conditions would correct this problem. Indeed, Lanzendorf and coworkers (1996) demonstrated that embryos derived from Prophase I oocytes differ in culture requirements than embryos derived from Metaphase I or II oocytes in the cynomolgus monkey.

We also caution that our findings may not apply to all Prophase I oocytes obtained following COH. In a study performed at the Jones Institute, Moffitt and coinvestigators (1993) found that Prophase I oocytes from large cohorts and cohorts with a greater percentage of Prophase I oocytes may have a greater

developmental potential than those from smaller cohorts and cohorts with a greater percentage of Metaphase I and II oocytes.

In summary, the present findings suggest that few embryos derived from Prophase I oocytes are normal, perhaps explaining in part why they rarely establish pregnancies in our IVF program. Comparison of the incidence of nuclear abnormalities observed in blastomeres in the two groups revealed that, although there was no difference in the incidence of multinucleated blastomeres, embryos derived from Prophase I oocytes had a significantly higher incidence of both anuclear blastomeres and blastomeres with a fragmented nucleus than their counterparts derived from mature oocytes. Because nuclear fragmentation is a hallmark of programmed cell death via apoptosis, which has been implicated in the processes of follicular atresia in vivo and cleavage arrest iv vitro, we speculate that Prophase I oocytes obtained following COH originate from follicles in early stages of atresia.

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## **APPENDIX A**

### **Ultrastructural and Molecular Evaluation of Preembryos Resulting from Immature Human Oocytes**

The following protocol (Appendix A1) and consent forms (Appendices A2 and A3) were submitted to and approved by the Institutional Review Board of Eastern Virginia Medical School on September 27, 1994. The consent form in A2 was used to obtain immature oocytes. The consent form in A3 was used to obtain semen for the insemination of donated oocytes. These forms were distributed and the nature of the study explained to all donors. Information concerning donor identity was kept confidential.

## **APPENDIX A1**

### **ULTRASTRUCTURAL AND MOLECULAR EVALUATION OF PREEMBRYOS RESULTING FROM IMMATURE HUMAN OOCYTES**

#### **Background**

Before ovulation, the human oocyte, located within the follicle of the ovary, is immature and cannot be fertilized. The nucleus of the immature oocyte, defined as the germinal vesicle (GV), contains chromosomes arrested at the prophase I stage of meiosis. This arrest must be overcome and meiosis must continue before the oocyte can support the development initiated by a penetrating spermatozoon. While arrested, the oocyte increases in size and develops associated cells (granulosa and theca cells) and membranes. Appropriate hormonal stimulation at the time of ovulation initiates cytoplasmic and nuclear maturation. The nuclear membrane disappears and the nucleus completes the first meiotic division. The progression of the oocyte to the metaphase II (MII) stage of development confers "fertilizability" to the oocyte, allowing it to take part in sperm incorporation, the cortical reaction, and decondensation of sperm chromatin.

In vitro fertilization (IVF) protocols routinely utilize exogenous hormonal stimulation to increase the number and growth of ovarian follicles. As a result of

the complexities of folliculogenesis, even in spite of adequate stimulation, oocytes retrieved will vary in maturational stage (Testart *et al.*, 1983; Veeck, 1986). Mature preovulatory oocytes are typically inseminated soon after collection and, in humans and monkeys, demonstrate the highest rate of fertilization and pregnancy following transfer.

Oocytes deemed immature at the time of recovery may be cultured *in vitro*, allowing for the completion of nuclear maturation. However, these *in vitro* matured oocytes demonstrate low rates of fertilization and pregnancy (Testart *et al.*, 1983, Veeck, 1986, 1989; Lanzendorf *et al.*, 1990). Therefore, the stage of oocyte maturity at collection plays a large part in IVF outcome, and hyperstimulation protocols are used which provide the greatest number of mature oocytes.

#### Gene Expression in the Maturing Oocyte

In the early 1970s it was discovered that cytoplasm of the mature frog oocyte activated meiosis and maturation when injected into the immature oocyte. This activating agent was termed maturation promoting factor (MPF) and was found to be contained in the mitotic cells of all eukaryotes tested including the human. Following its purification, MPF was found to be a high-molecular-weight protein with protein kinase activity. Studies performed in the sea urchin uncovered a protein referred to as cyclin, which accumulated in dividing cells in

a cyclic fashion, disappearing at the metaphase-anaphase transition. In a series of experiments, cyclin was found to cause cell division. Further studies indicated cyclin exists in a protein complex with protein kinase activity and that this complex is MPF (Draetta *et al.*, 1989).

The cellular counterpart of a viral proto-oncogene, *c-mos* has been shown to phosphorylate cyclin. In the mouse, expression of *c-mos* is restricted to a few tissues, such as the ovary and testis (Propst & Vande Woude, 1985). *In situ* hybridization studies also suggest that *c-mos* is expressed in developing oocytes of the mouse (Goldman *et al.*, 1987). During meiotic maturation to metaphase II, the level of *c-mos* transcripts decrease by approximately 20% (Mutter *et al.*, 1988). This level of transcripts continues to fall after fertilization and is undetectable from the 2-cell to blastocyst stage (Goldman *et al.*, 1988). In the mouse, lactate dehydrogenase (LDH) activity is found at very high levels in grown oocytes. The synthesis of LDH accounts for as much as 1.8% of total protein synthesis during oocyte growth and has been shown to decrease 7 and 20-fold during meiotic maturation and fertilization, respectively (Cascio & Wassarman, 1982). The steady-state level of this energy metabolism gene decreases by only 20% during meiotic maturation, however, LDH synthesis falls 7-fold. Similar patterns of synthesis and mRNA levels during meiotic maturation

are seen with beta-actin and are attributed to deadenylation (Bachvarova *et al.*, 1985; Paynton *et al.*, 1988)

### Hormonal Supplementation of Culture Medium

Studies to improve *in vitro* maturation using media supplementation with gonadotropic hormones, such as follicle-stimulating hormone (FSH) and/or luteinizing hormone (LH), have been performed using many species, including the mouse, cat, cow, rhesus monkey and human (Jagiello *et al.*, 1975; Shea *et al.*, 1975; Prins *et al.*, 1987). The majority of these studies have demonstrated an increase in maturation, fertilization and cleavage rates. However, despite some limited success, adequate controlled studies have not been performed in the human.

Jagiello and coworkers (1975) studied the effects of hormone additives on the maturation of human oocytes, in a limited study where sizes were small. Additives such as LH, estradiol, human chorionic gonadotropin (hCG), cyclic AMP, prostaglandins E1 and E2, and prolactin were investigated; only LH, estradiol and cyclic AMP were found to be beneficial. In another study, Shea and investigators (1975) added progesterone to culture medium and found no significant effect on germinal vesicle breakdown or on the number of oocytes which matured to MII. Prins and coworkers (1987) cultured immature oocytes in medium supplemented with LH and FSH and demonstrated a significant

increase in the rate of maturation and fertilization compared to control oocytes.

No conclusions were made on later development *in vivo* with these studies.

### Coculture of Oocytes and Preembryos

In recent years, investigators have utilized coculture techniques to enhance the *in vitro* environment of gametes and preembryos. Coculture typically involves the production of an anchorage dependent cell culture (feeder cells), such as epithelial cells, upon which another cell type is grown. An increase in fertilization rates has been demonstrated in bovine and human (Dandekar *et al.*, 1991) oocytes following their maturation on granulosa cell cultures obtained from mature, preovulatory follicles. Therefore, the presence of granulosa cells during maturation may induce or assist in nuclear and/or cytoplasmic maturation *in vitro*.

Investigators have also demonstrated an increase in cleavage and implantation when bovine preembryos, derived from oocytes matured *in vitro*, were cultured with feeder cells (Eyestone & First, 1989; Wiemer *et al.*, 1991). It is believed that oviductal cells may secrete certain factors which are beneficial to the developing preembryo and provide an environment similar to that *in vivo* (Gondolfi *et al.*, 1989).

### Specific Aims

The goal of the work proposed is to investigate mechanisms for the decreased developmental potential of human oocytes matured *in vitro*. In addition, studies will be performed to optimize rates of *in vitro* maturation, fertilization and cleavage in vitro. The specific aims of this study are:

1. To demonstrate that *in vitro* matured oocytes and resulting preembryos exhibit decreased levels in the expression of genes important for cell cycle control and energy metabolism. Levels of *c-mos*, cyclin, actin, and LDH will be determined in immature, *in vitro* matured and *in vivo* matured human oocytes, as well as embryos resulting from *in vivo* and *in vitro* matured oocytes.
2. To demonstrate that supplementation of culture with gonadotropins, growth factors and feeder cells enhances the rate of *in vitro* maturation and fertilization, and results in development comparable to that achieved by *in vivo* matured oocytes. Experimental endpoints will include both ultrastructural, molecular and biochemical analysis of oocytes and preembryos.

### Preliminary Data

Preliminary investigations have begun to study the expression pattern of *c-mos* in the multiple tissues of the cynomolgus monkey and single oocytes of

the monkey and human (Heikinheimo *et al.*, in preparation). Using amplification of cDNA reverse transcribed from RNA (RT-PCR), expression of *c-mos* could be detected in monkey ovary, testis, pituitary and hypothalamus. Lung, spleen, adrenal, kidney and muscle did not reveal any *c-mos* expression. RT-PCR amplification of  $\beta$ -actin, a house-keeping gene, was used as a reference and internal control in the experiments. Equal intensity of the PCR amplified  $\beta$ -actin suggested that approximately equal quantities of starting mRNA was used. In addition to the expected size, the identity of the PCR product was verified by Southern-hybridization analysis of the amplified DNA fragment using oligonucleotide probes internal to the PCR primers. As previously demonstrated in the mouse model, we found strong expression of *c-mos* also detected in single monkey and human oocytes. However, no *c-mos* mRNA could be detected in granulosa cells alone. From these findings, we conclude that *c-mos* is an important, highly oocyte specific maturation factor present in the primate oocyte.

Studies are also planned for the evaluation of cyclin B1 in human oocytes and early preembryos. We currently have the cyclin B1 primers and have successfully amplified cyclin mRNA in as few as two monkey oocytes.

Basic research studies involving the improvement of immature oocyte potential has been performed in the monkey model. The monkey model allows

us the unique opportunity to compare the developmental potential of embryos resulting from immature oocytes to that of mature oocytes from the same animal. We have established a protocol for obtaining immature, germinal vesicle-intact oocytes from one ovary of hyperstimulated cynomolgus monkeys by laparoscopic aspiration 16 hours after hCG administration. At 34 hours after hCG, another aspiration is performed and mature oocytes are collected from the other ovary. *In vitro* and *in vivo* matured oocytes are inseminated at the same time using the same sperm sample and fertilization and cleavage rates are evaluated and compared between the two groups. Using this protocol, we have investigated the beneficial effect of feeder cells on the development of preembryos resulting from immature oocytes. While the data show no significant benefit to embryos resulting from mature, metaphase II oocytes, only those preembryos from immature oocytes cocultured with feeder cells reached the expanded blastocyst stage (75%) compared to 0% cultured in medium alone. These results suggest that coculture may enhance *in vitro* development of embryos resulting from immature oocytes.

### Experimental Design

**Specific Aim 1. To determine if *in vitro* matured oocytes and resulting preembryos exhibit decreased levels in the expression of genes important for cell cycle control and energy metabolism. Levels of *c-mos*, cyclin, actin, and LDH will be determined in immature and *in vitro* matured oocytes, as well as preembryos resulting from *in vivo* and *in vitro* matured oocytes.**

For this study, RT-PCR techniques will be utilized to measure quantitatively the expression of these cell cycle control and energy metabolism genes in human oocytes and preembryos. The levels of expression in immature oocytes will be compared to those found in *in vitro* matured and *in vivo* matured oocytes. It is expected that levels of expression in *in vitro* mature oocytes will be significantly lower than those of *in vivo* matured oocytes. The expression of these genes will also be documented in the fertilized oocytes as well as cleaving preembryo to determine at what cell stage their expression is turned off, and if preembryos resulting from *in vitro* matured oocytes are deficient in levels of these genes.

**2. To determine if supplementation of culture medium with gonadotropins, growth factors and feeder cells enhances the rate of *in***

***vitro* maturation and fertilization, and results in development comparable to that achieved by *in vivo* matured oocytes. Experimental endpoints will include both ultrastructural, molecular and biochemical analysis of oocytes and preembryos.**

Immature oocytes will be cultured in medium supplemented with gonadotropins and/or growth factors. Controls will consist of oocytes cultured without these supplementations. Differences in rates of maturation, fertilization and cleavage between treatment and control groups, and fertilization and cleavage between treatment groups, controls, and *in vivo* matured oocytes will be recorded for statistical comparison. In addition, resulting preembryos will be divided and examined using the following techniques:

- A. Ultrastructural evaluation - electron and immunofluorescent microscopy will be utilized to examine ultrastructure and cell numbers in test and control preembryos. In addition, preembryos will be examined for genetic abnormalities using karyotypic analysis.
- B. Biochemical evaluation - hCG production of cultured preembryos will be performed on culture media using RIA techniques and compared in test and control preembryos.

- C. **Molecular evaluation - preembryos included in this specific aim will be evaluated for DNA and mRNA expression using PCR and RT-PCR technology.**

**Specific Aim 2 will also examine the supplementation of culture with feeder cells. Maturing oocytes will be cocultured in wells seeded with granulosa cells collected from the patient's own follicles at aspiration. Maturation rates will be compared to control oocytes cultured in medium only. Comparisons will be made between rates of maturation, fertilization, and development between treated, control, and oocytes matured *in vivo*. The effects of coculture in the presence of exogenous gonadotropins as well as the appropriate time for removal of the oocytes from the granulosa cell environment will also be determined. Preembryos resulting from oocytes matured *in vitro* will be cultured with feeder cells and development compared to control preembryos and preembryos developing from oocytes matured *in vivo*. Oocytes used in this study will include those resulting from the previous experiment (treated and controls) to demonstrate an additive effect by both coculture treatments. In addition, resulting preembryos will undergo further evaluation with ultrastructural, biochemical and molecular techniques as described on page 9.**

**For both specific aims, successful experimental outcome will be achieved when the *in vitro* results are statistically comparable to earlier results obtained**

with *in vivo* matured oocytes. In addition, procedures with successful outcomes will be utilized together to determine if combining protocols will produce maximum success.

For this study, oocytes will be fertilized using one of two methods:

1. the addition of husband's sperm to the culture dish containing the oocyte(s); or
2. fertilization by intracytoplasmic sperm injection (ICSI).

ICSI is currently used clinically to fertilize oocytes for infertile couples. This technique, which involves direct injection of a single sperm into the egg, has proved to be very efficient in the fertilization of human oocytes.

### Consents

For this study, human preembryos resulting from *in vitro* and *in vivo* matured oocytes will be examined. Evaluation of the preembryos will render them nonviable, therefore, they will not be used for initiation of pregnancy.

Two groups of preembryos will be examined: (1) those resulting from *in vitro* matured oocytes (immature at aspiration); and (2) *in vivo* matured oocytes (mature at aspiration). Patients donating the *in vitro* matured oocytes will be consenting to their fertilization with husband's sperm and then use in an assay which will render the preembryos nonviable (proposed consent attached).

The second group of preembryos will be donated by patients who no longer wish their frozen preembryos to be stored by cryopreservation. These preembryos will include those resulting from both immature and mature oocytes. At the patients request, they will be sent a form entitled "Authorization for Utilization of Cryopreserved Pre-zygote(s) at the Jones Institute" (see copy included). One option that the couple may choose is the use of the stored pre-zygotes for approved research. It is that approval we are requesting in this protocol.

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## **APPENDIX A2**

Medical College of Hampton Roads  
Approval Date: September 27, 1994  
IRB # 07-08-93-0043  
Revision Date: August 2, 1995  
Re-Approved: August 22, 1995

### **SUBJECT CONSENT FORM**

**TITLE:** Ultrastructural and Molecular Evaluation of Preembryos Resulting from Immature Human Oocytes

**INVESTIGATORS:** Susan Lanzendorf, Ph.D., Suheil J. Muasher, M.D., William E. Gibbons, M.D., and Jacob F. Mayer, Ph.D.

**TELEPHONE:** (804) 446-8948 which answers 24 hours a day in case of questions or problems.

**SOURCE OF SUPPORT:** The Jones Institute for Reproductive Medicine

**DESCRIPTION:** I am being asked to participate voluntarily in this research study, the purpose of which is to investigate the developmental potential of immature (Prophase I) oocytes (eggs). I understand that many patients have a number of Prophase I eggs. These immature eggs can often be matured in the laboratory and may sometimes undergo fertilization and early division. Nonetheless, they do not have an equal potential to develop into pregnancies as do eggs recovered in a mature state.

The main purpose of this study is to investigate methods to improve the developmental capacity of immature human oocytes. As a participant in this study, I am being asked to donate immature (germinal vesicle-bearing) eggs which I do not require for transfer as a patient undergoing an in vitro fertilization (IVF) attempt through the IVF program at the Medical College of Hampton Roads (MCHR).

I understand that the egg(s) which I donate will be immature and will be matured in vitro, using standard maturation techniques employed by the Jones Institute. These immature eggs can often be matured in the laboratory and may sometimes undergo fertilization and early division. Once matured, all the eggs will be inseminated by my husband's sperm and allowed to develop in vitro to determine whether or not they are capable of development. During development, preembryos resulting from the immature oocytes will be evaluated using techniques which will render the

preembryo(s) non-viable. At no time will resulting preembryos be used to initiate a pregnancy in myself or anyone else.

**EXCLUSION CRITERIA:** I understand that I am being asked to donate immature eggs only in the event that I have no history of fertilization failure. If on the day of aspiration, I have six (6) or more mature eggs, my immature eggs may be used for this protocol.

**RISKS:** There are no known risks to me at this time; however, there may be risks not yet identified.

**BENEFITS:** I understand that the pregnancy potential of immature eggs is very low and that preembryos resulting from this study will probably not increase my chances of achieving a pregnancy. However, much information can be gained from this study, which may enable investigators to improve the limited functional capacity of immature eggs to produce pregnancy. Scientific information from this study may have widespread application for future IVF cycles.

**ALTERNATIVE TREATMENT:** I have been informed that the only known alternative treatment is not to participate in this study.

**COSTS AND PAYMENTS:** I understand that there is no additional cost to me for participating in this research project, including the cost of future intrauterine replacement of preembryos obtained during this cycle. I further understand that I will receive no reimbursement for my participation in this study.

**NEW INFORMATION:** I understand that any new information obtained during the course of the research that may affect my willingness to continue participation in this study will be provided to me or to my legal representative.

**CONFIDENTIALITY:** I understand that any information concerning me which is derived from this study will be kept confidential, including answers to questionnaires, history, laboratory data findings, or physical examination(s) will be kept strictly confidential, and that my records will be protected within the limits of the law.

I also understand that the data derived from this study could be used in reports, presentations or publications, but that I will not be individually identified. I understand that, in order to ensure that Food and Drug Administration (FDA) regulations are being followed, it may be necessary for a representative of the FDA to review my medical records. **FREE WITHDRAWAL:** I understand that I am free to refuse to participate in this study or to withdraw at any time and that my decision will not adversely affect my care at this institution or cause a loss of benefits to which I might be otherwise

entitled. If I do decide to withdraw, I agree to undergo all trial evaluations necessary for my safety and well-being, as determined by my physician.

**COMPENSATION FOR ILLNESS OR INJURY:** I understand that in the unlikely event of a physical injury or physical illness resulting from the research procedure, no monetary compensation will be made, but any immediate emergency medical treatment which may be necessary will be made available to me without charge by the investigators. I am advised that if any injury should result from my participation in this research project, Medical College of Hampton Roads (MCHR) provides no compensation plan or free medical care plan to compensate me for such injuries. In the event I believe I have suffered injury as a result of my participation in any research program, I may contact Dr. Gerald Pepe, phone (804) 446-8423, an employee of MCHR, who will be glad to review the matter with me.

**VOLUNTARY CONSENT:** I certify that I have read the preceding or it has been read to me, that I understand its contents, and that any questions I have pertaining to the research and my rights as a research subject have been answered by Susan Lanzendorf, Ph.D., Suheil J. Muasher, M.D., William E. Gibbons, M.D., or Jacob F. Mayer, Ph.D., whose phone number (804) 446-8948. I have been given a copy of the signed informed consent. My signature below means that I have freely agreed to participate in this experimental study.

\_\_\_\_\_  
Wife's signature

\_\_\_\_\_  
Date

\_\_\_\_\_  
Husband's signature

\_\_\_\_\_  
Date

\_\_\_\_\_  
Witness' signature

\_\_\_\_\_  
Date

I certify that I have explained to the above individual the nature and purpose, the potential benefits, and possible risks associated with participating in this research study, have answered any questions that have been raised and have witnessed the above signature. I have explained the above to the volunteer on the date stated on this consent form.

\_\_\_\_\_  
Investigator's signature

\_\_\_\_\_  
Date

**APPENDIX A3**

Medical College of Hampton Roads  
Approval Date: September 27, 1994  
IRB # 07-08-93-0043  
Revision Date: August 2, 1995  
Addendum Approval Date: August 22, 1995

**ADDENDUM TO SUBJECT CONSENT FORM**

**TITLE:** Ultrastructural and Molecular Evaluation of Preembryos Resulting from Immature Human Oocytes. II Semen Donors

**INVESTIGATORS:** Susan Lanzendorf, Ph.D., Mahmood Morshedi, Ph.D., Suheil J. Muasher, M.D., William E. Gibbons, M.D., and Jacob F. Mayer, Ph.D.

**TELEPHONE:** (804) 446-8948 which answers 24 hours a day in case of questions or problems.

**SOURCE OF SUPPORT:** The Jones Institute for Reproductive Medicine

**DESCRIPTION:** I am being asked to participate voluntarily in this research study, the purpose of which is to investigate the developmental potential of immature (Prophase I) oocytes (eggs). I understand that many in vitro fertilization patients have a number of Prophase I eggs. These immature eggs can often be matured in the laboratory and may sometimes undergo fertilization and early division. Nonetheless, they do not have an equal potential to develop into pregnancies as do eggs recovered in a mature state.

The main purpose of this study is to investigate methods to improve the developmental capacity of immature human oocytes. As a participant in this study, I am being asked to consent to the use of my sperm (or a portion thereof) which has been previously donated or will be donated in the future for the insemination of immature eggs for research purposes.

I understand that resulting preembryos will be evaluated using techniques which will render the preembryo(s) non-viable. At no time will resulting preembryos be used to initiate a pregnancy in anyone.

**RISKS:** There are no known risks to me at this time; however, there may be risks not yet identified.

**BENEFITS:** I understand that much information can be gained from this study, which may enable investigators to improve the limited functional capacity of immature eggs to produce pregnancy. Scientific information from this study may have widespread application for future IVF cycles.

**ALTERNATIVE TREATMENT:** I have been informed that the only known alternative treatment is not to participate in this study.

**COSTS AND PAYMENTS:** I understand that there is no cost to me for participating in this research project. I also understand that, because I have been reimbursed for the donation of my semen for initiation of pregnancy, and the semen to be used for this study is excess from my previous donation, I will therefore not be reimbursed for consenting to the use my semen in this study.

**NEW INFORMATION:** I understand that any new information obtained during the course of the research that may affect my willingness to continue participation in this study will be provided to me or to my legal representative.

**CONFIDENTIALITY:** I understand that any information concerning me which is derived from this study will be kept confidential, including answers to questionnaires, history, laboratory data findings, or physical examination(s) will be kept strictly confidential, and that my records will be protected within the limits of the law.

I also understand that the data derived from this study could be used in reports, presentations or publications, but that I will not be individually identified. I understand that, in order to ensure that Food and Drug Administration (FDA) regulations are being followed, it may be necessary for a representative of the FDA to review my medical records.

**FREE WITHDRAWAL:** I understand that I am free to refuse to participate in this study or to withdraw at any time and that my decision will not adversely affect my care at this institution or cause a loss of benefits to which I might be otherwise entitled. If I do decide to withdraw, I agree to undergo all trial evaluations necessary for my safety and well-being, as determined by my physician.

**COMPENSATION FOR ILLNESS OR INJURY:** I understand that in the unlikely event of a physical injury or physical illness resulting from the research procedure, no monetary compensation will be made, but any immediate emergency medical treatment which may be necessary will be made available to me without charge by the investigators. I am advised that if any injury should result from my participation in this research project, Medical College of Hampton Roads (MCHR) provides no compensation plan or free medical care plan to compensate me for such injuries. In the event I believe I have suffered injury as a result of my participation in any research program, I may contact Dr. Gerald Pepe, phone (804) 446-8423, an employee of MCHR, who will be glad to review the matter with me.

**VOLUNTARY CONSENT:** I certify that I have read the preceding or it has been read to me, that I understand its contents, and that any questions I have pertaining to the research and my rights as a research subject have been answered by Susan Lanzendorf, Ph.D., Mahmood Morshedi, Ph.D., Suheil J. Muasher, M.D., William E. Gibbons, M.D., or Jacob F. Mayer, Ph.D., whose phone number (804) 446-8948. I have been given a copy of the signed informed consent. My signature below means that I have freely agreed to participate in this experimental study.

\_\_\_\_\_  
Donor's signature

\_\_\_\_\_  
Date

\_\_\_\_\_  
Witness' signature

\_\_\_\_\_  
Date

I certify that I have explained to the above individual the nature and purpose, the potential benefits, and possible risks associated with participating in this research study. I have answered any questions that have been raised and have witnessed the above signature. I have explained the above to the volunteer on the date stated on this consent form.

\_\_\_\_\_  
Investigator's signature

\_\_\_\_\_  
Date

## **APPENDIX B**

### **Authorization for Utilization or Disposition of Cryopreserved Prezygote(s)/Preembryo(s) at the Jones Institute**

**Appendix B contains a form distributed to IVF patients of The Jones Institute no longer wishing to keep their embryos in cryostorage. Under the protocol mentioned above (Appendix A1), we were able to use embryos donated for research in our study.**

**AUTHORIZATION FOR UTILIZATION OR DISPOSITION OF  
CRYOPRESERVED PREZYGOTE(S)/PREEMBRYO(S) AT THE JONES  
INSTITUTE**

We: \_\_\_\_\_ hereby forever irrevocably donate and transfer to The Jones Institute all of our joint and several rights, titles, and interests in and to our \_\_\_\_ (number) fertilized human egg(s) - prezygote(s)/preembryo(s) which were frozen and stored (cryopreserved) at The Jones Institute Cryopreservation Laboratory on \_\_\_\_, in IVF cycle number \_\_\_\_ for the following purpose.

**Instructions:**

- Select below a single (ONE) option agreed to by both husband and wife.
- Circle that ONE option and insert both husband and wife's initials next to YES.
- Circle the remaining options as NO and insert both husband and wife's initials next to NO.
- If you choose to donate to another couple please follow the remaining instructions after that option.

<b>YES</b> _____ initials	<b>NO</b> _____ initials	For use in approved research.
<b>YES</b> _____ initials	<b>NO</b> _____ initials	For thawing without undergoing any further development or utilization.
<b>YES</b> _____ initials	<b>NO</b> _____ initials	For use by another couple selected by The Jones Institute, the identity of such couple to be forever unknown to us. **

**\*\* If you have chosen to donate to another couple indicate below a secondary option to be implemented 2 years from this date if we cannot find another couple who will accept your frozen embryos.**

<b>YES</b> _____ initials	<b>NO</b> _____ initials	For use in approved research.
<b>YES</b> _____ initials	<b>NO</b> _____ initials	For thawing without undergoing any further development or utilization.

We make this gift and transfer freely, without inducement or compensation, and with full knowledge of the finality of our gift.

\_\_\_\_\_  
(Wife's signature) (date)

\_\_\_\_\_  
(Husband's signature) (date)

State of \_\_\_\_\_

City of \_\_\_\_\_, to wit:

Subscribed and sworn before me this \_\_\_\_\_ day of \_\_\_\_\_, 19\_\_\_\_ by

\_\_\_\_\_ and \_\_\_\_\_

Notary Public \_\_\_\_\_ My commission expires: \_\_\_\_\_

## **APPENDIX C**

Table 6 presents the results of the FISH analysis of each individual blastomere in 65 human embryos derived from oocytes that were either Metaphase I or Metaphase II at aspiration. Table 7 presents the results of the FISH analysis of each individual blastomere in 61 human embryos derived from oocytes that were Prophase I at aspiration. We include this information because as our knowledge of human embryology increases, our concept of a “normal” embryo is likely to change. We may wish to reevaluate these data.

**TABLE 6. FISH Analysis of Embryos in the Mature Group**

<b>Embryo # <sup>a</sup></b>	<b>Total # Cells <sup>b</sup></b>	<b># Anuclear Cells</b>	<b># Nucleated Cells</b>	<b>Complement for X,Y and 18 <sup>c</sup></b>	<b>Classification</b>
1-1	4	0	4	XX1818, XX1818, XX1818, XX1818	Normal
1-2	6	3	3	XXXX18181818, X18, XXXX18181818	Mosaic
1-3	2	0	2	XY1818, MNB <sup>d</sup> (XY1818, XY1818)	Abnormal
1-4	1	0	1	MNB (X1818, X18)	Didn't Cleave
1-5	6	2	4	MNB (- <sup>e</sup> , XX), XXX181818, MNB (-, X18), X18	Abnormal
1-6	4	2	2	XXXY181818, MNB (XY1818, XXX18)	Abnormal
1-7	3	0	3	MNB (XY1818, XY1818), XY1818, XY1818	Mosaic
1-8	5	3	2	LOST, XX18	Tech Failure
1-9	2	0	2	XX18, MNB (-, XX, X)	Abnormal
1-10	1	0	1	XXYY18181818	Didn't Cleave
1-11	4	0	4	XX1818, XX1818, XX1818, XX1818	Normal
1-12	2	1	1	Frag <sup>f</sup> (XY1818)	Abnormal
1-13	6	3	3	XX1818, XX1818, X18	Mosaic
1-14	2	1	1	Frag <sup>f</sup> (X[16] <sup>g</sup> 18[16])	Abnormal
1-15	8	4	4	MNB (XX1818, XX1818), X1818, MNB (XX1818, XX1818), MNB (XX1818, XX1818)	Abnormal
1-16	3	2	1	Frag <sup>f</sup> (XXYY18181818)	Abnormal
1-17	6	4	2	MNB (XXYY1818, 1818), XXYY18181818	Abnormal
1-18	4	2	2	Frag <sup>f</sup> (XXY), X[5]Y[5]18[9]	Abnormal
2-19	7	2	5	XXXX181818, XXXX18181818, X18, X18	Mosaic
3-20	6	1	5	XY1818, XY1818, X1818, XY1818, XY1818	Normal
3-21	3	0	3	XY1818, MNB (XY1818, X1818), XY1818	Mosaic
3-22	7	0	7	XX1818, XX1818, XX1818, XX1818, X1818, XX1818, XX1818	Normal
4-23	4	1	3	X1818, XY1818, XY1818	Normal
4-24	2	0	2	MNB (XX1818, XX1818), MNB (XXX1818, XX1818)	Abnormal
4-25	4	0	4	XY1818, XY1818, XY1818, XY18	Normal
5-26	10	0	10	XY1818, X18, XY1818, XY18, XY1818, Y18, XY1818, 18, XY1818, X18	Mosaic
5-27	10	7	3	MNB (XX1818, 1818), Frag <sup>f</sup> (X181818), Frag <sup>f</sup> (XX1818)	Abnormal

**TABLE 6. FISH Analysis of Embryos in the Mature Group**

Embryo #	Total # Cells	# Anuclear Cells	# Nucleated Cells	Complement for X,Y and 18	Classification
5-28	5	0	4	XY1818, XY1818, XY1818, XY1818	Normal
6-29	8	0	8	X1818, X18, X1818, XX1818, XX1818, X181818, X1818, X1818	Mosaic
6-30	4	0	4	XX1818, XX1818, XX1818, XX1818	Normal
7-31	8	0	5	XY1818, XY1818, XY1818, XY1818, XY18	Normal
8-32	4	3	1	MNB (XY1818, XXY181818)	Abnormal
9-33	12	2	10	XY1818, X1818, XY18, X1818, Y, Y, X1818, X1818, XY1818, X18	Mosaic
9-34	6	1	5	XY1818, XY1818, MNB (XXXYYY18181818, XXYY18181818), XY1818, XY1818	Mosaic
10-35	6	0	6	MMB (Y, Y, X18), XY1818, X1818, MNB (X18, -), Y18, 18	Mosaic
10-36	6	4	2	1818, MNB (XX1818, X)	Abnormal
10-37	8	7	1	LOST	Tech Failure
11-38	6	0	6	XXX181818, X1818, X1818, XXX181818, XX18, XX18	Mosaic
11-39	5	0	5	XX1818, XX1818, XX1818, XX1818, XX181818	Normal
11-40	4	0	4	MNB (XY1818, X18), MNB (XYY1818, XYY1818), MNB (XY1818, XY1818), MNB (XY1818, cyto)	Abnormal
11-41	4	1	3	XY1818, MNB (XY18, X181818), XY1818	Mosaic
11-42	7	0	7	MNB (X1818, XY1818), XY1818, XY1818, X1818, XY1818, XY1818, XY1818	Mosaic
11-43	5	0	5	MNB (XY1818, X1818), MNB (XY1818, XY1818), MNB (XY1818, XY1818), XY1818, XY1818	Abnormal
12-44	7	0	7	XX1818, XX1818, XX1818, XX1818, XX1818, XX1818, XX1818	Normal
12-45	6	1	4	X18, XY1818, MNB (XY1818, XY1818), meta:XY18	Mosaic
12-46	5	1	4	XX1818, XX1818, XX1818, MNB overlapping (XXXX181818)	Mosaic
12-47	4	0	4	MNB (XX18, 18, XX1818), XX1818, XX1818, X1818	Mosaic
12-48	1	0	1	XXXY18181818	Didn't Cleave
12-49	10	2	8	XX1818, MNB (X18, X18), X18, XX1818, XX1818, XX1818, XX1818, X18	Mosaic
12-50	7	0	7	XX1818, XX1818, XX1818, XX1818, XX18, XX1818, XX1818	Normal
12-51	6	1	5	-, MNB (X1818, X18), Y18, XY18, Y18	Mosaic
13-52	7	3	4	MNB (XX1818, X18, 18), MNB (X, XX18, -), XX18, X18	Abnormal

**TABLE 6. FISH Analysis of Embryos in the Mature Group**

<b>Embryo #</b>	<b>Total # Cells</b>	<b># Anuclear Cells</b>	<b># Nucleated Cells</b>	<b>Complement for X,Y and 18</b>	<b>Classification</b>
13-53	4	0	4	Frag (XY18), Frag (18), Frag (XY), Frag (XY18)	Abnormal
13-54	7	1	6	XY1818, MNB (X, Y, -), XY18181818, XY181818, MNB (X18, X), XY18181818	Mosaic
14-55	5	1	4	XY1818, XY1818, XY1818, meta:XY18	Normal
14-56	6	0	6	XX1818, XX1818, X18, XX1818, MNB (X18, X18), XX1818	Mosaic
15-57	4	1	3	XXX1818, MNB (18, X18, X1818), XX1818	Mosaic
15-58	7	3	4	XX1818, XX1818, XX1818, XX1818	Normal
15-59	2	0	2	MNB (X1818, XX18, XX1818, XX1818), XX18	Abnormal
15-60	3	2	1	Multiple signals for X,18 (no Y)	Abnormal
16-61	3	1	2	MNB (X18, XX18), Frag (XXX181818)	Abnormal
16-62	3	0	3	MNB (XYY181818, 1818), X18, XXY1818	Mosaic
16-63	4	1	3	XY1818, XY1818, XY1818	Normal
17-64	6	5	1	MNB (XXX181818, -, X181818, XX181818)	Abnormal
17-65	4	3	1	XXX181818	Abnormal

<sup>a</sup> : The first number indicates the number of the patient from which the embryo was obtained, and the number following the hyphen represents consecutive numbering of the embryos analyzed.

<sup>b</sup> : When the Total # of Cells is greater than the sum of the # Anuclear and the # Nucleated, one or more blastomeres were lost during analysis.

<sup>c</sup> : Genetic complements of the individual blastomeres are separated by commas

<sup>d</sup> : MNB indicates a multinucleated blastomere. The genetic complements of each individual nucleus is contained within the parenthesis separated by commas.

<sup>e</sup> : "-" Indicates nuclei in which there were no signals.

<sup>f</sup> : Frag indicates a blastomere with a fragmented nucleus. The total number of signals observed is contained within the parenthesis.

<sup>g</sup> : The number in the brackets indicates the total number of signals for the preceding chromosome.

**Table 7. FISH Analysis of Embryos in the Immature Group**

<b>Embryo # <sup>a</sup></b>	<b>Total # Cells <sup>b</sup></b>	<b># Anuclear Cells</b>	<b># Nucleated Cells</b>	<b>Complement for X,Y and 18 <sup>c</sup></b>	<b>Classification</b>
1-1	4	1	3	Frag <sup>s</sup> (XXYY18181818), XY1818, 1818	Mosaic
1-2	5	1	4	Frag <sup>s</sup> (XX1818), Frag <sup>s</sup> (XX1818), Frag <sup>s</sup> (XX1818), Frag <sup>s</sup> (XX1818)	Abnormal
1-3	1	0	1	Frag <sup>s</sup> (-) <sup>e</sup>	Didn't Cleave
2-4	1	0	1	Frag <sup>s</sup> (-)	Didn't Cleave
2-5	4	2	2	MNB <sup>d</sup> overlapping (X[8] <sup>e</sup> Y[4]18[12]), Frag <sup>s</sup> (X[4]Y[1]18[6])	Abnormal
2-6	5	2	3	Frag <sup>s</sup> (X[4]Y[8]18[4]), Frag <sup>s</sup> (18[7]), MNB (18, XX)	Abnormal
2-7	4	3	1	XXYY181818	Abnormal
3-8	8	3	5	XX1818, XXXX181818, MNB (XX18181818, XX1818), XX1818, Frag <sup>s</sup>	Mosaic
4-9	8	4	4	XX1818, XX1818, XX1818, XXX1818	Normal
4-10	13	10	3	Frag <sup>s</sup> (-), Frag <sup>s</sup> (XY1818), MNB (XY18, 1818)	Abnormal
4-11	5	3	2	XXXXYY18181818, Frag <sup>s</sup> (XXYY18[5])	Abnormal
5-12	6	2	4	XXYY181818, XY1818, XXXYY181818, XY1818	Mosaic
5-13	5	2	3	XXYYY18[8], XXX, X[9]	Mosaic
5-14	5	4	1	Frag <sup>s</sup> (X[8]18[6])	Abnormal
5-15	2	1	1	MNB (X[5]Y[3]18[3], XXX)	Abnormal
6-16	8	0	8	LOST, XXX181818, XXX181818, LOST, XXX181818, XXX181818, LOST, XXX181818	Aneuploid
6-17	12	2	10	XXY181818, XY1818, XY1818, XY1818, Frag <sup>s</sup> (-), Y1818, YY18[5], MNB (XY18, X), LOST, LOST	Mosaic
6-18	6	3	3	XXXXYY18[7], MNB (XY1818, XY1818), MNB (XXYY18181818, XXYY18181818)	Abnormal
6-19	4	1	3	MNB (X[6]1818, XYYY18[5], XYY18, YY18), XXXY18, MNB (Y18, 1818, 18, -,-)	Abnormal
6-20	9	2	7	XY1818, XY1818, XY1818, MNB (XYY181818, -, XY1818, -), Frag <sup>s</sup> (XY1818), XY1818, XY1818	Mosaic
7-21	8	4	4	Frag <sup>s</sup> (-), Frag <sup>s</sup> (X1818), Frag <sup>s</sup> (XY18), Frag <sup>s</sup> (1818)	Abnormal
8-22	7	0	7	LOST, MNB (XY, 18), MNB (-,XY18), XY1818, XY1818, XY1818, Frag <sup>s</sup> (XY1818)	Abnormal

**Table 7. FISH Analysis of Embryos in the Immature Group**

Embryo #	Total # Cells	# Anuclear Cells	# Nucleated Cells	Complement for X,Y and 18	Classification
9-23	5	4	1	Frag (XXXYYY181818)	Abnormal
10-24	12	0	12	XX1818, XX1818, XX18, XX1818, X18, XX18, X1818, MNB (XX181818, X), X1818, XX1818, meta:XX1818, XX1818	Mosaic
10-25	8	3	5	MNB (XXX181818, XXXX18), Frag (X18), MNB (XX1818, -), MNB (X18, X, XX18), 1818	Abnormal
10-26	7	5	2	Frag (XXXYYY18181818, Frag (XY181818)	Abnormal
11-27	5	2	3	X1818, MNB (X18, 18, -), XY1818	Mosaic
12-28	8	1	7	-, XX1818, Frag (X18), Frag (X18), Frag (XX18), Frag (XX1818), XX18	Abnormal
12-29	3	2	1	XX1818	Abnormal
12-30	8	1	7	XX1818, cyto, XX1818, XX181818, XX1818, XX1818, XX1818	Normal
13-31	8	6	2	MNB (1818, XX, X), Frag (XXX1818)	Abnormal
13-32	2	0	2	meta: X18181818, Frag (X[10]18[10]	Abnormal
14-33	4	1	3	MNB (meta: YY1818, X1818), Frag (-), Frag (-)	Abnormal
15-34	3	0	3	Frag (X1818), MNB (X, XY18, 1818, -), X18	Abnormal
15-35	5	4	1	Frag (XY1818)	Abnormal
16-36	2	0	2	X[8]18[5], MNB (XXX1818, XXX18181818, XXX18181818)	Abnormal
16-37	7	2	5	18, Frag (XY1818), Frag (XY18), Frag (XYY18181818), X1818	Abnormal
16-38	2	1	1	LOST	Tech Failure
17-39	4	2	2	MNB (XX18181818, X), X18	Abnormal
18-40	2	0	2	Frag (XX1818), Frag (X)	Abnormal
19-41	4	3	1	Frag (XX1818)	Abnormal
20-42	2	1	1	MNB (X18, XY1818, Y18, -)	Abnormal
21-43	8	2	6	XXY18181818, MNB (XX, XX1818, 1818), Frag (XX181818), YYY1818, XXY18181818, XYYY1818	Mosaic
21-44	6	0	6	XY1818, Frag (XYY1818), MNB (XY1818, XY1818), Frag (-), MNB (181818, XY), XY1818	Abnormal
21-45	7	2	5	MNB (1818, X), Frag (XXXYY181818), XXXYY1818, meta:-, meta:Y18	Abnormal
21-46	4	0	4	Frag (YY), Frag (XXXYY18181818), Frag (XX), meta:-	Abnormal

**Table 7. FISH Analysis of Embryos in the Immature Group**

Embryo #	Total # Cells	# Anuclear Cells	# Nucleated Cells	Complement for X,Y and 18	Classification
21-47	5	1	4	XX18, Frags (XXXX1818), Frags (X1818), Frags (18)	Abnormal
21-48	1	1	0		Didn't Cleave
22-49	4	1	3	XXXX18181818, Frags (X1818)	Abnormal
22-50	7	3	4	Frag (XXYY1818), Frags (XYY181818), Frags (XXYY181818)	Abnormal
23-51	7	0	7	MNB (-, XXX18[6]), XX18, XX18[5], XX18, XX18, meta: XX18, XX181818	Mosaic
24-52	5	1	4	Frag (Y18181818), Frags (XXY), MNB (XXYY, 18181818), Frags (XXYY181818)	Abnormal
25-53	8	0	8	X1818, XYY18181818, X1818, MNB (XYY1818, X18), X1818, MNB (Y1818, XY, Y), X1818, XY1818	Mosaic
25-54	8	3	5	X[6]1818, Frags (XX1818), XXXX1818, X1818, Frags (X18)	Mosaic
25-55	8	0	8	X[6]18181818, MNB (XX, 18, XX1818, -), XX1818, meta: XX1818, XX1818, XXX181818, XX1818, XX1818	Mosaic
26-56	5	0	5	MNB (XXX181818, XX18), MNB (X18, -), XX18181818, MNB (1818, -), 1818	Abnormal
26-57	4	0	4	XYY18[6], XXXYYY181818, XY181818, X1818	Mosaic
26-58	6	1	5	X1818, meta: X1818, X1818, Frags (XXX18), Frags (X18)	Aneuploid
26-59	5	3	2	MNB (Y18, -, 18), Frags (X[5]18[5])	Abnormal
26-60	5	1	4	Frag (XX181818), XXX18, MNB (XXX18181818,18), MNB (X18, X, XX18, 1818, X1818, -)	Abnormal
27-61	8	7	1	MNB (XXY18181818, X[6]Y[7]18[9])	Abnormal

<sup>a</sup> : The first number indicates the number of the patient from which the embryo was obtained, and the number following the hyphen represents consecutive numbering of the embryos analyzed.

<sup>b</sup> : When the Total # of Cells is greater than the sum of the # Anuclear and the # Nucleated, one or more blastomeres were lost during analysis.

<sup>c</sup> : Genetic complements of the individual blastomeres are separated by commas.

<sup>d</sup> : MNB indicates a multinucleated blastomere. The genetic complements of each individual nucleus is contained within the parenthesis separated by commas.

<sup>e</sup> : "-" Indicates nuclei in which there were no signals.

<sup>f</sup> : Frags indicates a blastomere with a fragmented nucleus. The total number of signals observed is contained within the parenthesis.

<sup>g</sup> : The number in the brackets indicates the total number of signals for the preceding chromosome.

## **VITA**

**Constance Montalto was born October 26, 1966, in Queens, New York to parents Orlando and Christine Montalto.**

**Ms. Montalto graduated from Siena College with a Bachelor of Science degree in Biology in May, 1988. She married Lieutenant Dominic DeScisciolo, USN, in August of the same year.**

**Following a year of work as a cytogenetic technologist at the Hospital of the University of Pennsylvania in Philadelphia, Ms. DeScisciolo began working in the Embryology Laboratory at The Jones Institute for Reproductive Medicine. One year later, in the fall of 1990, she began study in the joint Old Dominion University and Eastern Virginia Medical School Biomedical Sciences Program while continuing her work at The Jones Institute. While in the program, Ms. DeScisciolo held several Graduate Research Assistantships.**