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The state of the art of *in vitro* fertilization

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1. ABSTRACT

Since the first description of successful human *in vitro* fertilization in 1978, researchers and clinicians have been striving to improve the efficacy and safety of the technique. Advances in technology and in our understanding of human reproduction have contributed to increased success rates and decreased rates of higher order multiple births. However, there is still room for improvement as 'unexplained infertility' still affects many couples, and the incidence of twin pregnancies remains elevated. This review will discuss some of the recent advances in the fields of molecular genetics, proteomics and oocyte culture that will ultimately enhance the clinical practice of preimplantation genetic diagnosis, embryo selection and *in vitro* maturation. It will also discuss the potential for these advances to improve both the safety and efficacy of *in vitro* fertilization in the near future.

2. INTRODUCTION

The incidence of infertility continues to increase worldwide, and assisted reproductive technologies are being utilized to meet the demands of this ever-increasing population of patients. However, the overall live birth rate is only 41.3% in women under 35 years old, while the multiple birth rate for the same group is 35.2% (1). A major contributing factor to these figures is our limited knowledge of the myriad factors that determine successful embryo development and implantation. However, recent technological and scientific advances are currently being applied to the realm of *in vitro* fertilization to bring about significant improvements.

Advances furthering the field of assisted reproductive technology fall into two main categories: those leading to greater efficacy, and those leading to

improved safety. In actual practice, these two categories overlap to a large extent, and the pursuit of both goals continues to drive the progress of *in vitro* fertilization techniques. Specific advances will be discussed in detail below. In general, however, advances in science and technology have improved our understanding of the mechanisms underlying embryonic development and the pathophysiology of infertility. Enhanced techniques of preimplantation genetic diagnosis have improved that technology's diagnostic accuracy and the breadth of its applicability, while the study of proteomics has increased our understanding of the growth milieu and secretory profile of normal versus abnormal embryos. The information obtained from these studies ultimately can lead to the improved ability to select the 'best' embryos for transfer, thus providing the greatest chance of success. Improvements in embryo selection in turn lead to improved safety as fewer embryos are implanted to achieve the same, if not increased, effect.

Another fairly recent technique, the *in vitro* maturation of oocytes prior to fertilization, has provided an opportunity to avoid ovarian stimulation in patients at risk for hyperstimulation, and the more widespread application of this technique in the future should improve the overall safety of *in vitro* fertilization in these and other patients at similar risk (e.g. polycystic ovarian syndrome). Finally, advances in oocyte cryopreservation are bringing this technology closer to widespread application for fertility preservation. This review will discuss the above advances and how they serve to move *in vitro* fertilization into the future.

3. PREIMPLANTATION GENETIC DIAGNOSIS

In broad terms, preimplantation genetic diagnosis involves the removal and evaluation of DNA-containing material from an embryo. While there are many variables within the process, the overall goal of preimplantation genetic diagnosis is to determine which embryos carry a specific genetic defect so that we can avoid the transfer of these embryos into the uterus. This allows clinicians and patients the opportunity to determine the genetic composition of the embryo prior to implantation. The alternative approach would be prenatal testing (chorionic villus sampling or amniocentesis) with possible pregnancy termination for affected pregnancies. Most commonly, preimplantation genetic diagnosis is performed for patients at high risk of transmitting disease to their offspring, including chromosomal abnormalities, X-linked diseases, or specific monogenic disorders. This use of preimplantation genetic diagnosis must be distinguished from preimplantation genetic screening, performed for patients with unexplained infertility, recurrent pregnancy loss, or repeated IVF failures in an attempt to improve their chances for successful pregnancy.

The first case of pregnancy resulting from preimplantation genetic diagnosis was reported by Handyside *et al* in 1990 and was performed in couples at risk of transmitting X-linked diseases (adrenoleukodystrophy and X-linked mental retardation) (2). Embryos were fertilized via *in vitro* fertilization and biopsied at the six- to eight-cell stage. Gender determination was performed via DNA amplification of a portion of the Y chromosome, followed by transfer of only female embryos. Since that time, scientists, clinicians and embryologists have explored different methods of embryo biopsy and diagnostic technique in attempts to improve the accuracy and safety of the process.

3.1. Embryo Biopsy

The first step in preimplantation genetic diagnosis is embryo biopsy in order to obtain the necessary genetic material for diagnostic testing. Depending on the indications for the preimplantation genetic diagnosis and the experience of the performing center, the biopsy can be taken from different components of the oocyte or embryo at different developmental stages.

3.1.1. Polar Body Biopsy

The earliest possible stage at which a genetic sample can be obtained is following oocyte retrieval, at which point the first polar body can be removed after breaching the zona pellucida (usually performed mechanically or via laser). Alternatively, the second polar body (or the first and second) can be removed following fertilization at the zygote stage. The advantage to this technique is that it enables genetic analysis without necessitating a reduction in embryo cell volume. Furthermore, zygotes do not exhibit chromosomal mosaicism, a significant source of complication in the analysis of later-stage biopsied material. The main disadvantage to polar body biopsy is that only the maternal genetic component of the embryo can be evaluated, as the polar bodies are derived solely from the mother. While the vast majority (>90%) of aneuploidies are of maternal origin (especially when dealing with advanced maternal age) (3), trisomies resulting from paternal inheritance have been reported and would be missed by this technique (4). Polar body biopsy may be better suited for preimplantation genetic screening as opposed to preimplantation genetic diagnosis, and indeed the European Society of Human Reproduction and Embryology has begun a pilot study to determine whether polar body biopsy followed by an array-based analysis of the complete chromosome complement is a feasible approach to preimplantation genetic screening (5).

3.1.2. Cleavage-Stage Biopsy

Currently, the most common method of embryo biopsy for preimplantation genetic diagnosis is cleavage stage biopsy of the embryo on the morning of day 3 post-fertilization. This technique consists of removing one or two blastomeres after opening the zona pellucida, a step usually performed using acidic Tyrodes solution. The benefit of this procedure is that both the maternal and paternal genetic contributions can be analyzed. As mentioned above, however, the risk of chromosomal mosaicism is high at this stage, with rates up to 50% noted during preimplantation genetic screening (6).

A recent prospective cohort study analyzed developmental and birth outcomes for embryos biopsied

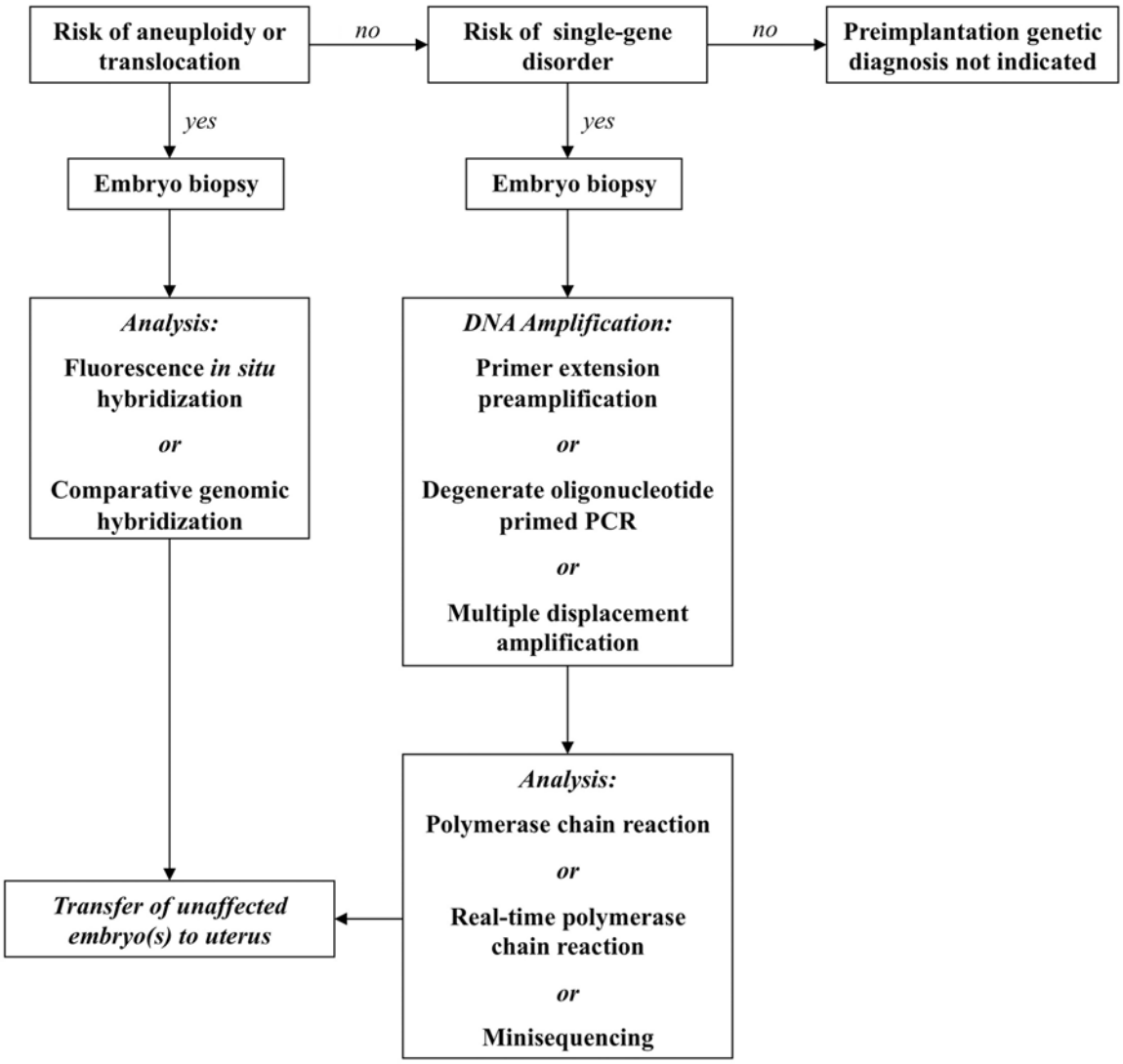

Figure 1. Algorithm for preimplantation genetic diagnosis.

(one or two blastomeres) at the cleavage stage and subsequently transferred on day 5 (single embryo transfer) (7). The overall live birth rate was comparable in the single-blastomere biopsied embryos compared to non-biopsied intracytoplasmic sperm injection controls. Compared to the single-blastomere biopsy, the live birth rate was significantly lower for the embryos from which two blastomeres were removed despite seemingly equivalent development on day 5. The obvious benefit of removing two blastomeres at the time of biopsy is the acquisition of more genetic material for subsequent testing, but this must be weighed against the possibility of embryo damage and reduced implantation rates.

3.1.3. Blastocyst Biopsy

As an alternative to blastomere biopsy, blastocyst biopsy can be performed in order to obtain more cells for genetic diagnosis without disturbing as much of the overall embryo volume. In this procedure, zona pellucida breaching is performed on day 3 post-fertilization, and herniating trophectoderm cells are biopsied on day 5-6. One limitation to this approach is the risk that few to no embryos survive to the blastocyst stage, resulting in the inability to perform preimplantation genetic diagnosis. Another limitation is the timing of the procedure, as biopsy on day 5 leaves little time to perform genetic testing and obtain results before the window for embryo transfer has closed. This necessitates the cryopreservation of biopsied embryos. With conventional slow-freezing techniques, the survival rate of biopsied embryos has been significantly lower than non-biopsied embryos (8,9). However, the recent application of vitrification to the cryopreservation of biopsied embryos has resulted in greatly improved embryo survival. In a prospective trial of vitrification of biopsied embryos at different developmental stages, Zhang *et al* demonstrated a 95% survival rate of biopsied blastocysts after warming (10). This technique thus holds promise for preserving biopsied embryos until reliable diagnostic

results can be obtained, and coupled with the improving speed of genetic testing, should make blastocyst biopsy a reliable option in the future.

For successful pregnancies resulting from biopsied embryos, recent data reveal no difference in birthweight or risk of major malformation between neonates resulting from biopsied embryos versus those subjected to intracytoplasmic sperm injection alone (11). However, a higher perinatal death rate was noted in post-preimplantation genetic diagnosis/screening multiple pregnancies compared to intracytoplasmic sperm injection multiple pregnancies. In a separate study, the same investigators found similar growth and health outcomes in 2-year-old children when comparing those with a history of embryo biopsy versus intracytoplasmic sperm injection alone (12).

3.2. Genetic Diagnosis

The technique used for genetic analysis in preimplantation genetic diagnosis depends upon the condition in question. Questions of chromosome number can be answered via karyotyping the embryo, while single-gene disorders are generally diagnosed by polymerase chain reaction. More recently, the technique of comparative genomic hybridization has shown great potential in providing a more thorough and accurate analysis on a genome-wide scale. For all of these different methods, various technological and scientific advances are being applied to improve their speed, accuracy and range of applicability.

3.2.1. Fluorescence *In situ* Hybridization

Whether the indication for preimplantation genetic diagnosis is a parental chromosomal translocation, screening for aneuploidy, or determining the sex of the embryo in light of an X-linked disorder, a determination of ploidy and chromosome makeup is often necessary. Fluorescence *in situ* hybridization involves the hybridization of fluorescent probes to interphase chromosomes, thereby allowing probed chromosomes to be identified and counted. While this technique has proved useful in preimplantation genetic diagnosis and prenatal diagnosis for detecting common aneuploidies, it has not been without its limitations. First, properly fixing the cell can be difficult, and in the case of preimplantation genetic diagnosis when only one or two blastomeres may be available, there can be very little margin for error. Second, analysis of fluorescence *in situ* hybridization results involves visualizing and counting fluorescent signals under microscopy. Overlap of chromosomes (and thus overlap of signals) is a recognized source of false-positive readings, while a split signal can lead to false-negative results (i.e. identifying two chromosomes when only one is present). Additionally, this method of analyzing results limits the number of probes which can be used, since a large number of different-colored probes can render the data difficult to interpret.

Recent advances in fluorescence *in situ* hybridization have addressed some of these limitations and have made the technique more applicable to various diagnostic situations. A three-dimensional fluorescence *in situ* hybridization has previously been described in which the cell is fixed in paraformaldehyde without first removing the cytoplasm (13). This allows for the visualization and probing of the interphase nucleus as well as cytoplasmic components and removes some of the technically more difficult aspects of traditional fluorescence *in situ* hybridization (like cytoplasm removal). This technique has recently been performed on blastomeres for preimplantation genetic diagnosis and shows promise for allowing a more comprehensive approach to studying not only chromosomal makeup but nuclear architecture as well (14).

Due to the limited number of available fluorochromes, a maximum of five can be used in each panel of fluorescence *in situ* hybridization. More rounds are thus needed to analyze more chromosomes, and currently nine chromosomes are analyzed in most fluorescence *in situ* hybridization protocols (X, Y, 13, 15, 16, 17, 18, 21, 22). Increasing the number of chromosomes analyzed would obviously enhance our understanding of that embryo's genetic makeup, but it would come at the expense of more rounds with the inherent problems of increased time and decreased diagnostic accuracy. Investigators have recently addressed these problems by using slightly larger probes, thereby allowing for faster hybridization, and by employing a technique known as 'no results rescue' (15). In this method, chromosomes for which fluorescence *in situ* hybridization results are inconclusive are reprobed at a different location, thereby improving the diagnostic accuracy of the procedure (16). By employing the above methods, Colls *et al* were able to test 12 chromosomes (the nine standard chromosomes plus 8, 14, and 20) in three panels of fluorescence *in situ* hybridization, with the information provided by the added information leading to an increased pregnancy rate (15).

Preimplantation genetic diagnosis has been used to detect cancer predisposition syndromes and usually involves multiplex polymerase chain reaction for the specific genes of interest (17). However, Vanneste *et al* have recently utilized fluorescence *in situ* hybridization for the detection of microdeletions implicated in neurofibromatosis type I and Von Hippel-Lindau syndromes (18), and others have employed the technique in the diagnosis of other microdeletion syndromes (19,20). Ultimately, the technique must be matched to the indication for preimplantation genetic diagnosis, but advances in fluorescence *in situ* hybridization technology are widening the range of applications for which it can be used.

3.2.2. Polymerase Chain Reaction

The first reported cases of preimplantation genetic diagnosis involved amplification of DNA via polymerase chain reaction (2), and this remains the technique of choice for diagnosis of single gene disorders. Embryo biopsy usually provides 1-5 cells for genetic analysis, so the starting amount of available DNA is small. Therefore, the first step in all polymerase chain reaction-based preimplantation genetic diagnosis techniques involves the amplification of the gene(s) of interest, followed by nested polymerase chain reaction to further

amplify the region of interest. Mutation analysis of this region is then performed by various methods, discussed below.

Because PCR involves the amplification of very small amounts of DNA to diagnose genetic disorders, the problem of allele dropout can have an enormous impact on the overall outcome of preimplantation genetic diagnosis. This phenomenon involves the failure of polymerase chain reaction to amplify one of the two alleles present in the cell, causing a heterozygous cell to appear homozygous. For a recessive disorder, this complication may result in the exclusion of that embryo for transfer even though it is an unaffected carrier. For dominant conditions, however, allele dropout can result in the transfer of an affected embryo that appears normal due to the preferential amplification of only the wild type allele. Improvements in polymerase chain reaction methods thus aim to minimize this risk while increasing speed and diagnostic accuracy.

Since the early days of preimplantation genetic diagnosis, investigators have utilized restriction endonucleases to distinguish between different alleles, as sequence variations lead to the production of different restriction fragment length polymorphisms upon enzyme digestion (21,22). More recently, a technique known as minisequencing has been applied to preimplantation genetic diagnosis for the diagnosis of multiple mutation-based disorders including sickle cell anemia, cystic fibrosis and β-thalassemia (23). This method involves the sequential addition of fluorescent-labeled nucleotides to a specific primer sequence followed by an automated determination of the sequence of interest. Specific mutations can then be detected by analyzing the DNA sequence, derived from the pattern of fluorescence. This approach enables the simultaneous analysis of multiple mutation sites by using different primers, equivalent to multiplex polymerase chain reaction. It also allows for the concurrent amplification of polymorphic markers to assist in the detection of allele dropout. Furthermore, it provides an alternative to restriction endonuclease-based methods of preimplantation genetic diagnosis for cases in which a mutation does not alter a restriction site.

Real-time polymerase chain reaction offers another approach to genetic diagnosis. By utilizing molecular beacons for the identification of specific alleles, investigators have demonstrated the technique's ability to rapidly and accurately detect mutations with a decreased allele dropout rate (24,25). Because it can quantify amplicon copy number, real-time polymerase chain reaction also shows promise for the detection of mitochondrial diseases in which the amount of mutant DNA predicts disease severity (26).

3.2.3. Genome Amplification

As mentioned above, the first step in a polymerase chain reaction-based diagnostic approach involves amplification of the gene of interest, followed by successively more focused amplification in preparation for mutation analysis. With minute amounts of starting DNA from a single cell, this approach can often only be used to investigate a single gene, as there is not enough template to reliably amplify several regions at the same time. As a solution, investigators have employed methods of whole genome amplification to replicate, aiming to reliably replicate from a single cell the entire genome rather than one region of interest.

Until recently, whole genome amplfication for preimplantation genetic diagnosis was performed by either primer extension preamplification or degenerate oligonucleotide primed polymerase chain reaction. In primer extension preimplification, DNA obtained from embryo biopsy undergoes polymerase chain reaction cycling with Taq polymerase and random 15-base oligonucleotide primers, resulting in widespread amplification. The technique has been utilized to identify various disorders, including cystic fibrosis, Tay-Sachs, and Duchenne muscular dystrophy (27,28,29). However, the entire genome may not be reliably replicated, and ADO can occur (reviewed in 30).

Like primer extension preamplification, degenerate oligonucleotide priming is polymerase chain reaction-based but involves the use of a partially degenerate primer which binds at multiple sites throughout the genome at low temperatures. As the temperature increases, more specific binding and amplification occurs, ultimately providing an accurate, high-copy yield from minute amounts of starting DNA (30). Degenerate oligonucleotide primed polymerase chain reaction has been used for the diagnosis of several monogenic disorders (31) and has been demonstrated as a reliable method of genome amplification prior to comparative genomic hybridization analysis (32,33,34). The technique has also been shown to produce greater amounts of DNA compared to primer extension preamplfication (33). However, as in primer extension preamplification, amplification bias and allele dropout can affect the products of degenerate oligonucleotide primed polymerase chain reaction, especially when starting with very small amounts of DNA. Additionally, both methods produce relatively short fragments and can introduce mutations into the amplified product due to imprecise replication.

In 2002, a novel method of whole genome amplification known as multiple displacement amplification was introduced (35). Utilizing the Phi29 DNA polymerase and random exonuclease-resistant hexamer primers, the entire procedure is performed at 30°C and does not require thermal cycling (Figure 2). The Phi29 DNA polymerase holds several other advantages over Taq polymerase, including a lower error rate (36,37) and less amplification bias (35). Multiple displacement amplification also generates larger DNA fragment sizes (>10kb) than polymerase chain reaction-based whole genome amplification methods (35). Despite these benefits, however, amplification failure and allele dropout do occur (38,39). Multiple displacement amplification has been successfully performed prior to polymerase chain reaction-based (40,41) as well as array comparative genomic hybridization-based preimplantation genetic diagnosis (42). Further refinement of the technique,

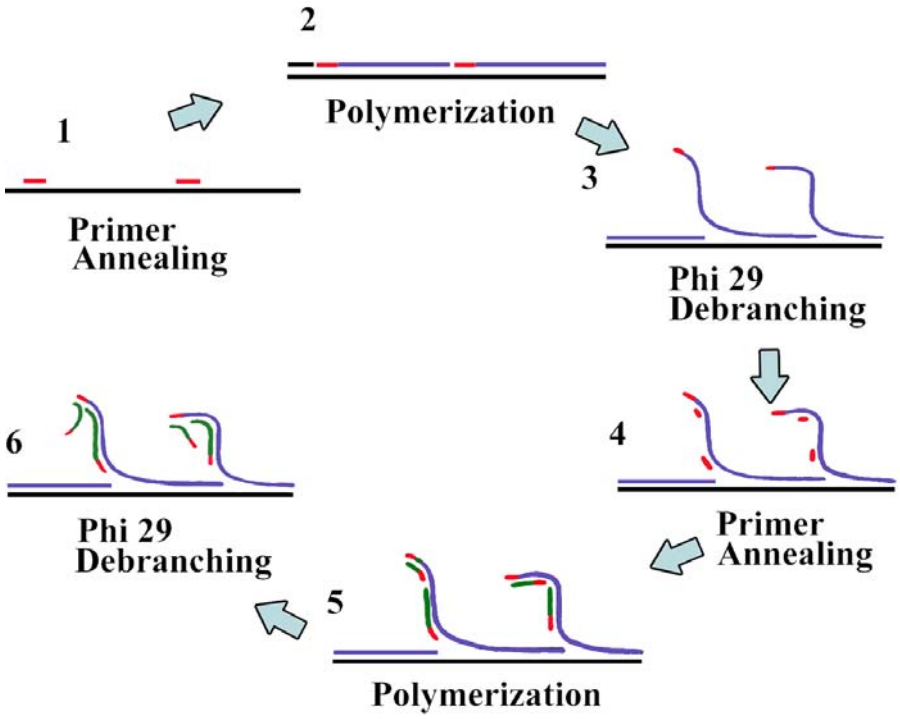

Figure 2. Multiple displacement amplification with Phi29 DNA polymerase.

including improvements in the rate of allele dropout and amplification failure, should result in the widespread application of the technique for preimplantation genetic diagnosis in the future.

3.2.4. Comparative Genomic Hybridization

As discussed above, fluorescence *in situ* hybridization techniques for aneuploidy detection are limited by the number of chromosomes that can be analyzed at one time and by the potential difficulty in preparing cells for analysis. Initially developed in 1992 (43), comparative genomic hybridization enables investigators to analyze a sample's entire chromosomal complement. In this method, green fluorescent-labeled sample DNA is mixed with red fluorescent-labeled DNA from a chromosomally normal control and applied to fixed metaphase chromosomes from a normal male control (46XY). The red and green-labeled DNA compete to hybridize with the chromosomes, which should reveal an even distribution of red and green in the case of normal sample DNA. In the case of aneuploidy, the sample DNA will bind to a greater or lesser extent than the control DNA, resulting in more or less green fluorescence. Results are analyzed by computer and have been shown to be highly accurate in various sample types (reviewed in 44).

Comparative genomic hybridization has been successfully applied to preimplantation genetic diagnosis and has allowed for complete karyotyping of a biopsied embryo (45,46). In the case of blastomere or blastocyst biopsy, comparative genomic hybridization has been performed following whole genome amplification, as described above (47,48). However, the application of traditional comparative genomic hybridization to preimplantation genetic diagnosis is limited by the time required to perform the procedure and obtain results, which can take as long as 72 hours. If the embryo was biopsied at the cleavage or blastocyst stage, this waiting period would necessitate cryopreservation of all embryos as they would not be karyotyped prior to closure of the implantation window. Cytogenetically normal embryos could then be thawed and transferred, resulting in possible damage to the biopsied embryo as discussed above. One solution to this problem has been to perform comparative genomic hybridization on polar bodies, which would allow ample time for diagnosis prior to embryo transfer. This technique has been successfully applied in a clinical setting (49), and the European Society for Human Reproduction and Embryology pilot study on polar body biopsy is utilizing this diagnostic approach (5).

Another possible solution is the use of microarray technology in which specific DNA sequences, instead of whole chromosomes, are affixed to a slide. As in conventional comparative genomic hybridization, the array of cloned sequences is exposed to labeled sample and control DNA, and the ratio of green to red fluorescence indicates the chromosomal status of the cell(s). However, hybridization occurs in approximately 24 hours, allowing for a biopsied embryo to be transferred during the same cycle. Furthermore, microarray comparative genomic hybridization offers higher resolution in detecting genomic imbalances involving small regions due to the use of mapped sequences instead of whole chromosomes (50). Investigators have successfully used this technique following genomic amplification of DNA from polar

bodies and blastomeres (47,49), and with further refinement this technique should offer new and better karyotyping options for preimplantation genetic diagnosis and screening.

4. PROTEOMICS/SECRETOMICS

Regardless of the technique used for genetic analysis, preimplantation genetic diagnosis is invasive as it requires the removal of cell volume from the developing embryo. And while the analysis provides information on the genetic makeup of each embryo tested, it does not necessarily indicate the developmental or implantation potential of the embryos. For this, clinicians and embryologists still rely most heavily on morphologic criteria for grading embryo quality and determining which are suitable for transfer (reviewed in 51,52). However, morphology alone is not highly predictive of subsequent implantation (52). The establishment of a reliable, predictive and noninvasive method of evaluating an embryo's developmental potential could have a positive impact not only on *in vitro* fertilization success rates, but on the incidence of multiple births as well by further optimizing single embryo transfer.

To gain a better understanding of embryonic cellular function, investigators have turned to proteomics, which involves the analysis of an embryo's protein content and the creation of a protein profile. Protein profiles of different embryos can then be compared to determine the effects of various interventions, such as *in vitro* fertilization culture conditions or cryopreservation (reviewed in 53). Proteomic analysis requires cell lysis, however, and thus could not be used to evaluate an embryo prior to transfer. For this purpose, proteins secreted into *in vitro* fertilization culture media have been analyzed (secretomics), resulting in a secretory profile for a given embryo that can be correlated to its reproductive outcome. By comparing, for example, profiles between embryos which do and do not successfully implant, biomarkers can potentially be identified which can be used to prospectively select embryos for transfer.

In addition to proteins, metabolites in spent culture media are also being investigated (metabolomics) as they reflect the physiologic and metabolic state of an embryo. As in secretomics, the comparison of profiles from viable and nonviable embryos can determine markers associated with improved reproductive potential, ultimately providing more accurate methods by which embryos can be selected for transfer in *in vitro* fertilization cycles.

4.1. Techniques

4.1.1. Proteomics/Secretomics

Earlier proteomic research in mouse embryos involved the use of two-dimensional gel electrophoresis (54), Western blot (55), or enzyme-linked immunosorbent assay (56) to identify expressed proteins. However, these techniques require a large amount of starting material and are limited either by their sensitivity (electrophoresis) or by the number of proteins that can be identified at one time (Western blot, enzyme linked immunosorbent assay).

More recently, the use of mass spectrometry for embryonic proteomics has enabled the simultaneous identification of numerous proteins, representing a significant advance in the field. The most commonly used technique, surface-enhanced laser desorption/ionization coupled to time-of-flight analysis, involves the laser ionization of bound proteins which produces various gaseous ions. These ions then travel down a vacuum time-of-flight tube towards a detector plate, with the ions traveling at different speeds depending on their size. They are separated according to their mass-to-charge ratio, thereby providing a profile of the proteins being analyzed. Using this method, Katz-Jaffe *et al* demonstrated differences in protein expression profiles between developing and degenerating embryos (57). They also showed protein differences among embryos with similar morphology, highlighting the limitations of morphologic assessment alone in embryo selection. Since some of these proteins are secreted into surrounding media, investigators have also analyzed embryo culture media to identify specific proteins and to create a profile of an embryo's secretome. Using surface enhanced desorption/ionization with time-of-flight analysis, Katz-Jaffe *et al* identified distinct secretomic profiles for embryos at various developmental stages and identified ubiquitin as a protein biomarker associated with blastocyst development (58).

Protein microarray technology has recently been utilized to compare secretomic profiles from culture media of implanted versus non-implanted blastocysts, revealing significantly decreased levels of granulocyte-macrophage colony-stimulating factor (GM-CSF) and chemokine (C-X-C motif) ligand 13 (CXCL13) from embryos that successfully implanted (59). Unlike mass spectrometry in which unknown proteins can be analyzed and later identified, microarray applications require the use of antibodies for specific proteins of interest and thus are better suited to the identification of individual markers rather than the creation of a protein profile.

4.1.2. Metabolomics

Just as spent *in vitro* fertilization culture media can be assayed for proteins by various methods, so too can metabolites be identified that can provide insight into the metabolic status of the embryo. While mass spectrometry coupled with chromatography for sample separation can be used for this purpose (as in secretomic profiling), the majority of studies to date have employed various spectroscopic techniques to identify and compare metabolites between samples.

Near infrared and Raman spectroscopy are both vibrational spectroscopic techniques which produce metabolite profiles based on the vibrational characteristics of molecules present in a given sample. While these techniques are based on similar principles, they have distinct advantages and disadvantages over one another with respect to the intensity of signal produced and the specificity for identifying various sample components. Utilizing both techniques, Seli *et al* identified differences in the metabolite profile between embryos that did and did not implant (60). The investigators then used this information

to create viability indices by which the reproductive potential of other embryos could be measured. Ultimately, both techniques predicted embryo viability with fairly good sensitivity and specificity, albeit in a retrospective fashion.

Other types of spectroscopy have also been employed for determining embryo quality based on a metabolomic profile. Identifying metabolites based on molecular behavior within a magnetic field, nuclear magnetic resonance spectroscopy has been used to identify differences in metabolite profiles between embryos with different reproductive potential (61). Utilizing nuclear magnetic resonance spectroscopy, Seli *et al* demonstrated higher glutamate concentrations in culture media from embryos that resulted in pregnancy compared to embryos that did not implant. In addition, a trend towards lower alanine, pyruvate and glucose was found in media from embryos that successfully implanted, although these results did not reach statistical significance. Based on their findings, the authors created a viability index that was predictive of reproductive success with a sensitivity and specificity of 88.2%. While this predictive value is comparable to that obtained with near infrared and Raman spectroscopy, the technique does not lend itself as readily for widespread clinical application as it is generally more expensive and more time-consuming with respect to data collection and analysis (62). The ideal analytical technique for metabolomic profiling of embryo culture media in a clinical setting thus has yet to be determined, and ultimately some combination of multiple tests may be necessary to provide the appropriate predictive value.

4.2. Clinical Applications

Since secretomic analysis of culture media is not invasive of the embryo itself, it lends itself to assisted reproductive technology applications, and findings from secretomic analyses have already found clinical utility. In 2002, Fuzzi *et al* found a correlation between soluble human leukocyte antigen G (HLA-G) in IVF culture media and successful embryo implantation (63). Based on these findings, Sher *et al* selected embryos for transfer after IVF based upon HLA-G status, as determined by enzyme linked immunosorbent assay of the culture media (64). Those embryos expressing HLA-G had significantly higher implantation and pregnancy rates than those embryos that were HLA-G negative, demonstrating the predictive value of the biomarker.

While metabolomics has yet to be applied to the selection of embryos for transfer in a prospective fashion, the technique has shown promise as several recent studies have demonstrated its potential clinical utility. Scott *et al* showed the ability of Raman spectroscopy to predict, in a prospective blinded fashion, the reproductive potential of day 3 and day 5 embryos based on a calculated viability index (65). Blinded to the ultimate pregnancy outcome, the investigators were able to predict delivery or failed implantation with a diagnostic accuracy of 80.5%. The technique was also shown to be rapid and convenient in assessing metabolites in culture media and thus has potential for use in embryo selection prior to transfer.

Two additional studies demonstrated the benefit of judging embryo quality on metabolomic assessment in addition to the traditional morphologic grading in the setting of single embryo transfer (66,67). Both investigations showed the predictive value of metabolomic profiling while revealing a lack of correlation between metabolomic-derived viability indices and morphologic grades of day 2 or 3 embryos. And while both studies were performed in a retrospective fashion, Seli *et al* did demonstrate the ability to predict an embryo's reproductive potential while blinded to the ultimate pregnancy outcome (67). Taken together, these data show the potential benefit to adding metabolomic assessment to the process of embryo selection. As the authors explain, this method would be especially useful in cases of single embryo transfer when choosing between two embryos of equal morphologic grade as it may improve our ability to select the one with the greater reproductive potential.

5. IN-VITRO MATURATION

In order to perform *in vitro* fertilization, oocytes must be retrieved and fertilized, followed by transfer of the resultant embryo(s) into the uterine cavity. Traditionally, exogenous gonadotropins are administered to stimulate the development of multiple oocytes, which are then retrieved via transvaginal aspiration. During this process, antral follicles are recruited by the action of follicle stimulating hormone (FSH) and undergo maturation to the Graafian stage prior to retrieval. The benefit of this method is that it somewhat mimics the normal *in vivo* development of follicles, albeit under supraphysiologic conditions. However, the process of controlled ovarian stimulation has several negatives, including the high cost of medications and the risk of ovarian hyperstimulation syndrome, which can be as high as 10% in certain at-risk populations (68).

As an alternative, a process of *in vitro* maturation has been developed utilizing unstimulated antral follicles, thereby foregoing the need for gonadotropin treatment. These antral follicles contain oocytes arrested in prophase of meiosis I and are cultured *in vitro* for 24-48 hours until they reach metaphase of meiosis II, the corresponding developmental stage to an oocyte ovulated *in vivo*. The mature cultured oocyte then undergoes fertilization, either via standard insemination or, more commonly, by intracytoplasmic sperm injection (reviewed in 69,70).

The first live birth resulting from *in vitro* maturation was reported by Cha *et al* in 1991 (71). Since then, improvements in our understanding of folliculogenesis have led to modifications in the process of *in vitro* maturation, including culture conditions (e.g. contents of culture media, culture techniques) and aspects of oocyte retrieval (e.g. pre-retrieval priming with FSH or human chorionic gonadotropin, hCG). Additionally, investigators are exploring the possibility of *in vitro* follicular growth from ovarian tissue prior to *in vitro*

maturation and subsequent *in vitro* fertilization (discussed below).

5.1. Culture Media

The environment in which oocytes are cultured during *in vitro* maturation plays a significant role in their development, and investigators have studied various culture methods across different species in an attempt to determine optimal conditions (reviewed in 69). Studies have compared various culture media on the maturation of human oocytes (72,73), and while no clear consensus has been reached, it is clear that the choice of media can influence oocyte maturation rate and energy consumption (73).

Investigators have shown pyruvate to be the preferential energy source of human oocytes during *in vitro* maturation (73), and as such it is often supplemented in maturation media. Due to the role of gonadotropins in the *in vivo* maturation of oocytes, recombinant FSH and luteinizing hormone (LH) or hCG are usually added to *in vitro* maturation culture media. While animal studies demonstrate improved oocyte maturation and fertilization when FSH and LH/hCG are added to culture media (74,75), supporting data for this practice in human clinical applications is limited (74,76). Further studies are necessary to determine the exact role of gonadotropins in *in vitro* human oocyte maturation.

Serum is also often included in culture media as a source of albumin as well as steroid precursors and growth factors. However, extended culture in serum-containing media has been shown to adversely affect the epigenetic profile of bovine oocytes (77). If serum is omitted from culture media, albumin must be supplemented (69).

In general, concerns have been raised as to the effects of *in vitro* maturation, and specifically culture conditions, on gene imprinting as oocytes undergo epigenetic modifications during maturation (69,78). More research needs to be performed to further elucidate the impact of *in vitro* maturation on epigenetic modifications, and in the future epigenetic profiling of oocytes may become necessary prior to fertilization and transfer.

5.2. Oocyte Retrieval

While the technology behind oocyte retrieval for *in vitro* maturation is similar to that used in standard *in vitro* fertilization, enhancements in pre-retrieval treatment regimens have led to higher success rates. Several studies have shown that priming with hCG *in vivo* prior to oocyte retrieval improves *in vitro* oocyte maturation rates (79,80,81), and this practice is now being employed for patients with and without polycystic ovarian syndrome (82,83).

Data demonstrating the effect of *in vivo* FSH pretreatment on oocyte maturation and subsequent pregnancy rates have been somewhat less consistent. Several investigators have shown that FSH priming increases both the number of oocytes retrieved and the maturational potential of these oocytes in healthy women (84) and those with polycystic ovarian syndrome (85). However, other studies have failed to demonstrate an improvement in maturation, fertilization or pregnancy rates after FSH pretreatment in these two groups of women (86,87). Most recently, Fadini *et al* conducted a prospective analysis of various gonadotropin priming regimens, including no treatment, FSH alone, hCG alone, and FSH plus hCG, in normovulatory women (81). Oocyte maturation rates were higher in those groups receiving hCG (with or without FSH). Most clinically relevant, however, the group receiving FSH plus hCG showed the highest clinical pregnancy rate (29.9%) of all groups, suggesting a benefit to the combination of gonadotropins.

Recent data has also demonstrated the importance of the timing of oocyte retrieval on *in vitro* maturation success. Comparing the relationship of dominant follicle size at the time of retrieval to *in vitro* maturation outcomes in polycystic ovarian syndrome patients, Son *et al* found higher implantation and clinical pregnancy rates in oocytes collected from follicles less than 14mm compared to those greater than 14mm (88). While these data need to be confirmed by additional studies, ideally in a prospective fashion, they suggest yet another way to improve outcomes and to further refine and enhance *in vitro* maturation protocols.

5.3. Clinical Applications

Because *in vitro* oocyte maturation avoids the need for extensive gonadotropin stimulation, it provides a safer alternative to controlled ovarian hyperstimulation in patients at risk of ovarian hyperstimulation syndrome. Thus, the majority of studies investigating the clinical application of *in vitro* maturation have been performed in patients with polycystic ovarian syndrome or others at increased risk of ovarian hyperstimulation syndrome. These studies report fertilization rates up to 73.3% (83), implantation rates up to 21.6% (85), clinical pregnancy rates up to 40.3% (88), and live birth rates as high as 15.9% (89).

In addition to these over-responders, *in vitro* maturation has also been successfully applied to patients with a poor response to ovarian stimulation (90). Typically, a lack of adequate oocyte growth or number in response to exogenous gonadotropins results in the cancellation of that cycle. However, Liu *et al* demonstrated in these patients that immature oocytes could be aspirated (from follicles ≤14mm in diameter) and matured *in vitro*, resulting in a 78.8% fertilization rate, a 20% implantation rate, and two live births (and a third ongoing pregnancy) in eight total cycles (90). *In vitro* maturation thus offers a novel therapeutic option for poor responders in addition to hyper-responders.

5.4. *In vitro* Follicular Growth

While the *in vitro* maturation of oocytes offers a promising option for those at risk of hyperstimulation or of poor response to gonadotropins, the procedure requires that the follicles already be at the antral stage. *In vitro* growth of follicles, on the other hand, involves the harvesting and *in vitro* development of primordial or preantral follicles.

The ultimate goal of this technique is the growth of follicles to the antral stage, at which point they can undergo *in vitro* maturation and ultimately fertilized. An effective method of *in vitro* growth followed by *in vitro* maturation would offer a revolutionary option for fertility preservation in patients undergoing chemoradiation, and although a successful human pregnancy has yet to result, investigators are making strides in human and animal studies (reviewed in 69).

Briefly, the initial step in the process involves the biopsy of ovarian cortical tissue, which in human studies has been performed at the time of gynecologic surgery or elective cesarean delivery (91,92,93). At this point, follicles can be isolated either mechanically (94) or by enzymatic digestion of the surrounding ovarian stroma (91) prior to being cultured. While the follicle yield from these procedures is generally good, follicles isolated in this manner undergo atresia in culture within several days, possibly due to the loss of ovarian stromal support. As a result, investigators have cultured strips of ovarian cortex, with the primordial follicles remaining *in situ* (92,93). Using this method, one study demonstrated viability in 66% of follicles within strips of ovarian cortex after 4 weeks in culture, with most of them progressing to the primary or secondary stage of development (93). Whether these oocytes maintain the developmental potential to mature further and be successfully fertilized remains to be determined.

Using a two-step culture system, Telfer *et al* were able to successfully mature human primordial/primary follicles *in vitro* to the antral stage (92). Strips of biopsied ovarian cortical tissue were cultured for 6 days in serum-free medium, after which preantral follicles were isolated and cultured in microwells with activin A. Although only 30% of cultured oocytes demonstrated antral formation, the technique shows promise as a potential method for the *in vitro* growth of follicles prior to *in vitro* maturation.

Strips of baboon, bovine and ovine ovarian tissue have also been successfully grafted to the chorioallantoic membrane of chick embryos, a highly vascular structure known to support transplanted tissue (95,96,97). Unlike follicles cultured *in vitro*, those cultured in this manner do not undergo spontaneous development beyond the primordial stage, but do retain their ability to develop to the primary stage once removed from the chorioallantoic membrane (97). This technique could thus serve as a potential model for understanding the factors responsible for triggering the maturation of oocytes.

More recently, investigators demonstrated neovascularization and follicular survival in cryopreserved human ovarian tissue following transplantation to the chorioallantoic membrane of chick embryos (98). While the developmental potential of these follicles remains to be determined, this technique could serve as a model for studying human follicular maturation and ultimately improve our ability to develop follicles *in vitro* from the primordial stage to ovulatory follicles that can be successfully fertilized.

If using any of the above applications for *in vitro* growth in preparation for *in vitro* maturation, it must be kept in mind that while human oocytes preferentially utilize pyruvate as an energy source, somatic cells (such as the granulosa and theca cells which surround the oocyte within a follicle) require glucose. Thus, media supplementation must be changed appropriately to provide the proper growth environment.

6. CONCLUSION

Despite the breadth and diversity of the above advances, they all contribute to the same overall purpose: To increase success rates while improving patient safety. With multiple births one of the biggest safety concerns in assisted reproduction, single embryo transfer currently represents the most promising option for reducing the incidence of twins and higher order multiples. With the exception of countries in which it is mandated by law, however, single embryo transfer will not be widely practiced until it can produce success rates comparable to those from transferring multiple embryos. By improving our ability to select genetically healthy embryos with the best reproductive potential, the above advances in preimplantation genetic diagnosis and secretomics could further optimize single embryo transfer and bring it closer to reality. Additionally, innovations in *in vitro* maturation techniques address the other major safety concern, ovarian hyperstimulation syndrome, while potentially providing new options for poor responders and those in need of fertility preservation. Although further research is required prior to widespread clinical application, the recent advances discussed above show great potential for the widespread improvement of assisted reproductive technology.

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