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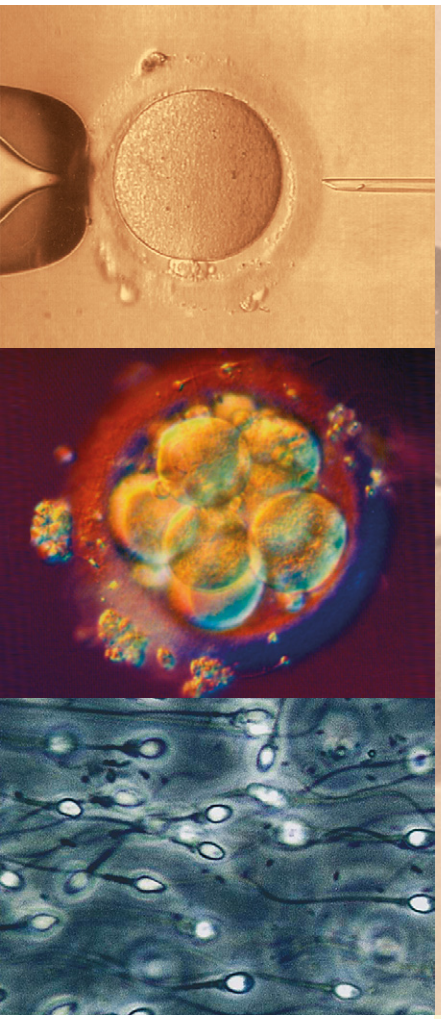

IFFS 2010 - Trilogy 5: Choosing the Best Embryo

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Non-invasive Approach to Embryo Selection: Non-invasive Assessment of Oocytes

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Summary Human oocytes and embryos, derived from in vitro fertilization (IVF) treatments, vary greatly in their capacity to produce a child. This presents a problem for fertility clinics, since they must decide which embryos should be transferred to the uterus in order to provide the greatest chance of a pregnancy. Current methodologies for the assessment of embryo viability, based upon analysis of morphology, provide only a weak guide to potential. Invasive tests, utilizing embryo biopsy followed by screening for chromosome abnormalities, may provide more definitive results. Embryos diagnosed aneuploid using such tests have little chance of producing a baby. However, the problem with invasive approaches to embryo testing is that poor biopsy technique can lead to embryo damage and loss of viability, potentially negating the benefits of improved embryo selection. The need for a safe, simple method providing improved embryo evaluation has led to research into a variety of non-invasive tests. Some research has focussed on proteomic or metabolomic profiling of embryos or the culture medium they are grown in, while other investigations have looked at mRNA transcripts in oocytes or their associated cumulus cells. This review discusses work towards non-invasive embryo evaluation, paying particular attention to recent transcriptomic analyses of oocytes and cumulus cells.

Introduction Assisted reproductive treatments utilizing in vitro fertilization (IVF) typically produce several embryos. In order to reduce the risks that a high-order multiple pregnancy will occur, the number of embryos transferred to the uterus are usually kept to a minimum. Multiple pregnancies are considered undesirable due to the increased incidence of serious complications for the mother and babies [1]. In some countries, it is already the case that embryos may only be transferred one at a time and there is also growing pressure on IVF providers in other countries to also move towards single embryo transfer (SET).

Since only a minority of the embryos produced during an IVF cycle are ultimately transferred, it is important that those chosen have the greatest potential for producing a live birth. The standard means of assessing embryo viability, carried out in all IVF laboratories, is to conduct a morphological assessment. However, the embryo grading systems used can vary significantly between different clinics and there is no consensus concerning which method is the best for this purpose. While microscopic analysis certainly provides some assistance in gauging embryo viability, it is acknowledged that morphology is only weakly correlated with embryo potential. The poor ability of current methods to discriminate viable from non-viable embryos explains, at least in part, the relatively low pregnancy rates achieved using IVF.

Invasive Methods of Embryo Evaluation and the Impact of Embryo Biopsy Apart from morphological analysis, the only methods widely used for embryo assessment are invasive, involving the removal and testing of cells from preimplantation embryos. In most cases the biopsied cells are subjected to chromosome analysis. The aim is to identify euploid embryos which can then be prioritized for transfer to the uterus. Chromosomally abnormal embryos are predicted to have little if any ability to produce a child and are therefore excluded from transfer [2–6]. Unfortunately, attempts to utilize embryo chromosome screening clinically have not always yielded the anticipated improvements in IVF pregnancy and birth rates [7–10]. There are a variety of biological and technical explanations for the poor outcomes reported by some aneuploidy screening studies [11, 12]. However, one of the most important reasons is likely to be damage done to the embryo during biopsy. Clearly, if embryo viability is compromised by biopsy the benefits of identifying chromosomally normal embryos will be reduced or eliminated.

To what extent might removal of a cell reduce implantation potential? Cryopreservation of cleavage stage embryos is often associated with the loss (degeneration) of one or more cells. One study has demonstrated that the loss of a single cell via this mechanism causes a 9.5 % reduction in embryo implantation, while if two cells are lost implantation rates decline by 54 % [13]. It is likely that a similar situation exists for embryo assessment methods that involve cell biopsy. Indeed, embryo damage may explain the results of two key studies that reported no benefits associated with chromosome. In one case, two cells were removed from each embryo in an effort to maximize diagnostic accuracy [7]. However, subsequent data from the same clinic has recently confirmed that biopsy of two cells leads to a significant reduction in implantation and birth rates [14]. The other study involved removal of a single cell, but the extremely low implantation rates of embryos that underwent biopsy, compared to those that did not provided a strong indication that embryos were seriously impaired by the biopsy procedure [9, 11, 12].

Although biopsy of a single cell, undertaken by experienced embryologists, appears to have little impact on embryo implantation [13, 14], there may be a small reduction in viability even for the most expert technicians. In experienced hands any negative effect of biopsy may be more than compensated for by improved selection of viable (i.e. chromosomally normal) embryos. However, techniques allowing embryos to be screened without removal of cells might be expected to yield even better results. Non-invasive approaches are also likely to occupy less of the embryologist's time compared with methods requiring biopsy, thereby reducing the costs associated with testing.

Non-invasive Strategies of Embryo Evaluation A variety of different strategies for the non-invasive assessment of oocytes and embryos have been proposed. Some research has focussed on collecting the medium in which each embryo is cultured and measuring the amounts of different molecules [15–18]. It is hoped that quantification of nutrient uptake from the medium and/or excretion of metabolites and other molecules into the medium might provide clues concerning the health of an embryo. Proteomic or metabolomic approaches of this kind typically involve analysis using mass spectrometry or alternatively near infrared or Raman

spectrometry. The former provides a detailed insight into the amounts of individual molecules in the medium, helping to identify key factors of potential significance for embryo assessment [18]. However, mass spectrometry is technically demanding and requires expensive equipment, and consequently its utilization will likely remain confined to biomarker discovery and other research rather than routine clinical use. Conversely, near infrared spectrometry and Raman techniques are cheaper and relatively easy to perform, but analysis of a complex mixture, such as culture medium, yields a complicated spectrum, making it difficult to clearly identify individual molecules. Although near infrared/Raman spectroscopy methods may be less useful for the discovery of specific biologically important molecules, algorithms to assess the spectra produced nonetheless show promise for the identification viable embryos [15, 16].

An alternative to screening media for viability markers is to undertake direct analysis of oocytes/embryos, in the hope that activity of specific cellular or developmental pathways may turn out to be indicative of potential. To date studies have focused on quantities of proteins in developing embryos [19] or the levels of different mRNA species in oocytes [20]. Aneuploidy is one of the most important defects affecting oocytes and embryos. It is extremely common, affecting more than half of all oocytes from women over 39 years of age and is almost always lethal to the embryo. For these reasons, one recent study attempted to find an association between altered amounts of mRNA transcripts in oocytes and aneuploidy [20]. Transcriptomic analysis, using microarrays to simultaneously interrogate ~30,000 genes, revealed 327 genes displaying statistically ($p < 0.05$) significant differences in transcript levels. The results indicated that oocyte aneuploidy is associated with altered mRNA transcript levels affecting a subset of genes. The possibility that different transcript levels in the oocyte affect the function of cellular pathways remains to be proven. However, it may be significant that some of the highlighted genes produce proteins involved in spindle assembly and chromosome alignment, key processes for maintaining accurate chromosome segregation [20]. In terms of the development of non-invasive assays, it is noteworthy that several genes displaying abnormal transcript numbers produce cell surface or excretory molecules. These molecules are readily accessible and may therefore serve as targets for non-invasive oocyte aneuploidy assessment.

Analysis of Cumulus Cells Transcriptomic analysis has also been applied to cumulus cells in an effort to gain an insight into oocyte and embryo viability. As oocytes mature they become surrounded by cumulus cells (CCs). The association between the CCs and the oocyte is an extremely close one, cytoplasmic projections pierce through the zona pellucida and form gap junctions at their tips with the oocyte [21]. This intimate association allows CCs to fulfil vital roles, supporting the maturation of the oocyte and relaying endocrine and other environmental signals. Cumulus cells metabolize the bulk of glucose consumed by the cumulus-oocyte-complex and supply metabolic intermediates like pyruvate to the oocyte [22]. Other substrates of low molecular weight, such as amino acids and nucleotides, are passed to the oocyte for its own synthesis of macromolecules. Cumulus cells are usually stripped away from the oocyte and discarded during IVF treatment and thus represent a promising target for the development of non-invasive assays.

Among the cellular components that have been assessed in CCs, antioxidants have received particular attention due to their importance in maintaining a healthy follicular environment. One class of antioxidant molecules, superoxide dismutases, are postulated to protect the oocyte from damage caused by reactive oxygen species. Superoxide dismutase activity in CCs shows a direct relationship with IVF outcome, decreasing with advancing female age and having higher activities in cycles with successful outcomes [23]. Glutathione S transferases, another class of enzymes known to protect cells from reactive oxygen species, have been shown to be good indicators of age related infertility, specifically glutathione S transferase theta 1 (GSTT1) [24]. Not only does this data emphasize the influence of oxidative stress on oocyte viability, but it also suggests that superoxide dismutases and GSTT1 in CCs might serve as potential biomarkers of prognostic significance.

Cumulus cell apoptosis rates have also been linked to the outcome of IVF treatments. Elevated levels of apoptosis in CCs are associated with the production of morphologically abnormal oocytes [25]. Additionally, an increase in CC apoptosis has also been associated with oocyte immaturity, impaired fertilization [26], suboptimal blastocyst development [27] and poor IVF outcomes [28, 29]. Whether abnormal/poor quality oocytes induce apoptosis in their associated CCs or whether high levels of CC apoptosis are symptomatic of a suboptimal follicular environment, leading to impaired oocyte development, remains unclear at this time. However, since tests for apoptosis are relatively straightforward to perform, the relationship between CC apoptosis and outcome presents an attractive target for development of diagnostic assays.

Cumulus cells are constantly responding to the intrafollicular environment, adjusting gene expression in order to maximize oocyte support and minimize damage caused by extrinsic factors. Data from an ongoing study in our laboratory has indicated that the follicular microenvironment might even play a role in the origin of oocyte meiotic chromosome abnormality, one of the most important causes of oocyte incompetence. The study indicated that cumulus cells associated with aneuploid oocytes have characteristic deviations in their gene expression profile [30]. Pathways related to cellular stress (e.g. hypoxia) displayed alterations in gene activity, suggesting an association between meiotic aneuploidy and suboptimal environment. Some of the abnormally expressed genes were involved in hormonal response, potentially providing a link between the increased frequency of aneuploidy seen with advancing age and the altered hormonal milieu in the ovaries of older women. Additionally, a number of genes with roles in apoptotic pathways were abnormally expressed, in concordance with previous studies suggesting an association between CC proliferation and/or apoptosis and poor IVF outcomes [26–28, 31].

Microarrays have also been utilised by several other groups for the analysis of CCs and oocytes. A study by Gasca and colleagues [32] identified several marker genes associated with oocyte maturation [32]. The genes were involved in processes such as cell cycle checkpoints and DNA repair, and included BARD1, RBL2, RBBP7, BUB3 and BUB1B. Although not yet clinically proven, it seems plausible that these genes may have relevance to oocyte quality. Another study, conducted by Assou et al [33], searched for an association between patterns of CC gene expression and embryo morphology or pregnancy outcome. Up-regulation of BCL2L11 (involved in apoptosis) and PCK1 (involved in gluconeogenesis) and down-regulation of NFIB (a transcription factor) were identified as potentially linked to outcome and proposed as biomarkers of oocyte potential [33].

Additional candidate CC markers of oocyte/embryo viability include STAR, COX2, AREG, SCD1 and SCD5 [34]. These genes were found to have mRNA transcript levels that were lower and distributed over a narrower range in CCs enclosing oocytes achieving blastocyst development compared with CCs associated with oocytes that failed to produce blastocyst stage embryos. PTGS2 (Prostaglandin-endoperoxide synthase; cyclooxygenase), HAS2 (hyaluronic acid synthase 2) and GREM1 (gremlin 1) have also been identified as potential markers of oocyte quality [35, 36]. CCs associated with oocytes that produced high quality cleavage stage embryos were found to have greater numbers of transcripts from these genes compared to CCs from oocytes that produced poor quality embryos. Early cleavage following fertilization, a characteristic considered to be a positive indicator of IVF outcome, may also be associated with characteristic CC gene expression patterns. In particular, mRNA levels of the genes CXCR4, GPX3, DVL3, HSPB1, CCND2, TRIM28, DHCR7 and CTNND1 appear to be useful predictors of early cleavage [37]. Finally, altered expression of the CDC42, 3βHSD, SERPINE2, FDX1 and CYPA191 genes have been shown to be associated with follicles that produced pregnancies, indicating that analysis of these genes in CCs may provide valuable clinical information [38].

Conclusion Techniques capable of supplementing routine morphological analysis, revealing additional information concerning embryo viability without harming the em-

bryo, are urgently required in order to improve IVF success rates, especially as clinics increase the proportion of single embryo transfer cycles. Non-invasive methods of oocyte and embryo assessment are particularly attractive, since such tests are unlikely to have any impact on viability. Although non-invasive techniques are still in their infancy, initial research has yielded encouraging data. A number of potential biomarkers of oocyte and embryo viability have already been identified and their clinical efficacy is currently being evaluated. It is not yet clear whether any of these new markers (or any combination of markers) will provide a definitive assessment of oocyte/embryo viability. It seems likely that, as with morphological analysis, levels of protein or mRNA detected from key genes will provide an indication of viability rather than an absolute diagnosis. Nonetheless, there can be little doubt that the disclosure of previously invisible aspects of oocyte/embryo biology, made possible by non-invasive quantification of biomarkers, will greatly enhance our ability to recognize embryos with a high potential for producing a child, leading to improved outcomes following IVF treatment, particularly for SET cycles.

Relevancy to Practice

- Existing methods are unable to accurately distinguish viable embryos produced using IVF from non-viable.
- Improved embryo selection is required if IVF success rates are to be increased. This is particularly important for cycles involving single embryo transfer.
- Invasive methods of embryo testing may be beneficial if optimal techniques are used, but non-invasive methods are likely to be less labour intensive and represent little or no cost to the embryo.
- Gene expression analyses of oocytes and cumulus cells have already revealed several promising candidates for the evaluation of oocyte/embryo viability. Further studies, to define the efficacy of screening using these markers are now required.

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Non-invasive Approach to Embryo Selection: Proteomics and Metabolomics

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The high success rates seen following in vitro fertilization (IVF) are attained in many cases through the simultaneous transfer of multiple embryos at the expense of multiple pregnancies. Multiple pregnancies, in turn, are associated with significant morbidity and mortality, primarily due to their propensity to result in preterm birth. Consequently, decreasing multiple gestations while maintaining or improving overall pregnancy rates remains the most significant contemporary goal in the treatment of infertility. In order to achieve this goal, an improvement over our current embryo assessment strategies largely based on embryo morphology and cleavage rates would be useful.

Proteomics The proteome is the entire complement of proteins expressed by a genome, cell, tissue or organism, at a given time and under defined conditions, while the secretome is the subset of the proteome that is actively exported from the cell. Proteomics is the study of a biological system's complete complement of proteins and has recently been applied to the analysis of embryos and embryo culture media in order to identify novel biomarkers associated with embryo development and viability.

Katz-Jaffe et al. first determined the protein biomarkers of development in mouse and human embryos. More recently, they used proteomic profiling of the spent embryo culture media via time-of-flight mass spectrometry to identify markers of embryo viability. They identified different secretome profiles at different embryonic developmental stages, and identified a correlation between upregulation of ubiquitin, a protein implicated in the implantation, and ongoing blastocyst development. These findings will need to be validated in blinded studies.

Metabolomics In 2007, Seli et al. reported the results of a proof-of-concept study where they collected day 3 spent culture media of individually cultured embryos with known pregnancy outcome and analyzed the samples using near infrared (NIR) and Raman spectroscopy [Seli et al. Fertil Steril 2007]. The mean spectrum for embryos that resulted in live birth was determined and compared to the mean spectrum obtained from embryos that failed to implant. Using a mathematical model, the regions in the spectrum most predictive of pregnancy outcome were identified. Next, based on these regions in the spectrum, an algorithm to calculate a viability index for each individual embryo reflective of its reproductive potential was developed. In this initial study, using both NIR and Raman spectroscopy, the mean viability index of embryos that implanted and resulted in a live birth was significantly higher ($p < 0.01$) compared to the mean viability index of embryos that failed to implant. Raman and NIR spectroscopies achieved a sensitivity of 76.5 % and 83.3 %, and a specificity of 86 % and 75 %, respectively. Moreover, the test was rapid (< 1 minute per sample) and required a very small sample volume (< 15 ml). Subsequently, the algorithm described above was tested in prospective blinded trial [Scott et al. 2008] and successfully predicted the pregnancy outcome for embryos transferred on day 3 and day 5 ($p < 0.05$).

The initial studies described above were followed by studies with larger sample size from centers that routinely perform single embryo transfer (SET) on day 2, 3, or 5. They found higher mean viability indices in culture media of embryos that resulted in a pregnancy, compared to those that did not. These studies have also shown that the metabolomic profile of embryo culture media was independent of morphology, therefore providing an independent parameter, and that a positive correlation existed between increasing viability index values and the reproductive potential of individual embryos ($p < 0.001$).

These data strongly suggest that in vitro cultured embryos that have a high reproductive potential alter their environment differently compared to embryos that do not result in a pregnancy, and that the difference is detectable by metabolomic profiling of spent culture media using spectroscopy and bioinformatics. Further investigation is necessary to validate the proposed models in different types and volumes of media, collected following embryo transfer at different stages of pre-implantation development, and to determine whether an on-site testing will be clinically valuable in aiding morphologic assessment.

Microarrays and CGH for PGD of Chromosome Abnormalities and Gene Defects

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Preimplantation genetic diagnosis for aneuploidy has become a fairly routine part of assisted reproductive technology worldwide. Although the theory of aneuploidy screening makes scientific sense, no randomized controlled trial (RCT) has shown a benefit of screening embryos for common chromosome anomalies. Here we discuss both the positive and negative aspects of current technology and review the literature to this point. Topics such as mosaicism of early embryos, the limitations of current fluorescence in situ hybridization (FISH) technology, as well as poor technique in the laboratories carrying out the embryology and genetic diagnostics, are discussed. New array-based testing platforms that allow for testing of all 24 chromosomes have shown promise in a few recent publications and abstracts at scientific meetings. The two principal array-based platforms are explained, then compared and contrasted, followed by a discussion of validation strategies for new technologies. Finally, the current state of the clinical use of array-based testing is reviewed. While no RCT has shown a benefit of aneuploidy screening to date, array-based testing platforms show great promise.

Introduction More than 50 % of cleavage-stage embryos produced in vitro are chromosomally abnormal, increasing to up to 80 % in women 42 and older [1–4]. Although some aneuploid embryos arrest during extended culture, most do not, and even at the blastocyst stage more than half of all embryos are abnormal (mean maternal age 38 years) [5]. The majority of numerical chromosome abnormalities detected in embryos are not compatible with implantation or birth thus negatively affecting the success of assisted reproductive treatments. The detrimental effect of aneuploidy is illustrated by the high prevalence of chromosome abnormalities detected in spontaneous abortions, exceeding 70 % in some studies [6–11]. It has been hypothesized that selection of embryos for transfer based on chromosome normalcy (euploidy) could improve success rates in assisted reproductive procedures [12]. This process is known as preimplantation genetic diagnosis (PGD) of aneuploidy or preimplantation genetic screening (PGS).

Shortcomings of Pre-Array Technologies The first PGD strategies to be described employed fluorescence in situ hybridization (FISH) analysis of cells biopsied from day-3 embryos [12–16], trophectoderm cells biopsied from blastocyst stage embryos [17] or polar bodies biopsied from oocytes or zygotes [18–22]. The FISH methods allowed analysis of 5–12 chromosomes in each oocyte or embryo, but were unable to provide a full evaluation of the chromosome complement.

Some studies utilizing FISH-based strategies reported an improvement in implantation rates, reduction in spontaneous abortions and/or an increase in take home baby rates [15, 21, 23–32]. However, these studies were not randomized. Other studies, some performed in a randomized fashion, did not produce significant improvements or showed a detrimental effect of PGD for aneuploidy [33–36]. Several reasons for these conflicting results have been advanced.

The biological argument, which does not explain differences in reports, but attempts to understand why some studies observed a negative effect on outcome, argues that cleavage-stage embryos have such high rates of chromosomal mosaicism that any analysis based upon a single cell is unreliable. Although it is true that mosaicism is common in cleavage-stage embryos (about 30 % according to FISH analyses) [1–4], the majority of these embryos display chromosome abnormalities in every cell. In such cases, the biopsied cell may not be chromosomally identical to the remaining cells of the embryo, they may contain errors affecting different chro-

mosomes, but the clinical diagnosis of 'abnormal' is still valid. Large follow-up studies of preimplantation embryos diagnosed using FISH estimate only a 5–7 % error caused by mosaicism [15, 37], and thus mosaicism is unlikely to be the primary cause of poor outcomes following PGD.

The most probable cause of inter-center differences in PGD results are variations in the embryological and genetic technologies employed. These encompass all aspects of the process and have been previously reviewed [38]. Here we will discuss briefly only a few of the key factors. Probably the most important variable in PGD is the embryo biopsy itself. One of the studies showing no difference in IVF outcome following PGD involved biopsy of two cells from each cleavage-stage embryo [33]. However, the same group later reported that two-cell biopsy, in contrast to single-cell biopsy, is detrimental to embryo development [39].

Even biopsying one cell in sub-optimal conditions could be extremely damaging to embryo potential. A study conducted by Mastenbroek et al. [35] reported an astonishingly high rate of diagnostic failure (20 %), resulting in many embryos being transferred without a diagnosis. The implantation rate of these undiagnosed embryos was 59 % lower than the control. In this case, the only difference between the control and test groups appears to have been the biopsy, suggesting that embryo viability was drastically reduced by the biopsy procedures used in the clinics involved.

The second most important factor in obtaining good results can be summarized in the "error rate". The steps after biopsy involve fixation, FISH with a variety of potentially different protocols and probes, and cell scoring. However, the overall accuracy of these steps can be summarized in a single number, which is the error rate of a PGD laboratory. This error rate can be obtained by reanalyzing all the cells of non-replaced embryos (abnormal embryos and arrested normal embryos) and determining if the original diagnosis was correct. Unfortunately error rates vary widely, ranging from 4–5 % [2, 15] to 40–50 % [40, 41] depending on the PGD laboratory. As shown in a recent review, error rates around 50 % will in fact decrease implantation rates [38].

When performed using appropriate, well validated methods, FISH can detect 90 % of the chromosome abnormalities detected by CGH [16, 42], and some PGD laboratories do appear to obtain consistently good results with FISH and cleavage-stage embryo biopsy. Regardless, the field of PGD is evolving away from biopsying at this stage of embryo development and is increasingly focusing on biopsy of polar bodies from oocytes or zygotes or removal of trophectoderm cells from blastocysts. These embryonic stages may be more resilient to technical manipulation. Additionally, the limited chromosomal screening conveyed by FISH is increasingly being replaced by comprehensive methods of DNA analysis, which detect close to 100 % of chromosomal abnormalities. The new wave of aneuploidy testing technologies are extremely redundant (each chromosome tested multiple times at different sites), readily automated, less subjective, and theoretically less prone to errors.

Comprehensive DNA Analysis Techniques Here we will cover three techniques that are currently being used for PGD of chromosome abnormalities.

Comparative Genome hybridization (CGH) was first applied to day-3 embryo biopsies [43–47]. However, CGH is time consuming and is incompatible with day-3 biopsy and transfer by day-5, necessitating cryopreservation of embryos while testing is carried out. At the time that it was first applied embryo freezing was a relatively inefficient technique and the low survival rate of thawed embryos likely neutralized any beneficial effects of CGH. For these reasons, CGH was temporarily abandoned and not applied again until the development of vitrification [48]. In conjunction with vitrification, CGH has been clinically applied to polar bodies [49–51] and blastocyst biopsies [5, 52]. The combination of CGH, Blastocyst biopsy and vitrification significantly improved implantation rates in a recent study, from 46.5 % in controls to 72.2 % in cycles with screening, with nearly 100 % of blastocysts surviving biopsy [5]. However, many clinics are not yet proficient at blastocyst culture and vitrification. Furthermore, freezing adds extra cost to the cycle and a majority of patients prefer to have a fresh cycle. Thus, for the time being, day-3 biopsy combined with comprehensive chromosome analysis remains the choice for most physicians and patients.

Two other techniques, microarray CGH (array-CGH or aCGH) [53–57] and single nucleotide polymorphism (SNP) microarrays [58–60], can be used for comprehensive chromosome analysis of single cells from day-3 biopsy and yield results in 24 hours. The rapid turnaround time for these methods eliminates the need to cryopreserve embryos while testing is carried out.

Array-CGH (aCGH) is already widely used for the cytogenetic analysis of prenatal and postnatal samples [61–66] since it is rapid, cost effective and allows chromosomal regions to be screened at high resolution. Several types of aCGH platform are available for the purposes of aneuploidy screening. The variety most commonly used for the purpose of PGD utilizes bacterial artificial chromosome (BAC) probes, about 150,000 bp in length, covering all chromosome bands and giving a 4MB or lower resolution. Even higher resolutions are achievable but not generally recommended since at that level the difference between clinically significant duplications/deletions and normally occurring copy number variations is less clear. A microarray recently validated for PGD had 4,000 probes and thus covered ~25 % of the genome sequence [58]. Microarray-CGH has a similar accuracy rate to conventional CGH, and should therefore be capable of producing similar results to those obtained in the promising CGH study performed by Schoolcraft et al. [5].

CGH and aCGH provide a quantitative analysis based on comparing the relative amount of DNA from two different sources, one from the clinical sample (e.g. a cell from an embryo) and another from a chromosomally normal individual. DNA samples from the two sources are differentially labeled and hybridized to either metaphase chromosomes (CGH) or probes on a microarray (aCGH). In the case of aCGH, each probe reveals the relative amounts of these two DNAs at a single chromosomal site. Since multiple copies of each probe are placed on the microarray and each chromosome is tested at many distinct loci, the diagnosis is very accurate.

Chromosome imbalances (aneuploidies, unbalanced translocations, deletions and duplications) are easily detected using CGH and aCGH, but a limitation of these approaches is that diploidy cannot be distinguished from changes involving loss or gain of an entire set of chromosomes (e.g. haploidy, triploidy, tetraploidy, etc). How important is this? Ina recently submitted paper (Munne et al, personal communication) about 7.7 % (n = 91,073) of the supposedly 2PN embryos tested were polyploid or haploid but the majority of them had additional abnormalities detectable by CGH or array-CGH and only 1.8 % of all embryos were homogeneously polyploid or haploid. Furthermore, of those, the majority arrested by day 4, leaving only 0.2 % of developing embryos uniformly polyploid or haploid. This suggests that failure to detect polyploid embryos may rarely lead to a misdiagnosis, but is unlikely to have a significant impact on the clinical efficacy of the screening using aCGH or CGH.

Single nucleotide polymorphisms are areas of the genome where a single nucleotide in the DNA sequence varies within the population. Most SNPs are biallelic, existing in one of two forms, and are found scattered throughout the genome. By determining the genotype of multiple SNPs along the length of each chromosome a haplotype (a contiguous series of polymorphisms on the same chromosome) can be assembled. This ultimately allows the inheritance of individual chromosomes or pieces of chromosomes to be tracked from parents to embryos. Current SNP microarrays simultaneously assay hundreds of thousands of SNPs, while utilizing powerful software to distinguish how many copies of each chromosome was inherited by an embryo [58, 60, 67].

All of the new generation of chromosome screening methods (CGH, aCGH and SNP-microarrays) rely on whole genome amplification (WGA) to amplify DNA from the single cell or small number of cells removed from a developing embryo [68]. CGH can be performed in combination with a variety of WGA methods, however, SNP-microarrays are more sensitive to the type of amplification technique used and are not compatible with all methods. Currently, WGA methods like multiple displacement amplification (MDA), GenomePlex and PicoPlex are most commonly used for SNP-microarrays. These amplification methods allow for better over-

IFFS 2010 – Trilogy 5

all coverage of the genome compared with earlier WGA methods (e.g. degenerate oligonucleotide primed PCR) and are less inclined to preferentially amplify some parts of the genome while leaving others unamplified or under amplified.

Currently, a few PGD groups around the world are validating SNP-microarrays and analysis software for clinical use in PGD for aneuploidy screening. It is expected that data from the clinical use of SNP-microarrays will closely match the data from CGH and aCGH testing. While the technologies differ greatly, both types of arrays (CGH-based and SNP-based) are trying to answer the same question; how many copies of each chromosome is present in a sample?

The small size of the SNP array probes, can lead to poor hybridization efficiencies and low signal intensities for individual probes. This factor, coupled with the failure of WGA methods to amplify the entirety of the genome, can lead to many probes yielding no result (i.e. a low "call rate"). Also, allele drop out (ADO) and/or preferential amplification (PA) of one SNP allele versus another can lead to a great deal of 'noise' in the system which requires sophisticated interpretation. Several methods for the cleaning up of data from SNP-microarrays have been developed: Qualitative methods, looking only at the inheritance of specific SNPs and requiring comparison with parental DNA samples; quantitative approaches, assessing only the intensity of SNP calls; and techniques combining qualitative and quantitative methods, using both SNP intensity calls and inheritance patterns.

For qualitative approaches it is necessary to assess parental DNA prior to clinical embryo testing. The key requirement is the deduction of the four parental haplotypes for each chromosome. Embryo testing is then focused on detecting the individual parental haplotypes, revealing how many chromosomes were inherited from each parent i.e. Karyomapping [58]. This approach has the disadvantage that mitotic abnormalities, in which only two haplotypes are present in a trisomy (i.e. caused by duplication of one of the two chromosomes in the embryo after fertilization), will not be detected. This can misdiagnose a substantial amount of embryos since 30 % of aneuploid embryos contain mitotic abnormalities (mosaics) [15]. A quantitative approach compares the intensity of each SNP against the other SNPs. A purely quantitative approach for aneuploidy screening may not require parental testing ahead of the cycle, however, this approach would not be compatible with combination testing of single gene defects with aneuploidy screening (discussed below). This approach is currently the least developed. A qualitative/quantitative approach has also been applied clinically, and probably can obviate the issues mentioned above for purely qualitative or quantitative approaches [60, 67]. All of the analysis approaches still share one limitation and that is the diagnosis of tetraploidies. In a tetraploid cell, only two haplotypes are present (i.e. a postmeiotic duplication of an euploid cell), therefore all SNPs will have the same intensity.

SNP-based microarrays offer some advantages over aCGH: a) If qualitative analysis is employed, SNP-based microarrays can also detect the parental origin of any chromosome abnormalities. This may be valuable in rare instances of young couples producing many chromosome abnormalities, but of little relevance to cases of advanced maternal age where at least 90 % of the aneuploidies will be maternal in origin; b) SNP microarrays applied to PGD for chromosome rearrangements can differentiate between normal and balanced (carrier) embryos. However, because the rate of abnormalities in translocation cases is generally very high (> 80 %) [69], the great majority of PGD cycles do not have a surplus of embryos with a balanced chromosome constitution. In most cases whatever balanced embryos are available are needed for transfer; c) SNP arrays can directly produce a fingerprint of the embryo, allowing for assessment of which of the transferred embryos led to a pregnancy. However, if a laboratory is using aCGH, a similar test can be performed by utilizing a small aliquot of the DNA produced by WGA to perform conventional DNA fingerprinting; d) Finally, qualitative SNP arrays can also detect uniparental disomy (UDP), although this is a very rare event (e.g. uniparental disomy 15 occurs in 0.001 % of newborns [OMIM]). A major disadvantage of a qualitative or combination approach to SNP array analysis is the need to assess parental DNA ahead of the PGD cycle. This complicates patient management, adds substantially to the cost of the test, and precludes ad hoc decisions on biopsy for PGD. Approximately 20 % of IVF cycles with planned PGD are cancelled on day three due to low embryo numbers. Thus, these patients would have spent money on pre-cycle parental testing that was ultimately unnecessary.

Validation of aCGH and SNP Arrays Due to the intrinsic and often unforeseen problems with every new technology, a novel method should always be validated against other, more established methods. Assessing a new approach against itself may preclude the detection of technique related flaws. Thus, validation by inadequate methods such as the analysis of cell lines with defined chromosome abnormalities which cannot mimic mosaicism and other peculiarities of the cell being tested; analysis of eggs or embryos by one technique with analysis of polar bodies or the remainder of the embryo by the same technique which will preclude identifying abnormalities not detectable by that technique; blindly replacing undiagnosed embryos (either by single embryo transfer or fingerprinting the embryo) and following pregnancies and clinical losses to determine the fate of each tested embryo which does not account for the status of non-implanted embryos; or using the SNP calls in one chromosome as internal controls for other SNPs in that same chromosome [60] may lead to false assumptions. In addition, the use of analysis tools that are qualitative in nature will miss the presence of two chromosomes of the same grandparental origin, and the errors caused by mosaicism will not be taken into account in this validation mode, resulting in bogus 99.9 % confidence results.

In our opinion, the optimal method for validating a new technique is to reanalyze those embryos that were not transferred to the patient, either because they underwent arrest or because they were diagnosed chromosomally abnormal. The reanalysis of these embryos should be done with another well established technique, the 'gold standard'. This would discern shortcomings of the new method under evaluation and account for issues related to embryo biology, such as mosaicism. The only problem with this approach is that euploid arresting embryos may become abnormal (karyokinesis without cytokinesis) from day-3 to day-5 before reanalysis [70, 71] and there is a scarcity of non-replaced normal embryos.

To simplify comparison between studies, an error should be classified as diagnosing an embryo as euploid when reanalysis shows that it was abnormal or vice versa. Due to the extent of mosaicism, an error rate per chromosome has questionable relevance and no clinical importance compared to an error rate per embryo.

SNP-microarrays have undergone a variety of validation experiments, such as comparison of PGD results and analysis of babies born [67, 72], SNP-microarray reanalysis of embryos previously analyzed by SNP arrays [59], and using data from one set of SNPs as internal controls for another set of SNPs. To date, no studies have confirmed the original diagnosis by reanalyzing the remaining embryonic cells with a different technique.

Microarray-CGH for PGD has been validated by analysis of single cells from known cell lines (Dagan Wells, personal communication) and by analyzing eggs with aCGH and comparing them to the results obtained using aCGH of the corresponding PBs (Montag and Gianaroli, personal communication). In a recent study, day 3 embryos analyzed by PGD with aCGH that were not replaced because of chromosome or morphological abnormalities were reanalyzed in most of their remaining cells by FISH using 12 probes for the most common chromosome abnormalities plus probes for any chromosomes found abnormal according to aCGH. Only 1.9 % (1/54) of embryos were found to be incorrectly diagnosed [57].

Clinical Results Of the techniques discussed, CGH is the one for which the greatest quantity of clinical data is available [5, 49, 50, 52]. Sher et al. [49] detected a 74 % ongoing pregnancy rate per transfer and 63 % per retrieval in women with an average age of 37.5 years. For patients of a similar age, receiving blastocyst transfer, Schoolcraft et al. [5] detected a significant increase in implantation rates, from 46.5 % to 72.2 % ($p < 0.001$) following embryo selection using CGH. Interestingly both studies showed high implantation rates and both avoided cleavage

stage embryo biopsy and transferred embryos that had previously been cryopreserved in a later cycle. In addition to the potential benefits of transferring euploid embryos, there may be additional advantages associated with transfer in a non-stimulated cycle [73]. Loss of blastocyst stage embryos after devitrification in the study by Schoolcraft et al was minimal (0.7 %) [5].

Less clinical data is available from SNP- and CGH-microarrays. In presentations at ASRM, Schlenker et al. [74] reported that CGH- and SNP-microarrays provided the same high implantation rates after blastocyst biopsy and vitrification.

Regarding day-3 biopsy followed by aCGH and day-5 replacement, our most recent data [75] showed that only 118/151 PGD cycles had normal embryos for transfer in a population 38 years of age. The pregnancy rate was 59 % per transfer compared with 38 % in controls with a transfer ($p < 0.001$). The ongoing pregnancy rate for the PGD group was 54 % per transfer, compared with 31.1 % in controls with a transfer ($p < 0.001$). These results are encouraging, but not as impressive as the day-5 (blastocyst) biopsy results. It is probable that the difference between clinical results obtained using CGH and aCGH is related to the stage at which biopsy was carried out rather than to differences in the method of chromosome screening. It is very likely that aCGH will replicate the results obtained by CGH when applied in conjunction with blastocyst biopsy. In summary, although data on the clinical application of comprehensive chromosome analysis techniques is preliminary, all studies suggest a significant improvement in ART results.

Microarrays for PGD and Preconception Screening of Gene Defects Neither CGH-microarrays nor the SNP-microarrays in current use can directly detect gene defect mutations. However, SNP-microarrays can be used to indirectly infer the presence or absence of a chromosome segment containing a mutant gene (i.e. identification of the same SNP haplotype as the parental chromosome carrying the mutation). A diagnosis can be performed based upon this sort of information [58], indeed, this approach has recently been applied clinically for the simultaneous detection of gene defects and chromosome abnormalities [76].

In the case of aCGH, although gene defects cannot be detected directly, enough DNA is produced during the WGA step of the procedure that an aliquot can be used for aCGH analysis of chromosome abnormalities and another taken for PCR-based analysis of gene defects.

The high levels of ADO recorded after WGA mean that direct detection of a mutation using a microarray is likely to be less reliable than existing forms of PGD. Microarray-based diagnosis will be safer using approaches such as Karyomapping, where conclusions are based upon the results from multiple linked SNPs, rather than a single mutation site. While the sort of microarrays used for preconception screening are not currently suitable for PGD, it is anticipated that their use will significantly increase the identification of high risk couples and therefore lead to an increase in the usage of genetic testing modalities such as prenatal testing and PGD.

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