

Diagnostic Techniques to Improve the Assessment of Human IVF Embryos: Genomics and Proteomics

Mandy G Katz-Jaffe

Introduction

A fundamental component of assisted reproductive technologies (ART) is the selection, from a cohort of embryos, the most competent for transfer. Principally, comprehensive morphological assessment is used to determine the embryo/s with the highest implantation potential [8, 29]. Though relatively successful, morphology-based selection has limitations, with more than 70 % of in vitro fertilization (IVF) embryos failing to implant. It is likely that this failure is due to the absence of developmentally competent embryos in a cohort as well as our inability to precisely select the most competent embryo. Therefore, the field of ART would be at a significant advantage with more precise and quantitative methods of embryo viability determination. The ability to select the most developmentally competent embryo in a cohort should improve pregnancy rates while allowing for routine single embryo transfers [41]. Improvements in platform sensitivity and cost effectiveness of omics technologies, including genomics and proteomics, has enabled the investigation of new approaches other than morphology to assess human IVF embryos.

Genomics

Chromosome aneuploidies, defined as the gain or loss of an entire chromosome, contribute to the vast majority (~70%) of pregnancy losses and congenital birth defects in both natural and ART conceptions. In fact a chromosomally aneuploid embryo or fetus will never result in a normal healthy pregnancy or baby. Advanced maternal age (AMA) is the most significant risk factor for chromosome aneuploid

M. G. Katz-Jaffe (✉)

National Foundation for Fertility Research, Lone Tree, CO, USA

e-mail: MKatz-Jaffe@FertilityResearch.org

Colorado Center for Reproductive Medicine, Lone Tree, USA

D. Sakkas et al., *Gamete and Embryo Selection*, SpringerBriefs in Reproductive Biology,
DOI 10.1007/978-1-4939-0989-6_2, © Springer Science+Business Media New York 2014

pregnancies. Furthermore, aneuploidy rates are higher in oocytes and embryos from women in their forties as they near the end of their reproductive lifespans [26]. Only a weak association has been observed between embryo morphology and chromosome constitution, thereby supporting the hypothesis that pre-implantation genetic diagnosis (PGD) for aneuploidy screening should improve reproductive outcomes during IVF.

PGD for Aneuploidy Screening

In the beginning, PGD for aneuploidy screening involved a blastomere biopsy from a cleavage-stage embryo with fluorescent in situ hybridization (FISH) examining a selected panel of chromosomes. The chromosomes most commonly observed in pregnancy loss and aneuploid deliveries were chosen for analysis, particularly chromosomes 13, 18, 21, X and Y. Initial retrospective studies were promising however, eleven randomized control trials (RCTs) and a meta-analysis showed no beneficial effects following FISH screening of a biopsied blastomere [23]. The lack of benefit has been attributed to a combination of concerns, specifically the high incidence of mosaicism in the cleavage stage embryo which questions the value a single cell may have as the representative of the whole embryo. Other concerns include the negative impact blastomere biopsy may have on the future developmental competence of the embryo, technical errors associated with the FISH technique itself and the lack of assessment of all 23 chromosome pairs. There was one group that was able to report a benefit from blastomere biopsy with FISH for repeated implantation failure (RIF) patients <40 years old and AMA patients 41–44 years old [31]. Utilizing blastomere FISH they conducted two randomized trials and observed a significant increase in live birth rates in both the RIF study (47.9 vs. 27.9%; $P < 0.05$) and the AMA study (32.3 vs. 15.5%; $P < 0.01$) with PGD for aneuploidy screening [31]. Nevertheless, it was evident that PGD for aneuploidy screening required a technique that could analyze all 23 pairs of human chromosomes.

Advances in molecular biology and cytogenetic platforms have now allowed for comprehensive chromosomal screening (CCS) or full karyotyping of biopsied material from human embryos. The first studies involved metaphase comparative genomic hybridization (CGH) [10, 34, 50] and have extended on to array based CGH [14, 40], SNP array technology [35, 37], and more recently quantitative real-time PCR [43] (Fig. 1). Independent of the molecular platform developed for CCS, the technique needs to be reliable, highly accurate, cost effective, and fully validated.

When should an Embryo be Biopsied?

There are three time points during in vitro embryonic development that allow for the biopsy of genetic material for CCS. The earliest time point involves the biopsy of the oocyte's polar bodies and has been applied by several groups including ESHRE PGS

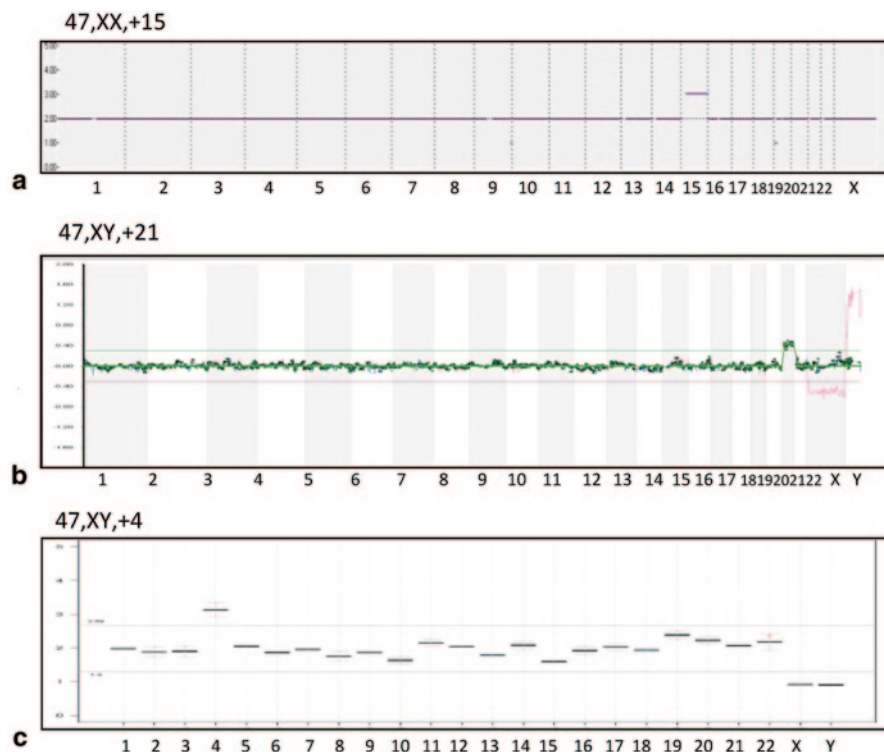
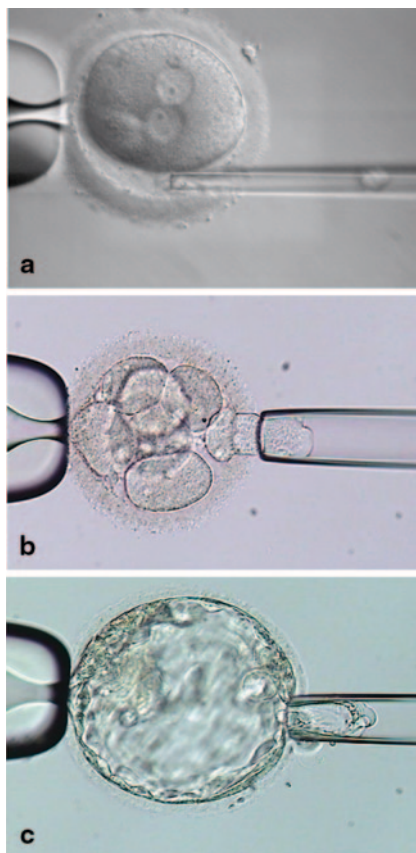


Fig. 1 **a** SNP array profile, **b** Array based CGH profile, and **c** Quantitative real-time PCR profile

Task Force Group [11, 12, 15, 22, 25]. Polar bodies are by products of the oocyte's meiotic divisions and are not required for either fertilization or embryonic development. Polar body biopsy is viewed as an indirect and less invasive approach of analysis of an oocyte's chromosomes (Fig. 2a). A pilot study published by the ESHRE PGS Task Force Group in AMA patients reported 76% aneuploid oocytes with a 6% discrepancy between the aneuploidy status of a polar body and the corresponding fertilized oocyte. Following embryo transfer of euploid oocytes a 30% pregnancy rate was recorded [12, 22]. Another detailed cytogenetic analysis was performed on 308 first and second polar bodies biopsied from fertilized oocytes generated by 70 infertile women of advanced maternal age (mean=40.8 years). The aneuploidy rate for this cohort of oocytes was 70%, with slightly more MII than MI errors [11]. Handyside et al. also observed over half of the aneuploidies were from the by-products of female meiosis resulting from errors in the second meiotic division [15]. In addition, they noted that most abnormal zygotes had multiple aneuploidies [15].

Interestingly, these published studies have recognized that chromosomes of all sizes participate in oocyte chromosome errors, endorsing the requirement for 23 chromosome testing. The mechanisms underlying the chromosome errors observed in oocytes include both whole chromosome non-disjunction and premature separation of sister chromatids. Further, premature separation of sister chromatids is the

Fig. 2 **a** Polar body biopsy, **b** Cleavage stage biopsy, and **c** Blastocyst biopsy



primary cause of MI errors in embryos from women with advanced maternal age and that reciprocal loss or gain of the same chromosome typically results in euploid embryos and even a live healthy delivery [9, 15, 38]. Nevertheless, it is important to note that polar bodies are difficult cells to work with due to their inherent nature to degrade which directly affects DNA quality and the coherence of chromatids. Additionally, polar body CCS does not allow for the identification of either paternal meiotic errors or embryonic mitotic errors. These concerns surround the clinical utilization of polar body biopsy for CCS and whether it is a viable option for the improvement of IVF outcome.

Embryo biopsy can involve either a cleavage stage blastomere biopsy, or a blastocyst stage trophectoderm biopsy. Cleavage stage biopsy has been to date the most common time point for aneuploidy screening (Fig. 2b). However, recent publications question its clinical utility owing to the high incidence of mosaicism and potential damage to the developing embryo from the biopsy procedure. A systematic review and meta-analysis of studies on embryonic chromosomal constitution revealed 73 % of cleavage stage embryos were mosaic, with diploid-aneuploid mosaicism the most common chromosomal constitution observed (59 %) [45].

Diploid-aneuploid mosaic embryos contain a mixture of both diploid (euploid) and aneuploid blastomeres. The accuracy and clinical viability of CCS would be significantly compromised if the biopsied blastomere did not represent the chromosome constitution of the remaining blastomeres in the embryo. A randomized and paired clinical trial investigating the impact of embryo biopsy showed an adverse effect from blastomere biopsy with 39% of cleavage stage embryos losing their ability to implant and sustain further development [39]. Only 30% of biopsied cleavage stage embryos had sustained implantation that resulted in live births, compared to 50% of unbiopsied cleavage stage embryos [39].

In contrast, it appears that limited harm is caused after biopsy at the blastocyst stage (Fig. 2c), with no measurable impact observed between biopsied and unbiopsied blastocysts in relation to IVF outcome [39]. Schoolcraft et al. observed that the probability of an individual blastocyst successfully establishing sustained implantation was 68.9%, an implantation rate 50% higher than for an individual blastocyst transferred without CCS [34]. Chromosomal mosaicism also looks to be less common at the blastocyst stage as compared with earlier embryonic stages. A recent report that reanalyzed 70 aneuploid blastocysts by isolating the inner cell mass and three segments of trophectoderm, showed a high accuracy of diagnosis with only 11 blastocysts that were mosaic (15.7%) and only two blastocysts classified as diploid-aneuploid mosaics (2.9%) [2]. Additionally, no preferential allocation was observed of aneuploid cells between the inner cell mass and the trophectoderm [2]. Altogether, these data point towards the use of blastocyst biopsy as a practical and effective time point for chromosome screening of IVF embryos.

Evaluation of Clinical Efficacy of Comprehensive Chromosome Screening

The predictive value of embryonic reproductive potential as well as RCTs to establish efficacy are essential in order to ultimately accept the clinical validity of CCS in ART practice. A prospective, double-blinded, non-selection study using a SNP array platform for CCS was performed to measure the negative and positive predictive value of CCS in relation to embryonic reproductive potential [37]. A total of 255 IVF embryos were cultured, biopsied and selected for transfer without knowing the result of the aneuploidy screening. CCS and DNA fingerprinting were performed after transfer allowing for implantation outcome to be calculated in relation to chromosome constitution, as well as embryo to fetus identification. Results revealed that CCS was highly predictive of clinical success, with 41% of euploid embryos resulting in ongoing implantation and 96% of aneuploid predicted embryos failing to implant [37]. There are already a few published RCTs to date revealing a significant benefit using trophectoderm biopsy with CCS. One of them evaluated single embryo transfer of fresh blastocysts, with or without biopsy, in good prognosis IVF patients of young maternal age. Even in patients that did not have an increased risk for aneuploidy, the CCS group showed a significantly higher clinical pregnancy rate (69.1%) compared to the control group (41.7%) of fresh blastocyst transfer

using morphology selection alone [51]. In the second RCT, IVF patients of advanced maternal age (>35 years) were randomized into either the control group of fresh blastocyst transfer based on morphology alone, or the test group of trophectoderm biopsy with CCS and blastocyst vitrification followed by a subsequent frozen embryo transfer. The ongoing clinical pregnancy rate was significantly lower at 40.9% in the control group compared to 60.8% in the CCS group [36]. Miscarriage rates were observed to be significantly lower for patients that had euploid blastocysts transferred in the CCS group compared to the control group that had traditional blastocyst morphological based selection [36]. Scott et al. [39] also showed significant improvement with CCS following fresh blastocyst transfer in a group of infertile women with a mean maternal age ~32 years. Delivery rates per cycle were significantly higher in the CCS group recorded at 84.7% compared to 67.5% for the control group without CCS [39]. These results are very encouraging and represent preliminary data of the clinical efficacy and validity of blastocyst CCS. Future completion of active RCTs involving CCS technologies is anticipated to build on this initial success, reflecting a significant improvement in the reproductive outcome for a range of infertility patients.

The future of CCS technologies lies with the rapidly developing whole genome analysis approach of next generation sequencing (NGS). NGS provides the opportunity to sequence millions of reads of DNA allowing for the simultaneous analysis of CCS and potential single-gene disorders. Recent developments in bench top sequencing platforms and sophisticated bioinformatics tools are leading the way to providing clinical CCS by NGS in the near future. A couple of publications have recently shed light on this future transition. The first study performed low coverage NGS on trophectoderm biopsies from 38 blastocysts [52]. A combination of euploid, aneuploid and structurally unbalanced blastocysts were identified by NGS and confirmed by SNP array. Only one blastocyst with different sizes of an unbalanced structural rearrangement was not confirmed by SNP array [52]. The second study published in 2014 investigated NGS for monogenic diseases on trophectoderm biopsies revealing 100% reliability with two conventional methods of single-gene mutation analysis [44]. Ongoing improvements in NGS protocols and technologies are encouraging with the potential of faster turnaround times, smaller requirements of DNA input, and less cost per run and even per embryo.

CCS goes beyond the analysis of chromosomes and allows for the development of additional markers to identify viable IVF embryos. As important as it is for all 23 pairs of chromosomes to be present for viable fetal development, not all euploid embryos, even at the blastocyst stage, will implant. Successful implantation depends on the synchronization and molecular crosstalk between a developmentally competent embryo and a receptive maternal endometrium. Any abnormality attributed to the embryo or endometrium will result in implantation failure. Utilizing other OMICS platforms, like proteomics, molecular biomarkers can be investigated to distinguish between a euploid blastocyst that has the ability to successfully implant and result in a live, healthy delivery, versus a euploid embryo that results in a negative pregnancy (Fig. 3).

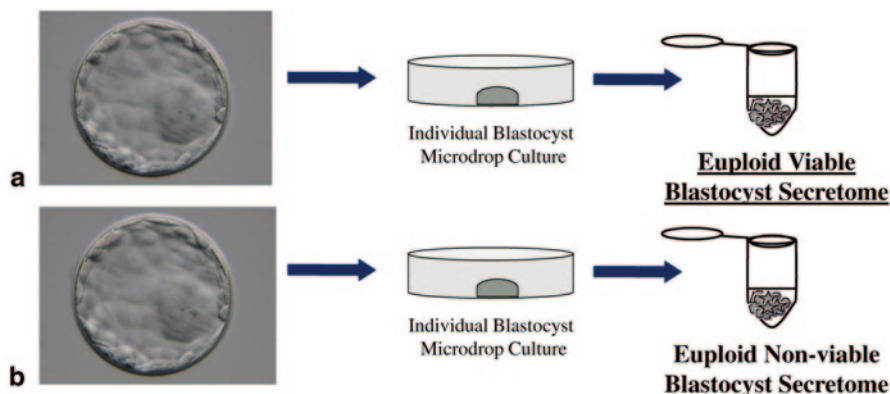


Fig. 3 Investigations to distinguish the protein secretome profiles of viable (a), and non-viable (b) blastocysts

Proteomics

The proteome represents all the proteins translated from a cell's transcriptome that are responsible for cellular function. Hence, in order to fully understand cellular function and comprehend biological processes, an investigation of a cell's proteome is vital. The proteome is complex and dynamic, constantly changing through both internal and external interactions and stimuli. The transcriptome does not always predict protein presence or abundance due to mechanisms that degrade mRNA transcripts prior to translation. Knowledge of the human oocyte and embryonic proteome is very limited even despite recent advances in proteomic technologies. The main hurdles include limited template, low protein concentration, deficient platform sensitivity, and limited protein database information.

Non-Invasive Proteomic Secretome Approach

Of particular interest to researchers trying to identify proteins involved in specific disease states is the secretome, defined as those proteins produced by cells and secreted at any given time [21]. In ART, the secretome includes those proteins that are produced by embryos and secreted into the surrounding culture medium. Analysis of the embryonic secretome would represent a non-invasive approach to embryo assessment [19]. Defining and characterizing the embryonic secretome that reflects developmental competence may improve ART outcome but will also advance our knowledge of early embryogenesis and the embryonic role during implantation [18]. To date, this has proven to be a challenging task due to the complexity and diversity of the human embryo, but holds promise with recent developments of increased sensitivity for both targeted and proteomic profiling approaches.

Early studies of the human embryonic secretome involved targeted analysis of individual proteins or molecules. The soluble factor, 1-o-alkyl-2-acetyl-sn-glycero-3-phosphocholine (PAF), was one of the first molecules to be identified in the human embryonic secretome. The release of PAF influences a range of maternal physiology alterations including platelet activation and maternal immune function [28]. Leptin, a 16 kDa small pleotrophic peptide has also been observed in embryonic conditioned medium [13]. Leptin has been hypothesized to initiate and establish a molecular dialogue with leptin receptors in the maternal endometrium during the window of implantation [3]. Competent human blastocysts secrete higher leptin concentrations into the surrounding medium than arrested embryos. Another reciprocal embryo-endometrial interaction that could transform the local uterine environment, impacting both embryo development and the implantation process, is HOXA10. HOXA10 is expressed by epithelial endometrial cells and its regulation is modulated by an unknown soluble molecule secreted by human blastocysts [32].

The presence of soluble human leukocyte antigen G (sHLA-G) in embryo spent culture media has been reported in several publications to be associated with successful pregnancy outcome [20, 27]. Indeed, HLA-G has been hypothesized to also play a role during the maternal embryonic interface of implantation. However, these results have not been absolute, with pregnancies established from sHLA-G negative embryos and studies revealing undetectable levels of sHLA-G in embryo spent culture media [42, 46, 47, 49]. There are numerous factors that could influence the presence of sHLA-G in embryo spent culture media including the culture system itself, the extent of cumulus removal, single versus group embryo culture, media composition, microdrop volume and the day of media collection [47, 49]. Another explanation for the lack of reproducibility and association observed to date could be the lack of sensitivity of the current sandwich ELISA assays used for most sHLA-G analysis. It would appear that a more sensitive (picogram level) and reproducible quantitative method for analysis is required in order to determine the significance of sHLA-G in relation to embryo development and implantation outcome [47].

Human chorionic gonadotropin (hCG) is produced by trophoblast cells and is an earlier marker for the establishment of pregnancy. Investigations for the presence of intact hCG and hCG isoforms in the human embryonic secretome have revealed promising but inconsistent results. A recent study showed that intact hCG is only detected at the time point of embryo hatching and that a significant proportion of the hCG immunoreactivity is associated with hyperglycosylated hCG (hCG_h), which may suggest a role with potential implantation and thus could be further investigated as a biomarker of IVF outcome [1].

Mass Spectrometry Analysis of the Embryo Secretome

The above studies have focused on only a single protein or molecule, however, it would be reasonable to assume with the complexity and multifactorial nature of embryonic development that more than one protein or molecule would be required to predict developmental competence and/or implantation potential. Mass

Spectrometry (MS) has rapidly become an important technology in proteomics research. Searching for reliable and reproducible changes in protein expression have revealed underlying molecular mechanisms of physiological processes and disease states [5]. Using Surface Enhanced Laser Desorption and Ionization MS, Katz-Jaffe et al., were the first to successfully analyze the protein secretome profile of individual human embryos [17]. The authors observed distinctive protein secretome signatures every 24 h during preimplantation development, from the time of fertilization to the blastocyst stage. Maternal proteins were observed during the first 24 h of development and unique embryonic proteins were observed in the human embryonic secretome after the activation of the embryonic genome post day 3. In addition, they reported a clear association between protein expression profiles and morphology, with degenerating embryos exhibiting significant up-regulation of several potential biomarkers that might be involved in apoptotic and growth-inhibiting pathways. Ubiquitin, a component of the ubiquitin-dependent proteasome system that is involved in a number of physiological processes including proliferation and apoptosis, was observed to have increased expression in the secretome of developing blastocysts when compared to the secretome of degenerating embryos. Secreted ubiquitin has been shown to be up-regulated in the body fluids of certain disease states and this accumulation provides evidence for an increased protein turnover [4, 33]. Ubiquitin has also been implicated in playing a crucial role during mammalian implantation by controlling the activities and turnover of key signaling molecules [48]. Two-dimensional (2D) gel electrophoresis and tandem MS have also been utilized to identify proteins in spent embryo culture media. In this study, increased levels of Apolipoprotein A1 (ApoA1) were identified in the embryonic secretome of blastocysts with higher morphological grade [53]. The presence of ApoA1 mRNA was also confirmed to be expressed in blastocysts, but not early cleavage stage embryos, suggesting that ApoA1 is a part of the embryonic transcriptome and secretome [53]. However, in relation to IVF outcome, no association was observed with ApoA1 levels in the embryonic secretome.

The incorporation of aneuploidy screening with a non-invasive method for embryo viability would be a significant advantage. Initial investigations of the blastocyst protein secretome in relation to chromosome aneuploidy have been performed using an LC-MS/MS platform. The protein profile of a euploid blastocyst secretome was markedly different from the protein profile of the aneuploid blastocyst secretome. Nine, novel, candidate biomarkers characteristically classified chromosome aneuploidy in a cohort of transferable-quality blastocysts. Lipocalin-1 was identified as the first potential biomarker for noninvasive aneuploidy screening and confirmed using an ELISA assay [24]. Lipocalin-1 has a large variety of ligands and is overproduced under conditions of stress, infection and inflammation. The increased secretion of Lipocalin-1 from an aneuploid blastocyst could represent an overall compromised state of the embryo itself that reflects the aneuploid chromosome complement. The ability to non-invasively assay for embryonic developmental competence, that included euploidy, would represent a powerful selection tool in ART.

Protein Microarray Analysis of the Embryo Secretome

Another proteomic technology that has been investigated in the characterization of the embryonic secretome is protein microarrays. In a retrospective study by Dominguez et al in 2008, protein microarrays that contained 120 targets were used to compare pooled, conditioned media from implanted versus non-implanted blastocysts following single embryo transfer [6]. Results revealed two proteins significantly decreased in the conditioned media of implanted blastocysts, CXCL13 and GM-CSF, with no proteins observed to be significantly increased. The authors hypothesized that the decrease in CXCL13 and GM-CSF indicated consumption of these proteins by the human blastocyst. Indeed, GM-CSF has been shown to promote embryo development and implantation when present in both human and murine blastocyst culture media [30].

In a subsequent study by the same group comparing protein secretome profiles between the endometrial epithelial cell (EEC) co-culture system and sequential microdrop culture media Interleukin-6 (IL-6), PLGF and BCL (CXCL13) were increased, while other proteins were decreased such as FGF-4, IL-12p40, VEGF and uPAR. IL-6 displayed the highest protein concentration in the EEC co-culture system, and upon assessment of the sequential culture media secretome using an IL-6 ELISA assay, viable blastocysts displayed an increased uptake of IL-6 compared to blastocysts that failed to result in a pregnancy, thereby suggesting a potential role for IL-6 in blastocyst development and implantation [7].

In summary, proteomic analysis is a promising technology for the development of non-invasive methods for embryo selection in ART. However, the challenge ahead still includes the reliable and reproducible identification of proteins and/or molecules associated with embryo viability and IVF success. This is a challenging task due to the complexity, heterogeneity and diversity of human embryos. Nevertheless, once these proteins and/or molecules are identified, there are sensitive, high throughput and cost effective methods available for application in an IVF clinical setting including immunodetection using ELISA or radioimmunoassays.

Conclusions

Ongoing developments in OMICS technologies are promising and are paving the way for the introduction of more quantitative, invasive and non-invasive methods for embryo selection in the field of ART. Noteworthy developments in genomics technologies, including microarrays, qPCR and NGS, have allowed comprehensive chromosome screening technologies to enter into the clinical setting and contribute to significant improvements in IVF success. The molecular assessment of the human embryonic secretome will further enhance our understanding of preimplantation embryonic development and viability. Together, the combination of a clinically proven robust quantitative non-invasive assay alongside CCS and detailed morphology assessment could represent the greatest improvement in embryo selection techniques allowing for successful routine single embryo transfers with healthy singleton deliveries.

Gamete and Embryo Selection
Genomics, Metabolomics and Morphological
Assessment

Sakkas, D.; Katz-Jaffe, M.; Sueldo, C.

2014, VII, 45 p. 10 illus., 2 illus. in color., Softcover

ISBN: 978-1-4939-0988-9