OMICS in assisted reproduction: possibilities and pitfalls

Emre Seli¹, Claude Robert², and Marc-Andre Sirard²*

¹Department of Obstetrics, Gynecology, and Reproductive Sciences, Yale University School of Medicine, New Haven, CT, USA. ²Titulaire de la Chaire du Canada en génomique animale, Centre de Recherche en Biologie de la Reproduction, Département de Sciences Animales, Université Laval, Ste-Foy Québec, QC, Canada G1K 7 P4

*Correspondence address. Tel: +1-418-656-7359; Fax: +1-418-656-3766; E-mail: marc-andre.sirard@fsaa.ulaval.ca

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ABSTRACT: A key step in assisted reproduction is the assessment of oocyte and embryo developmental potential in order to determine the embryo(s) most likely to result in pregnancy. Currently used embryo assessment strategies are largely based on embryo morphology and cleavage rate. Although these systems have been successful in improving pregnancy rates and reducing multiple gestations, their precision is still insufficient. Therefore, development of an objective, accurate, fast and affordable test that can aid in the assessment of oocyte and embryo developmental potential is a significant aim of reproductive medicine. Recently, global assessment strategies involving genomic, transcriptomic, proteomic or metabolomic profiling of oocytes, granulosa or cumulus cells, embryos or culture media have been applied to assisted reproduction. These technologies are at different stages of development and present unique advantages as well as limitations.

Key words: OMICS in embryo assessment / fluorescent in situ hybridization / comparative genomic hybridization / single-nucleotide polymorphism / microarray

Introduction

Infertility is a common disorder affecting ≏15% or reproductive age of couples, and has significant medical, social and financial implications. Assisted reproduction offers the highest success rates to couples with infertility and its use is steadily increasing since its inception more than 30 years ago.

A key step in assisted reproduction is the assessment of oocyte and embryo viability to determine the embryo(s) most likely to result in a pregnancy. Unfortunately, our understanding of molecular determinants of embryo viability is limited. Consequently, current embryo assessment strategies in clinical settings largely rely on embryo morphology and cleavage rate. These systems have been successful in improving pregnancy rates and reducing multiple gestations. However, their precision is far from ideal, as two out of three infertility type reactions) is the study of oocytes (or the somatic cells around it) and embryo developmental potential within the context of identifying the embryos with the highest potential to result in a pregnancy in clinical practice.

Emergence of omics as an investigational and diagnostic tool in assisted reproduction

The emergence of genomic, transcriptomic, proteomic and metabolomic tools (the omics) is slowly changing our approaches towards mammalian physiology. The previous paradigm used to study a given disease or to assess the efficiency of a medical procedure involved phenotyping as the major source of information (e.g. evaluating responses to a drug by measuring hormone levels, number of oocytes retrieved or pregnancy rates).

Although this remains a valuable approach, new powerful tools are becoming available to further expand phenotype description (by measuring RNA, protein or metabolite levels), and the use of this information in the new context of genotype–phenotype interactions rapidly overwhelms our modest mammalian brains with its complexity.

This reality also applies to the field of reproduction where specific problems should benefit from these technical advances. The first obvious field to benefit from the ability to amplify DNA (using PCR type reactions) is the study of oocytes (or the somatic cells around it) and embryos where the limited amount of material considerably...
reduces the information available when compared with somatic tissues. This is still reflected today by the number of unknown genes found to be expressed in oocytes or cumulus cells compared with granulosa cells. The same holds true for metabolomic and proteomic approaches where mass spectrometry (MS) is slowly becoming sensitive enough to analyze embryo/cumulus/granulosa biopsies or spent culture media. Therefore, the omics are becoming potentially very useful diagnostic tools to investigate differences among follicles, oocytes and embryos. These differences could serve as criteria for the selection of oocytes/embryos or be used to develop individualized treatment strategies.

Before using these tools in a clinical environment or even for research in reproduction, it is relevant to explore their strengths and weaknesses, as well as their current applicability. The following sections will analyze the different omics approaches with an emphasis on their actual respective values.

**Genomic analysis: studying the DNA constitution of cells**

**Genomic assessment of viability in assisted reproduction**

DNA determines the sequence of transcripts and is central to protein synthesis and phenotype determination. The existence of genetic determinants for embryo viability is therefore conceivable and could be identified by analyzing an individual’s DNA. However, specific DNA variant sequences associated with increased viability (independent of known mutations that are lethal) have not yet been identified. In addition, the variability in the DNA within a cohort of embryos generated by a given couple will be limited to meiotic recombination. Therefore, whether an analysis of embryonic DNA sequence can generate information on embryo viability is currently unknown.

Whereas DNA sequence analysis is not a likely strategy for embryo viability assessment, chromosome numbers and integrity have been successfully investigated as a determinant of embryo viability. The theoretical basis for this approach is the following:

(i) An abnormal number of chromosomes (aneuploidy and polyplody) is most of the time not compatible with the development of a healthy individual and occurs more commonly in humans compared with other species (Hassold et al., 1996; Hassold and Hunt, 2001).

(ii) The most common cause of spontaneous embryo loss is aneuploidy (Hassold and Chiu, 1985).

(iii) Aneuploidy is even more common (compared with spontaneous miscarriages) in preimplantation embryos generated by IVF (Munne et al., 1995).

(iv) Incidence of aneuploidy in oocytes, preimplantation embryos and in spontaneous miscarriages increases with female age (Hassold and Chiu, 1985; Munne et al., 1995; Dailey et al., 1996).

On the basis of these strong arguments, the number of chromosomes in the embryo has been analyzed in an effort to increase IVF success by identifying and excluding aneuploid embryos. The analysis is invasive and requires biopsy of blastomere(s) or trophectoderm from the embryo, 3 or 5 days after fertilization, respectively. Alternatively, removal of the polar body extruded from the oocyte during meiosis has been performed to test for oocyte aneuploidy. It is noteworthy that when embryo is biopsied on Day 5 after fertilization followed by analysis, fresh embryo transfer cannot be performed and cryopreservation and transfer in a subsequent cycle becomes necessary.

These approaches have recently been categorized as preimplantation genetic screening (PGS) by the European Society for Human Reproduction and Embryology (ESHRE) to distinguish them from preimplantation genetic diagnosis, performed for patients that are at high risk of transmitting a genetic or chromosomal abnormality to their children, which include single gene defects (autosomal dominant, autosomal recessive, X-linked disorders) and chromosomal abnormalities (translocations, inversions, etc.; Thornhill et al., 2005). In this section, we will review techniques that are currently being applied and promising new approaches that are being developed for assessment of chromosome number in embryos in order to improve the outcome of IVF.

**PGS with fluorescent in situ hybridization**

The method of choice for PGS has been fluorescent in situ hybridization (FISH). For FISH, the biopsied cell is fixed on a glass slide. DNA probes for 9–12 chromosomes to be tested are labeled with different fluorophores and hybridized to the slide in two to three successive hybridizations. Signals are visualized using a microscope. The most commonly screened chromosomes are 13, 15, 16, 17, 18, 21, 22, X and Y (Lalioti, 2008).

The first reports of FISH in single cells were by Griffin et al. (1992) who used probes for the X and Y chromosomes, and then applied this methodology to determine the sex of the embryos from patients with X-linked recessive diseases (Griffin et al., 1993). Meanwhile, Munne et al. (1993) used the same approach to diagnose aneuploidy in embryos. Soon after these initial reports, multiple studies using FISH to identify and exclude aneuploid embryos in women undergoing infertility treatment with IVF reported increased implantation and decreased spontaneous miscarriage rates (Gianaroli et al., 1997; Munné et al., 1999, 2003, 2006).

However, subsequent randomized controlled trials showed no improvement in IVF outcome parameters and some found a decrease in implantation and pregnancy rates using this technique (Staessen et al., 2004, 2008; Mastenbroek et al., 2017). Finally, a recent meta-analysis (Checa et al., 2009) found that in women undergoing IVF, PGS for aneuploidy does not increase but instead is associated with lower rates of implantation, ongoing pregnancy and live birth. They concluded that aneuploidy screening with PGS using FISH in IVF practice does not appear to be justified. Both the American Society for Reproductive Medicine (ASRM) and ESHRE made similar statements (Practice Committee Opinion, 2008; Harper et al., 2010).

A multitude of factors have been proposed to explain the lack of benefit from PGS with FISH. Among these are potential injury to the embryo from blastomere biopsy, mosaicism of the embryo for aneuploidy (potentially leading to false positive diagnosis) and limited number of chromosomes studied (potentially leading to false negative diagnosis). Additional false negative findings may be due to the fact that FISH only detects the presence or absence of the chromosomal region targeted by the probe (usually at the centromere),

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and does not provide information about the remainder of the chromosome.

The causes of failure to achieve a benefit with PGS with FISH are worthy of investigation as they could lead to improved approaches for embryo assessment. In their study published in the current issue of MHR, Northrop et al. (2010) used single-nucleotide polymorphism (SNP)-based analysis of morphologically normal blastocysts, previously assessed by cleavage stage FISH, and found that as many as 58% of the embryos that develop into morphologically normal blastocysts, despite having been diagnosed as aneuploid by FISH, are in fact euploid for all 24 chromosomes. More importantly, they did not observe uniparental disomy of aneuploid chromosomes in these euploid blastocysts previously diagnosed as aneuploid by FISH. Therefore, correction of a mosaicism by extrusion (would correct trisomy but not result in uniparental disomy in one-third of the embryos) or duplication (would correct monosomy but result in uniparental disomy in all embryos) of aneuploid chromosomes does not seem to be a common mechanism as was previously proposed. Authors concluded that the misdiagnoses associated with FISH, are at least in part due to technical shortcomings.

Below we discuss novel approaches to PGS that utilize global assessment techniques for DNA analysis, and have the potential to overcome limitations associated with FISH.

**PGS using comparative genomic hybridization**

Comparative genomic hybridization (CGH) was initially developed by cancer biologists who needed to analyze aneuploidy in cells obtained from solid tumors (Kallionemi et al., 1992, 1993, 1994). CGH works by comparing the sample (or test) DNA with known normal (or reference) DNA [see (Thornhill et al., 2005; Wells and Levy, 2003; Wells et al., 2008) for review]. In conventional CGH, test DNA and reference DNA are labeled with a green and red fluorochrome, respectively, and then simultaneously applied on normal male metaphase chromosomes fixed on a microscope slide. The labeled test and reference DNA are allowed to hybridize for 2–3 days and then a computerized imaging system is used to analyze the red and green fluorescence along the length of the metaphase chromosomes. A number of metaphase spreads are analyzed and the fluorescence profiles for individual chromosomes are determined. If, for example, the test DNA is diploid for chromosome 21, an equal amount of red and green fluorescence will be observed (green:red fluorescence for chromosome 21 will be 1:1) on all copies of chromosome 21 on the slide. Therefore, the test DNA will be diagnosed as normal for chromosome 21. If, on the other hand, the test DNA was trisomic for chromosome 21, then there will be more green labeled chromosome 21 in the mixture. As a result, all the copies of chromosome 21 on the slide would have an increased green to red fluorescence ratio. This would be identified as a shift in fluorescence ratio > 1 and the test DNA would be diagnosed as having an extra copy of chromosome 21.

The obvious advantage of CGH over FISH is that the copy number of all chromosomes can be determined. Moreover, a more detailed analysis of the entire length of each chromosome is obtained with CGH compared with FISH, allowing detection of chromosome segments imbalance (Wilton, 2005).

**Conventional CGH**

Whereas CGH was originally developed to test for aneuploidy in samples containing small numbers of cells (Kallionemi et al., 1992, 1993), the amount of unamplified DNA in a single cell is insufficient for hybridization to target chromosomes as required by CGH [CGH requires ~1 µg of DNA whereas a single cell only contains 5–10 pg (Wells et al., 2008)]. Therefore, amplification of DNA is necessary.

Multiplex polymerase chain reaction (PCR) has been used successfully to amplify several loci from single cells. However, CGH requires non-specific amplification across the entire genome. A number of whole genome amplification (WGA) techniques have initially been tested for their ability to evenly amplify random sequences from single cells (Wells et al., 1999). Among these, degenerate oligonucleotide primed PCR [DOP-PCR, (Telenius et al., 1992)], and primer extension pre-amplification [PEP, (Zhang et al., 1992)] provided a more even amplification of the genome compared with tagged PCR and Alu-PCR (Wells et al., 1999). However, only DOP-PCR produced sufficient amplified products from single cells for use in CGH.

Wells et al. (1999) were the first to use CGH to identify trisomies in individual amniocytes and blastomeres. Their findings were confirmed by Voullaire et al. (1999, 2000) followed by successful clinical application for CGH for identification of euploid embryos in women undergoing IVF (Wilton et al., 2001, 2003).

A significant problem associated with CGH is the length of time required for the procedure (~4 days), which is incompatible with fresh embryo transfer when blastomeres are obtained on Day 3 or 5 (Wells et al., 2008). Although analyzing polar bodies may allow fresh embryo transfer following CGH (Wells et al., 2002), this approach will fail to detect aneuploidies that occur during meiosis II, and those of paternal origin. Alternatively, embryo biopsy and CGH analysis may be coupled with cryopreservation (Wilton et al., 2001, 2003). The main drawback of this approach is that conventional freezing and thawing methods lead to a reduction in embryo viability, especially following embryo biopsy. However, the recent development of vitrification techniques has provided a mean to cryopreserve bisected oocytes and embryos without a significant decline in survival rates (Schoolcraft et al., 2009).

In a recent prospective cohort study, Schoolcraft et al. (2009) used CGH in embryos biopsied on Day 5, which were then cryopreserved by vitrification and transferred in a subsequent cycle. They achieved implantation and pregnancy rates of 68.9 and 82.2%, respectively. Their findings are very encouraging in demonstrating that their approach may have overcome many of the problems that limited earlier aneuploidy screening techniques. CGH using microarrays

Although the use of vitrification seems to have overcome an important obstacle in the clinical application of CGH, the complexity of the method, requiring expertise in both molecular genetics and cytogenetic methods remains a challenge (Wells et al., 2008). Consequently, in clinical practice, conventional CGH is likely to be applied by specialized referral centers. In addition, the need for cryopreservation is likely to reduce its utilization. Therefore, development of a more simple and rapid application of CGH would be beneficial. To decrease the complexity of the procedure and the time necessary for analysis, a 'microarray' approach has been explored.
WGA is a key component of all array approaches directed at embryo analysis. Previously described PCR-based methods for WGA (such as DOP-PCR and PEP) were found to be sub-optimal for arrays as they can generate non-specific amplification artifacts, provide incomplete coverage and produce short products (<3 kb) that cannot be used in many applications. More recently, two new methods for WGA have been developed. The first, termed multiple-displacement amplification (MDA), uses the highly processive phi29 DNA polymerase and random exonuclease-resistant primers in an isothermal amplification reaction (Dean et al., 2002). The second method, OmniPlex (Rubicon Genomics, Inc., Ann Arbor, MI, USA), converts randomly fragmented genomic DNA into a library of inherently amplifiable DNA fragments of defined size (Langmore, 2002). This library can be effectively amplified several thousand fold with the help of a high-fidelity DNA polymerase. The library can be re-amplified to achieve a final amplification of over a million fold without degradation of representation (Fig. 1). Both approaches have been reported to achieve a concordance of >99.8% in genotyping results from genomic DNA and amplified DNA, strongly indicating the ability of both methods to amplify genomic DNA in a highly representative manner (Barker et al., 2004). Both technologies are commercially available [MDA, repliG (Qiagen, Valencia, CA, USA; omniplex, sureplex (BlueGene, Cambridge, UK), Genomeplex (Sigma, St Louis, MO)]. and can achieve amplification within 2 h.

Similar to conventional CGH, microarray-CGH involves the competitive hybridization of differentially labeled test and reference DNA samples. However, in this case, the labeled DNAs are hybridized to DNA probes affixed on a microscope slide rather than to metaphase chromosomes (Fig. 1). Each probe on the slide is specific to a different chromosomal region and occupies a discrete spot on the slide. Chromosomal loss or gain is detected by the color adopted by each spot following hybridization (i.e. ratio of fluorescence intensity for the two colors). Compared with conventional CGH, microarrays have the advantage that the evaluation of fluorescence ratios is simple and easily automated and that they require less time for hybridization. Indeed, using array-CGH, comprehensive chromosome analysis can be completed in <48 hs (in ~24 h, D. Wells, personal communication), allowing cleavage stage or polar body screening without requiring embryo cryopreservation.

A variety of microarray-CGH platforms are available, including those using chromosome specific DNA-libraries as probes, bacterial artificial chromosome (BAC) probes (150–200 kb), or oligonucleotide probes (25–85 nucleotides) [see (Wells et al., 2008) for comparative review]. Successful detection of aneuploidy in single cells obtained from aneuploid cell lines or embryo have been reported using these platforms (Le Caignec et al., 2006; Stuerwald et al., 2007; Hellani et al., 2008; Vanneste et al., 2009).

Each platform has advantages and disadvantages based on the size and the methods used for the preparation of probes, with implications on positive and negative predictive values. Consistency among batches of slides, as well as robustness of the WGA procedure, coupled to a specific platform are also important determinants of analysis quality.

**PGS using SNP-based arrays**

A SNP is a DNA sequence variation occurring when a single-nucleotide—A, T, C or G—in the genome (or other shared sequence) differs between members of a species, or between paired
chromosomes in an individual. For example, two sequenced DNA fragments from different individuals, AAGCcTA to AAGcTET, contain a difference in a single-nucleotide, forming a SNP.

SNPs may fall within coding sequences of genes, non-coding regions of genes or in the intergenic regions between genes. SNPs within a coding sequence will not necessarily change the amino acid sequence of the protein that is produced, due to the degeneracy of the genetic code. SNP is the most frequent type of variation in the genome, with ~10 million SNPs already identified in the human genome.

Most SNP-microarrays test for 10 000–500 000 individual SNPs (Fig. 1). Unlike the microarray platforms discussed for CGH, which utilize an approach similar to conventional CGH and involving simultaneous hybridization of differentially labeled test and reference DNAs to the same microarray, SNP-microarrays assess test and reference samples separately (in parallel). In SNP-arrays, chromosomal copy number can be determined in two ways. In the quantitative approach, fluorescence intensities measured for test and reference hybridizations are compared. If probes from a given chromosome display brighter signals for the test DNA than the reference DNA, an excess of chromosomal material (e.g. trisomy) is predicted. Conversely, reduced fluorescence for the test sample is associated with chromosome loss. In the qualitative approach, the alleles detected at each SNP locus are compared with those of the parents, revealing which parental chromosomes were inherited by the embryo. The inheritance of three distinct chromosomal haplotypes indicates the presence of a trisomic chromosome, whereas monosomes are revealed by homozygosity for all loci on the affected chromosome. It is noteworthy that the latter approach requires beforehand testing of parental DNA, which means cases have to be carefully coordinated and planned in advance. In clinical application, qualitative and quantitative approaches may be used alone or in combination (requires more time).

Two main SNP-array platforms have been used for aneuploidy testing in the setting of assisted reproduction. Using the Affymetrix (Santa Clara, CA, USA) 250K platform (contains 250 000 SNPs) to analyze single blastomeres biopsied on Day 3, Treff et al. (2010) successfully identified aneuploidy in blinded analysis of single cells from karyotypically defined cell lines and in discarded embryos. The technique also allowed the tracking of which embryos implanted (Treff et al., 2009a). They then adopted the same approach to analyze blastomeres biopsied from preimplantation embryos prior to transfer (Scott et al., 2008a). In this study, findings from the SNP-array were not used for embryo selection. Again, authors reported that the SNP-array provided a reliable DNA fingerprint, revealing the presence or absence of aneuploidy. Analysis of the microarray data revealed that some of the transferred embryos were actually aneuploid. Fetal DNA was obtained from pregnancies and a DNA fingerprint was produced for each fetus, allowing the embryos that formed pregnancies to be identified. The negative predictive value of aneuploidy screening was found to be 100% (0/31 embryos with aneuploidy detected in the biopsied cell formed an ongoing pregnancy, 26 failing to implant and 5 miscarrying). The positive predictive value was also encouraging, 42.9% of embryos diagnosed normal formed an ongoing pregnancy.

In addition to the Affymetrix platform, encouraging results were reported using the Illumina SNP-microarrays (370 000 SNPs). Accurate detection of chromosomal losses and gains in single cells derived from cytotogenetically characterized aneuploid cell lines and blastomeres derived from human embryos has been reported (Kearns et al., 2008; Rabinowitz et al., 2008; Handside et al., 2009; Johnson et al., 2010).

A significant advantage of SNP-microarrays is that, in addition to chromosome copy number information, the probes used provide genotype data, generating a DNA fingerprint for each embryo tested. The DNA fingerprint allows parental origin to be confirmed, reducing the risk that a laboratory error could lead to embryos being transferred to the wrong patient and help identify the parental origin of aneuploidy. In addition, ability to identify which embryo resulted in the pregnancy also makes it possible to study additional factors associated with embryo viability and death. A recent study from Treff et al. (2009b) showed that analysis of the first polar body by SNP-array can help determine which embryo implanted in women undergoing multiple embryo transfer, without the need for embryo biopsy.

Genetic mutations that result in disease cannot be detected directly using SNP-microarrays (Table I). However, they can potentially be detected indirectly via a linkage approach. This approach would require identifying SNPs located in close proximity to the mutation site, on the same chromosome (i.e. linked polymorphisms). It is usually possible to identify specific alleles, which do not cause the disease, but are always inherited along with it and can therefore be used as a marker to infer a diagnosis. Therefore, concomitant detection of chromosome anomalies and single gene disorders could potentially be achieved using SNP-microarray platforms (Wells et al., 2008).

It is noteworthy that SNP-arrays may fail to detect cells that are mosaic within a trophectoderm biopsy. As reported by Northrop et al. (2010), SNP-arrays may fail to detect a mitotic event that affects <40% of the entire biopsy. Mitotic error in a blastomere, on the other hand, would not be missed unless one is referring to a sampling error in which the euploid cell is biopsied from an aneuploid diploid mosaic embryo.

**PGS using high throughput PCR SNP allele ratio analyses**

A PCR-based approach to aneuploidy testing using high throughput PCR-based on chromosomal SNP allele ratio has also been reported. This approach requires preparatory analysis of parental DNA, and assesses copy number for each chromosome by relative quantification of SNPs. A high (>97%) single cell accuracy of assignment of chromosome aneuploidy is achieved in <4 h (Treff et al., 2009c). This promising approach is currently under investigation and its accuracy remains to be further elucidated.

**Trancriptomic analysis: the study of RNA expression in cells**

Although the genomic constitution of a cell can be informative to determine the DNA integrity of an embryo or the disease susceptibility of a patient, it is not sufficient to explain the phenotype. Each cell uses DNA information, in addition to environmental information, to create a phenotype or an output that will often be similar for groups of cells of the same differentiated state (tissue). This differentiation is embedded not ‘in’ but ‘on’ the DNA and is referred to as the epigenetic context.

Therefore, any cell reaction depends on its DNA make up, its epigenetic status and the environment acting on gene expression. This is
true for oocyte-embryo as well as for any type of somatic cells. Cells may react to an environment through either a genomic or a non-genomic response (requiring or not gene expression).

The first type of genomic response is RNA synthesis, which will generally directly induce protein synthesis in somatic tissues or can be stored in special tissues like oocytes. The RNA level or output can be measured with incredible precision with very little amount of biologic material, thanks to the development of quantitative methods such as real-time PCR that can handle minute amount of starting material. This requires that the RNA is first converted into complementary DNA (cDNA) through a reverse transcription (RT) step. This system is sensitive enough to measure the RNA output from a single gene but it is influenced by numerous factors that will be described in details below.

**Methods of transcriptomic analysis**

Simply put, the transcriptome is the RNA content of a cell. Transcriptomic analyses can be used to assess which genes are transcribed and efficiently. RNA integrity is thus affected within minutes when a cell dies or is homogenized. One must either use inhibitors or rapid cooling to inactivate RNAses. RNA must then be extracted with all the care possible to prevent losses. In most experiments in reproduction, an amplification step is required to reach the micrograms of RNA that microarray experiments necessitate from the picograms of RNA contained in oocyte/embryo/cumulus and small samples of granulosa. To ensure that the relative representation of a given gene is preserved, amplification must be as linear as possible. Real-time product analysis is now available to achieve such linearity (Gilbert et al., 2009).

**RNA extraction**

RNA purification is needed mostly to prevent it’s degradation by RNAses that are abundant and active in most cells. There are several ways to capture RNA. Phenol/chloroform and membrane columns are the most commonly used methods. Other approaches use microarrays onto beads that bind the poly-A tail, which prevents contamination by ribosomal or small non-coding RNAs (Figs 2 and 3). To achieve purification from very small amount of material, commercial kits are now available. It is often useful to spike the sample with known quantity of exogenous RNA before the extraction process in order to validate the extraction efficiency and to ensure representation across samples (see (Robert et al., 2010) in this issue).

**RNA reverse transcription**

The variability and poor efficiency of the reverse transcriptase can lead to differences among samples. One important source of variation with these enzymes comes from the primers used to seed the enzyme at its copying starting point. The use of an oligo-dT as a primer for the RT reaction often creates an over representation of the 3’UTR portion of the mRNA. On the other end, this will prevent the copy of ribosomal RNA, which is not useful when assessing a cell phenotype as cells normally have large amounts of ribosomal RNA. Another approach involves random priming, which will amplify all RNA in the sample with no consideration for the poly-A stretch. With this technique, every RNA present in the cell will be transformed into cDNA and available for analysis (Fig. 3).

**RNA amplification and analysis**

RNA from small samples such as cumulus cells or embryos must often be amplified either to detect a given gene or for microarray hybridization. Two major different approaches are available for RNA amplification: the first one is based on the addition of priming sequences on

### Table 1 Comparison of genomic approaches to aneuploidy testing.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>FISH</th>
<th>CGH</th>
<th>Array-CGH</th>
<th>SNP-Array</th>
<th>SNP-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Invasive</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Number of chromosomes detected</td>
<td>9–12</td>
<td>24</td>
<td>24</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>Completed in 2 days without need for cryopreservation of biopsied Day 3 embryos</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Parental DNA analysis is required</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Identifies parental origin of a chromosome</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Identifies the embryo that implanted following multiple embryo transfer</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Detects uniparental disomy</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Detects polyploidy</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>WGA is necessary</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Requires technically difficult nuclear fixation</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Requires complex statistical/computerized analysis†</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Detects single gene disorders</td>
<td>–</td>
<td>–</td>
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</table>

*Both CGH- and SNP-arrays can be completed in 24 h.
†Aneuploidy testing using high throughput PCR SNP allele ratio analyses can be completed in 4 h.
‡Parental DNA analysis is required when qualitative aneuploidy analysis is performed using the SNP-array comparing to paternal alleles (and not when quantitative analysis is performed).
*Computerized analysis could be considered an advantage as it is objective compared to the more subjective assessment associated with FISH.
*Array-CGH does not allow simultaneous testing of single gene disorders and aneuploidy, but the biopsied cell is subjected to WGA, which produces enough DNA for separate testing of genes and chromosomes.
*SNP-arrays are believed to have the potential to detect single gene disorders.
**Figure 2** The preparation of RNA material for transcriptome analysis of granulosa cells. Granulosa cells are recovered and frozen (1), and total RNA is extracted (2). Then, RNA is labeled (3), and hybridized (4) to DNA microarrays. The results are then analyzed by bioinformatics (5) to generate a list of genes. *Column-based RNA extraction generally has a size-exclusion cut-off that prevents the isolation of small size RNAs.

**Figure 3** The preparation of RNA material for transcriptome analysis of oocytes or embryos. Oocytes or embryos are recovered and frozen (1), and total RNA is extracted (2). Then, mRNA is then amplified (4), labeled (5), and hybridized to DNA microarrays (6). The results are then analyzed by bioinformatics (7) to generate a list of genes. *Column-based RNA extraction generally has a size-exclusion cut-off that prevents the isolation of small size RNAs.
the 3’ and 5’ of the cDNA (SMART, Clontech, Mountain View, CA, USA). Those sequences are then used for a PCR amplification step. This approach is exponential and the resulting product is double stranded DNA. The second approach involves an in vitro transcription-based amplification strategy that utilizes the T7 RNA polymerase recognition (Fig. 3). RNA amplification is therefore linear and the end product is anti-sense RNA. It is often used in microarray experiments, mainly for the linearity of the process that should preserve the relative amount of RNA across several genes within a sample. Multiple validation comparisons have been attempted to select the best amplification system for RNA/DNA and the matter has not yet been resolved. In vitro transcription-based approaches are widely accepted over PCR-based methods that are criticized for the potential bias created by the presence of a plateau phase and the fact that the reaction favors smaller size nucleic acids.

A key issue when it comes to transcriptome analysis is how to survey the RNA sample from these cells. The basic tool for this is microarray hybridization (Figs 2 and 3). There are several commercial arrays available for humans and these arrays are somewhat thorough as the complete human genome and its associated cDNA targets are freely available. The most popular system is the Affymetrix chips. The result of the statistical analysis is a list of genes that are more or less present in the two samples that have been hybridized and are being compared. This system is easy to use but may not be very sensitive for low level expressed genes (Hamel et al., 2008).

One of the major concerns with microarrays is normalization of the data to allow statistical analysis with minimum bias. The simpler approach uses ratios to identify genes that are differentially expressed between two or more conditions. This approach is appropriate when the goal of the experiment is to find markers for diagnostic purposes, as those will normally be confirmed by real-time PCR.

When the objective of a transcriptome analysis is to explore normal physiology or, for example, the effect of medical treatments, such as ovarian stimulation, the list of differently expressed genes is not sufficient to provide a representation of the overall functionality of the ovarian cells. New pathway analysis softwares, such as Ingenuity (IPA), which link all differentially expressed genes based on functionality, are becoming more and more powerful as their databases are constantly curated and upgraded with information extracted from publications. The algorithms on which these software’s rely are very efficient at finding links between two genes, proteins or products but cannot necessarily select the ones that make sense in a given tissue. The major advantages of using the complete set of up and down-regulated genes in a given experiment, rather than focusing on the most affected ones is (i) physiological changes are not often translated into huge or large variation in gene expression and (ii) many genes from the same family or pathway moving in a cluster are quite a good indication of a real physiological response. On the other hand, the use of data that do not reach the 0.05 false discovery rate limit may lead to misinterpretations.

In addition to microarrays, there is the emerging alternative of deep sequencing where all the material is being sequenced and most genes expressed can be revealed. This analysis can also be used to quantify expression levels if enough sequencing data, or reads, are extracted. This approach is becoming less expensive although, considering that a good transcriptome analysis would require several millions reads, the cost remains in the thousands of dollars per experiment.

Transcriptomic analysis of cumulus and granulosa cells

In the scope of this review, the following discussion on cumulus/granulosa cell samples will focus on the availability of these cells from IVF cycles and therefore through transvaginal aspiration. These cells could also be obtained from biopsies or through elective ovariectomy but these applications will not be discussed here.

Transcriptome analysis within the context of gametes and embryos can serve different purposes. It can be used to address the phenotype of a cell in order to understand a physiological process, mainly done in a research environment with animal or human samples, or it can be used for diagnostic purposes where the goal is to select either the best oocyte/embryo or to assess the hormonal stimulation response (Assou et al., 2008; Hamel et al., 2008).

Most normal somatic tissues are easier to analyze than embryos, mainly because they are more abundant and their RNA is a more direct representation of the protein content that drives the phenotype of the cell. However, the analysis of the somatic components of the follicle also poses unique problems that have to be addressed in the context of transcriptome studies.

The first issue is cell contamination. If a biopsy is taken mechanically from a cumulus mass to which the oocyte is attached, it is quite reasonable to estimate that transcriptome analysis will reflect the general cumulus phenotype. However, it has been known for decades that cumulus cells are not all alike and that there is an inside-out gradient in gene expression from the oocyte perspective (Hussein et al., 2005). This is even more obvious with corona radiata cells that may still be in direct contact with the oocyte through the zona pellucida. In the context of cumulus physiology, regional differences might matter and laser captured cells could be quite informative. However, in the context of oocyte/embryo selection, the cumulus cells under investigation should represent the general pattern for a given oocyte in order to be useful for comparison/selection. For this purpose, if recovered granulosa mural cells are still attached to the cumulus, they must be removed to prevent them from altering the transcriptomic profile, as it is very unlikely that all samples would be equally contaminated.

Contamination is a more relevant issue for granulosa cells and has been addressed by several publications in the past. It will be obvious to anyone working in IVF that the follicular content is generally contaminated with blood and that blood contains cells. One thesis has been devoted to comparing different methods of purification of granulosa cells from follicular aspirates (Quinn et al., 2006). The conclusion of this work is that besides removal of red blood cells, contamination, especially white blood cells, remains almost impossible to eliminate. Red blood cell removal is not problematic for transcriptome analysis as these cells do not have a nucleus and most of their RNA encodes hemoglobin. However, the same cannot be said for white blood cells. The impact of contamination is minimized by the observation that different follicular samples will have random amount of contamination not associated with the physiological process examined and therefore will cancel each other in comparative transcriptome studies.

There are no reviews yet on the use of follicular markers or cumulus markers as this field is still in its infancy, but several papers have found associations between specific markers and either embryo quality (McKenzie et al., 2004; van Montfort et al., 2008) or pregnancy (Assou et al., 2008; Hamel et al., 2008). To our knowledge, several
of these markers are actually being validated in larger clinical trials to assess their predictive value. The first challenge of those clinical trials is finding markers that will remain valid in all sorts of genetic backgrounds, hormonal treatments and clinical conditions. The second challenge is related to creating a robust process to assess the level of a given marker or a combination of them in a clinical environment. To achieve such robustness, it may be required that specific proteins translated from unique RNA are measured instead of the RNA itself to minimize the risk of degradation as RNA is a very sensitive molecule compared with DNA or proteins and requires very careful handling procedures (Robert et al., 2010, this issue).

The use or transcriptomic analysis to improve ovarian stimulation

One of the most disappointing moments for a clinician is when he/she meets a patient after an embryo transfer has failed. The embarrassment does not come from failure per se, as a natural cycle does not generate very high success rates either, but mainly from the ignorance of what went wrong or what could be improved. One of the promises of transcriptomics is to provide precise diagnostics in complex situations such as ovarian stimulation. It is quite possible that some patients do not respond properly to drugs or are induced to ovulate too early or too late. Since we do not yet have a clear image of the normal physiology for a non-stimulated follicle, we are still far from understanding the stimulated follicle. Do patients react differently to the same procedure repeated over and over again or are there patient patterns or profiles that could inform the clinician of the specific response of each individual? Transcriptome analysis should soon reveal the bases of the observed differences and eventually some explanation about the causes of these variations.

Transcriptomic analysis of oocyte and embryos

When studying somatic cells, the number of cells in the samples to be compared is not taken into consideration. In fact, comparisons are based on a fixed amount of starting material and it is taken for granted that the amounts of RNA per cell and the proportion of polyadenylated mRNA are similar (Fig. 3). This presumption does not apply in the context of oocytes and early embryos. These atypical cells require additional considerations to prevent aberrant transcriptomic results. It is most problematic when different stage embryos are compared, considering that the number and size of the cells they contain are dramatically different and that their transcriptional and translational activity evolves prior and after the maternal to embryonic transition (MET). The comparison of fixed numbers of embryos at different stages will lead to an amplification bias. Indeed, sample with the same number of embryos at different stages will contain different amounts of material, making comparison irrelevant. The comparison of fixed amount of material will also be physiologically questionable since the end-point values will be comparing different numbers of embryos. Should the amount of cyclin or actin be the same in 10 embryos at the 2 cells stage as in five embryos at the 4 cells stage? In this context, extreme care must be taken to avoid the introduction of methodological biases. As mentioned, the scarcity of this type of biological material necessitates the use of a global amplification method to yield sufficient material for downstream transcriptomic study. As such, PCR-based methods have been mostly discarded, as they are known to lead to a plateau phase, which can disrupt the relative abundance of RNA within a sample. As a consequence, linear methods based on in vitro transcription have taken the forefront of sample amplification. It has recently been shown that these methods, although mostly linear, also reach a plateau phase, which homogenizes the reaction’s output (Gilbert et al., 2009). This leads to an important distortion in the natural differences in RNA contents between distinct developmental stages. Failing to account for such important bias may lead to false discoveries.

Proteomic analysis

Proteomic analysis of oocytes or embryos

The first limitation of proteomic analysis is the requirement of large amounts of material. With conventional gel electrophoresis analysis, between 2000 and 4000 oocytes are required per gel, depending on the staining method used; silver nitrate or colloidal blue. With these stains, some 500 proteins can be visualized as single spots. With new dyes (Cy-3 and Cy-5) this number is close to 1000. Although it may seem adequate, this number has to be put in perspective considering that the theoretical number of proteins in a given cell is estimated to be well over 100 000. In a recent deep sequencing experiment that yielded more than 1 000 000 reads from bovine oocytes and early embryos, a total of 14 422 genes were identified out of a total of 24 000 genes producing RNAs in the bovine genome (Sirard, results not shown). If oocytes are similar to somatic tissues in the number of isoforms per gene, we should expect an average of at least 3.2 mRNA per gene for close to 50 000 different RNA and consequently at least this number of different proteins, excluding post-translational modifications. Some scientists estimate that the embryo proteome could contain as much as 1 million proteins (Katz-Jaffe and Gardner, 2007). Therefore, looking at 500 or even 1000 proteins on a gel is clearly a very small portion of the proteome. Although these proteins are the most abundant, they may have less impact on regulation compared with low abundance proteins and may be less influenced by subtle variations.

Another approach that allows monitoring changes in proteins whereas reducing the amount of material required is radio-labeling. Using methionine S-35, our laboratory has monitored the appearance of new proteins every 4 h during the in vitro maturation of bovine oocytes (Coenen et al., 2004). This experiment was aimed at the observation of the newly synthesized proteins and the pattern observed was completely different from the gels made with native oocyte proteins. This means that the stockpile of proteins in the oocyte is substantial, and if compared with matured oocytes (Meta-phase II) or early embryos (pre-MET) the pattern is very similar, indicating that the most abundant forms do not change much during these early steps. Newly made proteins therefore represent a minimal portion of the total protein stockpile.

More recently, a vast mass spectrometry (MS) analysis was done on bovine oocytes including cumulus cells: 4395 proteins were expressed in the cumulus cells whereas 1092 proteins were expressed in oocytes. Furthermore, 858 proteins were common to both cumulus and oocytes (Memili et al., 2007). This new powerful approach, which more than doubles the number of targets to analyze, is suitable for single time point measurements but becomes rather difficult/
still requires protein confirmation. This is especially true in the case of embryos, although our final understanding of physiological processes is limited source of information for understanding mammalian oocytes or ovarian cells of higher quality (Jarkovska et al., 2005).

As for the embryo proteome, a recent review stated that despite new advances in proteomic technologies, knowledge of the proteome of the mammalian preimplantation embryo (including mouse, porcine, rabbit and human) remains limited. It was concluded that the combined effect of limited template, low protein expression and the lack of sensitivity of proteomics platforms are the main hurdles (Katz-Jaffe and Gardner, 2007).

Therefore, it is quite easy to conclude that proteomics is still a very limited source of information for understanding mammalian oocytes or embryos, although our final understanding of physiological processes still requires protein confirmation. This is especially true in the case of embryos prior to the embryonic genome activation where the amount of RNA is not always indicative of protein content or activity.

Proteomic analysis of granulosa cells

Protein synthesis is the major outcome of gene expression and is directly linked to the observed phenotype, which is not always the case with RNA. It thus becomes clear that protein analysis should be the preferred end-point of all physiological analyses. Unfortunately, very few publications are displaying even a partial protein pattern for ovarian cells (Wang et al., 2009). A few more recent publications are addressing the protein changes either in ovarian cancers or for polycystic ovarian syndrome (Atiomo et al., 2009) (which will not be discussed here).

Proteomic analysis of follicular fluid

The follicular fluid proteome has attracted interest much earlier than the granulosa cell or ovarian proteome. This is because follicular fluid is relatively easy to obtain during oocyte retrieval and should contain a protein pattern a lot simpler than the somatic cell. Several publications have identified a number of proteins in this fluid and it has recently become a target for the identification of protein markers associated either with disease (Atiomo et al., 2009) or oocyte of higher quality (Jarkovska et al., 2010). One of the challenges that scientists face with follicular fluid is the abundance of albumin, immunoglobulin and other abundant proteins present in serum. Some new chromatographic approaches to remove such overwhelming protein contributors are allowing the discovery of new proteins by permitting a higher concentration of the product to be analyzed following the depletion of the major constituents (Jarkovska et al., 2010). Animal studies on the other hand, have contributed minimally to our knowledge of follicular fluid components as there is yet to be a complete study that describes the most abundant proteins and their respective functions.

Proteomic analysis of culture media

This section will describe the study of protein in culture media, which is different from metabolomic studies (see next section) as in this case, most of the metabolites will be peptide chains that do not form functional proteins. Strictly speaking, the secreted proteins should be called as such since the term secretome may include other components than proteins. Few investigators have explored the embryo peptide secretion and the sensitivity of electrophoresis has limited their investigation as embryos do not secrete large amounts of proteins. Therefore, the use of MS and hybridization to protein arrays have become preferred tools for looking at embryonic protein secretions. Of course, once the protein of interest is identified, immuno-detection using RIA or ELISA is very sensitive means to assess presence and quantity. The real challenge is to find the right target.

Katz-Jaffe and others have proposed that viable embryos possess a unique proteome and that some of these proteins are potentially secreted into the surrounding culture medium, contributing to the ‘secretome’. One of the earliest studies of the human embryonic secretome revealed the release of platelet activating factor (PAF), a soluble factor (not a protein) that is produced and secreted by mammalian preimplantation embryos (O’Neill, 2005). PAF release causes a range of alterations in maternal physiology, including platelet activation, changes in ovudtial, endometrial and maternal immune functions. PAF also acts in an autocrine fashion as a trophic/survival factor for the early embryo. In vitro, supplementation of culture media with PAF improves embryo development (O’Neill, 2005).

Leptin, a small pleiotrophic peptide, has also been observed (Gonzalez et al., 2000) in the conditioned medium of human blastocysts. It was shown that competent human blastocysts secrete higher leptin concentrations into the surrounding medium than arrested embryos. Cervero et al. (2005) hypothesized that the leptin secreted by blastocysts initiates a receptor-mediated effect in the maternal endometrium during the implantation window.

Katz-Jaffe, using SELDI-TOF-MS (Surface-enhanced laser desorption/ionization time of flight mass spectrometer), identified different secretome profiles for each stage of embryonic development. She also found that ubiquitin, an 8.5 kDa protein, was up-regulated in the secretome at Day 5 of blastocyst development (Katz-Jaffe et al., 2006a, b).

Recently, a study using protein microarrays that contained 120 protein targets was used to simultaneously compare two given conditions. This system has a detection limit of 10–250 000 pg/ml. They collected conditioned media from human embryos (implanted and non-implanted) before their transfer using a single embryo transfer (SET) program. Interestingly, proteins like CXCL13 (BCL, B lymphocyte chemoattractant), stem cell factor (SCF) and macrophage-stimulating protein-a (MSP-a) significantly (P < 0.005) decreased and were, therefore, consumed by the human blastocyst. In contrast, soluble tumor necrosis factor (TNF) receptor 1 (sTNFRI) significantly increased in the media where the blastocyst was present in comparison with the control media. Unfortunately none of the proteins investigated was significantly up-regulated/secreted by the implanted blastocyst (Domínguez et al., 2008).

Metabolomic analysis

Embryo metabolism as a determinant of viability

Proper metabolic turnover is essential for a preimplantation embryo to remain viable and develop into a successful pregnancy.
Consequently, nutrients and metabolites within the culture environment have been studied as potential predictors of embryo viability.

A key aspect of preimplantation embryo development is the switch from carboxylic acid to glucose metabolism, which occurs around the time of pre- and post-compaction, and is among the best characterized phenomena of embryonic metabolic regulation (Leese, 1995). Both aerobic glycolysis (also called the tricarboxylic acid cycle or the Krebs cycle) and anaerobic glycolysis (Emden–Meyerhof pathway) are required for energy generation during preimplantation development. Of these pathways, carboxylic acid-based metabolism predominates in the early stages of preimplantation development, when pyruvate and lactate constitute the main sources of energy for the embryo, and glucose uptake is minimal (Biggers et al., 1967; Gardner and Leese, 1988). As embryo development progresses from the zygote stage to compaction, glucose uptake increases and glucose metabolism becomes predominant at the blastocyst stage. This increase in glucose consumption in late-stage preimplantation embryos has been documented in a number of species, including sheep, pig, cow, mouse, rat and human (Leese and Barton, 1984; Brison and Leese, 1991).

The changing nutrient requirements of the developing preimplantation embryo seem to be reflected in the composition of the in vivo milieu of the embryo as it travels within the reproductive tract. During the pre-compaction stages, the embryo interacts with the oviductal fluid, which contains relatively high levels of pyruvate and lactate and low levels of glucose. On the other hand, the reverse is seen in uterine fluid, with higher glucose and lower pyruvate concentrations (Gardner et al., 1996; Leese et al., 2001).

In addition to pyruvate and glucose, amino acids are also essential for optimal embryonic development, and serve a broad spectrum of functions including protein synthesis, metabolism, chelation, pH regulation and acting as energy substrates (Bavister, 1995; Gardner and Lane, 1997).

In summary, normal preimplantation embryo development requires key nutrients, which, upon degradation generate metabolites that reflect the activity and efficiency of embryo metabolism. On the basis of these observations, a multitude of investigators proposed that the change in the levels of these nutrients and/or their metabolites would reflect embryo viability, and assessed these nutrients/metabolites as potential biomarkers.

**Assessment of culture media nutrients and metabolites**

**Pyruvate**

As stated previously, the early preimplantation (cleavage stage or pre-compaction) embryo uses mainly the tricarboxylic acid (Krebs) cycle for which the main substrates are lactate and pyruvate and not glucose. Therefore, pyruvate uptake by cultured embryos has been investigated as a potential marker of embryo viability and growth potential, especially for embryos at cleavage stage. Initial studies reported higher pyruvate uptake by human embryos that develop to the blastocyst stage (Hardy et al., 1989; Gott et al., 1990). A following study from Conaghan et al. (1993a, b) reported that pyruvate uptake by 2–8-cell embryos was inversely related with embryo viability and pregnancy. A subsequent report by Turner et al. (1994), suggested that embryos display a wide range of pyruvate uptake values, but the variation is reduced in those embryos that are capable of implantation. Interestingly, these studies were performed by the same group (The laboratory of Henry Leese, which made significant contributions to the field), using the same methodology to determine pyruvate uptake. However, there were differences in the embryo culture media used, which may in part explain contradictory findings.

Most recently, Gardner et al. (2001) reported that pyruvate uptake on Day 4 is significantly higher in human embryos that go on to form blastocysts compared with embryos that fail to develop to the blastocyst stage, consistent with initial reports (Hardy et al., 1989; Gott et al., 1990). Overall, the published data correlating embryonic pyruvate uptake to viability is not consistent and therefore pyruvate does not seem to be a reliable biomarker for prediction of embryo viability.

**Glucose**

Upon transition from the morula to blastocyst stage glucose utilization increases significantly and seems to reflect the embryo’s developmental potential and viability (Devreker, 2007). Renard et al. (1980) reported that bovine blastocysts that have higher glucose uptake develop more successfully compared with those with lower glucose uptake. Gardner and Leese (1987) then showed that Day 4 mouse embryos that result in term pregnancies have a significantly higher glucose uptake in culture compared with embryos that fail to progress. However, contrasting with animal studies, several studies using human embryos reported the lack of a relationship between glucose uptake and blastocyst development (Hardy et al., 1989; Gott et al., 1990; Jones et al., 2001). It is noteworthy that many of these studies were compromised by absence of pyruvate, lactate, amino acids and vitamins in culture media, potentially leading to metabolic stress for the embryos, making interpretation of the findings difficult.

A more recent study using advanced culture media (Gardner et al., 2001) reported that the glucose consumption by Day 4 of human embryos is higher in embryos that form blastocysts, consistent with findings in animal models. On the basis of the initial animal studies and this more recent report on human embryos, it seems likely that embryonic glucose uptake on Day 4 or 5 of culture can be consistently correlated with viability.

A key aspect of the studies measuring pyruvate, lactate and glucose in human embryo culture media, is that they uniformly used microfluorometric enzymatic assays. These assays, although accurate, are technically difficult and require significant expertise and time commitment, making them less suitable for clinical application. More recently, Urbanski et al. (2008) reported the development of a microfluidics system that allows simultaneous measurement of pyruvate, lactate and glucose in small volumes of mouse embryo culture media. This report is encouraging, as it constitutes a major improvement in clinical applicability of metabolite measurement.

**Amino acids**

Using high performance liquid chromatography-MS (HPLC-MS) (Table II), Houghton et al. (2002) quantified secretion and uptake of amino acids by human embryos at different stages of preimplantation development. They then determined changes in secretion and uptake of amino acids in the culture media that correlate with successful in vitro embryo development. They found that a lower uptake of glutamine, arginine and methionine and a lower release of alanine...
and asparagine by Day 2 and 3 embryos correlates with successful development to the blastocyst stage (Houghton et al., 2002), whereas in 8-cell and morula stage embryos, blastocyst development is associated with lower uptake of serine and release of alanine and glycine. In all stages examined, lower amino acid turnover calculated by determining the sum of depletion and appearance of all amino acids, was associated with better development, consistent with the ‘quiet embryo’ hypothesis (Leese et al., 2008).

In a subsequent study from the same group, Brison et al. (2004) determined the changes in the concentrations of amino acids in spent human embryo culture media and identified amino acids that correlate with clinical pregnancy and live birth. In this study, zygotes were individually cultured in drops of pre-equilibrated medium (4 ml) containing a physiological mixture of amino acids for 24 h. Transfer was performed on Day 2, and spent culture medium was analyzed by HPLC. They found that a decrease in glycine and leucine and increase in asparagine levels in the culture medium correlate with clinical pregnancy and live birth (Brison et al., 2004). They also found that embryos with greater viability had a lower or quieter amino acid metabolism than those that arrested, consistent with the previous report by Houghton et al. (2002).

In a more recent study, Seli et al. (2008) used proton nuclear magnetic resonance (1H NMR) and found an association between higher glutamate levels in the culture media and clinical pregnancy and live birth. In this study, embryos were cultured in 25 ml medium for 48 h prior to transfer on Day 3. The differences in the volume and type of culture medium used, length of culture and technology used to measure amino acids may explain differences in identified amino acid biomarkers.

In this issue, the paper from Picton et al., goes a little further by associating the ploidy status with amino acid levels. Asparagine, glycine and valine turnover was significantly different between uniformly genetically normal and uniformly genetically abnormal embryos on Day 2–3 of culture. By Day 3–4 of culture, the profiles of serine, leucine and lysine differed between the two groups (Picton et al., 2010).

### Metabolomic analysis of culture media

The complete inventory of small-molecule (<1 kDa), non-proteinaceous compounds including metabolic intermediates (amino acids, nucleotides, lipids), adenosine triphosphate (ATP), hormones, other signaling molecules and secondary metabolites that are found within a biological milieu constitute the metabolome (Botros et al., 2008; Pasikanti et al., 2008). These compounds have diverse physical and chemical properties, and are found in a wide concentration range. The systematic analysis of this inventory of metabolites as small-molecule biomarkers that represent the functional phenotype is called metabolomics (Botros et al., 2008). Metabolomics is a powerful tool for the investigation of metabolic regulation as a function of normal and abnormal health in a biological system.

There are more than 25 000 genes in the human genome, encoding for ~100 000–200 000 transcripts and 1 million proteins, whereas there may be as few as 2500–3000 metabolites that make up the human metabolome (Botros et al., 2008). As the number of metabolites is significantly lower than the number of genes and proteins in a cell or organism, thorough metabolomic analysis can be achieved in a relatively faster period of time than genomics experiments. Nevertheless, metabolomic studies require a multidisciplinary team, from sample collection and preparation through analytical operations to processing of raw data and analysis of the processed data.

Metabolomics uses spectroscopic/spectrometric and chromatographic techniques (Table II) as analytical technologies to investigate complex metabolic/metabolomic profiles of biological systems [see (Botros et al., 2008) for review]. The analysis of biological fluids by spectroscopic technologies is termed ‘biospectroscopy’. The scientific platform that incorporates both biospectroscopy and metabolic profiling is commonly referred to as ‘biospectroscopy-based metabolomics’ or ‘BSM’. Both non-optical and optical spectroscopies have been used as analytical technologies for BSM (Fig. 4).

Non-optical spectroscopies applied for BSM include NMR spectroscopy, and MS, which can be coupled with separation methods like HPLC-MS. Both NMR and MS can generate information on multiple compounds within a sample, and have been applied to the analysis of embryo culture media (Houghton et al., 2002; Brison et al., 2004; Katz-Jaffe et al., 2006a, b; Seli et al., 2008). Unfortunately, these approaches are costly, require trained personnel, expensive equipment and longer analysis-time.

Optical spectroscopies used in BSM include near infrared (NIR) and Raman spectroscopies, characterized by reduced instrumental complexity and cost, as well as lack of requirement for sample preparation.

### Table II Examples of spectroscopic analytic technologies applied for assessment of metabolites.

<table>
<thead>
<tr>
<th>Technology</th>
<th>LC-MS</th>
<th>H-NMR Spectrometry</th>
<th>Raman spectroscopy</th>
<th>NIR spectroscopy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Determines most metabolites</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Requires sample preparation</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Requires sample separation</td>
<td>Yes</td>
<td>Minimal</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Allows non-destructive analysis of sample</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Allows direct determination of analyte identities</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Expensive system</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Requires highly trained personnel for clinical application</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Long analysis time</td>
<td>Yes</td>
<td>Yes</td>
<td>No*</td>
<td>No*</td>
</tr>
</tbody>
</table>

LC-MS, liquid chromatography mass spectrometry; H-NMR, proton nuclear magnetic resonance; NIR, near infrared.

*Analysis time for Raman and NIR spectroscopy applied to spent culture media analysis is reported to be <1 min.
or separation [see (Botros et al., 2008) for review]. Recently, these technologies were applied to the assessment of embryo culture media metabolome in correlation with embryo viability.

The first application of this technology to develop a model for prediction of embryo viability used both Raman and NIR spectroscopies to analyze Day 3 spent culture media of individually cultured embryos with known outcome (Seli et al., 2007). In this study, mean spectrum of embryos that resulted in a pregnancy and live birth was compared with that of embryos that failed to implant, and spectral regions most predictive of pregnancy outcome were identified. Then, using algorithms based on these spectral regions, a viability score was calculated for each embryo. Authors found that the mean viability score of embryos that implanted and resulted in a live birth were significantly higher compared with those that failed to result in a pregnancy (Seli et al., 2007). 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 min per sample) and required a very small sample volume (15 µl).

The model developed in this initial study (Seli et al., 2007) was then tested in a blinded trial (Scott et al., 2008b) using Raman spectroscopy to analyze Day 3 and Day 5 spent culture media collected at a different center, where embryos were cultured in a different type of culture medium, and a different volume. In this study, the viability scores of Day 3 and Day 5 embryos that resulted in a live birth were significantly higher compared with the viability scores of embryos that failed to implant.

The initial studies described above were followed by studies with larger sample size from IVF centers that routinely perform SET (Vergouw et al., 2008; Seli et al., 2009, 2010). In these studies, NIR spectroscopy and bioinformatics were used to analyze spent culture media as previously described (Seli et al., 2007), and independent regression algorithms for NIR spectra were developed for embryos that underwent SET on Day 2, 3 and 5 analyzing more than 500 SET samples (Vergouw et al., 2008; Seli et al., 2009, 2010). The recent studies analyzing spent culture media from SET cycles reported findings similar to previously reported and consistently showed higher mean viability scores for embryos that resulted in a pregnancy with fetal heart activity, compared with those that did not (Vergouw et al., 2008; Seli et al., 2009, 2010). They also showed that metabolomic profiling of embryo culture media was independent of morphology, providing an independent parameter for embryo viability assessment.

The findings using NIR and Raman spectroscopy are encouraging, not only due to their reported accuracy but also because of their potential to become a rapid, non-invasive, affordable test that can be applied on-site. However, to date, all published studies were performed using embryo culture media samples that were frozen upon collection and transported for analysis to a central laboratory. Therefore, whether metabolomic profiling will prove to be a robust test when performed at the IVF site remains to be seen. In addition, IVF practices use different types of culture media, volume, and perform transfers on different days of preimplantation development. Although metabolomic profiling using normalization to a control seems to be predictive across different type and volumes of culture media used, whether the accuracy is affected by these factors will need to be further investigated. Finally, aneuploidy plays an important role in embryo loss in IVF, and the predictive power of any non-invasive test will in part be determined by its ability to identify aneuploidies.
Whether some or all aneuploidies will have metabolic/metabolomic profiles that can be detected by this approach will also need to be investigated.

The ideological concept of embryonic normalcy

In addition to the technical hurdles caused by the minimal amount of material in embryos, the question of what constitutes a normal embryo complicates the interpretation of results. In any field of research, clearly defining the most desirable phenotype is a required and essential step in order to claim any contribution at solving the issue at stake. However, in the context of either wanting to improve assisted reproductive technologies and increase the yields in the production of transferable embryos or to develop tools to select the embryo that would provide the best chances of pregnancy, the phenotypes at stake are unclear. In an attempt to segregate different but related phenotypes, it may be important to distinguish embryonic yields from embryonic quality. These two concepts are often merged under the terminology of developmental competence. The embryonic yield is indicative of in vivo developmental competence and can be measured as the amount of fertilized oocytes that reach later developmental stages, which is generally set to be the blastocyst stage, whereas a good quality (or viable) embryo is developmentally competent with a potential to result in a live birth. The two concepts are without a doubt partly intertwined but by all logic, an increase in the amount of embryos does not guarantee embryonic quality. Furthermore, the definition of this embryonic quality is still a very vague and undefined concept. What exactly is a ‘good’ embryo? On what basis should the embryos be grouped to be further compared and studied in order to highlight their underlying characteristics? This question is of prime importance and is still difficult to grasp with the current state of knowledge on early embryo development.

It is noteworthy that the scope of the present discussion is not to challenge any of the progress that has been achieved so far by testing different aspects of key developmental events, such as the speed of polar body extrusion or of cellular cleavage or any other morphological characteristics that have been proposed as selection markers to increase pregnancy rates. It is only fair to mention that these characteristics are currently the best tools available but that their performances could be improved. This is supported by the continuing and growing interest in finding other means for embryo selection.

Non-destructive approaches, such as the ones aiming at the identification of culture media metabolites indicative of the state of the embryo, or the analysis of aspirated granulosa cells offer the benefit of determining the downstream outcomes following transfer. However, even in this best-case scenario where embryo quality is retrospectively determined based on pregnancy outcome, investigators can only confidently infer the quality of embryos that result in a pregnancy. This is because in cases where pregnancy does not occur, embryo may still be of high quality, as implantation failure could be due to factors independent of the embryo such as uterine factors or poor transfer technique.

Using a different angle, many teams are investigating embryo quality using destructive strategies (often with animal models) aimed at characterizing the impact of the culture environment by profiling and comparing the transcriptome or proteome of embryos from different sources or of different nature. Without the potential to determine the outcome of these embryos, the true competence status can only be inferred by extrapolation from other embryos.

It is unsettling to realize that this unclear definition of the desirable phenotypes directly impacts the interpretation of results since we currently cannot appreciate their value without being able to put them into physiological context. For example, numerous studies on the impact of culture conditions are comparing the treatment to in vivo produced embryos, which are considered to be the gold standard of developmental competence/quality. However, it is not expected that embryos produced in an artificial environment exhibit the same profiles as the ones that have been grown in vivo. Moreover, it is not expected that embryo culture systems will one day perfectly mimic the in vivo conditions. As such, some perturbations in the gene expression/proteins profiles should be considered normal. The question then is rather to define the extent of these perturbations that are acceptable, that do not compromise embryonic viability or lead to deleterious long-term effects. Could the best embryo be the one offering the largest plasticity level and thus being able to adapt and cope with more intense environmental insults? It is expected that, as it is the case in all living cells, adaptation can be stretched to a certain limit, beyond which irreversible damage will occur. The definition of embryonic competence should therefore include the level of plasticity and should be envisioned as an interval of acceptance rather than a clearly defined threshold value, especially when considering that adaptation could take diverse means to cope with the same stresses, thus creating heterogeneous populations.

In the end, it is expected that the development of a reliable aid for the selection of high quality (viable) embryos will be multi-factorial. It is currently of interest to define the concept of embryonic plasticity in order to appreciate the results being reported where different treatments are causing perturbations in the profiles of metabolites, RNA or proteins. The main question remains: what exactly is a ‘good’, ‘high quality’, ‘developmentally competent’ embryo?

Summary

Genomic, transcriptomic, proteomic and metabolomic analysis strategies (the omics) are being rapidly applied to the field of oocyte and embryo assessment, leading to discovery of biomarkers associated with oocyte and embryo viability. In addition, the clinical predictive value of some of these approaches has been demonstrated in blinded trials. Whereas randomized prospective trials assessing implantation and pregnancy rates using these technologies (used alone or in combination with morphology), compared with conventional morphologic assessment are lacking, recent reports are quite encouraging. Therefore, a fast, inexpensive, easy-to-use and hopefully non-invasive test involving one or a combination of these parameters may soon be available in clinical practice.

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