Article

Predicting human embryo viability: the road to non-invasive analysis of the secretome using metabolic footprinting

Dr Daniel R Brison received his doctorate from the University of York, UK in 1993, following studies with Professor Henry J Leese on the non-invasive assessment of embryo metabolism and ion transport. He then moved to a post-doctoral fellowship at the University of Pennsylvania, with Professor Richard M Schultz. Dr Brison was subsequently appointed Scientific Director of the Department of Reproductive Medicine at St Mary's Hospital Manchester in 1997, where he has concentrated on developing state-of-the-art clinical IVF laboratories and establishing a research programme focussed on understanding cell fate and viability in early embryos and embryonic stem cells.

Dr Daniel R Brison

Daniel R Brison[1,3], Katherine Hollywood[2], Ruth Arnesen[1], Royston Goodacre[2]
[1]Department of Reproductive Medicine, St Mary's Hospital, Manchester M13 OJH, UK; [2]School of Chemistry and Manchester Interdisciplinary Biocentre, The University of Manchester, 131 Princess Street, Manchester, M1 7ND, UK
[3]Correspondence: Tel: +44 161 276 6612; Fax: +44 161 224 0957; e-mail: Daniel.Brison@manchester.ac.uk

Abstract

Infertility affects an increasing number of couples and for many the treatment of choice is IVF. However, the success rate remains relatively low, and, as typically two or more embryos are implanted, successful pregnancy often leads to multiple pregnancies with attendant complications. The major limitation in clinical IVF is the inability to predict which embryos are most viable, with the highest chance of implantation and development to a live baby. In principle, embryos can be selected for transfer based on data obtained at the genomic, transcriptomic, proteomic and/or metabolomic levels; however, these measurements cannot always be made directly on the embryo without invasive biopsy of cells. Alternative strategies are needed and this review considers the range of possibilities, with a focus on the analysis of the secretome from human embryos using metabolic footprinting.

Keywords: *chemometrics, embryo viability, metabolic footprinting, metabolomics, secretome*

Introduction

Infertility affects one in six couples in the UK and for many the treatment of choice is IVF. However, success rates remain relatively low; for example, in the UK only about 25% of treatment cycles started yield a live baby (Human Fertilisation and Embryology Authority, 2007). Each IVF cycle in the UK produces on average 10 oocytes, of which six on average will fertilize to form embryos. Two preimplantation embryos are usually replaced in each cycle, yielding an unacceptably high rate of twin pregnancies with attendant complications (Human Fertilisation and Embryology Authority, 2006). In other countries this problem can be considerably worse, with three or more embryos routinely replaced in each cycle (Reynolds and Schieve, 2006). Globally, multiple pregnancies following IVF would be virtually eliminated by replacing only a single embryo at a time (Bergh, 2005). However, a major limitation in clinical IVF is the inability to predict which single embryo of a cohort is the most viable, with the highest chance of implantation and development to a live baby. In principle, embryos could be selected for transfer based on data obtained at the genomic, transcriptomic, proteomic and/or metabolomic levels (**Figure 1**). These methods of analysis can also be subdivided into those which are invasive to the embryo, usually requiring analysis of biopsied cellular material, and those which are non-invasive, based on analysis of embryo culture medium (Leese, 1987; Sakkas and Gardner, 2005).

Invasive methods

Invasive analyses of oocyte/embryo viability can be carried out by removing the oocyte 1st polar body by biopsy prior to fertilization, or biopsy of the resultant embryo at the 8-cell

stage, where one or two blastomeres are removed, or at the blastocyst stage, with trophectoderm (TE) cells being collected (McArthur *et al.*, 2005; Donoso *et al.*, 2007). Oocyte polar body biopsy has the major advantage that it can be considered a relatively non-invasive method of establishing the likely genetic status of the metaphase II oocyte. However, the method suffers from the disadvantages that it obviously cannot provide information on the resulting embryo following fertilization. A further disadvantage is that removal of the zona pellucida and the polar body require quite a high degree of technical skill not necessarily routinely available in IVF laboratories. On the other hand, biopsy of 8-cell-stage blastomeres has the advantage that the embryo can be assessed following activation of the embryonic genome. However, removal of cells at this stage is clearly highly invasive (Cohen *et al.*, 2007). Although evidence exists that the remaining 6/8 or 7/8 embryo develops normally to the blastocyst stage (Hardy *et al.*, 1990), concerns remain about the safety of this technique in terms of longer term embryo and fetal development. These concerns are heightened by evidence in animals of the establishment of polarity among the cells of the preimplantation embryo (Gardner, 1999), and of fetal programming originating as early as the preimplantation stage (Kwong *et al.*, 2000). In addition, the information which can be derived from biopsied cleavage stage blastomeres in particular is confounded by the high incidence of mosaicism in early human development, which means that any cell removed by biopsy may not be representative, at the genomic, transcriptomic or proteomic level, of the remaining embryo (e.g. Krussel *et al.*, 1998a; Kuo *et al.*, 1998). Thus, in terms of risk–benefit analysis, any method would need to demonstrate a high level of clinical benefit in order to justify the risk of removal of significant cellular mass (1/8 or 1/4) at a potentially sensitive stage of embryonic development. TE biopsy is arguably less invasive, as the cells of the inner cell mass are not collected. However, the relevance of information derived from TE biopsy to the health of the inner-cell-mass-derived fetus is questionable, and of course it requires extended culture of embryos to the blastocyst stage, which is not performed routinely in most laboratories.

Genomic

At the level of the genome, it is known that human embryos contain a high incidence of chromosomal aneuploidy, and this has been proposed as a method of negative selection of embryos for transfer, based on the biopsy of oocytes and embryos described above. However, evidence of the effectiveness of aneuploidy screening is currently inconclusive (Twisk *et al.*, 2006) and falls short of the high level of clinical benefit required to justify biopsy of embryos at the 8-cell stage. Oocyte polar body and TE biopsy are less invasive and have some promise in this respect. The area of aneuploidy screening has been reviewed recently (Twisk *et al.*, 2006; Donoso *et al.*, 2007) and will not be considered further here.

Transcriptomic

The expression patterns of a number of genes have been studied in single human embryos at the mRNA transcript level (Hamatani *et al.*, 2006). A small number of publications have suggested that expression of particular candidate genes might act as markers of embryo viability, e.g. insulin-like growth factor-1 (Kowalik *et al.*, 1999), and interleukin-1 (Krussel *et al.*, 1998b). More recently, mRNA amplification techniques have allowed whole families of genes to be analysed in single embryos, e.g. cell adhesion and apoptosis regulatory molecules (Bloor *et al.*, 2002; Metcalfe *et al.*, 2004). The authors' group has to date analysed more than 60 genes in the same individual embryos at all stages of development from oocyte to blastocyst (unpublished data). A number of these gene families could be candidate markers of embryo viability, e.g. the apoptosis regulatory *BCL2* gene family, which governs the cell death response of the embryo (Metcalfe *et al.*, 2004). However, the traditional hypothesis-based approach of identifying individual or small families of related molecules for analysis is a relatively poor way to generate clinically useful markers of viability, since it is slow, labour intensive, and also runs the risk of selecting embryos biased towards a particular aspect of viability. A systems biology approach is required, in which the whole range of gene expression can be analysed and integrated, offering a higher probability of yielding useful markers, and of encompassing the entire range of embryonic viability. The application of complementary DNA (cDNA) microarray technology coupled with embryo cDNA amplification means that it is now possible to analyse tens of thousands of genes in individual embryos. A few studies have applied global transcriptome analysis to early cleavage and blastocyst stages of human preimplantation development (Dobson *et al.*, 2004; Adjaye *et al.*, 2005; Li *et al.*, 2006; Brison et al., unpublished data). There is no reason in principle why this technique could not be applied to material biopsied from embryos for the purpose of embryo selection, analogous to aneuploidy screening. As gene expression patterns could offer both positive selection for viability genes, as well as negative selection of embryos expressing, for example, stress-response genes, this analysis at the level of the transcriptome is potentially more powerful than aneuploidy screening.

Proteomic

The analysis of the protein composition (proteome) of the embryo involves a different challenge because of the low concentration and wide dynamic range of the population of molecules which need to be analysed, and because, unlike mRNA, it is not possible to amplify proteins for analysis. It is now possible to analyse the proteome of individual pre-implantation embryos using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectroscopy (Katz-Jaffe *et al.*, 2005). In principle there is no reason why this method could not be applied to single cells for the purposes of embryo selection, analogous to transcriptome analysis. However, for the time being, a lack of sensitivity is likely to limit the clinical application of this technology, compared with transcriptomic analyses or metabolomic analyses (below), and proteomics is not yet considered wholly quantitative.

Summary of invasive methods

Invasive embryo biopsy carries potential hazards and any technique must demonstrate a high clinical benefit in order to justify this risk. Of the available technologies, transcriptomic analysis seems the most promising for clinical application because it combines the extreme sensitivity of mRNA reverse

transcription-polymerase chain reaction amplification from single cells, with analysis of the entire transcriptome using cDNA arrays. However, mRNA molecules do not do the work in the cell, they need to be translated into functional products such as proteins or metabolites. As such the analysis of the proteome, or metabolome, will provide markers closer to the phenotypic health of the cell or embryo (**Figure 1**). However, none of these techniques has yet demonstrated the high level of clinical benefit required for routine use in embryology. Therefore, any analytical technique should ideally be non-invasive, in order to reduce risk to the embryo, to reduce the hurdle of technology and skill required to apply it, and to reduce the level of benefit required to justify its use in clinical practice.

Non-invasive assays

As a result of the considerations set out above, less invasive methods (Leese *et al.*, 1993) are clearly required to select embryos for transfer, and it is proposed here that the proteome or metabolome would provide an ideal approach. So-called non-invasive analysis of embryo culture medium fits this bill perfectly, as it does not involve the embryo at all, but merely analyses of constituents of medium taken up, or released, by the embryo. The range of constituents which can be assayed is wide, and historically has included uptake of energy substrates such as glucose and pyruvate, and formation of lactate and ammonium as by-products of metabolism (Leese, 1987). The release of human leukocyte antigen-G into medium has also been correlated with implantation success (most recently by Desai *et al.*, 2006; and reviewed in Fanchin *et al.*, 2007); however, some doubt has been cast on the reported estimations of embryonic protein secretion using traditional antibody-mediated enzyme-linked assays (Ménézo *et al.*, 2006). Non-invasive assays of this type have been reviewed (Leese, 1987; Sakkas and Gardner, 2005) and will not be discussed further here. However, as argued above for transcriptome analyses, a robust assay of embryonic health is unlikely to arise from an assay of single or a small number of molecules. At minimum it would seem sensible to analyse families of related molecules using techniques such as high-pressure liquid chromatography (HPLC) with UV or mass spectroscopy detection to separate and analyse the constituents of embryo culture medium. Leese and co-workers have used HPLC to analyse the amino acid composition of the culture medium in which the embryo develops, and have provided strong evidence that a number of amino acids are predictive of pregnancy and live birth (Houghton *et al.*, 2002; Brison *et al.*, 2004). Nevertheless, a method for analysing the entire spectrum of interactions between embryo and culture medium, analogous to studying the entire transcriptome (above), might provide more powerful predictive markers and would select viable embryos without bias.

Since embryo metabolism is thought to be a critical determinant of viability (Houghton and Leese, 2004), there is much potential in a metabolomics approach (Goodacre *et al.*, 2004; Kell, 2004; Dunn *et al.*, 2005; Villas-Bôas *et al.*, 2005; Weckwerth and Morgenthal, 2005; Hall, 2006). Metabolic fingerprinting of the follicular fluid in which the oocyte develops is feasible (Thomas *et al.*, 2000) and may predict embryonic viability. Thus, it is reasonable to assume that a viable human embryo will possess a unique metabolic fingerprint, and that this secretome will be expressed in culture medium as a metabolic footprint (reviewed in Hollywood *et al.*, 2006). Preliminary analysis of the secretome from human embryos analysed by the metabolic footprinting method of Fourier transform infrared (FT-IR) spectroscopy with multivariate statistical analysis is detailed below.

FT-IR spectroscopy

Typical FT-IR metabolic footprints from embryo culture media are shown in **Figure 2**. Embryos were cultured from day 1 to day 2 of development in 200 µl Universal IVF culture medium (MediCult a/s Møllehaven 12, DK-4040 Jyllinge, Denmark) under an overlay of light mineral oil (MediCult), at 37°C under 5% CO_2 in air. One or two embryos were selected for transfer to the recipient on day 2 or 3, using conventional criteria (Steer *et al.*, 1992). Embryos were graded for cell number (1–8) and morphological appearance (1–4, with 1 being the poorest score), and these were then multiplied to give an embryo 'score' e.g. a 4-cell embryo of grade 3 is denoted as '4.12'. Spent culture medium samples were stored at –80°C until analysis, when they were thawed, and any contaminating paraffin oil was extracted based on the isolation and purification technique provided by Folch *et al.* (1957). Aliquots (20 µl) of the methanol layers were evenly applied onto a zinc selenide microplate containing 96 wells and oven-dried at 50°C for 30 min (or until visibly dry). Each sample was analysed in triplicate. The FT-IR instrument was a Bruker EQUINOX 55 FT-IR spectrometer equipped with a deuterated triglycine sulphate detector and operating in absorbance mode (Goodacre *et al.*, 2000; Harrigan *et al.*, 2004). Spectra were collected over the wave number range 4000 cm^{-1} to 600 cm^{-1} at a rate of 20 s^{-1}. The spectral resolution used was 4 cm^{-1}. To improve the signal-to-noise ratio, 256 spectra were co-added and averaged. Each sample was thus represented by a spectrum containing 1764 points and spectra were displayed in terms of absorbance. The data were normalised using extended multiplicative scatter correction as detailed in Martens *et al.* (2003).

Multivariate statistical analyses

As the data qualitatively look very similar and cannot be separated by eye it is necessary to employ chemometric methods. Principal components-discriminant function analysis (PC-DFA; **Figure 3**) of the metabolic footprints from primary culture drops was performed as detailed elsewhere (Goodacre *et al.*, 1998, 2000) using Matlab (The Math Works, MA, USA). Briefly, the initial stage involved the reduction of the multidimensional FT-IR data by principal components analysis (PCA) (Jolliffe, 1986). PCA is a well-known technique for reducing the dimensionality of multivariate data whilst preserving most of the variance, and Matlab was employed to perform PCA according to the nonlinear iterative partial least-squares algorithm (Wold, 1966). Discriminant function analysis [DFA; also known as canonical variates analysis (CVA)] then discriminated between groups on the basis of the retained principal components and the *a priori* knowledge of which spectra were replicates, and thus this process does not bias the analysis in any way (Manly, 1994).

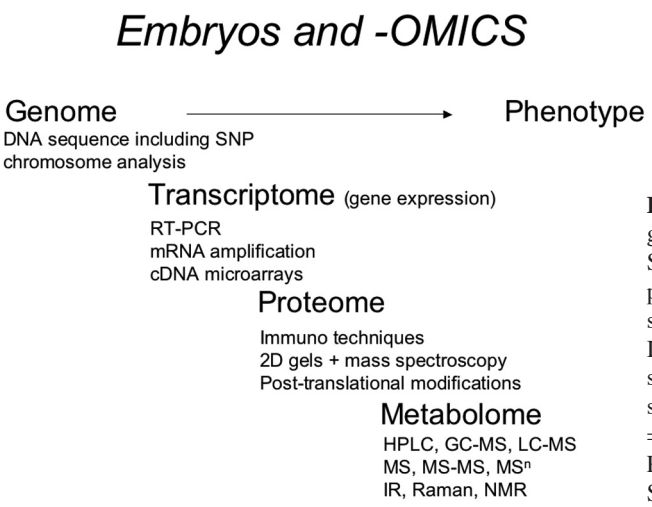

Figure 1. Post-genomic approaches to embryo selection, from genomic, transcriptomic, to proteomic and metabolomic. Shown under each of these techniques are the common analysis platforms employed. GC–MS = gas chromatography–mass spectrometry; HPLC = high-pressure liquid chromatography; IR = infrared; LC–MS = liquid chromatography–mass spectrometry; MS = mass spectrometry; MS–MS = tandem mass spectrometry; MS^n = mass spectrometry to the *n*th power; NMR = nuclear magnetic resonance; Raman = Raman spectroscopy; RT-PCR = reverse transcription polymerase chain reaction; SNP = single nucleotide polymorphism.

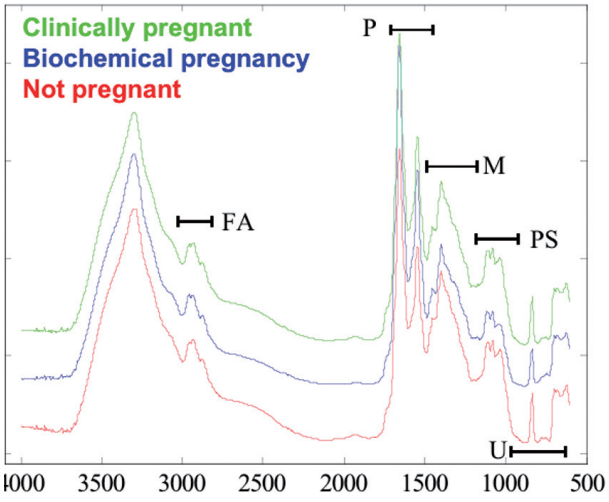

Figure 2. Typical Fourier transform infrared metabolic footprints from primary embryo culture drops. These spectra have been offset to see the features more readily. Key to vibrational bands: FA = fatty acid; P = protein; M = mixed region of fatty acids, polysaccharide, nucleic acids and proteins; PS = polysaccharide; U = unknown origin.

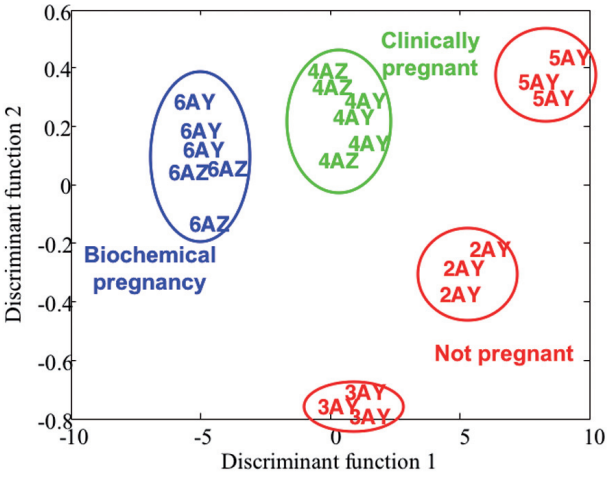

Figure 3. Principal components–discriminant function analysis of the metabolic footprints from primary culture drops showing the relationships between the seven samples (from five patients) analysed. Boundaries round the clusters are drawn manually and have no statistical significance. Principal components 1–5 were used for discriminant function analysis and these accounted for 98.4% of the variance. Each numeric code represents a single embryonic sample.

Discussion

Three typical FT-IR spectra from primary embryo culture for each of the above clinical outcomes are shown in **Figure 2**. These FT-IR spectra contain information on functional group vibrations resulting in the absorbance of infrared light at specific wave numbers (1/λ). The majority of these regions can be identified (Schmitt and Flemming, 1998; Ellis *et al.*, 2003) to be vibrational modes from water (O–H stretch centred at 3400 cm^{-1}), fatty acids (CH_x stretches at 2956–2850 cm^{-1}), proteins (amide I, C = O at 1652–1648 cm^{-1}; amide II, N–H, C–N at 1550–1548 cm^{-1}), polysaccharide (ring and C–O vibrations at 1085–1052 cm^{-1}), and there is also a mixed region from 1460–1110 cm^{-1} which contains information from fatty acids, polysaccharide, nucleic acids and proteins. Note that protein and peptide species, as well as nucleic acids, are detected because the sample preparation used a simple methanol:chloroform Folch-based extraction where the polar methanol fraction was analysed directly without any protein precipitation using, for example, acetonitrile.

Notwithstanding the above, these spectra can be considered as metabolic fingerprints (Fiehn, 2002; Kaderbhai *et al.*, 2003; Dunn *et al.*, 2005) since these spectra have been produced by employing a fingerprinting technology that is rapid but does not necessarily give specific metabolite information. Nevertheless, these and indeed the FT-IR metabolic footprint spectra from all embryo cultures showed broad and complex contours. There was relatively little qualitative difference between all the spectra and such data readily illustrate the need to employ multivariate statistical techniques for the analysis of these metabolic footprints.

The next stage was therefore to perform cluster analysis on all of the metabolic footprints (seven samples analysed three times by FT-IR) collected using PC-DFA as described above, and the resulting ordination plot is shown in **Figure 3**. Note that PC-DFA is a supervised method as *a priori* class information is used in the analysis. The information used here was the triplicate measurements collected from FT-IR (so called machine replicates) and so seven groups were used, one for each of the seven samples analysed. Therefore this process does not bias the analysis in any way, and one is seeking to observe the relationships within the cluster space – the closer samples cluster together the more biochemical similarity they possess.

The PC-DFA plot in **Figure 3** shows that the two separate samples (4AY and 4AZ) from the IVF patient who was clinically pregnant cluster together. This is highly significant because embryos from two different culture drops were transferred back to this mother, who later successfully gave birth to twins, demonstrating that both embryos implanted. This indicates that there are distinct chemical signals in primary embryo culture media which may indicate viability. The patient who also had two embryos replaced from separate embryo culture samples (6AY and 6AZ) also cluster together and this patient, whilst showing biochemical pregnancy signals, did not become clinically pregnant. Finally, the three patients with samples 2AY, 3AY and 5AY, who did not become pregnant, cluster into three separate groups, although these are located loosely together in the positive part of the first discriminant function score.

Whilst the sample size is small in this preliminary analysis of the primary embryo culture media in which embryos had been generated and cultured for 26 h, this approach does show great potential as a non-invasive screening technique. The metabolic footprints from viable embryos cluster together, as do those that initially show biochemical signs of successful implantation. That these are also located closely together and away from the patients who did not become pregnant indicates that the embryo culture medium contains chemical species that are indicative of embryo viability; a finding in common with targeting specific amino acids (Houghton *et al.*, 2002; Brison *et al.*, 2004).

Conclusion

A number of guiding principles can be used in the development of embryo selection criteria: (i) risk–benefit analysis; (ii) obtaining maximum information to aid the process of selection; and (iii) using cellular or metabolic information which is as near to phenotype as possible (**Table 1**). Risk–benefit analysis, by which any improvement in embryo selection should be weighed against the risk of applying the technique, in terms of loss of or damage to embryos, is clearly paramount. Applying this principle, non-invasive assays should be clearly favoured

Table 1. Comparison of various strategies for embryos selection, based on risk, nearness to phenotype, and amount of information obtained. (Risk increases from left to right, nearness to phenotype increases from top to bottom.)

	Culture medium assays (low risk)	*Oocyte PB/blast TE biopsy*	*Cleavage embryo biopsy (high risk)*
Genomic	N	Aneuploidy screening	Aneuploidy screening
Transcriptomic	N	Qualitative RT-PCR; quantitative RT-PCR; mRNA amplification + microarrays[a,b]	Qualitative RT-PCR; quantitative RT-PCR; mRNA amplification + microarrays[a,b]
Protein	Human leukocyte antigen-G	N	N
Metabolites	Amino acids[a]; infrared analyses[a]; mass spectroscopy[a]	N	N

[a]Systems biology approaches; [b]technologies that are theoretically possible but not yet demonstrated in principle.
Blast = blastocyst; PB = polar body; RT-PCR = reverse transcription polymerase chain reaction; TE = trophectoderm; N = not possible, at least with current technology.

over more invasive ones. Within the range of non-invasive assays, the second principle would clearly favour a systems biology approach over the more traditional hypothesis-based methods. Finally, the third principle would indicate assays at the proteomic or metabolomic rather than genomic or transcriptomic end of the spectrum. As a result of these considerations, it is proposed that wide-ranging assays of embryonic health are likely to prove most fruitful, and the data presented suggest that metabolic fingerprinting of the embryonic secretome may offer significant benefits.

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