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The biological basis of non-invasive strategies for selection of human oocytes and embryos

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There is a need for more accurate embryo selection in human assisted reproduction, if the goal of reducing the number of embryos used in embryo transfer is to be realized. Furthermore, any selection strategy should be non-invasive if the embryos are to be used in embryo transfer. Currently, the strategy is selection by one to three parameters in the cleaving- and blastocyst-stage embryo, sometimes with additional pronuclear selection. It is clear that no one system is ideal, as the vast majority of transferred embryos do not implant. As the health of the embryo is largely dictated by the originating gametes, the very early events in oocyte development should be considered. This review will point to the early biological events in the unfertilized and fertilized oocyte that can be scored non-invasively and which can have a profound effect on the later developmental stages. Using a sequential scoring system, with emphasis on the oocyte, a system for selecting the most viable single embryo for transfer may hopefully be achieved.

Key words: non-invasive embryo selection

Introduction

During the past two decades the practice of clinical assisted reproduction (ART) has grown from being conducted in a few clinics to a world-wide industry. Along with this growth there has been an expansion in the forms of infertility that are treated, from the initial criteria of tubal occlusion (Edwards *et al*., 1980) to include most reproductive disorders in both females and males. These can range from unexplained causes to endocrine dysfunction, decreased ovarian reserve, and physical blockage in male and female and disease states such as polycystic ovarian syndrome (PCOS). Third-party reproduction has been introduced, and in more extreme cases, ART coupled with genetic techniques has been employed to avoid devastating genetic disorders. Although in the early days of ART the success rates were low, today clinics report up to 50% clinical pregnancy rates per transfer. This increased success has come through a combination of clinical and laboratory improvements, which include improved drug regimes for ovarian stimulation, as well as simplified and more accurate methods of predicting follicle and oocyte maturity and oocyte harvesting. In the laboratory, the improvements have been primarily at the level of media, allowing development of embryos *in vitro* and selection at a more advanced stage (Bavister, 1969; Goddard and Pratt, 1983; Chatot *et al*., 1989; Leese, 1989; 1990; 1991; 1995; Lawitts and Biggers, 1993; Leese *et al*., 1993; Quinn *et al*., 1995; Gardner and Lane, 1997).

However, the reports of high clinical pregnancy rates can come at an extreme cost to the patient and society in that these are achieved by the use of multiple embryos in transfer, leading to a world-wide escalating incidence of both twin and high-order multiple pregnancies (Racowsky, 2002). The practice of replacing multiple embryos arises from the fact that the reported implantation rates for human embryos range from as low as 8% to a maximum of approximately 40%, implying that between 60 and 92% of all embryos transferred do not implant. Some countries have introduced mandates to limit the number of embryos that can be replaced (Ludwig *et al*., 2000a; b; Montag and van der Ven, 2001), while others have issued guidelines and recommendations for reductions. In order to comply with these regulations and meet the patient's and society's demands for treatment success, it is imperative that a reliable method of selecting one embryo with maximum implantation potential be devised.

To date, embryos have been selected primarily on morphological and development criteria, which are generally applied in a single, static observation. These have included pronuclear oocyte morphology (Payne *et al*., 1997; Scott and Smith, 1998; Ludwig *et al*., 2000a; b; Scott *et al*., 2000; Tesarik *et al*., 2000; Wittemer *et al*., 2000; Balaban *et al*., 2001; Zollner *et al*., 2002) and the early entry into the first mitotic division (Edwards *et al*., 1980; Shoukir *et al*., 1997; Bos-Mikich *et al*., 2001; Lundin *et al*., 2001; Petersen *et al*., 2001; Sakkas *et al*., 2001; Fenwick *et al*., 2002). The use of day 3 morphology and embryo scores have also shown correlations with implantation (Steer *et al*., 1992; Tan *et al*., 1992; Rijnders and Jansen, 1998; Van Royen *et al*., 1999; 2001; Desai *et al*., 2000; Rienzi *et al*., 2002). As embryos cleave, the form or evenness of cell division (Hardarson *et al*., 2001; Roux *et al*., 1995), the fragmentation patterns (Antczak and Van Blerkom,

1999; Alikani *et al*., 2000; Van Blerkom *et al*., 2001) and the rate of development (Racowsky *et al*., 2000; Shapiro *et al*., 2000; Check *et al*., 2001) have an impact on implantation. Blastocyst formation and morphology has also been related to implantation (Dokras *et al*., 1993; Gardner and Lane, 1997; Scholtes and Zeilmaker, 1998; Balaban *et al*., 2000; 2001; Gianaroli *et al*., 2000; Milki *et al*., 2000; Plachot *et al*., 2000; Blake *et al*., 2002).

However, with all these techniques there is still the limit of a maximum implantation rate, leading to the use of multiple-embryo transfers. Within a cohort of embryos transferred, either all, none or a subset may implant even if they were all scored as equal, indicating that at some non-observable level they are different. Embryos are on a continuum of development from the time of initiation of oocyte growth in the primordial follicle through implantation and fetal development. Many of the methods of selection rely on a static observation of the embryos, whereas the embryo itself, after fertilization, is on a very strict set of clocks governing division and initiation of key events from gene activation through compaction and blastulation (Johnson and Day, 2000). This would imply that to evaluate an embryo's potential reliably would need a sequence of reference points as it makes the necessary transitions from oocyte, through fertilization, initiation of RNA synthesis, switch from maternal to embryonic genome and the initiation of growth through protein synthesis and differentiation (Scott, 2003b).

The use of blastocyst transfers has been proposed as a means of selecting viable embryos that had made all of these transitions, leading to increased implantation potential (Vidaeff *et al*., 2000). The clinical use of blastocyst transfer has indeed shown higher implantation rates compared with day 3 results in selected groups of patients (Dokras *et al*., 1993; Gardner and Lane, 1997; Balaban *et al*., 1998; 2000; 2001; Scholtes and Zeilmaker, 1998; Huisman *et al*., 2000; Milki *et al*., 2000; Plachot *et al*., 2000; Scott *et al*., 2000; Kovacic *et al*., 2002), including older patients (Shapiro *et al*., 2002). In these studies, only patients with an adequate cohort of embryos with good day 1, 2 or 3 morphology were allowed to proceed to day 5 transfers. There are few controlled randomized studies comparing day 3 and 5 transfers and these show that, in good prognosis patients, the pregnancy rates are equivalent and that what differs is the implantation rate (Gardner *et al*., 1998a; Coskun *et al*., 2000). This is a start to solving the problem. However, the reported rates of blastocyst formation *in vitro*, using the culture systems that are currently available, range from 20 to 50% from both stimulated and unstimulated cycles. This means that there is great attrition from day 3 to 5 of embryos that may appear morphologically normal on day 3. There are no data available to show that these embryos could not implant if transferred at an earlier stage.

In two reports where all patients in an ART practice had day 5 blastocyst transfer, the data were not encouraging on a per embryo basis (Marek *et al*., 1999; Wilson *et al*., 2002). However, the first group (Marek *et al*., 1999) reported a slight improvement in implantation rates in women aged <35 years compared with their previous day 3 results (from 29.5 to 38.9%) with the use of two or more blastocysts on average. The implication here is that more than 60% of the blastocysts failed to implant. The second study also indicated an increase in implantation rate compared with previous day 3 rates in the younger age groups (from 30 to 50%), but with minimal increase for women aged over 35 years (Wilson *et al*., 2002). In both of these studies, ⩾50% of transferred blastocysts failed to implant.

A further study that may shed light on the problems faced by ART clinics dealt with oocytes from unstimulated cycles (Vlaisavljevic *et al*., 2001). In this study, patients were divided between day 2 and day 5 transfers. There was no difference in fertilization rate in the two groups or the achievement of an embryo on day 2. However, only 55% of embryos placed in extended culture grew to the blastocyst stage. This may indicate an intrinsic problem with a large cohort of retrieved oocytes that is exacerbated by ovarian stimulation, or a deficiency in the culture systems used. Overall, the implantation rates per embryo transferred were different between day 2 and day 5 (24 versus 40%), but per oocyte retrieved there was no difference (12 versus 14%). Even in these unstimulated cycles, 60% of blastocysts failed to implant, which is no different to that achieved with ovarian stimulation and a large cohort of starting material.

This would point to an underlying problem with >50% of all oocytes retrieved in human ART cycles. A major cause of implantation failure has been attributed to chromosomal aneuploidies (Gianaroli *et al*., 1997; Munné and Cohen, 1998; Verlinsky *et al*., 1998; Harper and Delhanty, 2000). These have been found in morphologically normal appearing embryos or from embryos with an obvious morphological abnormality (Pellestor *et al*., 1994; Magli *et al*., 1998; Sadowy *et al*., 1998; Munné *et al*., 1999; Hardarson *et al*., 2001; Nass *et al*., 2002) and in embryos from older patients (Gianaroli *et al*., 1999; Kahraman *et al*., 2000). The abnormalities are persistent through to the blastocyst stage in many instances (Magli *et al*., 2000; Sandalinas *et al*., 2001). Embryos can present with uniformly normal, uniformly abnormal, mosaic or chaotic chromosome constitutions. The mosaic type are of concern as it may take more than one cell in a biopsy to identify this condition (Kuo *et al*., 1998; Munné and Cohen, 1998; Magli *et al*., 2000).

The use of preimplantation genetic screening or diagnosis to select against embryos carrying some form of aneuploidy has been proposed as a means of increasing implantation in older women or those with repeated ART failure (Munné and Wells, 2002). However, the data available do not point to a great increase in the take-home-baby rate when this technique is applied in a clinical setting (Egozcue *et al*., 2002). The data from the two largest published studies show no dramatic improvement in the implantation or live delivery rates in older women (Gianaroli *et al*., 1999; Munné *et al*., 1999) or in the group of repetitive ART failures (Gianaroli *et al*., 1999). The issue of mosaicism could be contributing to this, but the use of too many cells from an embryo for accurate diagnosis is of concern for the developing embryo. Further, it is not a non-invasive strategy since the embryo is subjected to manipulation and cell removal, which could have severe consequences in later development when embryo polarity is considered.

Recent studies (Gardner, 2001) indicate that traditional ideas on the onset of polarity during early mammalian development need to be reconsidered. As with lower-order animals, mammalian embryos have been shown to have polarity and ordered directional development, from fertilization through compaction to the blastocyst stage. Although blastomeres from early embryos are said to be totipotent, and disaggregated cells can form small

blastocyst structures *in vitro*, they have little to no developmental potential. Increasing evidence is pointing to a very ordered and polarized progression from the oocyte to the fetus in mammalian development (Edwards and Beard, 1997; Gardner, 2001). In a very elegant study, it was shown that even as early as the oocyte stage there was differential distribution of STAT 3 and leptin within the human oocyte (Antczak and Van Blerkom, 1997). This continued in development, with differential distribution still being evident at the 8-cell stage. Further, it has been suggested that within the 4-cell embryo not all the blastomeres are equal (Gardner, 2002; Hansis, 2002). In a mouse study, it was clear that only one of the four cells has the polar body attached, that this is always the same cell (Gardner, 2002), and that later in development a progeny of this cell—still with the polar body attached—is always located on a particular point of the blastocysts, the embryonic axis (Gardner, 1997). In humans, it has been suggested that at the 4-cell stage, only one of the blastomeres continues to develop into the clone of cells that produces hCG (Hansis, 2002).

This would imply that the removal of cells from the early embryo could disrupt this order and even result in an embryo with decreased implantation potential due to the removal of a vital functional part. It has long been evident that mammalian embryos have plasticity in that they can develop after losing a few cells, but there must be a limit to how many cells they can lose without compromising viability. The later the cells are removed, the less likely it will cause harm (Gardner and Nichols, 1991; Gardner, 1996; 1999). However, the more cells the embryo has, the more the chances of selecting a normal versus abnormal cell in aneuploid mosaic cases.

If so few oocytes have the ability to develop into viable embryos, which can implant and form a fetus, what are the markers that can indicate this? Clearly the morphological criteria currently used are inadequate. Within the context of a clinical setting any selection system must be non-invasive. This review will outline some of the pointers that are currently available for selection on a continuous model, or through an elimination tree, from the oocyte through to the blastocyst, with emphasis on the early events, as these will dictate what happens in subsequent development. It will focus on the biology dictating the morphological feature described. No one selection criterion is better, but if possible a combination of one, two or even three selection points should lead to more accurate selection (Gerris *et al*., 2002). An embryo that does not meet a selection criterion at one point may meet it at another, but should be avoided, as it is unlikely that culture can correct an impaired embryo. The issue of selecting a viable spermatozoon will not be considered. The aim is to reduce the number of embryos used in transfer to one, while increasing the implantation rates and doing no harm to the embryo.

Ovarian factors and oocyte quality

It is well accepted that the developmental fate of the embryo is largely dictated by the 'quality' of the oocyte. If, as indicated above, so few oocytes result in viable embryos, having a measure of oocyte competence would enable better selection of the resulting embryo. However, the follicle and oocyte are not easily accessible for screening or assessment under clinical conditions. During ART, there is routine measurement of the levels of estradiol being produced by the follicles, which gives a fair indication of the health and functioning of the granulosa cells. There is also routine measurement of the growth of the follicle using ultrasound. Although these two parameters have enabled the harvesting of mature oocytes, which can fertilize and develop to form embryos, they give no indication of the health and competence of the oocyte. In some instances in humans during ovarian stimulation there is a complete uncoupling of the events of oocyte and follicle maturation, leading to arrested or immature oocytes originating from seemingly mature follicles (Hartshorne *et al*., 1999). This is in contrast to the mouse where this uncoupling cannot occur, with the oocyte driving the follicle and the follicle providing support to the oocyte (Albertini *et al*., 2001; Eppig, 2001). Other factors are needed that can indicate the health, maturity and competence of the oocyte.

This could potentially be available with pulsed colour Doppler ultrasound, whereby the blood flow to each follicle developing in the ovary can be quantified. Using this technique it has been shown that oocytes originating from follicles with reduced blood flow—and thus reduced oxygen delivery—give rise to embryos with reduced developmental competence and implantation (Nargund *et al*., 1996; Chui *et al*., 1997; Van Blerkom *et al*., 1997; Huey *et al*., 1999).

If the lack of adequate blood flow to the ovary is an issue, how exactly is it affecting the development of the oocyte and subsequent embryo? The use of oxygen for oxidative phosphorylation (OxPhos) was a defining event in the evolution of multicelluar and complex organisms, allowing sufficient energy generation for complex development. In all systems, metabolic substrates and oxygen need to be delivered to the cells, and the carbon dioxide waste product removed. Generally, the vascular system delivers oxygen to a tissue either directly or through diffusion via other cells. The vascular network in the body continually re-models in order to deliver the oxygen required. (Maxwell and Ratcliffe, 2002). If a cell which is normally in an oxygenated situation and utilizing OxPhos, is placed in low-oxygen conditions it will respond in one of two ways, depending on whether the condition of low oxygenation is acute or chronic (Kaelin, 2002). When the low-oxygen condition is acute, the response is to switch from OxPhos to glycolysis by up-regulating proteins involved in glucose uptake, metabolism and extracellular pH. This allows cells to continue with energy generation, although with vastly reduced amounts of ATP production (2 versus 38 molecules of ATP). If the condition of low oxygen becomes chronic, the response is to up-regulate proteins that are involved in angiogenesis and erythropoiesis, in an attempt to increase the delivery of oxygen to the cell through increased blood flow. Both of the responses to low oxygen are controlled or regulated by hypoxia-induced factor (HIF). This is located in the nucleus, and is a heterodimer consisting of alpha and beta subunits (Berra *et al*., 2000). HIF-1a is a highly phosphorylated moiety, and phosphorylation of HIF-1a induces changes in its electrophoretic migration (Richard *et al*., 1999a). HIF is only active under hypoxic or low-oxygen conditions (Berra *et al*., 2000). Hypoxia induced HIF-a is the primary mode of activation. HIF-1a has a short half-life but is maintained in continual low levels in cells under normoxic conditions. Control of HIF under normoxic conditions is through rapid ubiquitination followed by proteosome degradation, which is realized through the rapid degradation of the alpha unit by a polyubiquitination complex (Semenza, 2001; Yang and Kaelin,

2001). This complex contains the von Hippel–Lindau tumour suppressor protein (pVHL) and the nuclear phosphoprotein MDM2 (Maxwell *et al*., 1999; Ravi *et al*., 2000). The pVHL binds directly to the alpha subunit in an oxygen-dependent degradation domain (ODD), thereby rendering the molecule unstable in the presence of oxygen. This control is governed by the hydroxylation of a prolyl and asparaginyl residue in the HIF N-terminal transactivation domain (NTAD). This reaction is highly oxygen-dependent and is a highly conserved pathway. Hypoxia induces HIF-1a by inhibiting this degradation, and this is a key step in the hypoxia activation of HIF-1 (Salceda and Caro, 1997; Huang *et al*., 1998; Kalliho *et al*., 1999). Increased expression of HIF-a, which is the rate-limiting subunit, determines the activity of the whole complex (Richard *et al*., 1999b).

Up-regulation of HIF in response to hypoxia stimulates angiogenesis—the formation of new vasculature. When angiogenesis is deregulated it leads to a pathological condition, which is found in all tumours, and which is the rate-limiting step for all tumour growth (Berra *et al*., 2000). Vascular endothelial growth factor (VEGF) is the critical mediator of angiogenesis. VEGF is a member of secreted growth factors, is a mitogen for vascular endothelial cells, and an important part of blood cells. It is expressed in all tissues and is especially up-regulated in tumours (Berse *et al*., 1992). VEGF is regulated at multiple levels, such as transcription, post-transcription and at translation, and is controlled by a mitogen-activated protein kinase (MAPK) signalling cascade. Under normoxic conditions, VEGF is down-regulated through a p42/p44 MAPK-dependent pathway. Sustained activation of the p42/p44 pathway protects cells against apoptosis. Under hypoxic conditions, HIF-1 regulates VEGF through a VEGF promoter which has binding sites for HIF-1, and this is specifically the p42/p44 site. In culture, continual activation of the p42/p44 cascade fully protects cells against severe apoptosis initiated by a complete depletion of growth factors and extracellular matrix, and under hypoxic conditions this is switched off. Estradiol added to cells or media will activate and sustain the p42/p44 MAPK pathway protecting cells *in vitro* from apoptosis (Semenza, 2000).

Another aspect of HIF up-regulation is activation by p53, an important tumour suppressor protein that has a role in the G_1/S and G_2 /M cell cycle checkpoints (Meek, 2000). p53 specifically restricts the proliferation of cells with damaged spindles and nuclear structures, thus preventing aneuploidies and polyploidies. If fully deactivated, it will allow these cells types to form and continue with development. p53 does not control spindle assembly, but detects aberrant assembly and arrests the cell in G_1, thus preventing continued development. The process of p53 activation occurs subsequent to a stress such as hypoxia, metabolic change, or other damage specifically to the spindle, such as depolymerization (Sablina *et al*., 1999). When damage occurs, another p53-independent checkpoint is initiated, functioning at the metaphase–anaphase transition and preventing progression until the spindle is formed. This is not a strong block, and cells escape in a manner termed 'mitotic slippage', thus allowing mitosis to continue in which there is aberrant sister chromatid exchange (Meek, 2000). This induces p53, which puts up a G_1 growth arrest. A further arrest is also instigated at the G_2 phase. Under normal conditions, low levels of p53 are maintained by its rapid degradation via a proteosome system and specifically through MDM2 protein. In undamaged cells, MDM2 interacts with p53 and translocates it from the nucleus to the cytoplasm for ubiquitin-mediated degradation (Brune *et al*., 2001; Piret *et al*., 2002). Under conditions of insult, this does not happen and levels of p53 increase. One stress system that stabilizes p53 is that of hypoxia, and as levels of HIF-1a increase, so do levels of p53. The target genes for p53 are those such as p21, which are involved in apoptosis and cell cycle arrest. Disrupting the balance of p53 could allow more mitotic slippage or induce more cell cycle arrest.

Using the above concept, which is true for somatic cells, follicles that are well oxygenated should have HIF down-regulated, resulting in up-regulation of OxPhos and down-regulation of glycolysis. If the follicles (and therefore the oocytes) are under-oxygenated, then HIF would be up-regulated, VEGF up-regulated, glucose transport stimulated by Glut-1 and Glut-4 transcription (Behrooz and Ismail-Beigi, 1999), and glycolysis stimulated. This would result in OxPhos being decreased, and angiogenesis increased to cope with the acute lack of oxygen. This will in turn result in ATP depletion in the oocytes and follicles while resources are diverted away from follicle maturation to angiogenesis. Decreased ATP in the follicles and oocytes would lead to decreased viability, as has been documented (Van Blerkom *et al*., 1995a). Furthermore, if p53 is not being degraded there could be cell cycle arrest and induction of apoptosis, or a disruption of the cell cycle check on spindle formation leading to release of cells with aberrant chromosome constitutions (aneuploidy?).

Clinically, there are many reports linking increased VEGF levels in follicular fluid with decreased embryo development, embryo morphology and implantation rates (Van Blerkom *et al*., 1997; Barroso *et al*., 1999) and indicating that a state of poor oxygenation has been linked to poor oocyte potential. It has also been shown that, as stimulation increases, the concentration of VEGF in follicular fluid increases (Artini *et al*., 1998) with an increased risk of ovarian hyperstimulation syndrome (Neulan *et al*., 2001). Again, with increased stimulation and more follicles there will be a demand for more vasculature, which can be linked to the increase of VEGF in follicular fluid, with all the downstream effects on the follicle and oocyte. The balance between VEGF production and its receptor is critical for determining the response, with an excess of bioactive VEGF resulting in decreased conception (Neulan *et al*., 2001). There are also reports of increased VEGF levels in the follicular fluid from poor responders (Quintana *et al*., 2001). And finally, there is a correlation with increased age and the levels of VEGF in follicular fluid (Freidman *et al*., 1997), which has also been linked to lower estradiol levels, fewer oocytes and decreased implantation potential (Freidman *et al*., 1998). Again, the reported low implantation rates of embryos from older women, due to aneuploidy and other factors, can also be seen in the response of the ovary to stimulation and hypoxia.

Taken together, it would seem that a first-line, non-invasive technique of screening for competent oocytes is to screen for adequate follicle oxygenation through blood flow to the follicles using pulsed colour Doppler ultrasound, followed by follicular fluid screening for VEGF and perhaps also leptin (Van Blerkom *et al*., 1997; Barroso *et al*., 1999); in this way oocytes that have not

been challenged with hypoxia could be selected. This will ensure that the oocytes have adequate capacity to generate ATP via oxidative phosphorylation, that the HIF pathways are down-regulated, that cell cycle checkpoints preventing progression of oocytes with damaged spindles (aneuploidy) are operational, and that perhaps the p42/p44 cascade is left on, protecting the oocyte and subsequent embryo from apoptosis.

Metaphase II stage oocyte

Numerous reports have been made relating the morphological appearance of the oocyte to treatment outcome. The quantity and expansion of the cumulus cells has been used as an indicator of maturity, but this is inexact and may depend on the type of ovarian stimulation used. It is difficult to assess oocytes in routine IVF as the cumulus cells are left intact. During ICSI procedures, the cumulus cells are stripped off, allowing visualization of the oocyte. Assessment of the oocytes has resulted in a body of literature indicating that parameters such as the presence of vacuoles, cytoplasmic pitting and particles in the perivitelline space are associated with decreased pregnancy outcome.

During maturation of the oocyte, the metaphase spindle is formed, meiosis proceeds, and the polar body is extruded. Formation of the first polar body results from a highly asynchronous cell division at the end of the first meiotic division. The meiotic spindle forms near the centre of the oocyte and then tracks along its long axis to the oocyte periphery. This process is controlled by c-*mos* (cellular viral mos oncogene) and MAPK pathways (Choi *et al*., 1996; Verlhac *et al*., 2000). The tracking of the spindle to the periphery ensures that very little cytoplasm is lost when the first reduction division occurs, since the first polar body plays no further role in development (Araki *et al*., 1996). After extrusion, the polar body—which is complete by about 40 h after hCG—undergoes timed, programmed disintegration, which is again controlled by c-mos and MAPK (Choi *et al*., 1996; Verlhac *et al*., 2000). This is a natural phenomenon, and in the human oocyte is complete by approximately 20 h after extrusion (Ortiz *et al*., 1983). This could be a strategy to prevent sperm binding and entry to the polar body, as the chromosome complement in the polar body is normal. In fact, the first polar body can be successfully used in nuclear transfer experiments to form viable offspring (Wakayama and Yanagimach, 1998). Any oocytes presenting with very large first polar bodies are most likely abnormal, at the level of c-*mos* and MAPK expression or function. As this also directly relates to the spindle, where sister chromatid exchange occurs and is the most likely point of non-disjunction or aneuploidy formation, abnormal polar body size and appearance can indicate oocyte quality.

A further selection criterion that can be applied to oocytes, when the polar body can be visualized, is the state of polar body disintegration. As it is initially intact and begins to disintegrate with time, a fragmenting polar body at the time of ICSI (40–42 h post hCG) could indicate an early or abnormal extrusion of the polar body or a disruption of the c-mos-MAPK pathways, either of which could indicate aneuploidy or subsequent abnormal development.

Few reports have been made which link first polar body abnormalities (primarily the appearance of fragmentation or polar body disintegration) with lowered fertilization and reduced day 3 and/or day 5 development and implantation (Ebner *et al*., 1999; 2000). Thus, the state or morphology of the first polar body can provide a good insight to the molecular events that have occurred in its formation and which are very tightly linked with spindle formation and the most likely point of aneuploidy formation. This is a simple, non-invasive screening technique which is easily applied in ICSI cases but not in IVF. Further oocyte screening tools are needed for IVF oocytes.

A new technique available to screen metaphase II oocytes is visualization of the metaphase spindle. This can be accomplished non-invasively through a form of differential polarized light microscopy (Oldenbourg *et al*., 1998; Katoh *et al*., 1999). In light microscopy, all the light generally passes through an object and is gathered by the condenser such that an image is seen. Light passing through an object travels at different speeds depending on the path it takes, or on how much it is scattered. Light passing down an ordered structure will travel at a very different speed to that passing through a disorganized or chaotic structure. The difference in light speeds passing through an object produces birefringence, which is quantified using a polarizing microscope, to produce an image of the ordered structure (Oldenbourg *et al*., 1998; Katoh *et al*., 1999). Polarizing light microscopy makes use of the ordered form of the spindle. As it is merely light microscopy but with the light passing through the embryo being gathered differently, it is no more invasive than routine microscopy.

Spindles are formed from microtubules, which arrange in an array with an attachment site at each end (bipolar). Chromosomes attach to this array, through kinetechores, to short microtubules in the equatorial region of the long array. This attachment and alignment is controlled by checkpoints, which essentially prevent incomplete or incorrect events (such as faulty chromosome attachments) occurring in the cell (Nasmyth, 1996; Gorbsky, 1997). If there is damage or incorrect alignment or assembly of the spindle, then checkpoints will prevent further progression of meiosis until the correct spindle architecture is obtained (Steuerwald *et al*., 2001). Disruptions to the checkpoint mechanisms could allow meiosis to proceed with incorrect or incomplete spindle assembly. This ordered array of the spindle (and potential disruptions to it) can be detected using polarized microscopy. Non-bipolar or abnormal spindles can be distinguished from normal bipolar ones. If the spindle array is completely disorganized or disrupted, then the birefringent image will not be seen.

When polarizing light microscopy is applied clinically, oocytes without a birefringent spindle have lowered fertilization and slower rates of development *in vitro* (Wang *et al*., 2001a; b). It has also been shown that oocytes without birefringent spindles survive freeze–thawing less well than

those with spindles, and that much of the damage that occurs in oocytes during freezing occurs at the spindle level. However, oocytes without birefringent spindles can progress, even to the blastocyst stage (Wang *et al*., 2001a), but will have little to no developmental capacity. Thus, observation of the spindle structure by these non-invasive techniques could provide an early selection point against embryos with little developmental capability, even if they form morphologically normal blastocysts.

Fertilized oocytes, pronuclear stage

A mature oocyte has an intact polar body and is arrested at metaphase II of the second meiotic division. As the spermatozoon enters, the oocyte is released from the meiotic block, the second polar body is extruded (again in a highly asynchronous division to limit the amount of cytoplasm lost), the female pronucleus begins to form, and the sperm head decondenses, forming the male pronucleus. During its formation, there is differential condensation of chromatin in the two pronuclei (Van Blerkom *et al*., 1995b). In the female pronucleus, the chromatin decondenses on the side facing into the centre of the oocyte. In the male pronucleus it decondenses on the outside where the sperm tail was originally located, facing away from the centre. As the aster grows from the male centromere to capture and pull the female and male pronuclei together (Schatten, 1994), the male pronucleus rotates onto the female one, in order to align the chromatin onto the spindle, which will form between the two pronuclei (Van Blerkom *et al*., 1995b). This will ensure that all chromosomes are on the spindle for the subsequent mitotic division.

At the formation of the first polar body, the spindle is directly underneath. The spindle then moves away, around the periphery of the oocyte, in a time-dependent manner (Sousa and Tesarik, 1994; Silva *et al*., 1999; Hardarson *et al*., 2000). This is most likely due to cytoplasmic rotation (Payne *et al*., 1997). The point at which the second polar body is extruded indicates the position of the metaphase spindle at the time of fertilization. If this is highly displaced from the first polar body, it could indicate that the oocyte was aged at the time of fertilization or that there has been a disruption of the normal tracking of the spindle, all resulting in poor developmental outcome (Garello *et al*., 1999). Alternatively, it could indicate that the first polar body has become detached and is advanced in its disintegration, which again is a predictor of poor developmental outcome.

Once the nuclei have formed, the oocyte can be scored for a number of characteristics, all of which have been correlated with developmental potential and which have their basis in biological events. At 16–18 h after insemination/ICSI (about 56–60 h after hCG), the oocyte should have two centrally located pronuclei of approximately the same size. Any deviations will result in abnormal or arrested development (Scott, 2002a). Oocytes presenting with pronuclei that are not centrally located or together, probably have disruptions in the cytoskeleton (Sathanathan *et al*., 1988; Schatten, 1994), and those with very disparate size pronuclei are predominantly aneuploid (Munné and Cohen, 1998) with little developmental potential (Sadowy *et al*., 1998; Tesarik and Greco, 1999; Scott *et al*., 2000; Scott, 2003a).

In developing oocytes in antral follicles there is active RNA synthesis (Motlik *et al*., 1984a; b). With the onset of maturation, RNA synthesis ceases (Crozet *et al*., 1986). RNA synthesis occurs in the nucleoli, the sites of ribosomal gene transcription. Nucleoli are present in all mitotically active cells and number between two and seven in human cell nuclei. Nucleoli develop at sites on the DNA known as the 'nucleolar organizing regions' (NOR), and locate at the points where the ribosomal genes are located (this DNA is referred to as rDNA). Each nucleolus has three functional parts: a dense fibrillar component (DFC) which is required for transcription of the DNA; the fibrillar component (FC) which acts as the structural centre for transcription and storage of inactive transcription factors which initiate DNA transcription but which are not necessary for transcription; and the granular component (GC) which is a group of pre-ribosomes (Goessens, 1984; Schwarzacher and Mosgoeller, 2000). During mitotic cell cycles, the nucleoli fuse as the cell cycle proceeds and the chromatin condenses (Goessens, 1984). The nucleoli are the sites of protein synthesis and also of certain mitogenic and growth regulatory proteins, which are involved in cell-cycle control (Pedersen, 1998). There is always synchrony between the numbers and forms or sizes of the nucleoli in the two daughter cells, and any deviation from this is an indication of abnormality, usually in chromosome function (Goessens, 1984). Cervical, and other forms of cancerous cells, present mitotic daughter cells with unequal numbers of nucleoli. Another form of nucleoli abnormality is that seen with cell aging. As cells age and functions break down, the nucleoli can be seen to fragment (Guarente, 1997).

At the time of ovulation (mature metaphase II oocyte), all RNA synthesis has stopped and the nucleoli disaggregate into their component parts. Dense ovoid structures can be seen in pronuclei of fertilized pronuclear oocytes. These are a part of the nucleoli, the FC region, and are referred to as the nucleolar precursor bodies (NPB) (Tesarik and Kopecny, 1989; 1990). After fertilization, and with the onset of the mitotic divisions in embryos, the nucleoli begin to reform (Laurincik *et al*., 2000) and become fully functional as the embryo switches to complete reliance on the embryonic genome (Goessens, 1984; Flechon and Kopecny, 1998; Hyttel *et al*., 2000).

The distribution patterns and morphology (size) of the NPB in pronuclei can provide a good indication of the events of fertilization, the completion of meiosis and the cell cycle leading to the first mitotic division, the normality of the chromatin complement in the two nuclei, and the formation with chromosome attachment of the mitotic spindle. Pronuclear oocytes that present with NPB of the same number and with between two and seven in each nucleus, that have the same size and distribution pattern, at 16–18 h after insemination, have nuclei that are on the same cell cycle and are considered normal. If these NPB are also aligned into the furrow between the nuclei, they are considered to be

progressing at the appropriate time, with the chromosomes correctly positioned on the mitotic spindle ready for the first cleavage division. Oocytes that present with any other pattern are delayed or abnormal.

Those oocytes in which the chromatin and therefore the NPB, are on opposite sides of the furrow, have not had rotation of the male pronucleus onto the female pronucleus. This will present problems, as the centromere—which needs both to divide and be in the furrow to enable correct division and separation of the genetic material (Schatten, 1994)—cannot occur or will be very delayed. Oocytes that present with NPB patterns in which there is complete inequality in numbers and in distribution patterns between the nuclei most likely have asynchronous nuclei, checkpoints that have broken down, or are delayed in progression or show abnormal chromatin (aneuploidy?). This is reminiscent of the situation in most cancer cells. Oocytes that present with highly fragmented NPB may be showing signs of ageing and could be destined for early arrest.

Scoring the patterns and numbers of NPB has been used as a selection criterion in a number of instances (Scott and Smith, 1998; Tesarik and Greco, 1999; Scott *et al*., 2000). These systems have shown strong correlations with development potential (Scott *et al*., 2000; Balaban *et al*., 2001; Scott, 2003a) and implantation rates (Scott and Smith, 1998; Tesarik and Greco, 1999; Ludwig *et al*., 2000a; b; Scott *et al*., 2000; Tesarik *et al*., 2000; 2002; Wittemer *et al*., 2000; Balaban *et al*., 2001; Scott, 2002). In one report, it was shown that the use of embryos for transfer, resulting exclusively from pronuclear oocytes that had non-aligned and/or unequal numbers of NPB or very fragmented NPB, resulted in no live offspring (Scott, 2002). Further, in a selected group of patients, there was a strong correlation between poor NPB patterns and increased rates of aneuploidy (Kahraman *et al*., 2002). Considering the biology behind the patterns of the NPB, this is a very rapid, simple, early, non-invasive selection technique that enables the laboratory to separate oocytes with potential from those that clearly have a fundamental defect at a molecular and/or cellular level.

A further indicator of normal development at the pronuclear oocyte stage, that can be assessed non-invasively, is the appearance of a clearing or 'halo' on the periphery of the oocyte (Payne *et al*., 1997; Scott and Smith, 1998). The clearing is most likely due to the movements that occur in cells as they progress through the phases of development. It is a normal phenomenon in lower order animal oocytes and embryos (Seydoux and Fire, 1994; Kloc and Etkin, 1995) and has been documented by time-lapse video photography in human oocytes (Payne *et al*., 1997; Hardarson *et al*., 2002). In lower-order animals, streaming of the cytoplasm is involved in axis establishment and the movement of certain organelles. In some laboratory animals, the clearing has been associated with the redistribution of mitochondria during meiosis and mitosis (Muggleton-Harris and Brown, 1988; Barnett *et al*., 1996). This is also seen in human pronuclear oocytes, and has been associated with increased developmental (Scott, 2003a) and implantation potential (Payne *et al*., 1997; Scott and Smith, 1998). It is probably due to the fact that living cells are not static, that redistribution of organelles occurs in human oocytes just as it does in oocytes of other species, and this redistribution is an indication that the cell/oocyte is functioning normally. The lack of halo has been associated with slowed development and low blastocyst formation (Scott, 2003a). Again, screening for the halo is both rapid and non-invasive and provides a good indication of the health of the oocyte.

Completion of fertilization: the first mitotic cleavage

After alignment of the pronuclei, the nuclear membranes break down and the spindle rotates such that it is at right-angles to the axis defined by the second polar body. The position of the second polar body defines the cleavage plane, or the polar axis, of the mouse embryo (Gardner, 1996; 1997; Piotrowska and Zernicka-Goetz, 2001), though this has not yet been described in the human. Rotation onto the axis is most likely to occur after the nuclear membranes have broken down and cannot be seen. The result of the first mitotic cleavage, the completion of fertilization, is a 2-cell embryo in which the cells are not exactly the same size and are elliptical. The elliptical shape means they cannot rotate freely in the zona. Deviations from this pattern indicate abnormalities in the embryo.

When the final steps of oocyte maturation are triggered by the LH surge (hCG injection) a clock is set in the oocyte, which will mark the time since this event all through the development of the preimplantation embryo (Johnson and Day, 2000). Any delay in a developmental event will result in the embryo being out of synchrony with the clock. This fact can be taken into account in the timing of the first cleavage division. Early completion of the final events of fertilization—alignment of the pronuclei, alignment of the chromosomes on the metaphase spindle, and finally the first mitotic division—can be scored as early entry into the first mitotic division. The recognition of early nuclear membrane breakdown (Scott and Smith, 1998) and entry into the first mitotic division by 22–24 h after insemination (Edwards *et al*., 1980; Shoukir *et al*., 1997; Bos-Mikich *et al*., 2001; Lundin *et al*., 2001; Petersen *et al*., 2001; Sakkas *et al*., 2001) has been correlated with increased implantation rates. Checking embryos on day 1, at 22–24 h after insemination, is both simple and non-invasive, and may help to select embryos in which the time clock is advanced or at least not delayed.

Day 2 scoring: multinucleation

At 60–64 h post hCG (40–42 h post insemination), the embryo should be at the 2- to 4-cell stage. When there are so few cells, each cell and its nucleus can easily be visualized. Each cell should only have a single nucleus; multinucleation indicates a breakdown of one or more cellular events. One explanation is that it is not multinucleation but rather nuclei fragmentation, resulting from a total disintegration of the mitotic spindle. It could also be the result of an abnormal mitotic event involving the spindle, such as duplication of the microtubule organizing centre, a known feature of cells placed in culture (Sorimachi *et al*., 1998). Multinucleation has been correlated with decreased in-vitro development (Levy

et al., 1998; Hardarson *et al*., 2001), decreased or no implantation (Pickering *et al*., 1995; Gerris *et al*., 1999; Van Royen *et al*., 1999; 2001; Hardarson *et al*., 2001), and increased rates of aneuploidy (Kligman *et al*., 1996; Staessen and Van Steirteghem, 1998) However, if only one cell is multinucleate, normal development can result (Balakier and Cadesky, 1997). Coupled with day-3 morphology, multinucleation screening on day 2 has enabled the use of single embryo transfers without any decrease in pregnancy rates compared to the use of more than one embryo and a single selection criteria (Gerris *et al*., 1999; 2002; Van Royen *et al*., 2001). Multinucleation is often accompanied by embryo fragmentation and may be the causative event in this phenotype or be associated with apoptosis as the embryo attempts to rid itself of the abnormal cells. This is a simple non-invasive screen that can be used very early in culture to select against embryos that have no potential.

Cleavage-stage embryos

The second cleavage division occurs at right-angles to the first, with one blastomere dividing ahead of the other. In the mouse, the first of the two cells cleaves in a meridional manner and the second in an equatorial form, the result being a tetrahedron of the cells (Gardner, 2002). In the rabbit, both of the cells divide in a meridional manner, but the second cell to cleave rotates through 90° before cleaving, thus forming the tetrahedron shape (Gulyas, 1975). In human embryos, there is no evidence yet as to the cleavage planes and the fates of the cells. It is known that each cell in the 4-cell stage mouse embryo is not identical (Gardner, 2002), due to the polarized distribution of organelles in the oocyte (Antczak and Van Blerkom, 1997) and thus the 2-cell embryo. With the type of cleavage in the second mitotic division, these differences are more profound (Gardner, 2002). For instance, the second polar body—which remains intact and attached to embryo through to the blastocyst stage—is only attached to one cell. The progeny of this cell with the polar body still attached, ends up on the outside of the blastocyst at the junction between the inner cell mass (ICM) and the trophectoderm, and defines the embryonic axis (Gardner, 1997). In human embryos it has been shown that the blastomeres in the 4-cell embryo differ one from another, and that only one has the ability to form cells that can produce hCG at the blastocyst stage (Hansis, 2002). Thus, both the tetrahedron shape of the 4-cell embryo and the need for all the cells to be intact is a necessary aspect of normal development and subsequent blastocyst implantation. Screening for abnormal 4-cell embryos—those without the correct orientation—is simple and may eliminate a group of embryos that will have no potential in later development.

In normal human embryo development there is a degree of cleavage asynchrony, resulting in 3-, 5- and 7-cell embryos. This is a normal phenomenon and allows the correct spatial arrangement of the cells in the embryo as it divides (Roux *et al*., 1995). Thus, at any one point not all the cells will be the same size. However, in an embryo there should only ever be two sizes of blastomere, and between the 2- and 8-cell stages there can only be three sizes. Deviations from this will mean that the embryo is fragmenting or that one blastomere has arrested.

On day 3, embryos can be scored for morphological features and cell number. There have been many different scoring systems for cleaving embryos, all of which show some correlation with implantation (Steer *et al*., 1992; Tan *et al*., 1992; Dawson *et al*., 1995; Rijnders and Jansen, 1998; Van Royen *et al*., 1999; 2001). The rate of progression—that is, how many cells there are in the embryo—also has an impact on the implantation rate (Racowsky *et al*., 2000; Shapiro *et al*., 2000). When day 3 scoring is combined with early scoring in a combined system, implantation rates are even higher (Gerris *et al*., 1999; 2002; Scott *et al*., 2000; Van Royen *et al*., 2001).

The degree of fragmentation can also be used as means of embryo selection at the cleaving stages. Fragmentation has many phenotypes and should not be confused with the normal 'blebbing' that occurs as the cells divide, as these are transient and are reabsorbed (Antczak and Van Blerkom, 1999; Hardarson *et al*., 2002). Fragmentation could have many causes (Van Blerkom *et al*., 2001). In some instances it may be due to poor culture conditions, which induce multinucleation and cell death (Sorimachi *et al*., 1998) or apoptosis (Alikani *et al*., 2000). If fragmentation is due to apoptosis then it is a secondary event, the primary one being some other breakdown in cell structure or functioning resulting in apoptosis, such as multinucleation (Ochi, 2000), karyokinesis without cytokinesis (Pickering *et al*., 1995) or a chromosomal abnormality (Pellestor *et al*., 1994; Alikani *et al*., 2000). As discussed above, poor follicular development will lead to poor-quality oocytes resulting in embryos that will display anomalies, such as reduced ATP content (Van Blerkom *et al*. 1995a). If the embryo does keep developing, these fragments will be excluded from the embryo as it compacts and forms a blastocyst (Scott, 2002), and they play no further role in development. Whatever the cause, true fragmentation has a negative impact on development. If it is widespread, involving too many cells, it will prevent normal development. It is easily seen and scored as an additional selection criterion on day 3.

Compaction and blastocyst formation

At the 8- to 16-cell stage, the embryo will begin to compact and form tight junctions, and differentiation begins with the formation of two cell lines, the inside and outside. If compaction starts too early, it can lead to the formation of a trophoblastic vesicle, where all the cells are allocated to the outside, leaving none for the ICM (Johnson and Ziomek, 1981). As compaction progresses, refractile bodies in the cells begin to fuse in defined areas to form the blastocoele. The first fluid accumulates between the cells that define the inside and outside of the compacted morula (Calcaro and Brown, 1969). As the embryo grows, the blastocoele expands in volume and becomes one large central cavity. If the vesicles form too early, there is the appearance of a paving-stone formation, which has a negative impact on blastocyst formation and implantation.

When the blastocoele forms, the outside cells are totally committed to the outside and the ICM to the inside. The early blastocyst grows with cell proliferation but the late, expanded one does not (Gardner, 2000). In the early blastocyst there

should be a band of evenly sized and distributed cells on the periphery of the embryo and a distinct area of dense cells, the ICM. As the blastocyst expands, trophectoderm cells transform into giant cells, which presents as the elongation of the cells on the periphery of the blastocyst, especially in the mural region (away from the ICM). The trophectoderm cells in the region of the ICM (polar trophectoderm) remain mitotically active, with the cells migrating from this region to populate the mural region as the embryo expands (Gardner and Nichols, 1991). As the cells migrate to the mural end they are attached to the ICM through finger-like projections, which withdraw as they reach the junction between the polar and mural trophectoderm (Flemming *et al*., 1984; Gardner, 2000) and which break down as the cells move to a more mural position.

There are a number of different scoring systems for blastocysts, all of which take into account the features mentioned above, and show correlations with implantation (Dokras *et al*., 1993; Balaban *et al*., 2000; Gardner *et al*., 2000). As the blastocyst is very dynamic and grows rapidly, it needs to be scored sequentially. At approximately 154 h after hCG or 112–114 h after insemination, the blastocyst should have a defined blastocoele, a distinct ICM protruding into the cavity, and a ring of evenly spaced and sized trophectoderm cells. If the blastocysts are very expanded by this stage, they have lowered viability (Racowsky *et al*., 2000). Any blastocysts that have finger-like projections across the blastocoele and which do not break down with time should be avoided.

Metabolic markers as a selection criteria

Many elegant studies have been carried out on the metabolic requirements of cleavage-stage embryos *in vitro* (Leese, 1989; 1990; 1991; 1995; Lawitts and Biggers, 1993; Leese *et al*., 1993; Gardner *et al*., 1996; 1998b). The nutrient and amino acid needs and profiles of all stages of development from the 1-cell stage through to blastocyst have been described, allowing the development of media systems specifically tailored to the embryos needs. Understanding these metabolic parameters has also led to the concept of using metabolism as a specific marker of embryo health (Leese, 1987a; b; Leese *et al*., 1993). It has been established that, in the first phases of development, oocytes require and actively use pyruvate for maturation (Downs *et al*., 2002). Oocytes that are actively maturing and completing the transition from metaphase I to metaphase II transition use more pyruvate than immature or non-transitional oocytes. When the energy substrate dynamics were compared with meiotic status of the oocytes *in vitro*, pyruvate consumption was associated with the maturation process.

In the early stages of development the embryo uses pyruvate exclusively, switching to glucose as development progresses (Hardy *et al*., 1989; Gott *et al*., 1990). As the embryo begins to use glucose, it produces lactate. The use of pyruvate and glucose and the appearance of lactate have been linked with embryo health, and the suggestion has been made that embryos which metabolically are very active and have a high glycolytic activity (Gardner *et al*., 2001) are those with little potential (Leese, 2002). These metabolic assays are performed using microflurometric analysis, but this requires specialized equipment that is not normally found in a clinical IVF laboratory.

Another approach that is being developed with the increasing use of extended culture and blastocyst transfer is that of amino acid turnover (Houghton *et al*., 2002). This has been shown to be related to an embryo's ability to develop to a morphologically normal-appearing blastocyst. Only certain amino acids were shown consistently to predict blastocyst formation, with leucine being unique in that it was the only amino acid that was depleted for all blastocysts. Alanine was consistently produced by all blastocysts, appearing in increasing amounts during development. These assays are performed using high-performance liquid chromatography, a technique not readily available to most clinical IVF laboratories.

To use non-invasive metabolic markers or the turnover of amino acids for embryo selection requires that the tests be simple, readily available to clinical IVF laboratories, rapid, accurate and sensitive enough that they can be performed on single embryos. Since embryos change in their metabolic, anabolic and catabolic functions as they divide and differentiate, these tests necessarily need to be developmental stage-specific. Furthermore, the data need to be validated in a clinical setting where the relationship between measured parameter and outcome can be documented. With the developing area of proteomics and gene chip (micro-array) technology, these techniques may be realized in the near future and brought into the clinical setting. As they will be measuring the health and differentiation of the embryo—and also, potentially, gene function—they will be powerful, although at the present time they remain in the research setting and not available to the clinic.

Conclusions

Oocyte maturation, fertilization and embryo development are on a continuum, with the embryo changing morphology in a dynamic way at every stage. Selection criteria must take this, as well as the biology of the embryo, into account. The timing of development, from the time of hCG, must be known so that key developmental points can be scored within the correct window. No single point will provide a definitive picture of the embryo, and for successful selection of a viable embryo multiple parameters and time points should be used. The early events in oocyte and embryo development are probably the key to determining the later morphology and implantation potential, and more weight should be given to them. If the blood flow to each follicle is scored, followed by polar body morphology, NPB status and multinucleation at the 2- to 4-cell stages, a very good prediction of the embryo's ability to develop into a good grade 8-cell embryo and blastocyst may be achieved. Embryos at the 8-cell or blastocyst stage that had a poor prognosis at the earlier stages are most likely to be those in a cohort of similar grade embryos that fail to implant. In situations where there are strict laws regarding embryo use and disposal, more use of the early parameters may help in embryo selection. Where selection can occur at later stages, coupling of the early criteria and development at and beyond gene activation will build a better profile of the embryo.

Within the laboratory and ART practice there are many non-invasive, easy scoring points that can help in the selection of a viable embryo that will result in a healthy singleton offspring. Most require time and patience, with embryos being cultured in individual drops, but if the result is the ability to carry out single embryo transfer, then the extra time and effort is worthwhile.

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