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ORIGINAL ARTICLE *Embryology*

Developmental kinetics of cleavage stage mouse embryos are related to their subsequent carbohydrate and amino acid utilization at the blastocyst stage

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STUDY QUESTION: What is the relationship between cleavage stage embryo kinetics, blastocyst metabolism and subsequent embryo viability?

SUMMARY ANSWER: Embryos cleaving faster at the first cleavage division resulted in blastocysts with a larger inner cell mass (ICM), higher glucose consumption, lower glycolytic rate, higher aspartate uptake, lower global amino acid turnover and higher percentage of developing fetuses on E13.5 when compared with blastocysts that developed from slower cleaving embryos.

WHAT IS KNOWN ALREADY: Previous research has shown that morphokinetics, blastocyst carbohydrate metabolism and cleavage stage amino acid metabolism of the preimplantation embryo can be used independently as markers of its developmental competence and subsequent viability.

STUDY DESIGN, SIZE, DURATION: Morphokinetics of *in vitro* fertilized mouse zygotes were observed using a time-lapse imaging system and they were identified as 'fast' or 'slow' cleaving embryos. Spent culture media from resultant blastocysts were analysed for carbohydrate and amino acid utilization. Blastocysts either had their ICM and trophectoderm (TE) cell number determined, were cultured further in an outgrowth assay or transferred to a recipient female to assess implantation and fetal development.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Morphokinetics of *in vitro* fertilized C57BL/6xCBA (F1) zygotes individually cultured in 2 μl drops of G1/G2 media with HSA under Ovoil in 5% O_2, 6% CO_2 and 89% N_2 were analysed using a time-lapse incubator. At 72 h post-insemination, blastocysts were separated into quartiles derived from timing of the first cleavage division. Blastocysts were cultured for a further 24 h and spent media samples, including controls containing no embryos, were frozen and subsequently analysed for amino acid utilization using liquid chromatography-mass spectrometry. These blastocysts were then analysed over a further 1.5 h period for carbohydrate utilization and subsequently stained to determine ICM and TE cells. To analyse implantation potential, fetal quality and viability, additional 'fast' and 'slow' blastocysts were cultured further in an outgrowth model or transferred to recipient females.

MAIN RESULTS AND THE ROLE OF CHANCE: Embryos cleaving faster at the time of first cleavage (first quartile, designated 'fast') were on average 2.5 h ahead of slower embryos (fourth quartile, designated 'slow', 15.1 ± 0.1 versus 17.6 ± 0.1 h, $P < 0.001$). On Day 5 of culture, blastocysts developed from 'fast' embryos had a larger ICM number (17.4 ± 2.1 versus 7.4 ± 2.0, $P < 0.01$), a higher glucose consumption (21.2 ± 1.2 versus 14.3 ± 1.0 pmol/embryo/h, $P < 0.001$) and a lower glycolytic rate (expressed as the percentage of glucose converted to lactate) (49.6 ± 2.8 versus 59.7 ± 2.8%, $P < 0.05$) compared with 'slow' embryos. Further non-invasive metabolomic analysis revealed that 'fast' blastocysts consumed more aspartate (2.2 ± 0.1 versus 1.8 ± 0.1 pmol/embryo/h, $P < 0.05$) and produced little or no glutamate compared with 'slow' blastocysts (0.02 ± 0.07 consumed versus 0.32 ± 0.11 pmol/embryo/h produced, $P < 0.05$). Transfer of 'fast' blastocysts to pseudo-pregnant recipients resulted in higher fetal survival post-implantation compared with 'slow' blastocysts (69.6 versus 40.4%, $P < 0.01$).

LIMITATIONS, REASONS FOR CAUTION: The timing of the first cleavage division was used to classify blastocysts as 'fast' or 'slow' embryos; however, a combination of several developmental kinetic markers (cleavage time of 3- to 8-cell, duration between cleavage division times) may be used to more accurately determine an embryo as 'fast' or 'slow'. Only the fastest and slowest quartiles (those embryos with the fastest and slowest first cleavage division) were analysed in this study.

WIDER IMPLICATIONS OF THE FINDINGS: These findings show that kinetically different embryos develop into blastocysts with different metabolic profiles and viability. Work is now being undertaken to determine if using these viability markers in combination will increase embryo selection efficacy and further improve implantation and pregnancy rates.

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Key words: time-lapse / metabolism / selection / viability

Introduction

Although there have been several improvements in human IVF over the past three decades, live births from assisted reproductive technology (ART) remain low, on average ~30% per transfer (Ferraretti *et al.*, 2013; Sunderam *et al.*, 2013). Therefore, in order to attain acceptable pregnancy rates, it remains a common practice in many countries to transfer more than one embryo. For example, in 2010, in the USA, an average of two embryos were transferred per cycle amongst all age groups of patients, resulting in an incidence of multiple pregnancies of 46.4% (Sunderam *et al.*, 2013), the majority of these pregnancies being twins with fewer high-order multiple gestations. Complications associated with multiple gestations are thoroughly documented and include obstetric, perinatal and neonatal risks (Adashi *et al.*, 2003; Templeton, 2004). Consequently, transfer of a single embryo with the highest likelihood of establishing a pregnancy is the goal of human IVF. Although single-embryo transfer has been established in several countries, there remains a need to quantify embryo viability prior to transfer. Historically, selection of embryos for transfer has been based on morphological criteria such as degree of fragmentation and symmetry of blastomeres, and grading systems have been successfully applied to all stages of human embryo development (Scott and Smith, 1998; Van Royen *et al.*, 1999; Gardner *et al.*, 2000). Although relationships between embryo grade and outcome have been established, visual scoring remains subjective. Furthermore, morphology as a selection marker fails to convey information about physiology, which could reflect implantation potential and ongoing viability of the embryo. Hence, there has been considerable focus on identifying quantifiable biomarkers of developmental and implantation competence to improve embryo selection. Several areas have been considered, including preimplantation genetic screening, proteomics, metabolomics and the analysis of key metabolic pathways (Katz-Jaffe *et al.*, 2006; Mastenbroek *et al.*, 2007; Nagy *et al.*, 2009; Seli *et al.*, 2010; Gardner *et al.*, 2011; Tejera *et al.*, 2012).

During development, the preimplantation embryo utilizes both carbohydrates and amino acids. Time-dependent consumption of nutrients and production of metabolites has been shown to be an important physiological parameter related to embryo viability in the human and the mouse. Pyruvate is the preferred substrate for metabolism in the early stages of development (1- to 8-cell stage) with a switch to a more glucose-based physiology post-compaction (Leese and Barton, 1984; Gott *et al.*, 1990). Of significance, glucose consumption (Gardner and Leese, 1987) and lactate production, expressed as a ratio as an estimate of glycolytic rate (i.e. metabolic fate) have been related to embryo viability, with viable mouse blastocysts displaying a higher glucose uptake and a lower glycolytic rate, similar to the metabolic profile of *in vivo* blastocysts (Lane and Gardner, 1996). This has been confirmed in humans, where glucose consumption is positively correlated to embryo viability on days 4 and 5 of development, as embryos that give rise to a successful pregnancy following single blastocyst transfer consume significantly more glucose than those that fail to establish a pregnancy (Gardner *et al.*, 2011).

In addition to carbohydrate metabolism, the turnover of amino acids has been related to embryo viability, and Houghton *et al.* (2002) observed that human embryos that developed to the blastocyst stage have a lower amino acid turnover at the cleavage stage compared with arrested embryos. Brison *et al.* (2004) further revealed that the turnover of aspartate, glycine and leucine in cleavage stage human embryos was significantly correlated with higher viability. Analysis of amino acids would seem prudent, given that they play several functions in regulating embryo development, serving as osmolytes (Lawitts and Biggers, 1992; Dawson and Baltz, 1997), intracellular pH regulators (Edwards *et al.*, 1998), signalling during blastocyst implantation (Martin *et al.*, 2003) and as antioxidants and chelators (Lindenbaum, 1973; Liu and Foote, 1995). Furthermore, aspartate, which is the amino acid most highly consumed by the mouse blastocyst (Lamb and Leese, 1994; Wale and Gardner, 2012), has also been implicated in the regulation of carbohydrate metabolism, as it appears to be the rate-limiting factor in the malate-aspartate shuttle (MAS) (Lane and Gardner, 2005). Consequently, relationships between carbohydrate and amino acid utilization need to be characterized.

Thirty years ago, it was recognized that earlier cleavage was a strong predictor of embryo quality as transfer of early cleaving embryos resulted in higher implantation and pregnancy rates (Edwards *et al.*, 1984), an observation subsequently repeated several times (Shoukir *et al.*, 1997; Lundin *et al.*, 2001; Van Montfoort *et al.*, 2004). However, until recently, observation of embryo development was only performed at specific time points and involved frequent opening of the incubator, resulting in fluctuations in temperature and the gaseous environment of the culture system. Changes to levels of carbon dioxide directly affect pH, which can negatively impact on blastocyst development and cell number (Gardner and Lane, 1996; Zhang *et al.*, 2010). The recent integration of a high-frequency imaging system within an incubator has facilitated maintenance of a stable culture environment while monitoring embryo development, prompting renewed interest in quantifying developmental kinetics of embryos as a biomarker of viability (Arav *et al.*, 2008; Pribenszky *et al.*, 2010; Wale and Gardner, 2010; Meseguer *et al.*, 2011). Time-lapse imaging has revealed that embryo score can change within a short period of time due to the dynamic nature of embryo development (Montag *et al.*, 2011). Studies using such technologies have shown that cleavage division timings of mouse embryos are tightly regulated (Arav *et al.*, 2008) and an earlier first and second cleavage division is correlated to blastocyst development (Pribenszky *et al.*, 2010). In humans,

developmental events such as time of division to 2-cell, time of division to 5-cell and the interval between second and third cleavage event are associated with higher viability (Wong *et al.*, 2010; Meseguer *et al.*, 2011). It has been suggested that there is an 'optimal' range of time for developmental events as abnormally short cell cycles (e.g. cleavage of human embryos from 2- to 3-cells in <5 h) is consistent with significantly lower implantation rates (Meseguer *et al.*, 2011; Rubio *et al.*, 2012). Furthermore, Wong *et al.* (2010) have shown that embryos which take a significantly longer time to complete cytokinesis are more likely to arrest (Wong *et al.*, 2010).

To date, metabolism and morphokinetics as viability markers have been used independently as predictive markers of embryo viability. However, the correlation between them has not been investigated. In this study, we aimed to investigate the relationship between cleavage stage embryo morphokinetics, blastocyst carbohydrate and amino acid metabolism and their correlation to embryo quality and subsequent viability post-transfer.

Materials and Methods

In vitro fertilization

Oocytes were collected from 3- to 4-week old F1 hybrid female mice (C57BL/6 × CBA) stimulated with an intraperitoneal injection of 5 IU pregnant mare's serum gonadotrophin (Folligon, Intervet, UK) followed 48 h later with 5 IU hCG (Chorulon, Intervet). Oocytes were collected 15 h post-hCG in handling medium supplemented with 5 mg/ml human serum albumin (HSA) (G-MOPS PLUS, Vitrolife AB; Gardner and Lane, 2014) and transferred to 45 μl drops of fertilization medium modified without glutathione (Binder *et al.*, 2012) and cultured under paraffin oil (Ovoil, Vitrolife) in 6% CO_2, 5% O_2 and 89% N_2 at 37°C.

Sperm were collected from 8- to 12-week-old F1 hybrid male mice (C57BL/6 × CBA). Cauda epididymides and vas deferens were collected in 500 μl of fertilization medium and punctured with a needle to allow sperm to swim out. Collected medium with sperm was transferred to a 5 ml round bottom tube for swim-up procedure and capacitated for 1 h in fertilization medium. A Makler counting chamber was used to determine sperm concentration, and 1–2 million motile sperm per ml was added to one cumulus oocyte complex and fertilization allowed to occur over a 4 h period to mimic conditions used in human IVF. Previous detailed analysis in the laboratory confirmed that 95% of oocytes fertilize within the first 90 min. Following fertilization, pronucleate oocytes were washed once in both fertilization medium and G1 medium supplemented with 5 mg/ml HSA (G1 with HSA; Gardner and Lane, 2004, 2014) prior to culture.

Embryo culture and determination of morphokinetic events correlated to blastocyst cell number

Embryos were cultured individually in 2 μl drops of G1 with HSA under paraffin oil in 6% CO_2, 5% O_2 and 89% N_2 at 37°C in a humidified multi-gas imaging incubator (Sanyo MCOK-5M[RC], Japan) for 50 h and subsequently transferred to 2 μl drops of G2 medium supplemented with 5 mg/ml HSA (G2 with HSA; Gardner and Lane, 2004, 2014) for a further 48 h to the end of the culture period. Time-lapse images of individual embryos were generated every 15 min throughout culture. The timing of morphokinetic events, including syngamy, cleavage division from 2- to 6-cell stages, cavitation and first hatching were recorded. The interval between morphokinetic events (i.e. duration between syngamy to first cleavage division, duration between first cleavage division and second cleavage division etc.) were determined. Blastocysts at the end of the culture period were individually stained for total cell number to correlate to their respective morphokinetic event timings.

Embryo culture and analysis of morphokinetic relationship with carbohydrate and amino acid metabolism

The timings of the first cleavage division were recorded and embryos sorted into quartiles as previously described by Meseguer *et al.* (2011), with the fastest (1st) quartile designated 'fast' embryos and the slowest (4th) quartile designated 'slow' embryos. To quantify amino acid utilization during a 24 h period between days 4 and 5 of culture, at 72 h post-insemination, blastocysts from the same quartile were cultured in groups of three in 2 μl of G2 with HSA and cultured for a further 24 h, after which 1 μl aliquots of the spent culture media and a blank (control) media were collected and vacuum dried. Following the end of culture, blastocysts were either analysed for carbohydrate metabolism and dual stained for ICM and TE number, or their implantation potential was assessed using an *in vitro* outgrowth model or transferred to a recipient female to assess implantation and fetal development.

Carbohydrate levels were assayed by quantitative ultramicrofluorimetry (Leese and Barton, 1984; Gardner, 2007) using coupled enzyme-based reactions as illustrated below. Following final morphological assessment, individual blastocysts were placed into 95.8 nl drops of MOPS-buffered G2 with HSA (G2-MOPS), modified to contain 0.5 mM glucose as the sole carbohydrate source and incubated at 37°C. Serial 1 nl samples of media were taken every 30 min over a 1.5 h period. Concentrations of glucose and lactate in the same sample were assessed using independent assays. Glucose consumption and lactate production were expressed per embryo. Glycolytic rate was expressed as the percentage of glucose converted to lactate and was calculated on the basis that 2 mol lactate is produced per mol of glucose glycolysed by the embryo (Gardner and Leese, 1990).

For amino acid analysis, vacuum-dried spent media samples, including a control drop without embryos, were resuspended in 10 μl of MilliQ water, after which 70 μl of borate buffer was added to each sample, mixed by vortex and centrifuged for 1 min. To this, 20 μl of derivatization-labelling reagent 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (Aqc) was then added, vortexed immediately and then warmed on a heating block for 10 min at 55°C, with agitation. The final solution was then cooled to room temperature before liquid chromatography-mass spectrometry (LC-MS) analysis using an Agilent 1200 LC-system coupled to an Agilent 6420 ESI-QqQ-MS. LC-QqQ-MS. This enables the concentrations of coeluted fractions of a variety of amines to be resolved and quantified by comparison against a standard calibration curve (Boughton *et al.*, 2011). Final values of amino acid turnover of embryos were evaluated by standardizing results to control (blank) media drop. Stock solutions of 2.5 mM for each individual amino acid were prepared as a standard control, as previously described (Wale and Gardner, 2012).

Assessment of embryo cell numbers

Blastocysts were differentially stained to analyse the number of cells in the inner cell mass (ICM) and trophectoderm (TE) using a modification of a previously described protocol (Handyside and Hunter, 1984). TE nuclei were labelled with propidium iodide after permeabilizing the cell membrane using a complement reaction leaving the ICM nuclei unlabelled. Blastocysts were then treated with bisbenzimide in 10% ethanol to stain all nuclei. Blastocysts were subsequently mounted in glycerol and photographed under a fluorescence microscope and cell numbers counted.

Embryo transfer

Swiss female mice between 8 and 14 weeks of age were mated with vasectomized males to induce pseudo-pregnancy. Mating was confirmed by the presence of a vaginal plug. Embryos were then transferred 96–97 h post-insemination to the recipient female, at Day 3.5 of pseudo-pregnancy. Recipient female mice were anaesthetized with an intraperitoneal injection of ketamine (75 ml/kg Ketalar, Pfizer, Australia) and medetomidate (1 mg/kg Domitor, Pfizer). Ten embryos were transferred into each recipient female through a dorsal incision, with a glass pipette. Groups of five fast or slow blastocysts were transferred to the right or left uterine horn of each recipient, respectively, to avoid preferential implantation bias. Following embryo transfer, the skin wound was sealed with sterile surgical clips and the recipient underwent post-operative recovery with an intraperitoneal injection of antipamezole (1 mg/kg, Antisedan, Pfizer) to reverse the effects of the medetomidate, while the analgesic effects of ketamine remained. Pregnant recipients were killed on E13.5 and the number of fetuses or resorption sites was recorded to determine rates of implantation and fetal development. Fetal and placental weight, sex, crown-rump length, fetal eye and limb development were determined (Lane and Gardner, 1994).

Blastocyst outgrowths

Blastocyst outgrowth experiments were carried out at 96 h post insemination, as previously described (Hannan *et al.*, 2011). Briefly, flat-bottomed 96-well tissue culture dishes were rinsed with sterile PBS and coated with a solution of 10 μg/ml fibronectin (BD Biosciences, USA). Coated wells were rinsed with sterile PBS and incubated for 2 h with a solution of phosphate-buffered saline (PBS) containing 4 mg/ml bovine serum albumin (BSA). Wells were then rinsed with PBS and subsequently filled with 150 μl of G2 with HSA, overlayed with 120 μl of paraffin oil (Ovoil) and the plate equilibrated under 6% CO_2, 5% O_2 and 89% N_2 at 37°C. Expanded and hatching blastocysts were placed into each well and incubated for a further 72 h. Following blastocysts transfer into the plate, blastocyst outgrowths were imaged using an inverted epifluorescence microscope (T*i*-U eclipse, Nikon) at ×10 magnification at time points 48, 66 and 72 h. Images were obtained using NIS Elements BR 3.00, SP7 Laboratory Imagining software (Nikon) and outgrowth area was measured per image using Image J software (Image J).

Statistical analyses

All data were assessed for Gaussian distribution with the Shapiro–Wilk normality test. Data that did not meet the assumption of Gaussian distribution underwent non-parametric analysis using the Mann–Whitney test. Data that were normally distributed were statistically analysed with unpaired Student's *t*-test. Correlation between blastocyst cell number and the timing of the first cleavage division were not normally distributed and were analysed using Spearman's correlation coefficient. Cell number differences in quartiles of morphokinetic data were analysed by analysis of variance and between quartile differences determined using Bonferroni's multiple comparison test. Embryo transfer results were analysed using the χ^2 test.

All analyses were performed using GraphPad Prism version 5.04 for Windows (GraphPad Software).

Ethics statement

All mice were housed in a 12 h light/12 h dark photoperiod with food and water fed *ad libitum*. All mouse experimentation was approved by The University of Melbourne, Animal Ethics Committee (AEC).

Results

Correlation of morphokinetic events to blastocyst total cell number

The timing of morphokinetic events, including syngamy, cell division timings (2 cells, t2; 3 cells, t3; 4 cells, t4; 5 cells, t5; 6 cells, t6), cavitation, hatching, as well as the duration of syngamy to t2 (syn–t2), duration of second cell cycle (cc2 = t3–t2), duration of interval between 3-cell and 4-cell (s2) were calculated, as reported in Meseguer *et al.* (2011) and compared with the resultant blastocyst total cell number (Table I). The range of time over which each embryo reached every developmental

Table I The timing of morphokinetic events and their correlation with blastocyst total cell number.

Developmental event	Cell number	Event timing/ interval duration (hours PI)	Correlation to blastocyst cell number	Event timing standardized to syn–t2	Correlation to blastocyst cell number	Event timing standardized to t2	Correlation to blastocyst cell number
Syngamy	1-cell	14.6 ± 0.1	$P < 0.01$	—	—	—	—
syn–t2		2.2 ± 0.2	$P < 0.01$	—	—	—	—
1st cleavage (t2)	2-cell	16.8 ± 0.2	$P < 0.001$	2.2 ± 0.2	$P < 0.01$	—	—
cc2		24.3 ± 0.3	NS	17.7 ± 0.4	NS	—	—
2nd cleavage (t3)	3-cell	36.6 ± 0.2	$P < 0.001$	22.0 ± 0.1	NS	24.3 ± 0.3	NS
s2		1.5 ± 0.3	NS	—	—	—	—
t4	4-cell	37.9 ± 0.2	$P < 0.001$	23.2 ± 0.2	NS	25.7 ± 0.2	NS
3rd cleavage (t5)	5-cell	47.8 ± 0.2	$P < 0.001$	33.2 ± 0.2	$P < 0.01$	35.9 ± 0.2	$P < 0.01$
t6	6-cell	48.5 ± 0.2	$P < 0.001$	34.0 ± 0.2	$P < 0.05$	36.6 ± 0.2	NS
Cavitation		74.5 ± 0.4	$P < 0.001$	59.9 ± 0.4	$P < 0.01$	62.2 ± 0.4	$P < 0.05$
Hatching		80.5 ± 0.4	NS	64.3 ± 0.9	NS	66.9 ± 0.9	NS

Data are expressed as mean ± SEM. $n = 183$ embryos in total, five biological replicates. syn–t2 represents the duration from syngamy to cleavage to 2-cell, cc2 represents the second cell cycle of the embryo, s2 represents the interval between 3-cell and 4-cell (s2 = t4-t3), as reported in Meseguer *et al.* (2011).
PI, post-insemination; NS, not significant.

Table II Correlation of 2-cell cleavage division time to blastocyst total cell number.

Quartiles	1	2	3	4
Time of 1st cleavage division (hours post-insemination)	15.1 ± 0.08	16.0 ± 0.02	16.4 ± 0.02	17.6 ± 0.10
Total cell number	87.0 ± 4.3[a]	95.0 ± 3.9[b]	84.7 ± 3.6	71.3 ± 3.3[a,b]

Data are expressed as mean ± SEM. $n = 46$ (1st quartile), 43 (2nd quartile), 47 (3rd quartile), 47 (4th quartile), five biological replicates. There was a statistically significant difference between quartiles of total cell number as determined by one-way ANOVA analysis, ($P < 0.001$).
[a,b]Same letters within a row represent significant differences between quartiles.
[a]$P < 0.05$, [b]$P < 0.001$.

event increased as embryo development progressed. The number of cells in the blastocyst was highly correlated to timing of pronuclear syngamy, cleavage divisions t2, t3, t4, t5, t6 and cavitation (Table I), with an earlier timing of each respective event resulting in a greater blastocyst total cell number. The timing of hatching was not correlated to total cell number (Table I). A shorter syn−t2 was correlated to a larger total cell number ($P < 0.01$, Table I), while the time intervals cc2 and s2 were not significant.

To account for time of fertilization, data were standardized to syn−t2. The blastocyst total cell number was correlated to t2, t5, t6 and time of cavitation (Table I). As all other developmental events occurred post syn−t2, data were then further standardized to t2 to account for the possible impact of syn−t2 (Table I). It was found that only t5 and cavitation time remained significantly correlated to blastocyst cell number (Table I).

As t2 had a high correlation to blastocyst cell number (Table I) and is the earlier event compared with t5 and cavitation, it was chosen as the morphokinetic marker to identify 'fast' and 'slow' cleaving embryos. On Day 5 of culture, blastocysts were divided into four groups according to their quartiles derived from t2, as previously performed by Meseguer and colleagues (Meseguer *et al.*, 2011). There was a positive correlation with an early occurrence of t2 resulting in a higher blastocyst total cell number on Day 5 ($P < 0.001$, Table II). Quartiles 1 and 2 have a significantly higher blastocyst total cell number compared with quartile 4 ($P < 0.05$, <0.001, Table II). Embryos in all quartiles formed blastocysts at equivalent rates (Q1, 100%; Q2, 98%; Q3, 100%; Q4, 96%; respectively) and also had similar morphologies.

Effect of morphokinetics on blastocyst carbohydrate metabolism and cell lineage allocation

The rate of glucose consumption by 'fast' blastocysts was significantly higher than 'slow' blastocysts 98 h post-insemination ('fast' 21.2 ± 1.2 versus 'slow' 14.3 ± 1.0 pmol/embryo/h, $P < 0.001$, Fig. 1A). Similarly, the rate of lactate production by 'fast' blastocysts was significantly higher than 'slow' blastocysts ('fast' 21.5 ± 1.2 versus 'slow' 17.0 ± 0.9 pmol/embryo/h, $P < 0.05$, Fig. 1A). When expressed as a glycolytic rate, based on the assumption that 1 mol glucose produces 2 mol lactate (Lane and Gardner, 1996), 'fast' blastocysts exhibited a significantly lower glycolytic rate (% glucose converted to lactate) when compared with 'slow' blastocysts ('fast' 49.6 ± 2.8% versus 'slow' 59.7 ± 2.8%, $P < 0.05$, Fig. 1B). Following differential staining for ICM and TE cells, a significant difference in total cell number as seen between 'fast' and 'slow' blastocysts ('fast' 102.3 ± 7.4 versus 'slow' 71.4 ± 4.4, $P < 0.001$, Fig. 2). This was attributable to 'fast' blastocysts having a significantly larger ICM compared with 'slow' blastocysts. When the rates of glucose consumption and lactate production were normalized for cell number, there was no significant difference between fast and slow blastocysts; however, the per cent of glucose converted to lactate remained lower in the 'fast' blastocysts.

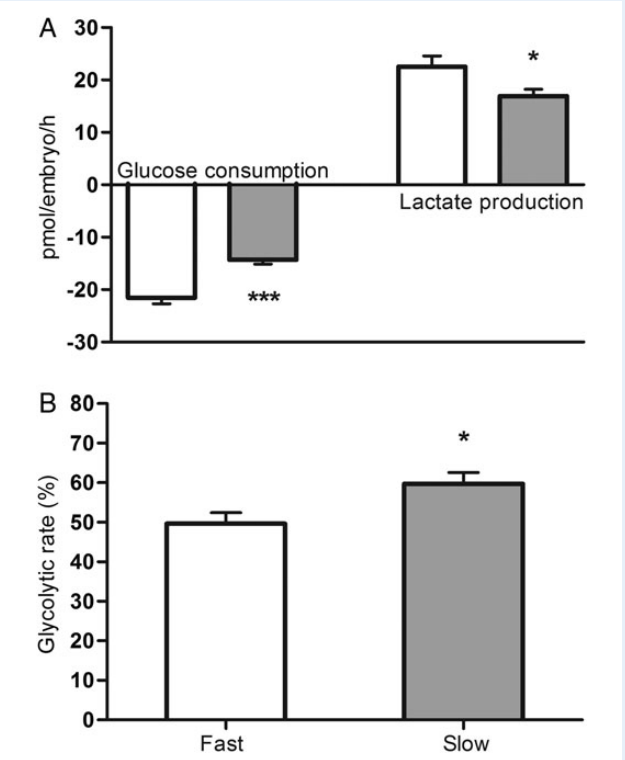

Figure 1 Correlation between morphokinetics and blastocyst carbohydrate metabolism. (**A**) Glucose consumption and lactate production, picomoles per embryo per hour; (**B**) Glycolytic rate of blastocysts, % of glucose converted to lactate. $n \geq 30$ embryos per group, 12 biological replicates, $^*P < 0.05$, $^{***}P < 0.001$. White bars represent 'fast' cleaving embryos and dark bars represent 'slow' cleaving embryos.

Effect of morphokinetics on blastocyst amino acid metabolism

There were significant differences in the amino acid metabolism profiles between 'fast' and 'slow' blastocysts (Fig. 3A). Of the 20 amino acids

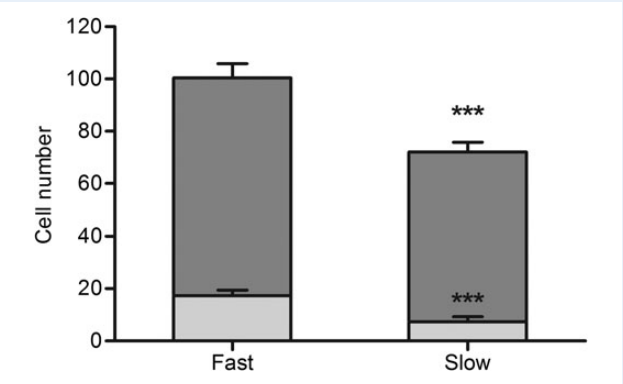

Figure 2 Correlation of morphokinetics and cell lineage allocation in the blastocyst. $n \geq 20$ embryos per group, 10 biological replicates, ***$P < 0.001$. Data are expressed as mean ± SEM. Dark portions of the bars represent the average number of TE cells and the light portion the average number of ICM.

present in the culture medium, arginine and aspartate were consumed by blastocysts while all other amino acids were released into the culture medium as metabolites. Blastocysts classified as 'fast' released significantly less glutamine and alanine compared with those designated as 'slow' (glutamine: 5.5 ± 0.9 versus 8.3 ± 1.0 pmol/embryo/h, $P < 0.05$; alanine: 3.6 ± 0.6 versus 6.2 ± 0.8 pmol/embryo/h, $P < 0.05$, Fig. 3A) and the variation in production of these amino acids from 'slow' blastocysts appeared larger. 'Fast' blastocysts consumed significantly more aspartate compared with 'slow' blastocysts (2.2 ± 0.1 versus 1.8 ± 0.1 pmol/embryo/h, $P < 0.05$, Fig. 3A). Interestingly, 'fast' blastocysts produced little or no glutamate compared with 'slow' blastocysts (0.02 ± 0.07 consumed versus 0.32 ± 0.11 pmol/embryo/h produced, $P < 0.05$, Fig. 3A). When total amino acid turnover was calculated, 'fast' blastocysts were found to have a lower release of amino acids and subsequently, a lower total turnover ($P < 0.01$, Fig. 3B).

Effect of morphokinetics on outgrowths and pregnancy

'Fast' blastocysts from the 1st quartile had a significantly larger outgrowth area after 72 h compared with 'slow' blastocysts from the 4th quartile ($P < 0.05$, Fig. 4). Following transfer of blastocysts to pseudo-pregnant recipients, there was no significant difference in implantation rate between 'fast' and 'slow' blastocysts. However, there was significantly higher fetal loss from implanted 'slow' blastocysts (Table III). Of those blastocysts that implanted, 69.6% of 'fast' blastocysts formed fetuses whilst only 40.4% of implanted 'slow' blastocysts formed fetuses ($P < 0.01$, Table III). Fetuses that had successfully developed were assessed using a morphological grading, including measurements of fetal and placental weight, crown-rump length, limb, eye morphology (Lane and Gardner, 1994) and determination of fetal sex. Of these parameters, there were no significant differences observed between fetuses from 'fast' or 'slow' blastocysts on E13.5 (Table III).

Discussion

This is the first study to incorporate time-lapse and metabolic analysis on the same embryo, demonstrating a correlation between morphokinetics, blastocyst ICM, total cell number and metabolic measures of blastocyst quality. Morphokinetic parameters that were significantly correlated to blastocyst total cell number included: timing of syngamy, first cleavage (t2), third cleavage (t5), cavitation and the duration between syngamy and t2 (syn−t2). Timing of the first cleavage division (t2) was chosen as the strongest morphokinetic marker to identify 'fast' and 'slow' cleaving embryos. 'Fast' embryos consumed significantly more glucose at the blastocyst stage compared with 'slow' embryos and of physiological significance, 'fast' embryos had a significantly lower glycolytic rate compared with 'slow' embryos. Blastocyst amino acid metabolism also differed significantly between 'fast' and 'slow' embryos, with 'fast' embryos consuming significantly more aspartate, and less glutamine, alanine and glutamate. Although there was no significant difference in blastocyst morphologies and subsequent implantation rates, 'fast' embryos had a significantly larger outgrowth area *in vitro* and significantly higher fetal development per implantation *in vivo*, indicating that there was more post-implantation fetal loss and resorption associated with 'slow' embryos. Overall, it has been demonstrated that embryos with different morphokinetics develop to blastocysts with significantly different metabolic profiles, cell lineage allocation and subsequent viability.

Studies in the mouse have demonstrated that timing of the first cleavage event is correlated with the embryo's potential to develop into a blastocyst, with earlier cleaving embryos more likely to develop (Arav *et al.*, 2008; Pribenszky *et al.*, 2010). Pribenszky *et al.* (2010) also identified that t2, cc2 and t3 were correlated with blastocyst development (Pribenszky *et al.*, 2010). In the present study, we further correlated morphokinetics to blastocyst cell number and additionally standardized timing to the first cleavage event to eliminate the effect of the time interval between syngamy to the first cleavage division. It was found that following standardization, only t5 and cavitation time remained significantly correlated to blastocyst cell number. This supports data previously reported from human morphokinetic studies, where t5 has been correlated to implantation potential (Meseguer *et al.*, 2011). Furthermore, we found that the differences in the timing of the first cleavage division led to significantly different rates of viability, with higher fetal development per implantation from faster cleaving embryos. Our morphokinetics data can be further related to previous reports (Grisart *et al.*, 1994; Arav *et al.*, 2008; Herrero *et al.*, 2013) showing that early divisions were closely synchronized in embryos and that later developmental events occur over a wider range. In humans, it was found that implanting embryos have a tighter range of timing and all developmental events (except for s2) follow a normal distribution compared with non-implanting embryos, where timings of developmental events do not follow a normal distribution (except for t5) (Meseguer *et al.*, 2011). In the present study, it was observed that timings of developmental events did not follow a normal distribution, presumably due to morphokinetic analysis conducted on embryos with unknown viability. Several morphokinetic parameters, including hierarchical selection models and algorithms, have been proposed to be predictive of viability (Wong *et al.*, 2010; Meseguer *et al.*, 2011). However, optimal morphokinetic parameters are variable between IVF clinics (Meseguer *et al.*, 2012), and variations in results may be related to culture conditions such as oxygen levels (Wale and Gardner, 2010; Kirkegaard *et al.*, 2013), oocyte, sperm quality and chromosomal abnormalities (Kirkegaard *et al.*, 2012). Additionally, the majority of time-lapse studies have investigated human embryos, whilst the present study focused on mouse embryos. As embryonic gene activation begins at 2-cell stage in mice and at 4-cell stage in humans, this may

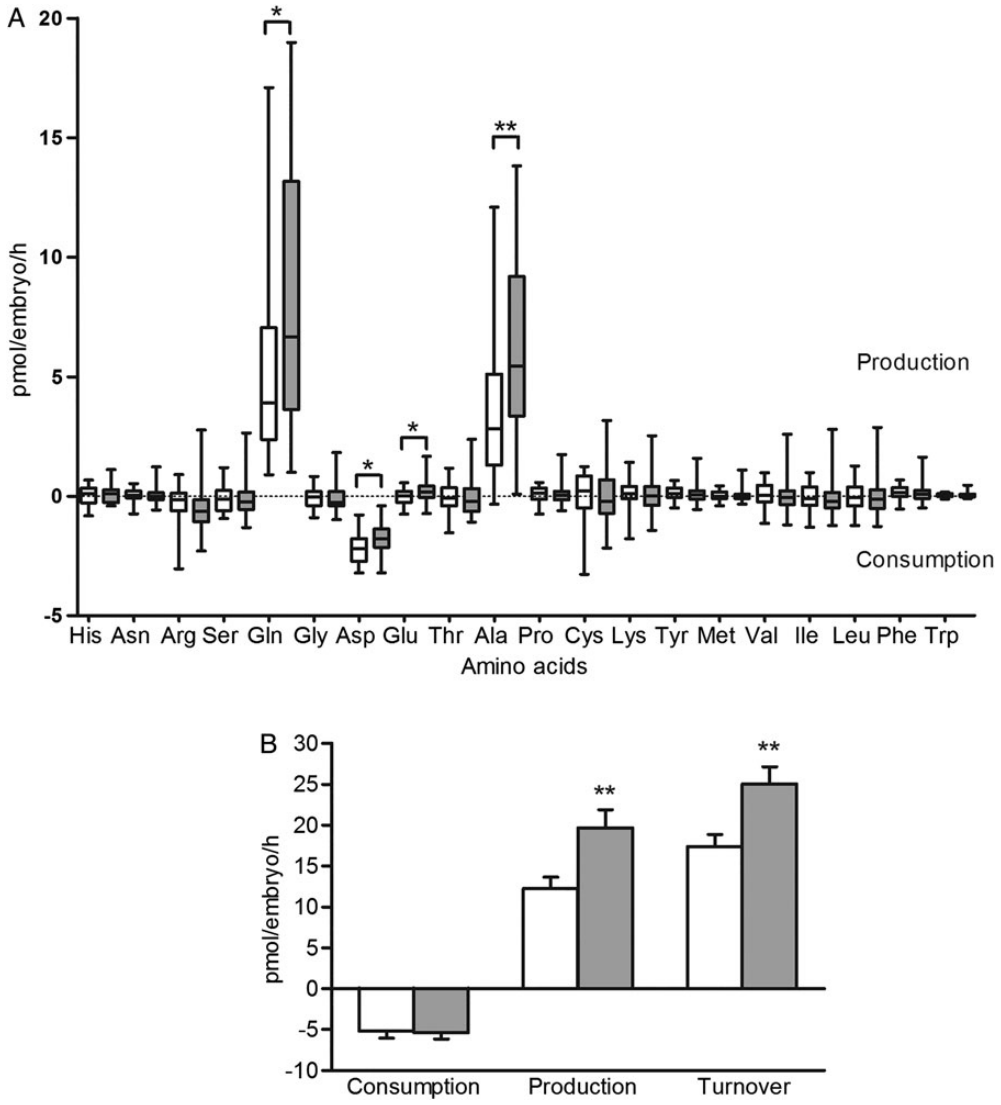

Figure 3 Correlation of morphokinetics and blastocyst amino acid metabolism. (**A**) Amino acid metabolism for blastocysts from day 4 to 5; Boxes represent the interquartile range (middle 50% of the data), whiskers represent the 5th and 95th percentiles. (**B**) Total amino acid consumption, production and turnover by blastocysts from day 4 to 5. Data are expressed as mean ± SEM. $n = 26$ samples per group. One sample consists of three embryos in a single incubation drop. Thirteen biological replicates, $^{*}P < 0.05$, $^{**}P < 0.01$, white bars represent 'fast' cleaving embryos and dark bars represent 'slow' cleaving embryos.

result in different interpretations of morphokinetics data and disparity between studies.

In the current study, blastocysts that developed from 'fast' embryos had significantly higher rates of glucose uptake compared with blastocysts that developed from 'slow' embryos, and previous studies on both the mouse and human have shown that a higher glucose uptake is predictive of a successful pregnancy (Gardner and Leese, 1987; Lane and Gardner, 1996; Gardner *et al.*, 2011). Analysis of both glucose uptake and lactate production in this study revealed that these 'fast' blastocysts exhibit a significantly lower glycolytic rate when compared with 'slow' blastocysts, even though they consumed more glucose. Additionally, this group of 'fast' blastocysts developed a larger outgrowth area and better fetal development per implantation when compared with 'slow' blastocysts. These findings support previous research that development of the ICM is positively correlated with viability while glycolytic rate is negatively correlated with subsequent viability (Lane and Gardner, 1996).

Analysis of amino acid metabolism showed that kinetically different embryos have different amino acid profiles. The production of glutamine and alanine, plausibly from the breakdown of alanyl-L-glutamine, was elevated in 'slow' blastocysts compared with 'fast' blastocysts and may therefore reflect differences in the ability of kinetically different blastocysts to regulate the utilization of alanyl-L-glutamine. Total amino acid turnover was found to be significantly lower in 'fast' blastocysts compared with 'slow' blastocysts and this difference can be attributed primarily to the production of more glutamine and alanine by 'slow' blastocysts. In clinical IVF media, alanyl-L-glutamine is commonly used in replacement of glutamine, which breaks down to form toxic ammonium in the culture

medium (Vickery et al., 1935; Lane and Gardner, 1994; Zander et al., 2006). In contrast to this study, Wale and Gardner (2012) used glutamine instead of alanyl-L-glutamine and demonstrated that post-compaction embryos cultured in atmospheric oxygen consume more glutamate and subsequently displayed a significantly lower amino acid turnover compared with embryos cultured in 5% oxygen (Wale and Gardner, 2012). Interestingly, the same group previously showed that embryos compromised by culture in atmospheric oxygen displayed a larger distribution of timings in morphokinetics, as opposed to faster and tighter division timings of embryos cultured in 5% oxygen (Wale and Gardner, 2010). Similarly, in this study, it was seen that slower cleaving embryos with a larger distribution of timings consumed less glutamate compared with faster cleaving embryos. Houghton et al. (2002) and Brison et al. (2004) have shown that cleavage stage human embryos that have a higher viability have a lower amino acid turnover (Houghton et al., 2002; Brison et al., 2004). However, embryos in both of the human studies employed atmospheric oxygen conditions (20% oxygen), which has been shown to affect amino acid utilization (Wale and Gardner, 2012, 2013) and has also been reported to have a negative impact on blastocyst and fetal development, as well as resultant blastocyst gene expression and proteome (Harlow and Quinn, 1979; Gardner and Lane, 2005; Katz-Jaffe et al., 2006). Of interest, Booth et al. (2007) conducted multiple observations on porcine embryos for cleavage time, and analysed these cleavage stage embryos for amino acid turnover. They found that faster cleaving embryos have a higher consumption rate of methionine, asparagine and arginine and a higher turnover rate compared with slower cleaving embryos (Booth et al., 2007). As their study was on cleavage stage porcine embryos and had different concentrations of amino acids compared with this study, direct comparisons are difficult as previous work has shown the relative concentration of amino acids present in culture medium affects overall turnover rates (Lamb and Leese, 1994; Gardner, 1998).

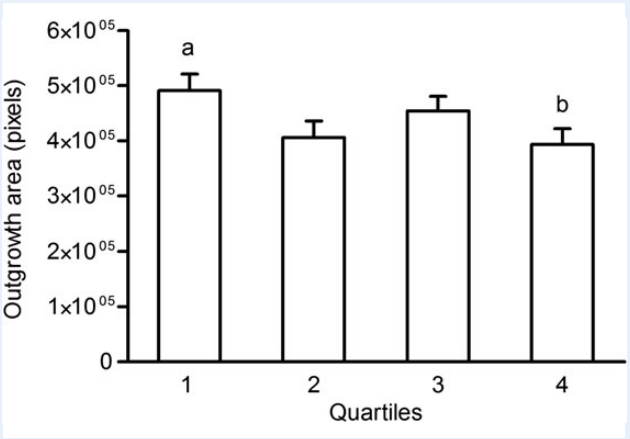

Figure 4 Correlation of morphokinetics and blastocyst outgrowth. Data are expressed as mean ± SEM. $n = 129$ embryos in total, five biological replicates. (**a** and **b**) Different letters represent significant differences between quartiles, $P < 0.05$.

Aspartate, the most highly consumed amino acid by the blastocyst, has been shown to regulate carbohydrate metabolism in the embryo via its own metabolism through the MAS (Lane and Gardner, 2005). The higher consumption of aspartate by 'fast' blastocysts may indicate an increase in the MAS activity. A high MAS activity would generate more intracellular NAD^+ to facilitate a greater glucose utilization through the Embden–Meyeroff pathway. In turn, this would mean that less pyruvate would be converted to lactate in order to generate the cytosolic NAD^+ required to maintain glucose flux. Consequently, this may go some way to explaining why 'fast' blastocysts have a lower glycolytic rate than 'slow' blastocysts as they also exhibit higher aspartate utilization.

Inhibition of the MAS at the blastocyst stage has been shown to compromise subsequent viability and fetal growth (Mitchell et al., 2009), and the present study demonstrates that although implantation rate was not different between 'fast' and 'slow' blastocysts, 'fast' blastocysts have a higher developmental potential, shown by reduced post-implantation fetal loss and a higher outgrowth area from 'fast' blastocysts compared with 'slow' blastocysts. However, of the 'slow' blastocysts that developed to fetuses, growth and normality were unaffected. In future work, assessment of the allocation of cells to trophoblast and epiblast tissue may increase the sensitivity of the outgrowth analysis and give a better insight into the resorption of 'slow' blastocysts after implantation.

Table III Correlation of morphokinetics and pregnancy.

Parameter	Fast	Slow
Implantation	62.2 ± 6.8%	63.3 ± 8.4%
Fetal development per implantation	69.6%	40.4%*
Fetal weight (mg)	128.3 ± 2.4	125.0 ± 3.5
Placental weight (mg)	96.8 ± 2.9	92.3 ± 3.3
Crown-rump length (mm)	10.0 ± 0.08	10.0 ± 0.13
Limb morphological grade	14.2 ± 0.08	14.1 ± 0.14
Eye morphological grade	14.7 ± 0.07	14.4 ± 0.15
Sex of fetus	53.8% F, 46.2% M	47.8% F, 43.5% M, 4.3% n/a, 4.3% Ex

Data are expressed as % mean ± SEM. $n = 90$ blastocysts transferred per group.
F, female; M, Male; n/a, undetermined; Ex, Exencephalic.
*$P < 0.01$.

In conclusion, this study has demonstrated for the first time that kinetically different cleavage stage embryos develop into blastocysts with significantly different metabolic profiles and viability. Independently, morphokinetics and metabolism have been previously shown to successfully correlate with viability; however, these biomarkers do not provide absolute certainties of the prospective viability of the embryo. Data presented in this study have shown that there is a relationship between the embryo biomarkers, morphokinetics and metabolism. This study goes some way to explain the reported success of using morphokinetics as a biomarker, as faster cleaving embryos were shown to have a pattern of carbohydrate and amino acid metabolism typically associated with viable embryos. Interestingly, within the 'fast' group, there was variability in the glycolytic rate of resultant blastocysts, indicating that not all 'fast' blastocysts have an equivalent metabolism. From this observation, it is plausible that utilizing these biomarkers in combination to select for an embryo will increase the accuracy of determining embryo viability, and hence increase IVF success outcomes.

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Authors' roles

All authors were involved in conceptualization of study design. Y.S.L.L. performed all embryo cultures, statistical analyses and drafted the manuscript. G.A.T. and D.K.G. provided input and a critical discussion of the data and in the writing of the manuscript.

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Conflict of interest

None declared.

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