Identifying Metabolic Determinants of Embryo Viability and Normality

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Abstract

Despite improvements in assisted reproductive technologies (ART) over the past three decades, live births remain low, at ~30% per embryo transfer. In order to attain acceptable pregnancy rates, it remains a common practice in many countries to transfer more than one embryo. However, this results in a high incidence of multiple pregnancies and associated complications. Therefore, there has been considerable focus on identifying quantifiable biomarkers of optimal developmental and implantation competence (viability) to improve the selection of preimplantation embryos produced using IVF. There is currently no reliable, quantitative technique based on embryo physiology to non-invasively select for the single most viable embryo for transfer.

The recent integration of imaging and incubation technology to allow continuous, non-invasive monitoring of embryo developmental morphology during culture has prompted renewed interest in quantifying developmental kinetics as a biomarker of viability. In mice, developmental kinetics has only been correlated to blastocyst development, which does not necessarily reflect blastocyst quality. Consequently, the first experiment of this thesis investigated correlations of quantifiable morphokinetics of cleavage stage embryos to blastocyst cell proliferation. It was determined that the earlier occurrences of syngamy, division to 2-cell and 5-cell, and time to cavitation were correlated to a higher blastocyst cell number.

On-time events that occur throughout preimplantation embryo development rely on optimal metabolic control for all cellular processes. Earlier studies of carbohydrate metabolism of mouse embryos have shown that higher consumption of glucose and lower production of lactate are related to increased embryo viability. In humans, it has also been shown that specific amino acids are related to increased embryo viability, following studies of global turnover. Independently, these metabolites have therefore been used as predictive biomarkers of embryo viability; however, their interrelationship has not previously been investigated, especially on the same embryo. Hence, the second experiment was aimed to determine the relationship between cleavage stage
morphokinetics, blastocyst metabolism, cell proliferation and post-implantation development. This study demonstrated for the first time that kinetically different cleavage stage embryos develop into blastocysts with significantly different carbohydrate and global amino acid profiles that correspond with different viability outcomes, despite appearing morphologically similar. Aspartate was also confirmed as the single most highly consumed amino acid, and is a quantifiable biomarker of viability at the single blastocyst level.

The mechanisms that cause variations in developmental kinetics of the embryo are not well understood, and to assess if cleavage stage embryo kinetics were related to subsequent blastocyst transcriptional patterns, the next study of the thesis analyzed the expression of a selection of genes involved in regulation of metabolism, cytokinesis, trophoblast and hormone receptor genes at the blastocyst stage and Day 14 fetal and placental tissues. It was found that genes involved in carbohydrate metabolism (Slc2a1, Slc2a3, Pkm2) were expressed at significantly higher levels in the group of blastocysts and placental tissue that were kinetically faster at the cleavage stage. Additionally, Got1, an enzyme involved in the aspartate metabolism, was expressed at increased levels from blastocysts and placental tissues developed from embryos identified to be kinetically faster and vice versa.

Collectively, these data suggested that at the blastocyst stage, aspartate metabolism is linked to glucose metabolism. Therefore, the aim of the fourth study was to develop a novel assay to quantitate aspartate metabolism, using ultramicrofluorescence. Subsequent results demonstrated that embryo consumption of aspartate was positively correlated to culture media aspartate concentration, with a proportional increase in glucose consumption, however lactate production was not different.

Overall, the research outlined in this thesis strongly suggests that glucose and amino acid metabolism of the embryo are biologically linked to developmental morphokinetics, and may be used to predict viability. Additionally, a potential new biomarker, quantification of aspartate metabolism has been identified. In combination, these
Abstract

parameters will facilitate the development of more reliable embryo selection methods to increase the accuracy and power of embryo viability assessments, to improve the success of clinical \textit{in vitro} fertilization outcomes, especially for single embryo transfer.
Declaration

This is to certify that:

i. The thesis comprises only my original work towards the PhD

ii. Due acknowledgement has been made in the text to all other material used

iii. The thesis is fewer than 100 000 words in length, exclusive of tables, figures, bibliographies and appendices

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Publications arising from thesis to date

Journal articles


Conference abstracts


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<td>Anti-DNP</td>
<td>Anti-dinitrophenyl</td>
</tr>
<tr>
<td>AOA</td>
<td>Aminooxyacetic acid</td>
</tr>
<tr>
<td>ART</td>
<td>Assisted reproductive technologies</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EAAT1</td>
<td>Excitatory amino acid transporter 1</td>
</tr>
<tr>
<td>EGA</td>
<td>Embryonic genome activation</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron transport chain</td>
</tr>
<tr>
<td>Glast</td>
<td>Glutamate aspartate transporter</td>
</tr>
<tr>
<td>hCG</td>
<td>Human chorionic gonadotropin</td>
</tr>
<tr>
<td>HSA</td>
<td>Human serum albumin</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<tr>
<td>ICM</td>
<td>Inner cell mass</td>
</tr>
<tr>
<td>IVF</td>
<td>In vitro fertilization</td>
</tr>
<tr>
<td>LC-MS</td>
<td>Liquid chromatography mass spectrometry</td>
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<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>MII</td>
<td>Metaphase II</td>
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<tr>
<td>MAS</td>
<td>Malate-aspartate shuttle</td>
</tr>
<tr>
<td>MEA</td>
<td>Mouse embryo assay</td>
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<tr>
<td>mMTF</td>
<td>Modified mouse tubal fluid</td>
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Abbreviations

NADH/NAD⁺ Nicotinamide adenine dinucleotide (reduced/ oxidized)

NIR Near infrared spectroscopy

PBS Phosphate buffered saline

PCR Polymerase chain reaction

PFK Phosphofructokinase

PK Pyruvate kinase

PGD Preimplantation genetic diagnosis

PGS Preimplantation genetic screening

PI Post insemination

PLC-ζ Phospholipase C-zeta

PMSG Pregnant mare’s serum gonadotrophin

PPP Pentose phosphate pathway

PVP Polyvinyl pyrrolidone

RCT Randomized controlled trial

RNA Ribonucleic acid

SEM Standard error of the mean

TE Trophoderm

TNBS Trinitrobenzenesulfonic acid

UMF Ultramicrofluorimetry

VSOAC Volume-sensitive organic osmolyte/ anion channels
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1. Literature review
1. Literature Review

1.1 Introduction

Recognised as a public health issue worldwide by the World Health Organization (WHO), infertility is defined as "a disease of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse." In Australia and New Zealand, one in six couples suffers from infertility, and it is estimated that 48.5 million couples worldwide are currently unable to have a child even after trying for five years (Mascarenhas, et al., 2012). The causes of infertility are complex, and include genetic and cytological factors, with sex specific problems such as ovulation problems and low semen quality, for example. Assisted reproductive technology (ART) and in particular, in vitro fertilization (IVF) is the primary treatment for infertility.

IVF is a process by which oocytes are retrieved surgically from the ovary prior to ovulation and are fertilized by sperm in vitro, followed by in vitro culture of the embryo. Fertility medication is typically used to stimulate the ovarian cycle to produce more oocytes to achieve a higher number of embryos per IVF cycle, thereby increasing the chance of producing a viable embryo. The developing embryo(s) is then selected for replacement into the uterus for the chance of implantation. In 1978 medical history was made following the birth of Louise Brown, the first IVF baby in the world (Steptoe and Edwards, 1978). Since then, considerable advances have been made in the field of ART and IVF, with global application leading to an estimated five million IVF babies born since 1978 (ICMART, 2012). Despite this impressive figure, IVF is by no means a perfected technology. Success rates of IVF average ~30 % per embryo transfer (Kupka, et al., 2014, Sunderam, et al., 2014), which only provides a 10 % increase in success rates when compared to the natural conception rate of ~20 % per month (Taylor, 2003).

It is important to understand that the IVF process is a system of many components, which can collectively impact on pregnancy rates over several cycles. Patient factors
may include suboptimal stimulation responses, or inadequate endometrial receptivity. Biological factors may include failure of fertilization or poor embryo development during culture, which depend directly on laboratory protocols for embryo culture, selection and cryopreservation. Success rates of IVF are impeded by differences between the \textit{in vivo} and \textit{in vitro} environment where fertilization and embryo development occurs, which represents one of the most critical variables. The reality of working \textit{in vitro} is that embryos are exposed to several stresses they do not experience \textit{in vivo}, such as oxidative stress and exposure to non-physiological conditions (Wale and Gardner, 2016). Current culture conditions, although advanced, do not fully represent the \textit{in vivo} embryo environment, with respect to the complexity of dynamic environments within the oviduct and uterus, and biological constituents of their fluids. Over the past four decades since the first IVF birth, extensive optimization and testing of culture conditions to mimic physiological conditions has led to an overall increase in pregnancy rates, but there remain several areas where further improvements can be implemented.

One particular area is the laboratory protocols for assessment and selection of embryos for transfer. In order to attain acceptable pregnancy rates, it remains a common practice in many countries to transfer more than one embryo, resulting in a dramatic increase in multiple pregnancies. In 2012 in the USA, 43.6 \% of babies conceived with ART were part of multiple pregnancies, compared with the natural multiple pregnancy rate of 3.4 \% (Sunderam, et al., 2015), with the majority of these pregnancies being twins, with fewer high-order multiple gestations. Complications associated with multiple gestations are thoroughly documented to be associated with obstetric, perinatal and neonatal complications (Adashi, et al., 2003, Templeton, 2004). Thirty seven per cent of triplets are born before 32 weeks of gestation, with over a third of these triples weighing less than 1.5 kg at birth. Unsurprisingly, the perinatal mortality rate of triplets is six times higher than that for singleton births. There are also maternal consequences in these cases, with higher risk of preeclampsia and eclampsia, placental abruption and primary post-partum haemorrhage (Templeton, 2004). Of the ART babies conceived in the USA, 62 \% of twins and 97 \% of triplets have been delivered preterm, corresponding to approximately 17,000 infants. Excess hospital costs for long-term care of pre-term twins
or triplets has been estimated to be US$ 51,600 per infant, amounting to a staggering US$ 1 billion financial burden due to multiple pregnancies. Furthermore, there is substantial emotional and financial burden on families raising twins or triplets (Connolly, et al., 2010, Thorpe, et al., 1991).

Consequently, the goal of IVF should be to transfer a single embryo with the highest likelihood of establishing a healthy pregnancy, culminating in the delivery of a healthy singleton child. Although single embryo transfer has been successfully established in several countries, including Australia, Finland and Denmark, there remains the necessity to quantify embryo viability prior to transfer, especially in countries with strict limitations on the number of embryos that can be cultured, such as in Germany, where no more than three fertilized oocytes can be cultured and transferred. Successful identification of the embryo with the highest implantation potential will contribute to reduction in IVF cancellation rates, the number of IVF cycles required to achieve pregnancy, and rates of patient drop out, which is as high as 17 % (Verberg, et al., 2008). In turn, reducing the number of IVF attempts to achieve a successful pregnancy will alleviate the immense emotional and financial stress placed on a family undergoing IVF.

Historically, selection of embryos for transfer has been based on morphological criteria, however, this allows for human subjectivity and variability of observation. Furthermore, morphology fails to convey information about embryo physiology, the key mechanisms of biological function that regulate ongoing and on-time development of the pre- and post-implantation embryo and fetus. Hence, there has been considerable focus on identifying quantifiable biomarkers of developmental and implantation competence to improve embryo selection (Gardner, et al., 2015, Gardner, et al., 2011, Katz-Jaffe, et al., 2006a, Nagy, et al., 2009, Tejera, et al., 2012).
Much of the methodology applied to the laboratory assessment of human IVF embryos has been developed using the mouse model. The *in vitro* human embryo takes five days to reach the blastocyst stage. Similarly, mouse embryo development occurs over a four day period prior to implantation, and forms an expanding blastocyst similar to that of the human. This is in contrast to the embryos of domestic animal species, such as the cow, which take seven days to form a blastocyst, and subsequently undergo greater proliferation and expansion prior to attachment to the uterine endometrium around day 14. The mouse genome is also highly conserved, with 95% similarity to that of the human. As such, the mouse is the most widely adopted and characterized model of mammalian preimplantation embryo development. Further advantages include cost-effective housing and rapid breeding times compared to ruminant species. Additionally, there is the ability to perform large-scale embryo transfers, as mice can have multiple (>10) offspring, as opposed to singleton or twin pregnancies in the sheep and cow. Furthermore, the mouse and human embryo share a similar metabolism during embryo development and display a switch in metabolite usage during the preimplantation period. The following literature review concentrates on current knowledge on human and mouse preimplantation embryo development, and additionally draws comparisons to other mammalian species.

1.2 Preimplantation embryo development

Following successful sperm penetration of the zona pellucida of mature metaphase-II (MII) oocyte after insemination, meiotic arrest is released, and extrusion of the second polar body then occurs. This key step in oocyte fertilization is initiated by the sperm-specific protein, phospholipase C-zeta (PLC-ζ) (Saunders, et al., 2002), which has been shown to trigger intracellular calcium (Ca\(^{2+}\)) oscillations that reactivate the meiotic cell cycle in the oocyte cytoplasm. This simultaneously induces cortical granule exocytosis, which causes glycoproteins in the zona pellucida to cross-link, thereby preventing multiple sperm entry and polyspermic fertilization (Kline and Kline, 1992). Decondensation of the sperm chromatin, disappearance and reformation of the sperm nuclear envelope and completion of nucleolar precursor development contributes to the
formation of the male pronucleus (Tesarik and Kopecny, 1989). Subsequently, the male pronucleus migrates to the centre of the oocyte, along with the female pronucleus, which was formed after decondensation of the meiotic spindle. At this stage, the embryo is termed a ‘pronucleate oocyte’ and undergoes DNA replication in preparation of the first mitotic division. Both the male and female pronuclei continue to move to the centre of the oocyte, where they eventually merge. The pronuclear membranes of the haploid male and female pronuclei then disassemble, mitotic spindle aligns (syngamy) the duplicated chromosomes and moves them to opposite poles of the cell, completing the first mitotic division to produce a 2-cell embryo (Fig. 1.1).

The preimplantation embryo continues holoblastic cellular division (Fig. 1.1), during which the overall size of the embryo remains the same, but blastomeres gradually decrease in cytoplasmic volume with ongoing mitotic divisions. During these early cleavage stages of development, also known as pre-compaction stages, the blastomeres of the 2- to 4-cell embryo are totipotent, able to form an entirely new blastocyst, as demonstrated for 2-cell and 4-cell embryos of mice and cattle, respectively (Johnson, et al., 1995, Tarkowski, 1959). Even in the 8-cell stage human embryo, the biopsy of one or two blastomeres for genetic analysis can be compensated for by the remaining blastomeres, resulting in an implantation competent blastocyst (Handyside, et al., 1989). These blastomeres are in contact with each other but not connected, and are contained by the zona pellucida. The large volume of oocyte cytoplasm divided into daughter blastomeres contains the precursors of all essential organelles for protein synthesis (rough and smooth endoplasmic reticulum, Golgi apparatus) and metabolism (mitochondria, peroxisomes) (Bachvarova and De Leon, 1980, Cascio and Wassarman, 1982), which remains at slower rate, while the blastomeres remain undifferentiated. Maternally expressed genes regulate these events until embryonic genome activation (EGA), when approximately 90 % of the maternal transcripts are degraded and de novo transcription commences under genetic control of the newly fertilized embryo (Hamatani, et al., 2006). Studies have shown that the timing the EGA is species specific, beginning at the 1- to 2-cell stage in the mouse, 4- to 8-cell stage in humans and 8- to 16-cell stage in the cow and sheep (Braude, et al., 1988, Flach, et al., 1982, Schultz, 1993, Telford, et al., 1990). Additional studies have shown that EGA in the mouse
occurs in a series of events, as opposed to a single simultaneous activation, corresponding to the late 1-cell stage, early 2-cell stage, late 2-cell stage and 8-cell stage of the mouse embryo (Aoki, et al., 1997, Bouniol, et al., 1995, Matsumoto, et al., 1994). The spermatozoon itself has little cytoplasm and contributes only its centriole in addition to its paternally imprinted genetic complement (Goto, et al., 2010, Schatten, 1994).

Following cleavage to the 8-cell stage, the embryo undergoes a process called compaction and is subsequently referred to as a morula, as its appearance resembles that of a mulberry (Latin, morula) (Fig. 1.1 and 1.2). Compaction is characterized by physical connection of adjacent blastomeres, and precedes the formation of a small internal cavity (Benos, 1981). During compaction, blastomeres express E-cadherin and form tight junction complexes, resulting in the formation of a primitive epithelium by the outer blastomeres (Fleming, et al., 2001, Handyside, 1980, Ziomek and Johnson, 1980). This process is critical for rearrangement and polarization of blastomeres, resulting in the first stages of differentiation. Through increased expression and activity of Na⁺/K⁺ ATPases on the basolateral membrane, an ionic gradient is formed and water flows into the embryo through aquaporins, resulting in accumulation of fluid in the cavity, now referred to as the blastocoel (Violette, et al., 2006, Watson, et al., 2004).

Cavitation marks the formation of the blastocyst, which contains two newly differentiated cell types, the inner cell mass (ICM), which remains pluripotent, and the trophectoderm (TE). The different cell types are derived from asymmetric divisions from the 8-cell stage that generates one daughter cell on the inside and one on the periphery of the embryo. Cells positioned in the centre of the embryo develop into the ICM, whereas outside cells develop into the TE (Fleming, 1987, Johnson and Ziomek, 1981, Pedersen, et al., 1986). The TE forms the outer lining of the blastocyst and eventually forms extra-embryonic tissue such as the placenta and yolk sac. The ICM eventually forms the post-implantation embryo and subsequent fetus, as well as contributing limited material to the yolk sac and allantois (Gardner, 1975). Throughout
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cavitation, cell division is continuous and relatively higher in the TE; with increased uptake of water through TE membrane aquaporins causing the blastocyst to expand.

**Figure 1.1:** Mouse preimplantation embryo development from Day 1 to Day 3

a. Pronucleate oocyte, b. 2-cell stage, c. 3-cell stage, d. 4-cell stage, e. 5-cell stage, f. 6-cell stage, g. 7-cell stage, h. 8-cell stage, i. Compacting morula, at initial stages of compaction (membrane definition becomes less distinct between blastomeres). (Magnification X400).
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Figure 1.2: Mouse preimplantation embryo development from Day 3 to Day 5.

The fully compacted morula begins the cavitation process to form a blastocoel and undergoes expansion and collapsing before hatching out of the zona pellucida. a. Morula, b. Beginning of cavitation, c. Cavitating morula, d. Early blastocyst, e. Expanding blastocyst, f. Collapsed blastocyst, g. Hatching blastocyst, h. Hatching blastocyst (partially hatched), i. Fully hatched blastocyst. (Magnification X400)
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Appearance of blastocoele

Zona pellucida
Enclosed by the zona pellucida, the blastocyst initiates the process of hatching, via a build up of internal pressure. Proteases secreted by the TE (and adjacent uterine epithelium *in vivo*) then act to digest and thin the zona pellucida, while repeated expansion and collapse allows the blastocyst to gradually escape (hatch) through the digested opening as it widens (Fig. 1.2). Once fully hatched, the blastocyst is then free to implant in the uterus lining, by physical and molecular interactions between the TE and uterine epithelium (Gardner, 2015b, Salamonsen, et al., 2013).

There are distinct differences between the pre- and post-compaction stages of preimplantation embryo development. The pre-compaction embryo has minimal cellular growth and slow cleavage division, uses maternally derived transcripts and is located within the oviduct during this developmental period. In contrast, the post-compaction embryo exhibits rapid cell growth, activates its own embryonic genes, a transporting epithelium and develops predominantly in the lumen of the uterus.

1.3 *In vivo* environment of the female reproductive tract

The milieu of the female reproductive tract is comprised of oviduct and uterine secretory fluids, which collectively support preimplantation embryo development. Analysis of fluid composition has led to a greater understanding of nutrient and metabolic requirements of the embryo, as well as improvements in culture media composition (Gardner, et al., 1996, Gardner and Leese, 1990). Fertilization of the oocyte takes place at the ampulla of the oviduct. Subsequently, the pre-compaction embryo undergoes cleavage division to the 8-cell stage (Fig. 1.1) between day 1 and 3, followed by compaction of cells to form the morula on late on day 3 / early day 4. The morula then passes through the utero-tubal junction and enters the uterus early day 4. Within the uterus, the post-compaction embryo initiates cavitation and expansion followed by hatching and implantation into the uterine epithelium.
The environment in which the early embryo develops is complex, comprising of not only nutrients in the form of carbohydrates and amino acids (the levels of which change during the cycle), but also key signalling molecules from the mother in form of hormones (Lavranos and Seamark, 1989), growth factors and cytokines (Laird, et al., 2006, Thouas, et al., 2015). However, the focus of the following discussion is on metabolic components of the female reproductive tract. Comparison of oviduct and uterine fluid composition has revealed that the environments are biochemically different and correspond to the metabolic requirements of pre- and post-compaction developmental stages of the embryo, respectively. For example, carbohydrate concentrations are significantly different between oviduct and uterine fluids. Oviduct fluid has high levels of tri-carboxylic acids such as pyruvate and lactate (Gardner, et al., 1996, Tay, et al., 1997). Gardner and Leese (1990) showed that the concentrations of pyruvate and lactate were ~0.4 mM and 4.8 mM respectively in mouse oviduct fluid (Gardner and Leese, 1990). In human oviduct fluid, while the pyruvate concentration was similar (~0.3 mM), the concentration of lactate was shown to fluctuate with respect to the menstrual cycle (Gardner, et al., 1996). In humans, lactate increases from 4.9 mM to 10.5 mM between the follicular phase to time of ovulation (Gardner, et al., 1996). Before fertilization, the cumulus cell mass surrounding the freshly ovulated MII oocyte has been shown to metabolize glucose into pyruvate and lactate, contributing to oviduct fluid levels (Gardner, et al., 1996, Gardner and Leese, 1990). Collectively, these oviduct fluid fluctuations in carbohydrates ensure that the ovulated oocyte and newly fertilized embryo is maintained in an environment high in pyruvate and lactate. By comparison, uterine fluid has lower levels of pyruvate and lactate in the mouse, human and cow (Gardner, et al., 1996, Hugentobler, et al., 2008, Tay, et al., 1997).

Relatively low levels of glucose are found in oviduct fluids (Gardner, et al., 1996, Tay, et al., 1997), and it was shown that the menstrual cycle similarly has an effect on glucose concentrations. Levels of glucose drop from 3.1 mM to 0.5 mM during the time of ovulation, significantly lower than lactate (~4.8 mM). Similar reduction of glucose concentration following ovulation was also reported in the pig (Nichol, et al., 1992). Dickens et al. (1995) on the other hand, reported low levels of glucose, 0.5 mM, regardless of menstrual cycle day in humans (Dickens, et al., 1995). In contrast, uterine
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fluid, was shown to have significantly higher levels of glucose, ~3.0 mM in human and mouse uterine fluid, and an increase to 4.5 mM in cow uterine fluid (Gardner, et al., 1996, Hugentobler, et al., 2008, Tay, et al., 1997). It can be concluded that during ovulation and pre-compaction embryo development, there are high levels of pyruvate, lactate and low levels of glucose and that the levels of these carboxylic acids are reversed in uterine fluid, during post-compaction embryo development.

Oviduct and uterine fluids also contain significant levels of amino acids (Menezo and Laviolette, 1972, Miller and Schultz, 1987). Those present in high levels in the oviduct include aspartate, glycine, alanine, glutamate and serine. Of interest, this group share a remarkable resemblance to those found in Eagle’s non-essential group, so named because they were not absolutely required to support the growth of cell lines in culture (Eagle, 1959). Essential amino acids are present in oviduct fluid as well, albeit at lower concentrations compared to the non-essential amino acids (Gardner and Lane, 1993, Miller and Schultz, 1987). Similar to the changes in carbohydrate concentrations, levels of specific amino acids change significantly from the oviduct to the uterus (Harris, et al., 2005). In uterine fluid extracted from excised tissues in the mouse and in flushed aspirates of uterine fluid, amino acids were detected at a significantly lower overall level compared to the oviduct fluid, with non-essential amino acids present at higher concentrations compared to essential amino acids (Gardner, et al., 1996, Harris, et al., 2005). Glycine is present in high concentration in oviduct fluids across several species, cow (14.1 mM), rabbit (18.4 mM), mouse (3.2 mM), pig (1.7 mM) (Elhassan, et al., 2001, Harris, et al., 2005, Iritani, et al., 1971, Li, et al., 2007, Miller and Schultz, 1987). In mouse oviduct fluid, the next highest concentrations are alanine (2.5 mM), glutamine (1.4 mM) and glutamate (1.3 mM) (Harris, et al., 2005). The abundance of glycine in oviduct fluid correlates to the high intracellular concentrations of glycine in the mouse embryo (Van Winkle and Dickinson, 1995). Glycine has also been shown to function as an organic osmolyte in mouse embryos, at the early cleavage stages (Dawson, et al., 1998, Van Winkle, et al., 1990), however as the embryo develops, its ability to utilize glycine transporters to accumulate glycine is lost (Dawson, et al., 1998, Hammer, et al., 2000). It has also been shown that human and mouse embryos possess similar glycine transport systems and regulation is disturbed by environmental stresses (Hammer, et al.,
Overall, these changing levels of carbohydrates and amino acids in the oviduct and uterine fluid closely reflect the nutrient requirements and developmental needs of the embryo, and will be discussed in the following section.

1.4 Optimization of preimplantation embryo culture

*In vitro* development of the preimplantation embryo exposes it to complex set of stresses, and these are associated with reduced cleavage and blastocyst development rates, perturbed metabolism, altered gene expression and genomic imprinting defects (Gardner and Lane, 1996, Gardner and Lane, 2005, Giritharan, et al., 2007, Lane and Gardner, 1996, Wale and Gardner, 2016). Previous studies suggest that IVF may carry an increased risk of birth defects (Anthony, et al., 2002, Hansen, et al., 2005) and increased occurrence of epigenetic disorders such as Angelman, and Beckwith-Wiedemann syndrome (Halliday, et al., 2004, Ludwig, et al., 2005). However, it was concluded that the increased risk was due to the advancing age of mother and condition of health between patients (Anthony, et al., 2002). This conclusion was further supported by a recent review of published literature, which concluded that there was no direct link between assisted reproduction and health-related risks in assisted conception children (Fauser, et al., 2014). It is paramount that culture systems minimize embryo stresses to ensure healthy embryo growth and development. Improving culture media composition and minimizing embryo toxins have mainly contributed to achieving this, which has recently been reviewed by Wale and Gardner (2015).

Over the years, such research into the nutrient requirements of the preimplantation embryo has resulted in the development of culture media that have improved embryo growth and implantation potential. Culture media have been formulated using two separate approaches. The first is based on the developmental needs of the embryo, according to measurements of embryo metabolism and physiology, and reflecting the changing composition of the oviductal and uterine tract (sequential media)(Gardner,
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1.4.1 Carbohydrates in *in vitro* culture

Early attempts to culture the preimplantation embryo date to over sixty years ago, when successful *in vitro* fertilization and culture of rabbit embryos was first achieved in 1955 (Chang, 1955). The following year, Whitten (1956) reported the growth of 8-cell mouse embryos to the blastocyst stage, cultured in a simple media based on Krebs-Ringer bicarbonate solution, supplemented with glucose, bovine albumin and a bicarbonate buffering system (Whitten, 1956). It was later shown that lactate, pyruvate, phosphoenolpyruvate and oxaloacetate were each able to support growth of 2-cell mouse embryos to the blastocyst stage (Brinster, 1963, Whitten, 1957) and further, glucose alone was
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unable to support mouse embryo development prior to the 8-cell stage (Brinster, 1965a, Brinster and Thomson, 1966). These foundational studies demonstrated that the pre-compaction embryo was unable to utilize glucose and required tri-carboxylic acids, specifically pyruvate and lactate. In fact, pyruvate was believed to be the sole carbohydrate supporting fertilized oocyte development to the 2-cell stage and beyond (Whittingham and Biggers, 1967), as lactate alone was unable to support embryo development past the 2-cell stage (Brinster, 1965a). This paradigm was changed in 2005 when it was discovered that the addition of exogenous aspartate (10 mM) facilitated mouse zygote development to utilize lactate as the sole carbohydrate in the absence of pyruvate (Lane and Gardner, 2005a) (discussed further in Chapter 1.5.3), and further reinforced the importance of the Krebs (tri-carboxylic acid) cycle in metabolic support of pre-compaction development. Today, all commercially available sequential culture media used to support human pre-compaction embryos contain pyruvate, lactate and low levels of glucose.

1.4.2 Amino acids in in vitro culture

Oocytes and embryos possess specific transport systems for amino acids and shown to maintain an endogenous pool of amino acids (Schultz, et al., 1981, Van Winkle, 1988). Hence, exogenous amino acids, like those in oviductal and uterine fluid, are likely to play important physiological roles in embryo development. Indeed, the addition of amino acids to embryo culture media has been shown enhance embryo development in different mammalian species, such as the hamster (Bavister and Arlotto, 1990, McKiernan, et al., 1995), cow (Takahashi and First, 1992), mouse (Gardner and Lane, 1993, Gardner and Lane, 1996) and sheep (Gardner, et al., 1994). Amino acids present at relatively high levels in the oviduct are those used during pre-compaction stages. Their presence in culture increase cleavage rates, leading to improved blastocyst formation, TE differentiation and hatching rates (Lane and Gardner, 1994, Lane and Gardner, 1997a, Lane and Gardner, 1997b).
Of note, essential amino acids display a negative effect during the pre-compaction stages, significantly reducing blastocyst cell number (Gardner and Lane, 1993, Lane and Gardner, 1997a, Steeves and Gardner, 1999). Specific essential amino acids, such as cysteine, isoleucine, leucine, phenylalanine, threonine and valine have an inhibitory effect on hamster embryo development when supplemented at the 1-cell stage (McKiernan, et al., 1995). In contrast to their overall negative effects on the pre-compaction embryo, essential amino acids have beneficial effects post-compaction, resulting in an increased development of the ICM in the blastocyst (Lane and Gardner, 1997a). Consistent with this, mouse blastocyst attachment and outgrowth, used as an in vitro model of implantation, is increased with the supplementation of essential amino acids (Spindle and Pedersen, 1973). Mouse blastocysts have also been reported to increase their uptake of essential amino acids between day 4 and day 5 by 170 %, as compared to 30 % for non-essential amino acids (Lamb and Leese, 1994).

Individual amino acids also have specific metabolic and non-metabolic roles that can affect preimplantation embryo development (metabolic roles will be discussed later, see Chapter 1.5.2). Non-essential amino acids such as glycine can act as osmolytes (Dawson and Baltz, 1997, Dawson, et al., 1998, Lawitts and Biggers, 1992), intracellular pH regulators (Edwards, et al., 1998a) and signalling molecules in blastocyst implantation (Martin, et al., 2003). Amino acids also act as antioxidants and chelators (Lindenbaum, 1973, Liu and Foote, 1995) and play a role in cell signalling, via the m-TOR (rapamycin) pathway, to regulate development of TE for blastocyst implantation (Murakami, et al., 2004). Only in vitro mouse embryos cultured in the presence of amino acids are able to form outgrowths, highlighting the importance of amino acids in culture media (Martin and Sutherland, 2001). Furthermore, leucine and in particular, arginine, stimulate TE adherence and invasion of the uterine lining (Gonzalez, et al., 2012, Martin, et al., 2003). The basic function of individual amino acids in the preimplantation embryo has been summarized in Table 1.1.
### Table 1.1: List of amino acids and their known functions.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>Sequestering of ammonium; transamination and Krebs cycle intermediates</td>
<td>Orsi and Leese, 2004</td>
</tr>
<tr>
<td>Glutamate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspartate</td>
<td>Regulation of Malate aspartate shuttle</td>
<td>Lane and Gardner, 2005</td>
</tr>
<tr>
<td>Arginine</td>
<td>TE adherence, invasion of uterine lining via mTOR pathway</td>
<td>Gonzales et al., 2012</td>
</tr>
<tr>
<td>Leucine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Taurine</td>
<td>TE adherence, invasion of uterine lining via mTOR pathway, antioxidant</td>
<td>Liu and Foote, 1995</td>
</tr>
<tr>
<td>Glutamine</td>
<td>Embryo development, blastocyst formation, TE cell formation, substitute for glucose, Krebs cycle intermediate</td>
<td>Lane and Gardner, 1997a, Gardner et al., 1989</td>
</tr>
<tr>
<td>Glycine</td>
<td>Osmolyte</td>
<td>Dawson et al., 1998, Van Winkle et al., 1990</td>
</tr>
<tr>
<td>All AAs</td>
<td>TE differentiation via mTOR pathway</td>
<td>Martin and Sutherland, 2001</td>
</tr>
<tr>
<td>All AAs</td>
<td>Chelators</td>
<td>Lindenbaum, 1973</td>
</tr>
<tr>
<td>Non-essential AAs</td>
<td>Intracellular pH buffer</td>
<td>Edwards et al., 1998</td>
</tr>
<tr>
<td>Non-essential AAs</td>
<td>Pre-compaction embryo development, increase blastocoel formation</td>
<td>Lane and Gardner, 1997</td>
</tr>
<tr>
<td>Essential AAs</td>
<td>Increase in ICM numbers post-compaction</td>
<td>Lane and Gardner, 1997</td>
</tr>
</tbody>
</table>
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As mentioned earlier, lactate can only support development from the 2-cell stage in the mouse (Whittingham, 1969) and pyruvate was believed to be the sole substrate that supports the fertilized oocyte prior to the 2-cell stage (Whittingham and Biggers, 1967). Following addition of exogenous aspartate (10 mM) to increase malate-aspartate shuttle (MAS) activity, the fertilized oocyte was able to utilize lactate as sole carbohydrate and develop normally past the 2-cell stage (Lane and Gardner, 2005a). The inability of the pre-compaction embryo to metabolize lactate was due to limited activity of the MAS, and that aspartate was the rate-limiting factor (Lane and Gardner, 2005a). With exogenous supply of aspartate, the MAS is able to better facilitate oxidation of lactate as an energy source (Quinn and Wales, 1973), which allows development past the 2-cell stage (Lane and Gardner, 2005a). Importantly, these results highlight the sensitivity of the embryo to specific substrates and that altering the levels and composition of nutrients within culture media has the potential to cause transient or lasting changes in both carbohydrate and amino acid metabolism of the embryo.

1.4.3 pH of embryo culture

Intracellular ions are universal regulators of cell function and are known to regulate protein synthesis, metabolism, biological and enzymatic reactions and play an important role in maintaining overall cellular homeostasis (Boron, 1986). Somatic mammalian cells therefore have a robust regulation of intracellular pH (pHi) that occurs at the cell membrane via the HCO$_3^-$ / Cl$^-$ (sodium/bicarbonate), Na$^+$ / H$^+$ exchanger and HCO$_3^-$ / Cl$^-$ exchanger. However, the oocyte and embryo are less equipped with the ability to regulate pHi (Dale, et al., 1998). Mature mouse oocytes are virtually incapable of regulating change in pH and therefore cannot recover from alkalosis (Phillips and Baltz, 1999). Even upon fertilization, it has been shown that the hamster oocyte lacks functional transport systems to regulate pHi for the first six hours (Lane, et al., 1999), highlighting the necessity to avoid fluctuations in the pH of media during oocyte retrieval and embryo culture. From the 2-cell stage, it has been shown that the early mouse embryo has a pHi of 7.17 - 7.22 and can recover from acidosis and alkalosis, however their mechanism for regulation differs from that of somatic cells (Baltz, et al., 1991a, Baltz, et al., 1991b, Edwards, et al., 1998b).
Baltz et al. (1991) demonstrated an activation of the HCO$_3^-$/Cl$^-$ exchanger to relieve alkalosis in the 2-cell mouse embryo (Baltz, et al., 1991a). However, the early embryo lacks active mechanisms to regulate acidosis. Instead, by inhibition of known intracellular regulatory mechanisms, such as the HCO$_3^-$/Cl$^-$ exchanger, it was shown that the recovery from acidosis was a passive process (Baltz, et al., 1991a). This capability to regulate against alkalosis more than acidosis is in consistent with the fact that the pH of oviduct fluid is more alkaline (7.6 - 7.9) (Iritani, et al., 1971, Maas, et al., 1977). Pre-compaction embryos use amino acids such as glycine and alanine, which exist as zwitterions, able to chelate or release H$^+$ ions to buffer pH. This ability is lost during the 8- to 16-cell stage due to a change from Na$^+$ independent to Na$^+$ dependent transport systems, such that the morula is able to regulate its pH independently. The generation of a permeability seal during compaction via tight junction formation, expression of acid-regulating pumps, and the subsequent formation of the TE epithelium for cavitation also enable the morula to regulate its intracellular pH (Dale, et al., 1998, Edwards, et al., 1998b).

Beyond acting as carbohydrate energy sources, pyruvate and lactate are also weak acids that may contribute to pH$i$. Lactate in particular, has been reported to cause a significant decrease in the pH$i$ of the early embryo when its extracellular concentration is higher than 5 mM (Edwards, et al., 1998a). Further, pH$i$ is a powerful regulator of glycolysis and downstream effects of disruptions to pH$i$ have been linked to disruptions in mitochondrial distribution and microfilament organisation in addition to altering the nuclear: cortical ratio of mitochondria within the embryo (Squirrell, et al., 2001). Hence, regulation of pH$i$ within an optimal range is vital for normal oocyte and embryo development.

**1.4.4 Oxygen**

Analysis of oxygen concentrations within the oviduct and uterus of hamster, rabbit and rhesus monkey have revealed that oxygen concentration lies in the range of 2 - 8 % (Fischer and Bavister, 1993, Maas, et al., 1976, Mastroianni and Jones, 1965). This was
in contrast to atmospheric oxygen concentration (~20 %), used traditionally in tissue cultures, which is considered hyperoxic. The first report of the beneficial effects of culturing embryos at physiological (normoxic) oxygen concentrations (5 %) was published in 1971, where Whitten demonstrated that mouse pronucleate oocytes survived and developed to the blastocyst stage when cultured at this level, whereas all pronucleate oocytes arrested when cultured under 20 % oxygen (Whitten, 1971). Subsequently, the culture of other mammalian embryos under physiological oxygen concentrations (5 - 7 %), was shown to improve embryo development in the sheep (Tervit, et al., 1972), cow (Thompson, et al., 1990), rabbit (Li and Foote, 1993), human (Kovacic and Vlaisavljevic, 2008, Meintjes, et al., 2009) and mouse (Quinn and Harlow, 1978), as opposed to culture under atmospheric oxygen. Even exposure to 20 % oxygen for as little as one hour followed by culture in 5 % oxygen, resulted in significant developmental delays at the morula and blastocyst stage in the mouse (Pabon, et al., 1989, Umaoka, et al., 1992). Consistent with these data, time-lapse microscopy observations of embryos confirmed that mouse pronucleate oocytes cultured in 20 % oxygen were delayed at the first cleavage stage by 0.45 h, and the delay was extended to 3.2 h by the third cleavage stage (Wale and Gardner, 2010). The same study showed that partial or complete culture in 20 % oxygen resulted in significant reduction in blastocyst cell numbers compared to embryos cultured under 5 % oxygen and that switching from 20 % to 5 % oxygen at the morula stage was unable to alleviate the perturbations induced by 20 % oxygen during the pre-compaction stages (Wale and Gardner, 2010). Similarly, time-lapse microscopy observations of human embryos cultured under 20 % oxygen reveal a delay in cleavage divisions from 4- to 8-cell stages, as compared to embryos cultured under 5 % oxygen (Kirkegaard, et al., 2013a).

In addition to negatively affecting embryo development to the blastocyst stage and reduction in blastocyst cell numbers, the culture of mouse embryos in the presence of atmospheric oxygen decreases fetal development after blastocyst transfer, when compared to blastocysts cultured in 5 % oxygen (Karagenc, et al., 2004). Furthermore, analysis of mouse blastocyst gene expression show that when mouse pronucleate oocytes were cultured to the blastocyst stage in 20 % oxygen conditions, there were greater perturbations in their global pattern of gene expression, as compared to embryos
cultured under 5 %, which closely resembles gene expression patterns of in vivo derived embryos (Rinaudo, et al., 2006). Analysis of the blastocyst proteome profiles generated from culture under 5 % similarly resemble that of in vivo produced embryos, whereas proteome profiles of embryos cultured under 20 % oxygen result in a down-regulation of ten proteins (Katz-Jaffe, et al., 2005). In addition to these transcriptomic and proteomic alterations, culture under hyperoxic versus normoxic conditions alters carbohydrate and amino acid utilization (Wale and Gardner, 2012a). Pre-compaction embryos consume significantly less pyruvate when cultured under 20 % oxygen as compared to culture under 5 % oxygen, and post-compaction embryos consume significantly less glucose. Decreased consumption of each pyruvate and glucose has been correlated to a decrease in embryo development and viability of human pre-compaction (Conaghan, et al., 1993a), and post-compaction embryos (Gardner, et al., 2011), as well as mouse post-compaction embryos (Lane and Gardner, 1996). Amino acid utilization is altered when embryos are cultured in 20 % oxygen, resulting in aspartate, glutamine and serine consumption by the pre-compaction embryo as opposed to production (Wale and Gardner, 2012a). Of significance, aspartate uptake is decreased and has been implicated in carbohydrate metabolism. This will be discussed further in Chapter 1.5.3.

Interestingly, despite evidence of detrimental effects of oxygen, preimplantation embryos of the human and other mammalian species have historically been cultured under atmospheric oxygen (~20 %) (Edwards, et al., 1981), as it follows a protocol derived from earlier somatic cell tissue culture techniques (Ham and Puck, 1962). Several studies have indicated that human embryo culture under low oxygen is not beneficial (Dumoulin, et al., 1999, Kea, et al., 2007, Kovacic, et al., 2010). In contrast, a more recent meta-analysis of studies on the effects of oxygen concentration and IVF outcomes concluded that embryo culture under low oxygen improves the success rate of IVF (Bontekoe, et al., 2012). Furthermore, given the available data of altered genetic, transcriptomic, proteomic, metabolic, developmental, implantation and post-implantation outcomes using 20 % oxygen, it follows that embryos of all mammalian species, including the human, should be cultured under 5 % oxygen. Subsequently,
conclusions drawn regarding the physiology of the embryo must take into consideration
the concentration of oxygen in which the embryo was cultured.

1.5 Metabolism of the preimplantation embryo

The preimplantation embryo metabolizes different energy sources at the pre- and post-
compaction stages, which coincides with the time the embryo enters the uterine lumen.
In the pre-compaction stages of development, the pronucleate oocyte and cleavage stage
embryo has a relatively quiescent metabolism, attributed to low levels of biosynthesis
and no net growth. This is a contrast to the post-compaction embryo, which is
characterized by a dramatic increase in metabolism, due to the energetically costly
processes of cavitation, TE proliferation and hatching. The key energy substrates that
regulate these processes are discussed below.

1.5.1 Carbohydrates: Pyruvate, glucose and lactate metabolism

Somatic cell glucose metabolism occurs via the Embden-Meyerhof (glycolysis)
pathway and subsequently, through the Krebs cycle to produce approximately 30
adenosine triphosphate molecules (ATP) from cellular respiration (Rich, 2003). In
contrast, the pronucleate oocyte and cleavage stage embryo are not yet able to utilize
glucose, and fail to develop in vitro if glucose is the sole substrate (Biggers, et al., 1967,
Brinster, 1965b). This is due to a higher ATP: Adenosine diphosphate (ADP) ratio in
the early embryo, as it has minimal cellular growth and does not require high energy
levels to undergo initial cleavage divisions. A high ATP: ADP ratio allosterically
inhibits phosphofructokinase (PFK), thus blocking the glycolysis pathway (Barbehenn,
et al., 1974, Leese, et al., 1984). Pyruvate is mostly converted directly to lactate, by
lactate dehydrogenase (LDH), while a small percentage enters the Krebs cycle for
energy production (Gott, et al., 1990). Consistent with their elevated concentrations in
oviduct fluid, pyruvate and lactate are therefore required to support development to
compaction (Brinster, 1969, Wales and Whittingham, 1973, Wales and Whittingham,
1970). Cumulus cells surrounding the ovulated oocyte in the human and mouse have
been shown to metabolize glucose into pyruvate and lactate (Gardner, et al., 1996,
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Gardner and Leese, 1990, Leese and Barton, 1985), contributing to the pool used by the pre-compaction embryo. This is of significance as it has been shown that the relative ratios of pyruvate to lactate directly affect the ratios of reduced and oxidized forms of nicotinamide adenine dinucleotide (reduced form; NADH, oxidized form; NAD\(^+\); NADH to NAD\(^+\) ratio), which regulates the redox state of the embryo (Lane and Gardner, 2000, Rieger, 1992). NAD\(^+\), produced upon conversion of pyruvate to lactate, is also required for the Embden-Meyerhof pathway to proceed, and the availability of which will affect the flux of nutrients metabolised through other cytoplasmic pathways, as well as mitochondrial pathways. In support of this, it was shown that a greater proportion of 2-cell embryos develop when both pyruvate and lactate are present as nutrient sources as compared to either source alone (Brinster, 1965b).

Up to the 8-cell stage in single mouse embryos cultured in vitro, pyruvate and lactate, instead of glucose, are utilized for energy production (Gardner and Leese, 1986). This then switches to glucose utilization, when the embryo reaches the 8-cell to morula stage, which correlates to the time it enters the uterus (Gardner and Leese, 1986, Leese and Barton, 1984). This metabolic switch represents an adaptation to the increased energy demands of compaction, TE differentiation, mitosis, and cavitation during blastocyst formation (Gardner, 1998, Gardner and Leese, 1988). Specifically, the ratio of ATP to ADP decreases as a result of the increased ATP usage, which subsequently leads to a positive allosteric effect on PFK, that facilitates glucose metabolism via glycolysis (Leese, et al., 1984). Additionally, activation of the embryonic genome allows utilization of other substrates such as malate and α-ketoglutarate via the Krebs cycle from the 2-cell stage onwards (Biggers, et al., 1967).

At the blastocyst stage, around 50 % of glucose is converted to lactate, or diverted to other cytoplasmic pathways, such as the pentose phosphate pathway (PPP) rather than through the Krebs cycle and electron transport chain (ETC) (Gardner and Leese, 1990, Lane and Gardner, 1996, Lane and Gardner, 1998, Wales, 1986). This is often described as aerobic glycolysis, and was first identified in highly proliferative cells such as cancer cells, lymphocytes and intestinal epithelial cells by Otto Heinrich Warburg, and termed
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the Warburg Effect (Hanson and Parsons, 1977, Loos and Roos, 1973, Warburg, 1956). In addition to cancer cells, aerobic glycolysis has also been identified in other cell types, such as embryonic stem cells (Harvey, et al., 2014, Turner, et al., 2014). Although energetically not as efficient as complete conversion to water and carbon dioxide, aerobic glycolysis ensures that reducing equivalents in the cytoplasm are available for biosynthetic pathways, such as DNA replication, RNA transcription (Gardner, 1998), and production of reduced glutathione, an intracellular antioxidant (Nasr-Esfahani and Johnson, 1992). In order for aerobic glycolysis to proceed, cytosolic NAD\(^+\) needs to be regenerated via cytoplasmic LDH. A secondary means of generating cytosolic NAD\(^+\) is via the MAS, and it has been shown that tumours undergoing aerobic glycolysis also have high levels of MAS activity (Greenhouse and Lehninger, 1977). The key regulatory enzyme of glycolysis is pyruvate kinase (PK) and an isoform of this enzyme; pyruvate kinase enzyme M2 (PKM2) has been identified in cancer cells (Christofk, et al., 2008) and the mammalian blastocyst (Redel, et al., 2012). Significantly, this particular isoform of PK has been shown to promote aerobic glycolysis and anabolic metabolism, further contributing to lactate production (Christofk, et al., 2008). Lactate itself has other roles, such as establishment of low pH around the embryo, which facilitates trophoblast invasion and may promote blastocyst implantation processes (Gardner, 2015b).

1.5.2 Amino acid metabolism

The oocyte and preimplantation embryo has the ability to transport and metabolize amino acids (Van Winkle, 1988) and creates an endogenous and specific pool within the embryo (Schultz, et al., 1981). Although initial culture experiments omitted the use of amino acids, it is evident that amino acids are highly beneficial to mammalian embryo development and regulation of embryo metabolism \textit{in vitro} (Bavister and Arlotto, 1990, Gardner and Lane, 1993, Gardner and Lane, 1996, Takahashi and First, 1992). When mouse embryos are exposed to a collection medium lacking amino acids for only brief periods (~5 min), they have a lower rate of blastocyst development, (Gardner and Lane, 1996), highlighting their importance in maintaining survival. \textit{In vivo} derived embryos
are exposed to abundant extracellular pools of amino acids in the reproductive tract, with oviduct fluid containing more amino acids as compared to uterine fluids (Gardner and Leese, 1988, Harris, et al., 2005). In relation to metabolism, glutamine is an important amino acid during pre-compaction embryo development (Carney and Bavister, 1987, Petters, et al., 1990). Glutamine can release 2-cell mouse embryos from developmental arrest (Chatot, et al., 1989) and significantly increase levels of blastocyst formation, hatching and TE cell proliferation (Lane and Gardner, 1997a). When present as the sole amino acid, glutamine is taken up and metabolized by preimplantation embryos (Gardner, et al., 1989) and can substitute for pyruvate in the Krebs cycle via α-ketoglutarate, following conversion to glutamate. The non-metabolic functions of specific amino acids have been discussed in detail in Chapter 1.4.2, and the following is specific to the amino acid aspartate, and its implication in carbohydrate metabolism.

1.5.3 Aspartate and the malate-aspartate shuttle

Aspartate is one of the most metabolically important amino acids in the preimplantation embryo (Brinster and Thomson, 1966, Lamb and Leese, 1994, Wale and Gardner, 2012a), and it has been demonstrated that exogenous aspartate increases the activity and metabolism of lactate as an energy source by the embryo (Lane and Gardner, 2005a). Aspartate is also transported into the Krebs cycle via the MAS across the impermeable inner mitochondria membrane for the production of ATP via oxidative phosphorylation (Fig 1.3). The function of the MAS is important, as it enables regeneration of NADH inside the mitochondrial matrix and NAD⁺ in the cytoplasm. As the embryo undergoes aerobic glycolysis, cytosolic NAD⁺ is produced via the conversion of pyruvate to lactate,
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**Figure 1.3:** Malate-aspartate shuttle. Schematic diagram of reactions in the malate-aspartate shuttle, which results in production of NAD\(^+\) in the cytosol and transfer of electrons from NADH across mitochondrial membrane.

The enzyme, malate dehydrogenase catalyzes oxaloacetate and NADH to produce malate and NAD\(^+\). Malate is formed via attachment of two electrons and H\(^+\) and is transported from the cytosol into the mitochondrial matrix. Within the cytosol, malate is converted by malate dehydrogenase into oxaloacetate, with the reformation of NADH and release of H\(^+\) for the electron transport chain. Oxaloacetate is then converted by glutamic oxaloacetic transaminase 1, Got1 (also known as aspartate aminotransferase) to aspartate, with the concomitant transformation of glutamate to α-ketoglutarate so as to supply an amino radical to oxaloacetate to form aspartate. Finally, aspartate is exported from the matrix to the cytosol and converted by aspartate aminotransferase to oxaloacetate. Hence, formation of NAD\(^+\) in the cytosol can be used for metabolism purposes, and formation of NADH in the mitochondrial matrix can be used to pass electrons to the electron transport chain for ATP production.
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![Diagram of metabolic pathways involving malate, oxaloacetate, glutamate, and aspartate.](image-url)
but importantly, the MAS can also regenerate NAD\(^+\), thus maximising the production of ATP for embryo development (Lane and Gardner, 2005a). Inhibition of the MAS in the pre- and post-compaction stages significantly reduces blastocyst development and quality, as well as subsequent fetal and placental growth (Mitchell, et al., 2009, Wakefield, et al., 2011). In addition, perturbation of the MAS during preimplantation embryo development has been shown to alter gene expression in the neonatal brain (Fullston, et al., 2011).

Aspartate is the key substrate involved in the MAS pathway, and is also the most highly consumed amino acid in mouse preimplantation embryos, cow and pig blastocyst (Humpherson, et al., 2005, Lamb and Leese, 1994, Lee, et al., 2015, Orsi and Leese, 2004, Wale and Gardner, 2012a). This has been determined using methods including high-performance liquid chromatography (HPLC) or liquid chromatography mass spectrometry (LC-MS) to determine global amino acid metabolism, which requires grouping of embryos and/ or long incubation periods (24 – 48 h)(Brison, et al., 2004, Lamb and Leese, 1994, Wale and Gardner, 2012a). Previously, consumption of radiolabelled amino acids (e.g. leucine and glycine) have been investigated by Brinster (1971) and determined to stimulate embryo culture, however, this requires culture of embryos in a medium containing only the radioactive labelled amino acid of interest, which may result in altered amino acid metabolism in the embryo due to lack of supporting nutrients (Brinster, 1971). With the exception of glutamine (Gardner, et al., 1989), measurement of individual amino acid metabolism, and in particular, aspartate metabolism have not been investigated yet. In addition to its contribution to the activity of the MAS, aspartate may also be involved in ammonium sequestration via its conversion to asparagine, catalysed by asparagine synthetase (Wale and Gardner, 2013).

In summary, substrates of the MAS pathway play multiple roles in the complex metabolic network, and further work is warranted to fully understand the significance and impact of MAS on embryo development and viability. However, current techniques (HPLC, LC-MS) are unable to target aspartate as an amino acid of interest, and insensitivity of these techniques does not facilitate measurement of aspartate metabolism in a single embryo.
1.5.4 Ammonium and ammonium toxicity

The effects of ammonium are directly related to amino acid metabolism, but also represent an important biochemical factor in suboptimal culture systems that have multiple negative impacts on embryo development and viability. Ammonium is released as a by-product of embryo amino acid metabolism, with mouse pre-compaction embryos producing approximately 0.1 pmol ammonium per embryo per hour, and increasing by 10-fold at the blastocyst stage (Gardner and Lane, 1993). In humans, ammonium production at the blastocyst stage is even higher, and has been determined to be around 33.1 pmol per embryo per hour (Gardner, et al., 2001). The beneficial effects of amino acids in embryo culture medium have been effectively demonstrated, however amino acids spontaneously break down in a time-dependent manner to form embryo-toxic levels of ammonium under standard culture conditions (Heeneman, et al., 1993, Vickery, et al., 1935, Virant-Klun, et al., 2006), with glutamine contributing the most to the break down (Tritsch and Moore, 1962, Vickery, et al., 1935). Media renewal is a method of preventing build up of ammonium to toxic levels, and Gardner and Lane (1993), who showed that change of media following the first 48 hours of development (pronucleate oocyte to morula stage in the mouse) can alleviate this ammonium build up without altering blastocyst formation, while also allowing inclusion of both essential and non-essential amino acids for the corresponding changes in post-compaction amino acid metabolism (Gardner and Lane, 1993). Replacement of glutamine with more stable isomers such as alanyl-L-glutamine or glycyl-L-glutamine furthermore significantly reduces the concentration of ammonium produced in culture media, to 20.2 µM, following 120 h of embryo culture (Biggers, et al., 2004a, Lane and Gardner, 2003, Summers, et al., 2005).

The toxicity of ammonium to somatic cells and embryos is well documented. Somatic cell metabolism produces ammonium as a by-product and its accumulation results in detrimental effects such as reduction in cell proliferation (Visak, et al., 1972). The toxicity of ammonium has also been demonstrated to result in cell death, increased oxidative stress and inhibited cell metabolism (Grivennikova, et al., 2008, Seo, et al.,
The dynamic in vivo environment of the female reproductive tract ensures that there is limited build up of ammonium (Lane and Gardner, 1995). However, current in vitro culture carried out in static media has an inevitable build up of ammonium from amino acids following an extended period of incubation at 37 °C (Gardner and Lane, 1993). Detrimental effects of ammonium on embryo culture and development include negative effects on blastocyst development, metabolism, embryo viability and gene expression (Gardner, et al., 2013, Lane and Gardner, 1994, Lane and Gardner, 2003, Zander, et al., 2006). Ammonium ions added directly to culture medium as ammonium chloride inhibit embryo development when concentrations are above 150 µM, with complete inhibition of blastocyst formation at 5 mM (Gardner and Lane, 1993). Blastocyst cell numbers are also significantly decreased when ammonium concentration is above 75 µM with further decrease in a dose-dependent manner with higher ammonium concentrations (Gardner and Lane, 1993).

Zander et al. (2006) found that the pre-compaction embryo mouse embryo is more sensitive to ammonium as compared to the post-compaction embryo, showing decreased cell numbers, but the rate of development to blastocyst was not affected (Zander, et al., 2006). This is interesting as exposure to ammonium has a negative impact on viability, yet the timing of development to blastocyst appears unaffected (Zander, et al., 2006). This may be due to the limited number of observations of embryo morphology conducted only at certain time points (e.g. 19, 43, 74 and 91 h of culture), and continuous observation under time-lapse analysis may reveal developmental delays.

Additionally, ammonium negatively affects fetal growth (Lane and Gardner, 1994) by down regulating the metabolism of aspartate by disrupting MAS activity (Lai, et al., 1989). In fact, ammonium has also been found to perturb pyruvate metabolism and glycolysis at all stages of mouse embryo development (2-cell, 8-cell and blastocyst) (Lane and Gardner, 2003). Other studies have found similar negative effects on cultured embryo development in the human and sheep (Virant-Klun, et al., 2006, Yuan and Krisher, 2010). Further experiments have revealed that ammonium can also affect embryo pH regulation and gene expression (Lane and Gardner, 2003, Zander, et al., 2006). Even at low concentrations (18.8 µM), embryos display a significantly decreased
level of pH compared to control embryos and exhibits further significant decreases in pH with increasing concentrations of ammonium (Lane and Gardner, 2003). Hence ammonium can alter basic embryo homeostasis.

Following gradual break down of amino acids in modified mouse tubal fluid (mMTF) culture media over a typical culture time (96 h), levels of ammonium were measured to increase linearly with time and reached a final concentration of 300 µM (Gardner and Lane, 1993). The production of ammonium may vary in different embryo culture media due to different amino acid compositions, however similar patterns of break down have been shown in potassium simplex optimized medium with amino acids (KSOM-aa) medium (Biggers, et al., 2004b). Of note, culture of mouse embryos in 300 µM of ammonium resulted in 37 % exencephalic fetuses (Lane and Gardner, 1994). Although Sinawat et al. (2003) and Biggers et. al (2004) did not observe the same level of fetal abnormality (2.3 % and <1 %), they confirm that the breakdown of glutamine into ammonium had a negative effect on blastocyst cell numbers, with serious downstream consequences on fetal normality (Biggers, et al., 2004a, Sinawat, et al., 2003).

Such differences in results are common, and may be due to differences in the genetic makeup of the mouse strains used, resulting in the discrepancies in this type of data between different groups (Biggers, et al., 2004b). Also, variable culture conditions between the research groups, for example, incubator oxygen (20 % vs. 5 %) and carbon dioxide (5 % vs. 6 %) concentrations, as well as quality of oil overlay may also play a role. In relation to this, it has now been shown that oxygen can affect the ability of mouse blastocysts to regulate exogenous ammonium (Wale and Gardner, 2013). Specifically, blastocysts cultured under 20 % oxygen and ammonium consume glutamine, whereas in the presence of 5 % oxygen and ammonium, mouse blastocysts alleviated ammonium stress by transamination of ammonium to glutamine (Wale and Gardner, 2013). Therefore, the high rates of exencephaly reported in Lane and Gardner’s study may be due to the embryo’s failure to utilize glutamine to alleviate ammonium stress, and is only able to do so when cultured at 5 % oxygen, such as in the study by Biggers et al. (2004a). Consequently, under stress induced by suboptimal
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culture conditions, the embryo may be more vulnerable to ammonium toxicity and subsequent viability will be affected.

1.6 Selection of embryos for transfer

Although there have been several improvements in clinical IVF over the past three decades, leading to routine embryo culture and successful cryopreservation, live births remain low, on average ~30% per transfer (Kupka, et al., 2014, Sunderam, et al., 2015). Despite the dangers of multiple pregnancies (Adashi, et al., 2003, Templeton, 2004), it remains a common practice in many countries to transfer more than one embryo to achieve acceptable pregnancy rates and to meet patient expectations. However, even for countries in which patients undergoes single embryo transfer, patients are subjected to multiple cycles of embryo transfer, with no predictive marker of embryo viability to ensure success of embryo transfer. Hence the identification of an embryo with the highest likelihood of establishing a healthy pregnancy is the primary goal of IVF, and several biological parameters have been considered, including proteomics, metabolomics, analysis of key metabolic pathways and morphokinetics (Gardner, et al., 2011, Katz-Jaffe, et al., 2006b, Mastenbroek, et al., 2007, Meseguer, et al., 2011, Nagy, et al., 2009, Seli, et al., 2010, Tejera, et al., 2012). The ideal technique should be non-invasive, accurate, reproducible and cost-effective.

1.6.1 Morphology

Historically, morphological criteria such as pronuclear morphology, degree of fragmentation and symmetry of blastomeres have been routinely used (Rienzi, et al., 2005). Grading systems have been successfully applied to all stages of human embryo development (Cummins, et al., 1986, Gardner, et al., 2000b, Scott and Smith, 1998, Van Royen, et al., 1999), and relationships between embryo grade and pregnancy outcome
have been established (Montag, et al., 2011). However, selection based on morphology alone is visually subjective and assessment is not always carried out at constant time points or for consistent durations, due to differences in insemination times and, embryology schedules. Hence, even a slight variation in the time of embryo scoring on day 2 may determine when embryos will be at the 2-cell stage or at 4-cell stage. Additionally, it has been shown that morphology of the pre-compaction embryo can change in a short period of time (<2 h)(Montag, et al., 2011), highlighting the technical difficulties associated with visual embryo scoring. Although scoring is conducted at a few discrete time points throughout embryo culture, these limited observations conceal what happens during the intervals between observations (Cruz, et al., 2012). While the passive selection of embryos by extended culture to the blastocyst stage eliminates those embryos that undergo developmental arrest during the early cleavage stages, the morphology of the blastocyst can be variable and subject to blastocoel collapse and expansion. Moreover, embryos that appear normal may be multipronucleate or have chromosomal abnormalities (Munne, et al., 2007). Therefore, assessment of morphology alone fails to convey important information about the physiological mechanisms that regulate pre and post-implantation embryo development.

1.6.2 Preimplantation genetic diagnosis and screening

Genetic analysis of embryos has provided an alternative method of embryo selection. Edwards and Gardner carried out the first known embryo biopsy on rabbit embryos, having successfully sexed live day 5 blastocysts by fixing and staining excised TE cells to visualize the sex chromosomes using fluorescence microscopy (Edwards and Gardner, 1967). The first report of preimplantation genetic diagnosis (PGD) on human embryos was published in 1990, by Handyside and colleagues, who screened 6- to 8-cell human embryos for the X-linked disease Lesch-Nyhan syndrome, culminating in successful normal pregnancies (Handyside, et al., 1990). These cycles of PGD were carried out to prevent transfer of embryos affected by an X-linked disease, using polymerase chain reaction (PCR) to amplify a repeat on the Y chromosome. More than two decades later, PGD is now an established clinical procedure widely applied in IVF worldwide with
over 27 000 cycles documented resulting in the biopsy of over 150 000 embryos (De Rycke, et al., 2015, Harper, et al., 2012). PGD has now evolved to be widely used for screening of IVF embryos for single gene mutations, chromosomal aneuploidies and single nucleotide polymorphisms (Harper, et al., 2012).

With the growing use of PGD for aneuploidy screening purposes, the term preimplantation genetic screening (PGS) was introduced by the ESHRE PGD consortium to specifically refer to techniques used to screen presumed chromosomally normal genetic parents for aneuploidy in embryos, as opposed to preimplantation genetic diagnosis, for cases of one or both parents being known to have a genetic abnormality. Though PGD and PGS has been widely adopted in clinical IVF procedures, the embryo biopsy procedure is highly invasive and involves the molecular analysis of genetic material directly extracted from embryos, commonly from polar bodies, individual blastomeres or TE cells (Dokras, et al., 1990, Handyside, et al., 1989, Verlinsky, et al., 1997). The biopsy procedure involves zona penetration by a micromanipulation needle (Cieslak, et al., 1999), acid digestion using Tyrodes solution (Tucker, et al., 1993), or using laser for thinning or removal of the zona pellucida (Boada, et al., 1998). Most importantly, PGD/PGD only allows elimination of chromosomally abnormal embryos from a cohort, but does not provide information regarding the potential viability of the remaining normal embryos, except for age-related indications.

1.6.3 Proteomics

Screening of the protein composition or secretion in human embryos has also provided an alternative method of embryo screening. Proteomics is the large-scale study of proteins, essential, as they are the main components driving critical functional mechanisms within the cell, such as metabolic enzymes and transporters, signalling proteins in the cell cycle. The embryonic proteome is dynamic and can vary with
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internal or external stimuli (Katz-Jaffe, et al., 2005), and thus analysis of spent culture media consisting of protein production by the embryo (secretome) is a potential method for embryo selection. Previously, detailed analysis of the embryonic proteome was not possible as the sensitivity of traditional methods, including 2D electrophoresis, was low (Latham, et al., 1992, Shi, et al., 1994). Furthermore, large sample sizes were required, such as a sample size of 500 mouse oocytes in one proteomic study (Vitale, et al., 2007) Following the application of spectroscopy tools, such as surface-enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI-TOF), sensitivity and accuracy was greatly enhanced. Proteins to be analyzed via SELDI-TOF are crystallized with UV absorbing compounds and vaporized with a pulsed UV laser beam, through which ions are accelerated through an electric potential and down a flight tube. The time taken for the ion to travel down the tube and its kinetic energy supplied by the electric field can be used to identify respective ions (Jr, et al., 1999). The study of the human and mouse embryonic proteome revealed that secretome profiles were similar between mouse and human, with unique profiles displayed by each stage of embryo development (Katz-Jaffe, et al., 2006b). Correlation of day 5 human secretome data with ongoing blastocyst development identified a 8.5-kDa protein whose abundance increased only in the secretome of developing blastocysts. It was determined that the most likely candidate for this 8.5-kDa biomarker was ubiquitin. Ubiquitin has been implicated in the implantation process, and it has been suggested that its involvement in mouse embryo implantation is via control of activities and turnover of key signalling molecules (Wang, et al., 2004).

Analysis of the human blastocyst secretome has revealed that blastocysts with similar morphology and grading have different protein profiles, and embryos that fail to develop exhibit significantly different protein profiles (Katz-Jaffe, et al., 2006a). Preliminary identification suggested that proteins involved in apoptotic and growth inhibiting pathways are up regulated in these embryos that fail to develop (Katz-Jaffe, et al., 2006a). In addition to mass spectrometry technology, a different approach using protein microarrays identified proteins (CXCL13 and granulocyte-macrophage colony-stimulating factor (GM-CSF)) that is significantly less in the spent media from blastocysts that implanted compared to those that failed, even though these blastocysts
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had similar morphological grading (Dominguez, et al., 2008). GM-CSF deficiency has been linked to adverse fetal and placental development, as well as altered neonatal growth (Robertson, et al., 2001, Sjoblom, et al., 2005).

Despite these advances in the field of proteomics, secretome analysis is impaired by the high concentrations of albumin, immunoglobins and other serum proteins present in commercial culture media, which makes it difficult to identify low levels of proteins, such as ligands or growth factors. Furthermore, the applicability of proteomics in clinical IVF is difficult, as it requires expensive equipment with standardized methodology of spent media collection and storage.

1.6.4 Metabolomics

Metabolomics is the systematic analysis of metabolites present in culture media that represents the end product of cellular metabolic processes, determined by gene expression. The use of metabolomics in embryo selection identifies and quantifies the change of a collective profile of metabolites of relevance to embryo selection, as opposed to a specific target molecule. Several quantitative spectroscopy platforms have been used, for example, Near Infrared (NIR) (Botros, et al., 2008, Nagy, et al., 2008), Raman (Seli, et al., 2007), and proton nuclear magnetic resonance (NMR) (Seli, et al., 2008) techniques. By measuring the absorption spectra of spent media from embryos that resulted in pregnancy against embryos that failed to implant, an algorithm was applied to provide a viability score (Botros, et al., 2008, Brison, et al., 2007, Seli, et al., 2007). In a retrospective study conducted by Seli et al. (2007), the viability scores of day 3 embryos correlated with pregnancy outcome (Seli, et al., 2007). Similarly, a correlation was observed between blastocyst viability score and successful pregnancy outcome (Ahlstrom, et al., 2011). The morphology of embryos and resulting viability score determined via NIR spectroscopy were also compared and proved significantly different, where the accuracy determined by metabolomic profiling to be 53.6 % as compared to morphological section, which was 38.5 % (Vergouw, et al., 2008),
highlighting that morphology does not necessarily convey all of the biological information related to embryo physiology.

Following the success of these retrospective studies, prospective randomized controlled trial (RCT) studies have been conducted to test the use of metabolomics as an embryo selection assay. However, the results of these RCT’s have shown no significant difference between control and study groups (Hardarson, et al., 2012, Sfontouris, et al., 2013), resulting in early termination of one trial (Hardarson, et al., 2012). The combination of metabolomics with traditional morphological scoring was determined to have a very small chance of improving pregnancy rates (Vergouw, et al., 2014). It was reported that instrument variability, threshold of analytical signal in the medium, instrument signal to noise ratio and variability between clinics might have impacted on development of a robust algorithm and subsequent failure of the application. Hence, there is a requirement for more robust and sensitive equipment to overcome these problems, as well as the development of highly reliable algorithms accounting for all possible bias.

1.6.5 Carbohydrate metabolism and relationship to embryo viability

Indirectly measurement of the consumption of specific substrates and release of products into the culture medium can be performed at the single embryo level, using biochemical principles. In particular, non-invasive measurement of metabolism can be used as a technique for determining embryo viability and has been one of the earliest techniques used to predict embryo viability in most mammalian species (Gardner and Leese, 1987, Renard, et al., 1980). The metabolism of an embryo significantly modifies the in vitro culture environment based on its given substrates (Fig. 1.4). Therefore, analysis of exhausted culture medium substrates reveals the metabolic state of the embryo.
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Figure 1.4: Example of incubation drop with a blastocyst metabolizing media
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At the pre-compaction stage, an inverse relationship, of which lower pyruvate consumption (22.9 ± 1.0 pmol/embryo/h) measured for day 1 to 2 embryos was shown to have a higher blastocyst development rate and pregnancy outcome as compared to embryos with an increased pyruvate consumption (~27.1 ± 0.6 pmol/embryo/h) (Conaghan, et al., 1993b). Despite this correlation, a wide range of pyruvate uptake amongst embryos was reported, with overlapping confidence limits, and it was concluded that pyruvate as a sole criterion was not suitable as a biomarker of viability (Conaghan, et al., 1993b). In the mouse, it has been shown that there is a significant difference in glucose uptake between viable and nonviable blastocysts (Lane and Gardner, 1996). It was further demonstrated in the human, that the level of glucose consumption is related to blastocyst viability and the sex of the embryo, with female embryos consuming higher levels of glucose, possibly due to two active X chromosomes which results in higher levels of enzyme glucose-6-phosphate dehydrogenase (G6PDH), a rate-limiting enzyme in glucose metabolism through the PPP (Gardner, et al., 2011).

As the embryo undergoes aerobic glycolysis at the blastocyst stage, almost all of the glucose consumed is converted to lactate, so glycolytic rate can be related directly to a 1 is to 2 ratio each (Lane and Gardner, 1996). A higher glucose utilization and subsequent lactate production, resulting in a low glycolytic rate, is related to higher viability of mouse blastocysts (Gardner, et al., 1996, Gardner and Leese, 1987). In vivo mouse blastocysts have a lower glycolytic rate of ~40 %, while in vitro cultured blastocysts have a wide range of 41 - 280 % (Gardner, et al., 1996, Gardner and Leese, 1990, Gardner and Sakkas, 1993), with percentages greater than 100 representing excessive lactate production. In a study by Lane and Gardner (1996), blastocysts were selected for transfer to pseudopregnant recipient mice to determine subsequent viability of blastocysts based on three categories; morphology, blastocysts with low glycolytic rate similar to in vivo blastocysts, and blastocyst with abnormally high glycolytic rate. Their results showed that based on morphology alone, only ~20 % of blastocysts implanted. However, when blastocysts with a low glycolytic rate were selected, 80 % of these blastocysts implanted as compared to 6 % of blastocysts with abnormally high glycolytic rate (Lane and Gardner, 1996). An abnormally high glycolytic rate is due to
low glucose consumption and high lactate production, and it was hypothesized that these blastocysts may be unable to implant in the uterus due to premature exhaustion of glycogen stores or fatty acid stores, leading to the abnormally high glycolytic rate (Lane and Gardner, 1996).

This has been confirmed in humans, where glucose consumption is significantly higher by embryos that went on to form blastocysts as compared to embryos that arrest (Gardner, et al., 2001). Similarly, a higher 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 50 patients that had a single blastocyst transferred, 56% resulted in a live birth rate, which is significantly higher than the average reported clinical IVF live birth rate of ~30% (Kupka, et al., 2014, Sunderam, et al., 2015). Of note, the relationship between glucose uptake and subsequent viability is independent of blastocyst morphology, suggesting that morphology alone does not convey reliable information regarding the implantation potential of the embryo. Data reported by Gardner et al. (2011) also reinforces conclusions drawn from mouse glucose metabolism and transfer experiments, and the potential of using glucose uptake as a biomarker of viability. Interestingly, the study by Gardner et al. (2001) reported that there was a considerable spread of glucose consumption values for individual human blastocysts of the same morphological grade, scored at the same time, from the same patient, thereby supporting the need for other quantitative biomarkers of viability (Gardner, et al., 2001).

1.6.6 Amino acid metabolism and relationship to embryo viability

Amino acid metabolism of the embryo has also been shown to predict subsequent viability. In a study to determine global amino acid turnover using HPLC, Houghton et al. (2002) found that human embryos that develop to blastocyst stage have a lower amino acid turnover compared to the embryos that arrest (Houghton, et al., 2002a). Of
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those embryos that developed to the blastocyst stage, 95% displayed a significant level of turnover specifically for alanine, arginine, glutamine, methionine, and asparagine (Houghton, et al., 2002b). Using a similar method, Brison et al. (2004) found that the turnover of asparagine, glycine and leucine was significantly correlated with higher pregnancy and live birth rate (Brison, et al., 2004). With the exception of asparagine, the amino acids that were predictive of viability were different to those that predicted blastocyst development. Upon comparison of experimental conditions, Houghton et al. (2002) examined embryo amino acid turnover between day 2 to 3, whereas Brison et al. (2004) examined the same between day 1 to 2. Of note, the human embryonic genome is not activated till the 8-cell stage (day 3), which may explain the difference in results between these two studies. Furthermore, it has now been shown that oxygen concentration significantly alters amino acid metabolism in the mouse, with pre-compaction embryos exhibiting a greater amino acid utilization in 20% oxygen as compared to those cultured under 5% oxygen (Wale and Gardner, 2012a). Consequently, the amino acids identified to be predictive of embryo viability by Brison et al. (2004)(asparagine, glycine and leucine), requires validation, as embryos used in this study were cultured under 20% oxygen. Interpretation of past and future research must take into consideration the culture conditions of the embryo, and future experiments should be conducted under optimal culture conditions in order to reflect the true amino acid profile of the embryo.

1.6.7 Developmental kinetics of the preimplantation embryo in vitro

The programming and mechanisms that regulate the sequence of events of preimplantation embryo development are precisely timed, and loss of viability is often associated with delay or complete arrest. Recently, there has been renewed interest in quantitation of cell division times as a predictor of embryo viability. Timing of early cleavage events correlates with embryo quality and subsequent pregnancy rates in the human (Edwards, et al., 1984, Lundin, et al., 2001, Sakkas, et al., 1998, Shoukir, et al., 1997). Assessment of these cleavage events is usually conducted by observations at specific time points, which may overlook important changes in developmental kinetics,
as pronuclear and embryo morphology can change within a short period of time (<2 h) (Montag, et al., 2011). Though frequent evaluation outside the incubator enables assessment of developmental events, it exposes the embryos to undesirable changes in environmental conditions such as gas composition, temperature and humidity (Zhang, et al., 2010), hence traditional observations are conducted sparingly. The integration of a high-frequency imaging system within the incubator allows a more detailed analysis of developmental kinetics, omitting the need for frequent disturbances to the incubator.

The use of time-lapse imaging to observe developmental kinetics dates back to 1929, when development of rabbit embryos was observed (Lewis and Gregory, 1929). Several other studies were conducted on cow, hamster and horse embryos (Gonzales, et al., 1996, Grisart, et al., 1994, Massip and Mulnard, 1980), evaluating development of embryos under various culture conditions. The first documented study of human embryos using time-lapse was in 1997, where Payne et al. (1997) observed that the variability in time taken to achieve the different stages of pronuclei development was different between embryos of good and poor quality based on cell number, shape and size of blastomeres, granularity of cytoplasm and fragmentation (Payne, et al., 1997).

Studies using such technologies have shown that the timing and synchrony of mouse embryo early cleavage stages are tightly regulated and gradually exhibit a wider distribution of cleavage times throughout development (Arav, et al., 2008, Grisart, et al., 1994, Wale and Gardner, 2010). While more advanced cleavage division correlates with blastocyst development in the mouse, it is uncertain which developmental event (e.g. time of division to 2-cell stage) can be used to reliably predict higher viability. Furthermore, a majority of previous studies of developmental kinetics in relation to blastocyst development have been under atmospheric oxygen (20 %) (Arav, et al., 2008, Pribenszky, et al., 2010). Investigations by Wale and Gardner (2010) have shown that atmospheric oxygen in itself can cause a delay in cleavage timing as early as the 1st cleavage stage (to 2-cell) (Wale and Gardner, 2010), due to oxygen toxicity. Similarly, culture of human embryos in atmospheric causes delay in the 3rd cleavage cycle (4- to 8-cell) and a reduced number of embryos that develop to the blastocyst stage, again highlighting the toxicity of atmospheric oxygen (Kirkegaard, et al., 2013a). Although
1. Literature Review

time-lapse imaging is not a novel technology, to date, the characteristics of mouse cleavage timing under ideal culture conditions and subsequent relationships to viability are yet to be fully defined. Additionally, in earlier studies, blastocyst development and formation was often chosen as an experimental end-point, which does not reflect implantation and potential viability.

In humans, several morphokinetic events have been proposed as predictors of blastocyst formation or pregnancy. As definitions of developmental events vary in the literature, for the purpose of this thesis, division is defined as a mitotic event that leads to the formation of two new cells from one cell. In contrast, a cleavage cycle refers to a cleavage division (mitotic event where a larger blastomere divides evenly into two smaller ones - i.e. 1st cleavage cycle yields a 2-cell embryo, 2nd cleavage cycle yields a 4-cell embryo and 3rd cleavage cycle yields a 8-cell embryo). In human embryos, the study by Meseguer et al. (2011) describes a correlate between morphokinetic and pregnancy (time of division to 5-cell stage and interval between division from 2- to 3-cell stage, and 3- to 4-cell stage), as well as identification of a set of negative predictive factors (direct cleavage from 1-to 3-cells, uneven blastomeres size at 2-cell stage, multinucleation at 4-cell stage). Thus, Meseguer et al. (2011) propose a hierarchical selection model to classify embryos according to their probability of implantation (Meseguer, et al., 2011). In another study, Wong et al. (2010) correlated blastocyst formation with the duration of first cytokinesis (defined as appearance of cleavage furrow to complete separation of daughter cells), the interval between 1- to 2-cell stage, and between 2- to 3-cell stage (Wong, et al., 2010). With the exception of the interval between 3- to 4-cell stage, these morphokinetic events correlated to blastocyst development were different to those described by Meseguer et al. (2011). One reason may be that none of the embryos in the study by Wong et al. (2010)’s study were transferred; highlighting that developmental event that correlated with high blastocyst development may not correlate with implantation and viability. Second, the comparison of these studies are complicated by the use of frozen-thawed embryos by Wong et al. (2010). In addition, the negative impact of 20 % oxygen on embryo development has been discussed (Chapter 1.4.4), and embryos in Meseguer et al. (2011)’s study were cultured under 20 % oxygen, as opposed to low oxygen concentrations used in Wong et al. (2010).
Interestingly, Conaghan et al. (2013) combined traditional morphology assessment with the use of a time-lapse system, and significantly improved the embryologist’s ability to identify embryos that would become usable blastocysts (Conaghan, et al., 2013), suggesting that a combination of both selection methods may be used to improve pregnancy rates of day 3 transfers, when the embryo has not developed to the blastocyst stage. Several other human embryo studies have reported other developmental events that correlate to pregnancy rate, such as the kinetics of pronuclear formation and fading (Azzarello, et al., 2012, Chamayou, et al., 2013), the timing between various cleavage stages (Dal Canto, et al., 2012, VerMilyea, et al., 2014), the duration of cleavage stages (Chen, et al., 2013), time to blastocyst formation (Campbell, et al., 2013b), and time to expanded blastocyst (Storr, et al., 2015). Despite identification of numerous morphokinetic markers, comparison between these studies is complicated by the influence of varying culture conditions, as gas composition (Kirkegaard, et al., 2013a), fertilization method (Freour, et al., 2013, Lemmen, et al., 2008), day of transfer, type of morphokinetic imaging system used and types of culture media (Ciray, et al., 2012). Due to specific differences between clinics, hierarchical models may not be easily applicable to all clinics. Recent data have also indicated that the morphokinetic markers may be influenced by patient or treatment factors, such as fertilization method (i.e. IVF vs. ICSI), age of patient or FSH dose (Kirkegaard, et al., 2015b), which challenges data collected from previous studies using time-lapse analysis, as these studies often include more than one embryo per patient, and requires re-interpretation of data that normalizes for grouping of embryos.

In an attempt to combine retrospective data collected from multiple clinics, a more recent study by Meseguer et al. (2012) uses a significantly larger cohort of 1474 embryos (as compared to 522 embryos in the 2011 study), in a multicentre comparison of implantation rates between embryos selected by either their hierarchical model or conventional morphology. Their results indicated that use of the morphokinetic model demonstrated an increased pregnancy rate of 15.7 % per embryo transfer (Meseguer, et al., 2012). These promising results led to a prospective RCT using the same model, reporting a more modest increase of 9.2 % per embryo transfer (Rubio, et al., 2014). However, in that study, embryos that were transferred based on conventional morphology were cultured in standard incubators, and hence, there may be potential
confounding of data compared to use of an undisturbed culture conditions in the time-lapse system. Additionally, embryos were cultured under atmospheric oxygen and implantation data was collected from both day 3 and day 5 embryo transfers (Rubio, et al., 2014). In another RCT, morphokinetic markers (duration of first cytokinesis, duration of the 3-cell stage and direct cleavage to 3-cells) were correlated to blastocyst development, however were not predictive of blastocyst implantation (Kirkegaard, et al., 2013b). The same group reported in another study that 50.6 % of embryos that were classified un-usable or low potential still resulted in pregnancy (Kirkegaard, et al., 2014), despite a significant increase in implantation rates using the morphokinetic prediction model. Due to the unavoidable variables (e.g. patient age, stimulation protocol, culture system) that come with data collected from human embryo culture, the use of developmental kinetics as a viability marker may benefit from further research conducted as a multicentre comparison, in parallel with studies using the same models in more detailed animal studies to enable better control of these variables.

1.7 Conclusion

As a means of increasing IVF success rates, the common practice of transferring more than one embryo has resulted in dramatically increased incidence of multiple pregnancies and its associated risks and implications. As such, the need for a reliable, accurate biomarker of the single most viable embryo has led to considerable focus on several areas, such as developmental kinetics and metabolism of the embryo, and both of these have since been clinically translated. In mice, developmental kinetics has only been correlated to blastocyst development (Arav, et al., 2008, Pribenszky, et al., 2010), which does not necessarily translate to viability of the embryo. The underlying reason for variable kinetics within the same cohort of embryos is also unclear, but may be related to suboptimal culture conditions or intrinsic genetic differences in the programmed development competence of the gamete. The in vitro produced preimplantation embryo is also undergoing morphogenesis and differentiation, and so, is highly susceptible to the slightest perturbations in culture conditions. Despite this, the developmental plasticity of the embryo allows adaptation of nutrient utilization and
subsequent blastocyst development. As discussed, atmospheric oxygen (20 %) has a negative impact on metabolism and cleavage rate in embryos (Kirkegaard, et al., 2013a, Wale and Gardner, 2012b), hence, it is important to note that a majority of studies on developmental kinetics of the mouse and human embryo have been conducted under atmospheric oxygen (Arav, et al., 2008, Lemmen, et al., 2008, Meseguer, et al., 2011, Pribenszky, et al., 2010), and conclusions regarding predictive blastocyst development markers from these studies require further investigation, under culture conditions that are closer to physiological conditions.

Independently, carbohydrate and amino acid metabolism, as well as developmental kinetics have been demonstrated to be predictive of embryo viability in the mouse and human. Given that metabolism is fundamental for energy production to support all mechanisms of embryo development, it may be possible that metabolic rates are responsible for regulating developmental kinetics. Additionally, the use of multiple viability markers, as opposed to a single measurement, may plausibly increase the accuracy of embryo selection. However, relationship between viability markers must first be identified and prospectively investigated. Additionally, understanding the transcriptome is essential in interpreting function aspects of the genome, and to date, the expression of genes for metabolism and cytokinesis in kinetically different mouse embryos have not been characterized.
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1.8 Hypotheses and aims

Embryos exhibit a range of cell division rates that may correspond to a range of subsequent levels of implantation potential. The underlying reason for variation in cleavage rates of embryos is unclear, although there is indirect evidence that metabolic control is an important regulator. As metabolism is critical for energy production for cell growth and differentiation, and is directly related to both developmental competence and viability of the embryo, it is therefore hypothesised that cleavage division and blastocyst developmental rates are correlated with metabolism of the embryo. Specifically, carbohydrate, total amino acid utilization and aspartate metabolism will be investigated, based on previous evidence of its importance in determining embryo viability. It is envisaged that an embryo with a glycolytic rate and/or amino acid utilization similar to that of in vivo embryos will exhibit more timely cleavage and higher viability.

1.8.1 Aims

1. To characterize developmental kinetics of mouse preimplantation embryos using quantitative time-lapse analysis
2. To characterize blastocyst metabolism of kinetically different groups of embryos (based on first cleavage division timing) selected with time-lapse imaging and subsequent correlation to embryo viability and normality
3. To identify genes potentially related to the developmental kinetics of the embryo and blastocyst metabolism
4. To investigate single embryo metabolism of aspartate and its relation to glucose, and potential of aspartate as a biomarker for embryo selection
2. Materials and Methods
2. Materials and Methods

2.1. Media preparation

Unless otherwise stated, all media chemicals were purchased from Sigma Aldrich (USA) and quality control tested with a 1-cell mouse bioassay prior to experimental use (Appendix A).

For preparation of culture media, all media components were weighed out (Sartorius Quintix, Australia) in a clean area into plastic ware (BD Falcon; BD Biosciences BD Falcon™, Australia). Ultra-pure water (Milli-Q) was used to make up the media solutions in a laminar flow hood. Stock solutions of respective media components were prepared before media preparation due to small amounts of media required each week for embryo handling and culture (Lane and Gardner, 2004), and preparation is outlined in (Appendix B). All stocks and media were sterilized by filtration through a 0.2 µm syringe filter (Pall Corporation, Australia) and subsequently stored at 4 °C in 5 ml tubes (BD Falcon).

The compositions of all media used in this project are shown in Table 2.1. The media used for oocyte insemination was IVF fertilization media (Table 2.1). Oocytes and embryos were collected in handling medium buffered with 3-(N-morpholino)propanesulphonic acid, and supplemented with 5 mg/ml of human serum albumin (HSA)(GMOPS with HSA; G-MOPS™ Plus, Vitrolife, Sweden). Unless otherwise stated, embryos were cultured in G1.2/G2.2 media (Table 2.1), a sequential system developed and optimized for mouse and human blastocyst culture (Gardner, et al., 2000a). Media changes in a sequential system minimize the adverse effects of toxic ammonium build up in the media generated by embryo metabolism of amino acids and their spontaneous amino acid breakdown at 37 °C. Quality control testing was performed using an amino acid free version of G1.2 (Simple G1.2, Table 2.1). All media were supplemented with HSA at a concentration of 5 mg/ml (denoted as G1.2 with HSA; G2.2 with HSA; G-MOPS with HSA) and pre-equilibrated in a humidified dual-gas incubator at 37 °C with a gas phase of 6 % CO₂, 5 % O₂ and 89 % N₂.
**Table 2.1:** Final concentrations in media solutions

<table>
<thead>
<tr>
<th>Component</th>
<th>IVF Media (mM)</th>
<th>G1.2 (mM)</th>
<th>G2.2 (mM)</th>
<th>Simple G1.2 (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaHCO(_3)</td>
<td>25.0</td>
<td>25.0</td>
<td>25.0</td>
<td>25.0</td>
</tr>
<tr>
<td>CaCl(_2)</td>
<td>1.8</td>
<td>1.8</td>
<td>1.8</td>
<td>1.8</td>
</tr>
<tr>
<td>Glucose</td>
<td>3.2</td>
<td>0.5</td>
<td>3.2</td>
<td>0.5</td>
</tr>
<tr>
<td>Sodium L-Lactate acid</td>
<td>10.5</td>
<td>10.5</td>
<td>5.9</td>
<td>10.5</td>
</tr>
<tr>
<td>Sodium Pyruvate</td>
<td>0.4</td>
<td>0.3</td>
<td>0.1</td>
<td>0.3</td>
</tr>
<tr>
<td>EDTA</td>
<td>-</td>
<td>0.01</td>
<td>-</td>
<td>0.01</td>
</tr>
<tr>
<td>NaCl</td>
<td>95.6</td>
<td>90.1</td>
<td>90.1</td>
<td>90.1</td>
</tr>
<tr>
<td>KCl</td>
<td>5.5</td>
<td>5.5</td>
<td>5.5</td>
<td>5.5</td>
</tr>
<tr>
<td>NaH(_2)PO(_4)</td>
<td>0.5</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>MgSO(_4).7H(_2)O</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>-</td>
</tr>
<tr>
<td>Glutamate</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>-</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>-</td>
</tr>
<tr>
<td>Arginine</td>
<td>-</td>
<td>-</td>
<td>0.6</td>
<td>-</td>
</tr>
<tr>
<td>Cystine</td>
<td>-</td>
<td>-</td>
<td>0.1</td>
<td>-</td>
</tr>
<tr>
<td>Histidine</td>
<td>-</td>
<td>-</td>
<td>0.3</td>
<td>-</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>-</td>
<td>-</td>
<td>0.4</td>
<td>-</td>
</tr>
<tr>
<td>Leucine</td>
<td>-</td>
<td>-</td>
<td>0.4</td>
<td>-</td>
</tr>
<tr>
<td>Lysine</td>
<td>-</td>
<td>-</td>
<td>0.4</td>
<td>-</td>
</tr>
<tr>
<td>Methionine</td>
<td>-</td>
<td>-</td>
<td>0.1</td>
<td>-</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>-</td>
<td>-</td>
<td>0.2</td>
<td>-</td>
</tr>
<tr>
<td>Threonine</td>
<td>-</td>
<td>-</td>
<td>0.4</td>
<td>-</td>
</tr>
<tr>
<td>Thiamine</td>
<td>-</td>
<td>-</td>
<td>0.0042</td>
<td>-</td>
</tr>
<tr>
<td>Pyridoxal</td>
<td>-</td>
<td>-</td>
<td>0.0049</td>
<td>-</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>-</td>
<td>-</td>
<td>0.00296</td>
<td>-</td>
</tr>
<tr>
<td>Gentomycin</td>
<td>10ug/ml</td>
<td>10ug/ml</td>
<td>10ug/ml</td>
<td>10ug/ml</td>
</tr>
</tbody>
</table>
2. Materials and Methods

2.2. Embryo culture

2.2.1. Animals and ethics statement

Mice used in this study were either bred at Monash University Animal Services, Walter and Eliza Hall of Institute of Medical Research or Florey Institutes of Neuroscience and Mental Health, and were housed in animal facilities at the Department of Zoology, the University of Melbourne, with a 12 h light/12 h dark photoperiod, and food and water fed ad libitum. All mouse experimentation was approved by The University of Melbourne, Animal Ethics Committee (AEC). Ethics application ID is 1112188.3.

2.2.2. Oocyte and sperm collection

MII oocytes were collected from pre-pubescent F1 hybrid female mice (C57BL/6 x CBA). 3 to 4 week old females were stimulated through intraperitoneal (IP) injection of 5 IU pregnant mare’s gonadotropin (PMSG) (Intervet, UK) and 5 IU human chorionic gonadotropin (hCG) (Intervet) per mouse. Females were injected with PMSG at 4 pm, followed 50 h later by hCG (6pm). Cumulus-oocyte complexes then were collected 14 h after hCG injection. Sperm was collected from 8 to 12 week old virgin F1 hybrid male mice (C57BL/6 x CBA).

On the morning of oocyte collection, superovulated female mice and a single male mouse were killed by cervical dislocation. The abdomen was sterilized with 70 % ethanol and a small incision was made in the ventral midline and dissected upwards, exposing the peritoneum. The peritoneum was cut using fine scissors to expose the digestive tract, which were moved aside to expose the reproductive tract. The oviducts were isolated carefully and dissected using microforceps (Watchmaker’s forceps, #5) and scissors. Subsequently, oocytes were isolated by rupturing the ampullary region of the oviduct (Gardner and Lane, 2004, Hogan, 1986).
2. Materials and Methods

For oocyte insemination, cumulus-oocyte complexes were collected 14 h post-hCG in 500 µl of equilibrated IVF media in an organ well dish. From there, intact complexes were transferred to a second dish containing a 45 µl drop of equilibrated IVF medium overlayed with 9 ml of Ovoil (Vitrolife).

2.2.3. Sperm collection and capacitation

The caudal epididymides with connecting vas deferens were collected in an organ culture dish containing 500 µl of equilibrated IVF media. A 27-gauge needle was used to puncture 8-10 holes in the isolated epididymides. The dish was then placed in an incubator for 15 min to allow the sperm to swim out. This sperm suspension was then transferred to a 5 ml round bottom test tube, to which 500 µl of equilibrated IVF medium was added, by dripping slowly down the side of the tube to form an overlay. The tube was then returned to the incubator, and left in an upright position for a further hour to allow sperm with higher motility to swim up. A 500 µl aliquot was then collected from the top fraction the tube containing capacitated sperm. Sperm density was then determined using the Makler chamber, with the number of spermatozoa counted in any strip of 10 squares determined as the sperm concentration in millions per ml.

2.2.4. In vitro fertilization

The final count of the capacitated sperm extract from Chapter 2.1.6 was adjusted by dilution in the range of 10-20 million motile sperm/ml, which was further diluted to a final concentration of 1-2 million/ml (5 µl of extract added to 45 µl of IVF medium). Cumulus-oocyte complexes containing MII oocytes were co-incubated with the sperm for 4 h in these 45 µl insemination drops. Following insemination, successful fertilization was determined by visible observation of the pronuclei and 2nd polar body. IVF pronucleate oocytes were then collected using a pulled borosilicate glass pipette with an internal diameter only slightly larger than the pronucleate oocytes to remove
sperm and residual cumulus cells that remained attached to the zona pellucidae. Washed pronucleate oocytes were then washed in G1.2 media with HSA and cultured as per embryo culture protocol (see below 2.2.6).

2.2.5. Pronucleate oocyte collection

Pronucleate oocytes were collected from pre-pubescent F1 hybrid female mice (C57BL/6 x CBA). 3 to 4 week old females were stimulated through intraperitoneal (IP) injection of 5 IU pregnant mare’s gonadotropin (PMSG) (Intervet) and 5 IU human chorionic gonadotropin (hCG) (Intervet) per mouse. Females were injected with PMSG at 12 pm, followed 48 h later by hCG (12 pm). These females were then housed with F1 hybrid male mice overnight and mating was determined the next morning by the presence of a vaginal plug. Mated female mice were killed following procedure outlined above (Chapter 2.2.2). Cumulus-oocyte complexes were collected 22 h post-hCG in 500 μl of pre-warmed G-MOPS with HSA (Vitrolife), under ambient air conditions (0% CO₂ levels), then denuded by addition of hyaluronidase (1 mg/ml, Bovine Testes, Sigma) to the same medium. After 1 min, when cumulus cells began to disperse, pronucleate oocytes were separated by repeated pipetting with a hand-pulled Pasteur pipette with an outer diameter slightly larger than that of the pronucleate oocyte, followed by rinsing 3 times in G-MOPS with HSA and once in pre-equilibrated G1.2 medium with HSA before allocation to culture drops. All oocytes were randomized into sample groups following oocyte collection. Whenever possible, oocytes were divided equally between groups.

2.2.6. Preimplantation embryo culture

Embryos derived from in vitro or in vivo fertilized pronucleate oocytes were cultured in 35 mm or 60 mm petri dishes (BD Falcon) containing 20 μl/ 2 μl droplets of culture
medium (G1.2/ G2.2 with HSA) overlayed with 3.5/ 5 ml Ovoil (Vitrolife), respectively to prevent media evaporation. Each culture dish had a minimum of 1 wash drop to facilitate embryo washing between culture medium. Specific dish set up for each culture procedure is outlined in Appendix D). Incubator conditions were defined above (Chapter 2.1). Pronucleate oocytes were classified as Day 1 post insemination (PI) embryos. Embryos were cultured for a total of 5 days, with media changes from G1.2 with HSA to G2.2 with HSA, performed 48 hours later. For in vivo fertilized embryos, only those that reached the 2-cell stage on Day 2 were allocated to experiments, as indicative of successful fertilization. All embryos were randomized into sample groups following embryo collection. Whenever possible, embryos were divided equally between groups.

2.3. Assessment of embryo development in vitro

Preimplantation embryo morphology was assessed at a magnification of 100X using a Nikon phase contrast microscope fitted with a warming stage previously calibrated to ensure that the temperature within culture droplets are maintained at 37 °C. Embryos were scored based on typical morphologies depicted in Fig 1.1 and Fig 1.2. Successfully fertilized pronucleate were identified by the presence of two pronuclei and extrusion of the second polar body. Cleavage stage embryos were assessed and scored according to the number of clearly visible blastomeres (2- to 8-cell embryos)(Fig 1.1). Compacting embryos were determined by a loss of defined cellular membranes, indicating that tight junctions had formed between cells. Finally, blastocysts are assessed based on the size of the blastocoe1 cavity (<50% early blastocyst, >50% blastocyst) and overall diameter (deemed ‘expanded blastocysts’ when larger than size of morula). Blastocyst hatching was determined upon observation of initial TE extrusion from the zona pellucida, with a fully hatched blastocyst indicated by complete zona absence (Fig 1.2).
2. Materials and Methods

2.4. Assessment of embryo cell numbers

2.4.1. Total cell number staining

Total cell numbers of blastocysts were determined by counting the total number of nuclei of day 5 embryos using the nuclear stain, bisbenzimide (Hoechst No 33258, Sigma, USA). Bisbenzimide bind to adenine-thymine rich regions of DNA and hence, nuclei will be labelled accordingly. Blastocysts were incubated at 37 °C in a solution composed of G-MOPS with HSA, 10% ethanol and 0.1 mg/ml bisbenzimide for 1 h, followed by rinsing in G-MOPS with HSA for 10 min. Stained blastocysts were then mounted in glycerol on glass microscope slides and photographed using a fluorescence microscope (Martin, et al., 1998). Cell numbers were counted using image processing software (Image-J, National Institutes of Health, USA).
2. Materials and Methods

2.4.2. Differential cell number staining

To analyze the number of cells in the ICM and TE, blastocysts were differentially stained as previously described (Handyside and Hunter, 1984), and further detailed in Appendix C. The TE nuclei were labelled with membrane impermeable propidium iodide by permeabilising the cells using pronase to lyse the outer membranes, but keeping the inner membranes intact, allowing inner cell mass cells to remain unexposed. The blastocysts were then counter-stained for all nuclei with bisbenzimide (which is permeable to all membranes but does not displace the propidium iodide bound to the nuclei), mounted in glycerol and photographed under a fluorescence microscope (Martin, et al., 1998). Using this method, blastocyst spreads have TE cells that appear pink (containing both propidium iodide and bisbenzimide) while the ICM cells appear blue (containing only bisbenzimide) (Fig 2.2). Cell numbers were then counted using Image-J program.
Figure 2.1: Fluorescence microscope image of a bisbenzimide-stained blastocyst mounted on a microscope slide. Blue nuclei represent total cell count inclusive of trophectoderm and inner cell mass. (Magnification X400)
2. Materials and Methods

Figure 2.2: Fluorescence microscope image of a differentially stained blastocyst mounted on a microscope slide.

Pink nuclei represent the trophectoderm and blue nuclei represent the inner cell mass. (Magnification X400).
2. Materials and Methods
2.5. Time-lapse imaging and analysis

The developmental kinetics of preimplantation mouse embryos derived from in vitro and in vivo fertilization, were analyzed using an incubator equipped with a microscope and digital camera, set to take photographs every 15 minutes (SANYO InCu-View Live MCO-5M, Japan)(Fig 2.3). The timing of images recorded at onset of key developmental events including pronuclear syngamy, first and subsequent cleavage events (2 cells; t2, 3 cells; t3, 4 cells; t4, 5 cells; t5, 6 cells; t6), blastomere compaction, cavitation and blastocyst hatching were then determined and expressed as hours PI (Binder, et al., 2012). The duration of syngamy to t2 (syn-t2), duration of second cell cycle (cc2; = t3-t2), duration of interval between 3- to 4-cell (s2) were calculated, as reported in (Meseguer, et al., 2011) and compared to the resultant blastocyst total cell number.

2.6. Analysis of carbohydrate metabolism

Concentrations of glucose and lactate were determined from spent media samples using ultramicrofluorimetry, as detailed previously (Gardner, 2007, Gardner and Leese, 1990, Leese and Barton, 1984). Metabolites are quantitated, using enzymatic reactions coupled to changes in fluorescence of pyridine nucleotide cofactors NADPH or NADH, when produced or consumed during the reaction. These pyridine nucleotides have an absorption maxima at ~340 nm, and when the reduced forms are excited with light in the ultraviolet range, they emit fluorescence at 459 nm and above (Gardner, 2007, Passonneau and Lowry, 1993). To be able to deliver volumes in the nanolitre range, micropipettes were created from borosilicate glass capillaries pulled over a flame. A microforge is used to place a constriction in the pipette, and micropipettes are siliconized with Sigmacote (Sigma) and tritiated water is used to calibrate the pipette (Gardner, 2007). A 20 nl droplet of modified G-MOPS with HSA (containing 0.5 mM glucose) is created, and a single blastocyst is held inside.
Serial 1.0 nl samples of media were removed at regular intervals (every 30 min or 1 h, depending on experiment) during the incubation period. Overall glucose consumption and lactate production is expressed as a percentage (glycolytic rate) on the basis that 2 moles of lactate is produced per mole of glucose consumed (Gardner, et al., 1996). To normalise for differences in blastocyst cell numbers, the rate of glucose consumption and lactate production were divided by number of cells in blastocyst as determined later by either total cell staining or differential cell staining. The concentrations of substrates and reaction conditions used in the assay are listed in Appendix E. The same approach was modified to determine aspartate consumption of individual blastocysts, and optimization, validation and reaction conditions are outlined in Chapter 6 and Appendix E.

For glucose consumption, the following enzymatic glucose assay was used:

\[
\text{Glucose} + \text{ATP} \xrightarrow{\text{Hexokinase}} \text{Glucose 6-phosphate} + \text{ADP}
\]

\[
\text{Glucose-6-phosphate} + \text{NADP}^+ \xrightarrow{\text{dehydrogenase}} 6\text{-phosphogluconate} + \text{NADPH} + \text{H}^+
\]

For measurement of lactate production, the following lactate assay was used:

\[
\text{Lactate} + \text{NAD}^+ \xrightarrow{\text{Lactate dehydrogenase}} \text{Pyruvate} + \text{NADH} + \text{H}^+
\]

For measurement of aspartate production, the following aspartate assay was used:

\[
\text{Aspartate} + \alpha\text{-ketoglutarate} \xrightarrow{\text{Glutamic-oxaloacetic transaminase}} \text{Oxaloacetate} + \text{Glutamate}
\]

\[
\text{Oxaloacetate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{Malate dehydrogenase}} \text{Malate} + \text{NAD}^+
\]
2. Materials and Methods

Figure 2.3: Photograph showing the inside of the SANYO InCu-View Live MCO-5M time-lapse incubator used in this project.

Dishes containing embryos were placed on the turntable, which rotates periodically every 15 minutes. A light source (metal cylinder) located above, shines 0.1W white LED illumination only when taking images, downward through the dishes and table holes to the digital camera located underneath. This allows images to be taken with the same camera using multiple dishes.
2. Materials and Methods

2.7. Analysis of amino acid metabolism

As a further measure of metabolic health of embryos, global amino acid utilization of early blastocysts (Day 4) was performed. Blastocysts were cultured in groups of three in 2 µl drops of G2.2 medium with HSA, dispensed using a positive displacement pipette (eVol, SGE Analytical Science, USA), in order to accurately dispense 2 µl drops. Following 24 h of culture, 1 µl aliquots of the same incubation media, as well as control drop that contained no embryos during this period, were collected and dried using a speed-vac (RVC 2-25, Quantum Scientific, Australia). Amino acid analysis was performed by mass detection, using a triple-quadrupole mass spectrometer (LC-QqQ-MS), as previously described (Wale and Gardner, 2012a).

Culture media samples were derivatized with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC), which enables concentrations of co-eluted fractions of amines to be resolved and quantitated using LC-QqQ by comparison against a calibration standard (Boughton, et al., 2011). A 2.5 mM stock solution was prepared containing the following amino acids; lysine, histidine, asparagine, arginine, taurine, serine, glutamine, glycine, aspartate, glutamate, threonine, alanine, proline, cysteine, tyrosine, methionine, valine, isoleucine, leucine, phenylalanine and tryptophan. Calibration standards for these amino acids were then prepared by diluting the stock solution to 150, 100, 50, 25, 10, 5, 1, 0.5, 0.1, 0.05, and 0.01 μM in water using volumetric glassware. Norleucine (25 μM) was used as an internal standard in borate buffer (pH 8.8) containing sodium borate (200 mM) the antioxidant ascorbic acid (1 mM) and the reducing agent tris(2-carboxyethyl)phosphine (10 mM).

Dried aliquots of spent culture media were resuspended in 10 μl of MilliQ water and 70 μl of borate buffer, vortexed for 20 sec, and centrifuged for 1 min at 18 000G. To this, 20 μl (10 mM) of AQC was then added, vortexed for 20 sec and subsequently warmed on a heating block (Thermomixer, Eppendorf, Australia) with shaking (1000 rpm) for 10 min at 55 °C. The final solution was cooled to room temperature and centrifuged for 1 min, followed by liquid chromatography-mass spectrometry (LC-MS) analysis using
an Agilent 1200 LC-system coupled to an Agilent 6420 ESI-QqQ-MS to determine mass spectra for all 20 amino acids in parallel.

2.8. Analysis of blastocyst outgrowth

As an indicator of blastocyst implantation potential, a plate-based outgrowth assays was used, as previously described (Hannan, et al., 2011), optimized from an earlier method (Cole, et al., 1965). Flat-bottomed 96-well tissue culture dishes were rinsed with sterile phosphate buffered saline (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 PBS containing 4 mg/ml BSA. Wells were then rinsed with PBS and subsequently filled with G2.2 with HSA, overlayed with Ovoil and the plate equilibrated under 6% CO₂, 5% O₂ and 89% N₂ at 37 °C. Expanded and hatching blastocysts were placed into each well and incubated for 72 h. Blastocyst outgrowths were imaged using an inverted epifluorescence microscope (Ti-U eclipse, Nikon) at x10 magnification at 48 h, 66 h and 72 h following blastocysts transfer into the plate, (Fig 2.4a). 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), (Fig 2.4b).
2. Materials and Methods

**Figure 2.4:** Example of blastocyst outgrowth image

a) Blastocyst outgrowth attached to the bottom of a culture plate. b) Outline drawn around blastocyst outgrowth using Image-J software to calculate outgrowth area. (Magnification x10). In the centre of the outgrowth, the dark mass represents inner cell mass and the surrounding are differentiating cell types.
2. Materials and Methods

a) 

b)
2. Materials and Methods

2.9. Mouse surgical procedures

All instruments for use in surgical procedures (vasectomies and embryo transfers) were washed with detergent and soaked in distilled water. Instruments were heat sterilized with an autoclave machine at 121 °C. Between animals, instruments were soaked in 70% ethanol and sterilized with heated glass beads at 250 °C. For surgical procedures, sterile gloves, laboratory coat and a surgical mask were worn and all surgical procedures were performed using a dissecting microscope. The microscope stage was washed with 80% ethanol and a sterile drape placed on the microscope base.

Anaesthesia was induced by one intraperitoneal injection of 75 mg/kg ketamine (e.g. Ketalar) and 1 mg/kg medetomidate (Domitor) in 0.4 to 0.6 ml. In later experiments, anaesthesia was administered by isoflurane inhalation, according to revised Ethics Committee recommendations for improved recovery. Lack of reflex reaction to small incisions and pinching of the tail was used as an indicator of successful anaesthesia, both immediately after anaesthesia and throughout the procedure. If a response was noted the procedure was halted and the animal was administered more anaesthetic before recommencing. The site of incision for both female and male mice was prewashed with 80% ethanol to ensure sterility.

2.9.1. Vasectomies

Male F1 hybrid mice (C57BL/6 x CBA) were vasectomised between 6 and 8 weeks of age, for mating with female recipients to establish pseudo-pregnancy. Male mice were anaesthetized as outlined above (Chapter 2.9), and placed in the supine position on the drape covering the microscope stage. Following swabbing of the scrotal sac using 80% ethanol, a small transcutaneous incision (~2 mm) was made. The fat pad below the caudal epididymis was located and used to gently pull the bursa sac containing the testes through the incision, to expose the testes and vas deferens. Using a fine pair of forceps and a pair of fine scissors, a 1 cm section of the vas deferens was excised. The
Materials and Methods

Testes were then eased back into the body cavity and similar to embryo transfers, the wound was then closed with an absorbable suture (Dexon, Covidien, Australia). Recovery process and monitoring process is detailed above (Chapter 2.9).

Males were allowed 3 weeks to recover from the surgery and were then assessed for sterility by mating with superovulated F1 female mice (C57BL/6 x CBA) overnight. Successful mating was determined by the presence of a vaginal plug the following morning. Confirmation of sterility was made when the pronucleate oocytes collected from the female failed to divide in culture.

2.9.2. Embryo Transfers

Embryo transfers allow the assessment of implantation and subsequent fetal and placental growth of treatment embryos. Female recipient mice (Swiss females or F1 hybrid females C57BL/6 x CBA) were mated to vasectomised F1 hybrid males (C57BL/6 x CBA), resulting in female pseudopregnant mice that allowed the implantation of IVF embryos. Following anaesthesia, the mouse was placed on a warm pad at 37 °C throughout the surgery period. A small incision was made on the dorsal side of the female mouse and the uterine horn was gently pulled out. A 27 gauge needle was used to make an opening the uterine horn and five blastocysts were transferred, using a borosilicate glass pipette, with a flame-polished opening to minimise trauma to the wound, into each uterine horn of the pseudopregnant female. The incisions were sealed with an absorbable suture (Dexon, Covidien), and additionally with sterile surgical clips. 1 mg/kg atipamezole was used to reverse the effects of medetomidate and awaken the mouse. The mouse was wrapped in sterile drapes and placed in an animal cage to recover from the anaesthetic. To maintain body temperature, half of the cage is placed on a heated stage at 37 °C. Health of the mouse was monitored every hour for the first 4 h and subsequently every day for the next week to ensure full recovery.
2. Materials and Methods

On day fourteen of after transfer, post-operative females were killed, and their abdomen sterilised with 80% ethanol. An incision was made along the ventral midline and across either side of the abdomen, exposing the body cavity. The internal organs were pushed aside to expose the uterine horns containing the foetuses. The reproductive tract was removed by cutting away from the ovaries and in the midline of the uterus, ensuring that the left and right sides were kept separate and the original orientation to determine control and test groups. Careful dissection of the uterine wall was made with a single cut along the lumen and the individual fetuses and subsequent placentas were isolated. Embryo transfer data was only collected when there was at least one site of implantation in the recipient mouse, indicating success of embryo transfers and to eliminate procedure errors. Using fine forceps, the extra-embryonic membrane was carefully torn and the umbilical cord was cut to separate the fetus and the placenta, which were removed from the extra-embryonic membranes. The fetuses and placentae were weighed individually and fetal crown-rump length was measured using vernier calipers. Developmental morphology of the eye, ear and finger digits were assessed for normality against expected observations for the current age of the fetus, as previously described (Lane and Gardner, 1994) (Table 2.1). The gonads of the foetuses were dissected out for the sexing of the fetuses. Resorption sites were identified by necrotic embryonic tissue (dark protrusions) in the uterine wall. Following all measurements, the liver tissue from the fetus and its respective placenta were each dissected out and stored in Eppendorf tubes, then snap frozen in liquid nitrogen for gene expression analysis.
Table 2.2: Morphological time scale of fetal development for mouse F1 blastocysts.

<table>
<thead>
<tr>
<th>Day of pregnancy</th>
<th>Eye features</th>
<th>Ear features</th>
<th>Limb features</th>
<th>Crown-rump length (mm ± SEM)</th>
<th>Fetal mass (g ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>Round, Cornea barely evident, Very fine ring of pigment</td>
<td>Distinct meatus and pinna, protrusion</td>
<td>F: Footplates</td>
<td>9.1 ± 0.3</td>
<td>0.1436 ± 0.007</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>H: Smooth</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Round, Faint cornea, Circle of pigment</td>
<td>Pinna growing out and forward</td>
<td>F: Separated digits</td>
<td>11.3 ± 0.2</td>
<td>0.1786 ± 0.005</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>H: Webbed digits</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>Beginning to elongate, Distinct cornea, Dark pigment</td>
<td>Distinct pinna flap almost over meatus</td>
<td>F: Splayed digits</td>
<td>14.1 ± 0.2</td>
<td>0.3255 ± 0.009</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>H: Separated and beginning to splay</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2. Materials and Methods

2.10. RT-PCR and qRT-PCR

2.10.1. RNA extraction from Day 5 blastocysts

Total RNA from Day 5 blastocysts was extracted using Absolutely RNA Nanoprep Kit (#400753, Agilent Technologies, USA), as per manufacturer’s instructions. Previously, Day 5 blastocysts were snap-frozen in lysis buffer with β-Mercaptoethanol (0.7 µl β-Mercaptoethanol to 100 µl lysis buffer, 2 µl of Lysis Buffer- β-Mercaptoethanol mixture per blastocyst). For RNA extraction, the blastocyst lysates were thawed and supplemented with an equal volume of 70 % ethanol, then mixed thoroughly by vortexing for 5 seconds. The mixtures were then transferred to an RNA-binding nano-spin cup and centrifuged for 1 min (12000G). This was followed by DNase treatment and subsequent washes for DNA-free total RNA. RNA concentration was quantified using a Nanodrop 2000 spectrophotometer (Thermo Scientific, USA).

2.10.2. RNA extraction from placenta and fetal liver samples

Frozen fetal liver and placenta samples were thawed on ice. For RNA extraction, 600 µl of Trizol (Life Technologies, Australia) was added to each tube containing respective the tissues and a pestle was used to grind the tissue until it had formed a homogenous suspension. Following this, 120 µl of chloroform (Chem Supply, Australia) was added and tubes, which were the inverted for 4 min and chilled on ice for a further 5 min. The tubes were then centrifuged at 4 °C at 13000G for 15 min and 300 µl of the supernatant was extracted and added to a fresh tube containing 300 µl of isopropanol. These tubes were chilled on ice for 10 min before centrifugation at 18000G for 10 min. Supernatant was then removed and discarded and 400 µl of 70 % ethanol was added followed by centrifugation at 18000G for 1 min. Again, supernatant was removed and discarded. DNase treatment was carried out using a DNase treatment kit (Ambion, Life Technologies). The RNA pellet centrifuged from the extraction step was resuspended in 20 µl of water and incubated in a heating block at 65 °C for 5 min. The mixture was then incubated with 2 µl of 10x DNase buffer and 1 µl of DNase at 37 °C for 45 min,
followed by addition of 2 µl of inactivating reagent. This was left at room temperature for 2 min before being manually flicked twice. Tubes were then centrifuged at 18000G for 1 min and supernatant (with RNA) was removed and stored in a fresh tube at -80 °C before cDNA synthesis.

2.10.3. cDNA synthesis

The M-MLV reverse transcriptase kit (#M1701, Promega, Australia) was used to synthesize cDNA. Water was added to RNA extracted as outlined in 2.9.1 and 2.9.2, to make up a total volume of 12 µl. To this mixture, 1 µl of Oligo-dt (Geneworks, Australia) was added, and the tube was incubated at 70 °C for 5 mins, then cooled to 4 °C. The following was then added to each incubation tube: 4 µl M-MLV 5x buffer, 0.5 µl RNase inhibitor, 1 µl dNTP, 0.4 µl M-MLV reverse transcriptase and 1.1 µl water. This mixture was then incubated at 37 °C for a further 90 min and stored at 4 °C. A control tube without M-MLV reverse transcriptase (-RT) was also set up in parallel with test samples, and cDNA was diluted 1 in 3 by the addition of 40 µl of water before use in subsequent PCR reactions.

2.10.4. qRT-PCR

5x HOT FIREPol EvaGreen qPCR Mix Plus (ROX) (Solis BioDyne, Estonia) was used for qRT-PCR reactions. The qPCR reaction mix per sample included: 2 µl 5x HOT FIREPol EvaGreen qPCR Mix Plus, 0.5 µl forward primer, 0.5 µl reverse primer, 6 µl water, 1 µl cDNA (as outlined in 2.9.2). Samples were amplified using ViiA 7 Real Time PCR System (Life Technologies). The samples were initially heated to 95 °C for 15 min followed by 40 cycles of the following conditions: 95 °C for 15 sec, 60 °C for 15 sec and 72 °C for 30 sec. The cycle threshold (Ct) values for GAPDH, β-actin and 18s Ribosomal RNA were each used to normalise against the sample results, and raw Ct values were analyzed using Q-Gene software package (Simon, 2003).
2. Materials and Methods

2.10.5. Forward and Reverse primers

Primers were synthesised by Geneworks (Australia) and sequenced to confirm product before usage. A list of primer sequences is shown in Table 2.3.
## 2. Materials and Methods

### Table 2.3: Primer Sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence 5' -&gt; 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Gapdh</em> F</td>
<td>CTTCACCACCATGGAGAAGGC</td>
</tr>
<tr>
<td><em>Gapdh</em> R</td>
<td>GGCATGGACTGTGGTCATGAG</td>
</tr>
<tr>
<td><em>β-actin</em> F</td>
<td>CTGCCTGACGGCCAGG</td>
</tr>
<tr>
<td><em>β-actin</em> R</td>
<td>GATTCCATAACCAAGAAGGAAGG</td>
</tr>
<tr>
<td><em>18S</em> F</td>
<td>GAAACGGCTACCACATCCAA</td>
</tr>
<tr>
<td><em>18S</em> R</td>
<td>CCTGTATTGTATTTTTTCGACTACCT</td>
</tr>
<tr>
<td><em>B3gnt5</em> F</td>
<td>AGGTGGGCTCTCTGTATTAGG</td>
</tr>
<tr>
<td><em>B3gnt5</em> R</td>
<td>GCTACATCTCTGGAGACAACATAGG</td>
</tr>
<tr>
<td><em>Eomes</em> F</td>
<td>GCAGGGCAATAAGATGTACG</td>
</tr>
<tr>
<td><em>Eomes</em> R</td>
<td>CTAAGTCTTGGAAGGGTCA</td>
</tr>
<tr>
<td><em>Wnt3a</em> F</td>
<td>TGTTCCTGGACAAAGCCACCC</td>
</tr>
<tr>
<td><em>Wnt3a</em> R</td>
<td>GATTCCATAACCAAGAAGGAAGG</td>
</tr>
<tr>
<td><em>Cdx2</em> F</td>
<td>CCCTAGGAAGCCAAGTGAAAAC</td>
</tr>
<tr>
<td><em>Cdx2</em> R</td>
<td>CTCCTTGCTCTGCCTGTTCTG</td>
</tr>
<tr>
<td><em>Glut1</em> F</td>
<td>CCAGCTGGGAATCGTCGTT</td>
</tr>
<tr>
<td><em>Glut1</em> R</td>
<td>CAAGCTCTGCTGCCCATGAT</td>
</tr>
<tr>
<td><em>Glut3</em> F</td>
<td>CGGTGATAGTCTCTTAAGCCTTCTTCT</td>
</tr>
<tr>
<td><em>Glut3</em> R</td>
<td>ATGGGGTCACCTTCGTTGTC</td>
</tr>
<tr>
<td><em>GOT1</em> F</td>
<td>CGTGGGCTATTTCGCTATTTG</td>
</tr>
<tr>
<td><em>GOT1</em> R</td>
<td>AGATTCCCCCCTCTCATTGAGA</td>
</tr>
<tr>
<td><em>PED</em> F</td>
<td>CAGATCCCAAGGCACAT</td>
</tr>
<tr>
<td><em>PED</em> R</td>
<td>TGCCAAGTCAGGATGTC</td>
</tr>
<tr>
<td><em>IGFR2</em> F</td>
<td>CTTGCCCTCCAGAAACGGAT</td>
</tr>
<tr>
<td><em>IGFR2</em> R</td>
<td>TGCTACACCACAGTTCGCT</td>
</tr>
<tr>
<td><em>Pkm2</em> F</td>
<td>TCTTCCTGTGCTGTGTAAGGA</td>
</tr>
<tr>
<td><em>Pkm2</em> R</td>
<td>CCACCCGGTCAGCACAAAT</td>
</tr>
</tbody>
</table>
2. Materials and Methods

2.11. Statistical analyses

All analyses were performed using GraphPad Prism version 5.04 for Windows (GraphPad Software) and all data was assessed for Gaussian distribution with the Shapiro-Wilk normality test. Data that did not meet the assumption of a Gaussian distribution underwent nonparametric analysis using the Mann-Whitney test. Data that were normally distributed were statistically analyzed with Student’s unpaired t-test.

Correlation between blastocyst cell number and morphokinetic data that was not normally distributed were analyzed using Spearman’s correlation coefficient. Cell number differences in quartiles of morphokinetic data were analyzed by analysis of variance (ANOVA) and post-hoc test between quartile differences determined using Bonferroni’s multiple comparison test. Embryo transfer results were analyzed using the Chi-square test. Differences in expression levels of genes between ‘fast’ and ‘slow’ blastocysts, placenta and fetal liver tissue samples were statistically analyzed with Student’s unpaired t-test. Correlation of aspartate consumption over increasing aspartate concentrations was determined using Pearson’s correlation. One-way ANOVA with post-hoc Bonferroni’s test were used to analyze the relationship of increasing aspartate concentrations to glucose consumption and lactate production. Glycolytic rate percentages were arc-sine transformed and compared using Student’s unpaired t-test.
3. Developmental Kinetics of the Preimplantation Embryo

Part of this chapter has been published as:

**Lee, Y. S., Thouas, G. A. & Gardner, D. K.** 2015. Developmental kinetics of cleavage stage mouse embryos are related to their subsequent carbohydrate and amino acid utilization at the blastocyst stage. *Hum Reprod*, 30, 543-52
3. Developmental Kinetics of the Preimplantation Embryo

3.1. Introduction

The transfer of a single embryo to the uterus following IVF has become a priority objective of assisted human conception (Gardner, 2000, Van Royen, et al., 1999), however, since the inception of human IVF, selection of embryos for transfer via assessment of morphology has been the primary method utilized by embryologists to assess embryo quality and viability (Cummins, et al., 1986). Grading systems based on observations such as degree of fragmentation and symmetry of blastomeres, have been successfully applied to all stage of human embryo development (Gardner, et al., 2000b, Scott and Smith, 1998, Van Royen, et al., 1999). However, visual scoring remains subjective to the endpoint user, while embryo morphology has been found to be highly dynamic and variable within short time intervals (<2 h) (Montag, et al., 2011). Consequently, it is difficult to interpret embryo morphology without multiple assessments of embryos at specific time points on each day throughout culture. Although discrete temporal assessments have shown that key morphological characteristics of the embryo are linked to developmental potential (Rhenman, et al., 2015), the removal of the embryo from the incubator for microscopic assessment leads to temperature and pH fluctuations, which can negatively affect embryo quality and development (Zhang, et al., 2010).

The recent integration of a high-frequency imaging system with 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 marker of viability (Arav, et al., 2008, Meseguer, et al., 2011, Pribenszky, et al., 2010, Wale and Gardner, 2010). Using time-lapse monitoring to study morphokinetics dates back to 1929, when rabbit embryos were first evaluated (Lewis and Gregory, 1929). Subsequently, several mammalian species embryos have been examined, such as cow (Grisart, et al., 1994, Holm, et al., 1998), hamster (Gonzales, et al., 1995) and pig (Booth, et al., 2007). In the mouse, studies using such technologies have shown that cleavage division timings of 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). Arav et al. (2008) identified that mouse
embryos with a cleavage time within a normal range (defined as an interval during which 50% of all embryos cleaved) had a significantly higher proportion of embryos that developed to blastocysts as compared to those with a delayed first cleavage (Arav, et al., 2008).

These results suggest that there is an ideal range of cleavage timings, outside of which blastocyst development is suboptimal. However, Pribenszky et al. (2010) found that the first embryos to reach the first and second cleavage stage correlated with increased blastocyst development (Pribenszky, et al., 2010). Similar correlations were observed in cow embryos, with embryos developing into morula-blastocyst cleaving significantly earlier at the first cleavage event (Grisart, et al., 1994, Holm, et al., 1998). However, it is difficult to draw conclusions from Arav et al. (2008) and Pribenszky et al. (2010)’s studies, due to differences between the two studies, such as strain of mouse, incubator models and culture media used. Of significance, both studies cultured embryos under atmospheric oxygen (20%), which is detrimental to embryo health and significantly delays cleavage timings (Wale and Gardner, 2010). Furthermore, the endpoint of both studies was percentage of blastocyst development, which does not necessarily relate to embryo viability, as some cow embryos that arrest at later stages had similar early cleavage timings as non-viable embryos (Grisart, et al., 1994). Hence, further studies are warranted to examine the variation of morphokinetics of mice embryos in relation to assessment of embryo quality, such as blastocyst cell numbers, which has been shown to be predictive of embryo viability post-transfer (Lane and Gardner, 1997a). The aim of this chapter is to characterize developmental kinetics of mouse preimplantation embryos under physiological oxygen concentrations (5%), and correlate developmental events to blastocyst development and total cell number on day 5.
3. Developmental Kinetics of the Preimplantation Embryo

3.2. Experimental design

3.2.1. Embryo collection

Oocytes were collected from 3 to 4 week old female virgin F1 (C57BL/6 x CBA) mice stimulated with 5 IU PMSG at 5 pm, followed by hCG 49 h later at 6 pm. Cumulus-oocyte complexes were collected 15 h post-hCG (8 am) in equilibrated fertilization medium and transferred to 45 µl drops of fertilization medium and cultured under Ovoil in 6% CO₂, 5% O₂ and 89% N₂ at 37°C. Sperm were collected from 8 to 12 week old F1 hybrid male mice (C57BL/6 x CBA), as per described in Section 2.2.3

3.2.2. Determination of time required for in vitro fertilization

Following oocyte collection and sperm preparation, 1 to 2 million motile sperm per ml was added to 1 cumulus oocyte complex and fertilization was allowed to occur over different incubation time periods (15 min, 30 min, 45 min, 60 min, 75 min and 90 min). Following the incubation period, oocytes were denuded by the addition of hyaluronidase (1 mg/ml) to the incubation drop. After a 1 min incubation, when cumulus cells began to disperse, pronucleate oocytes were separated by repeated pipetting with a hand-pulled Pasteur pipette with an outer diameter slightly larger than that of the pronucleate oocyte, followed by rinsing 3 times in IVF culture medium and once in pre-equilibrated G1 medium before allocation to culture drops. Successful fertilization was determined by visible observation of polar body extrusion and reconfirmed on Day 2 by observation for the 2-cell stage in the individual embryos.
3. Developmental kinetics of the preimplantation embryo

3.2.3. Imaging system and time-lapse evaluation of morphokinetic parameters

Pronucleate oocytes were cultured individually in 2 μl drops of G1 with HSA under Ovoil in 6% CO₂, 5% O₂ and 89% N₂ 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 with HSA 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 timings of developmental events were recorded, with time of cleavage defined as the first observed time point when the newly formed blastomeres are completely separated by confluent cell membranes, as defined by Meseguer et al. (2011). The time of all morphokinetic events were initially expressed as hours post insemination (hours PI).

The earliest recorded morphokinetic event was syngamy of the male and female pronuclei at the pronucleate oocyte stage (Fig 3.1). Following this, time of division of the pronucleate oocyte to 2-cell stage is annotated with a shorthand notation of t2. Subsequently, second division (to 3-cell stage) is annotated as t3, third division (to 4-cell stage; t4), forth division (to 5-cell stage; t5) and fifth division (to 6-cell stage; t6)(Fig 3.1). Subsequent cell divisions (to 7-cell and 8-cell stage) were difficult to observe due to overlap of blastomeres and focus of camera, and thus were not recorded.
3. Developmental Kinetics of the Preimplantation Embryo

**Figure 3.1:** Illustration of morphokinetic events that were recorded.

Syngamy represents the earliest observation of pronuclei disappearance. PI, post insemination. t2, t3, t4, t5 and t6 represents time of cleavage to the number of cells. 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 by Meseguer et al. (2011). Cavitation represents the earliest observation of blastocoel cavity appearance, and hatching is the earliest observation of cells hatching from the zona pellucida.
3. Developmental kinetics of the preimplantation embryo
3. Developmental Kinetics of the Preimplantation Embryo

Following compaction of the embryo, the time of cavitation and time of hatching of blastocyst was also recorded. With these recordings, the duration of interval between cell divisions was calculated, with the duration of syngamy to t2 annotated as syn-t2, duration of the second cell cycle, cc2 (cc2=t3-t2), duration of interval between 3-cell and 4-cell was defined as second synchrony, s2 (s2=t4-t3) (Fig 3.1), and as reported in Meseguer et al. (2011), whereby s2 represents the duration of the period as a three blastomere embryo. The blastocyst developmental rate was observed on the morning of Day 5.

3.2.4. Assessment of embryo cell numbers

All embryos that reached expanded blastocyst stage by Day 5 were stained for embryo cell numbers. Total cell numbers were measured in blastocysts by counting the total number of nuclei of day 5 blastocysts using the nuclear stain, bisbenzimide (Hoechst No 33258, Sigma, USA). For staining, blastocysts were incubated at 37 °C in a solution composed of G-MOPS™ Plus medium, 10% ethanol and 0.1 mg/ml bisbenzimide for 1 h, followed by rinsing in G-MOPS™ plus for 10 min. Stained blastocysts were then mounted in glycerol on glass microscope slides and photographed using a fluorescence microscope (Martin, et al., 1998). Cell numbers were counted using image processing software (Image-J, National Institutes of Health, USA).

3.3. Statistics

All data were assessed for normality with the Shapiro-Wilk test. Correlations between blastocyst cell number and the timing of all morphokinetic parameters that were not normally distributed were analyzed using Spearman’s correlation coefficient. Cell number differences in quartiles of morphokinetic data were analyzed by analysis of variance (ANOVA) and post-hoc tests between quartile differences determined using Bonferroni’s multiple comparison test.
3. Developmental kinetics of the preimplantation embryo

3.4. Results

3.4.1. Embryo fertilization, blastocyst development and insemination time

Over 95% of oocytes fertilize within the first 90 min of insemination (Table 3.1). However, in following experiments (Results 3.4.2 onwards), an insemination time of 4 h was used to represent those IVF clinics in which a short insemination protocol is utilised. Such short insemination incubation durations vary from 1 h to 6 h (Huang, et al., 2013, Zhang, et al., 2010), and it has been determined that sperm first appear in the human oocyte cortex at 4 h post insemination (Gianaroli, et al., 1996). For all biological replicates in this chapter, fertilization rate per replicate was > 90% and blastocyst development rate was > 95% of fertilized oocytes.

3.4.2. Correlation of morphokinetic events to blastocyst total cell number

The timing of morphokinetic events, including syngamy, cell division (2 cells; t2, 3 cells; t3, 4 cells; t4, 5 cells; t5, 6 cells; t6), cavitation and hatching, as well as the duration of syngamy to t2 (syn-t2), duration of second cell cycle (cc2; t3-t2) and duration of the 3-cell to 4-cell interval (s2) were recorded, averaged, calculated as hours post insemination (PI) and compared to the resultant blastocyst total cell numbers for the same embryos (Table 3.2). The distribution of morphokinetics for each event did not follow a normal distribution. For each developmental event, the range of timings (SEM) was found to increase as embryo development progressed (Table 3.2). Blastocyst cell number was correlated to the timing of pronuclear syngamy, cleavage divisions, t2, t3, t4, t5, t6 and cavitation, with an earlier occurrence of these events correlated to a higher cell number (Table 3.2). The timing of hatching was not related to total cell number (Table 3.2). A shorter syn-t2 was correlated to a larger total cell number while the time intervals cc2 and s2 were not significantly correlated (Table 3.2).
Table 3.1: Effect of insemination duration on fertilization rate

<table>
<thead>
<tr>
<th>Duration of insemination (min)</th>
<th>Number of fertilized embryos</th>
<th>Fertilization rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>4/34</td>
<td>9.9 ± 6.2</td>
</tr>
<tr>
<td>30</td>
<td>11/48</td>
<td>18.1 ± 9.5</td>
</tr>
<tr>
<td>45</td>
<td>28/65</td>
<td>40.0 ± 8.3</td>
</tr>
<tr>
<td>60</td>
<td>41/67</td>
<td>61.9 ± 3.73</td>
</tr>
<tr>
<td>75</td>
<td>48/75</td>
<td>63.8 ± 5.3</td>
</tr>
<tr>
<td>90</td>
<td>22/23</td>
<td>96.4 ± 3.6</td>
</tr>
</tbody>
</table>

n = 312 embryos in total, 6 biological replicates. Data is expressed as mean ± SEM.
3. Developmental kinetics of the preimplantation embryo

<table>
<thead>
<tr>
<th>Developmental event</th>
<th>Cell number</th>
<th>Event timing/ Interval duration (hours PI)</th>
<th>Correlation to blastocyst cell number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Syngamy</td>
<td>1-cell</td>
<td>14.6 ± 0.1</td>
<td>P &lt;0.01</td>
</tr>
<tr>
<td>syn-t2</td>
<td></td>
<td>2.2 ± 0.2</td>
<td>P &lt;0.01</td>
</tr>
<tr>
<td>1st cleavage (t2)</td>
<td>2-cell</td>
<td>16.8 ± 0.2</td>
<td>P &lt;0.001</td>
</tr>
<tr>
<td>cc2</td>
<td></td>
<td>24.3 ± 0.3</td>
<td>NS</td>
</tr>
<tr>
<td>2nd cleavage (t3)</td>
<td>3-cell</td>
<td>36.6 ± 0.2</td>
<td>P &lt;0.001</td>
</tr>
<tr>
<td>s2</td>
<td></td>
<td>1.5 ± 0.3</td>
<td>NS</td>
</tr>
<tr>
<td>t4</td>
<td>4-cell</td>
<td>37.9 ± 0.2</td>
<td>P &lt;0.001</td>
</tr>
<tr>
<td>3rd cleavage (t5)</td>
<td>5-cell</td>
<td>47.8 ± 0.2</td>
<td>P &lt;0.001</td>
</tr>
<tr>
<td>t6</td>
<td>6-cell</td>
<td>48.5 ± 0.2</td>
<td>P &lt;0.001</td>
</tr>
<tr>
<td>Cavitation</td>
<td></td>
<td>74.5 ± 0.4</td>
<td>P &lt;0.001</td>
</tr>
<tr>
<td>Hatching</td>
<td></td>
<td>80.5 ± 0.4</td>
<td>NS</td>
</tr>
</tbody>
</table>

n= 183 embryos in total, 5 biological replicates. Data is expressed as mean ± SEM. 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 by Meseguer et al. (2011). PI, post insemination; NS, not significant.
3. Developmental Kinetics of the Preimplantation Embryo

3.4.3. Morphokinetics of embryos standardized to time of syngamy

To account for possible variation in duration taken for fertilization to occur, the data was standardized to time of syngamy (Table 3.3). When time of fertilization was accounted for, the blastocyst total cell number was correlated to t2, t5, t6 and time of cavitation (Table 3.3). As compared to Table 3.2, there was a loss of statistical significance in the correlation between t3 and t4 and blastocyst cell number, as well as a reduced $P$ value across remaining significantly correlated events (t2, t5, t6 and cavitation), although significance was maintained (Table 3.3).

3.4.4. Morphokinetics of embryos standardized to 2-cell division time

To further identify if the significant difference in later developmental events (post syngamy) was partly due to a cumulative delay during syn-t2, data was further standardized to t2 to eliminate the impact this delay (Table 3.4). It was found that only t5 and cavitation time remained significantly correlated to blastocyst cell number, and there was a loss of statistical significance of t6 (Table 3.4).
### Table 3.3: The timing of morphokinetic events and their correlation with blastocyst total cell number, standardized to syngamy

<table>
<thead>
<tr>
<th>Developmental event</th>
<th>Cell number</th>
<th>Event timing standardized to syngamy</th>
<th>Correlation to blastocyst cell number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st cleavage (t2)</td>
<td>2-cell</td>
<td>2.2 ± 0.2</td>
<td>P &lt;0.01</td>
</tr>
<tr>
<td>cc2</td>
<td></td>
<td>20.0 ± 0.2</td>
<td>NS</td>
</tr>
<tr>
<td>2nd cleavage (t3)</td>
<td>3-cell</td>
<td>22.0 ± 0.1</td>
<td>NS</td>
</tr>
<tr>
<td>s2</td>
<td></td>
<td>1.2 ± 0.1</td>
<td>NS</td>
</tr>
<tr>
<td>t4</td>
<td>4-cell</td>
<td>23.2 ± 0.2</td>
<td>NS</td>
</tr>
<tr>
<td>3rd cleavage (t5)</td>
<td>5-cell</td>
<td>33.2 ± 0.2</td>
<td>P &lt;0.01</td>
</tr>
<tr>
<td>t6</td>
<td>6-cell</td>
<td>34.0 ± 0.2</td>
<td>P &lt;0.05</td>
</tr>
<tr>
<td>Cavitation</td>
<td></td>
<td>59.9 ± 0.4</td>
<td>P &lt;0.01</td>
</tr>
<tr>
<td>Hatching</td>
<td></td>
<td>64.3 ± 0.9</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data is expressed as mean ± SEM. n= 183 embryos in total, five biological replicates. Cc2 represents the second cell cycle of the embryo, s2 represents the interval between 3-cell and 4-cell (s2= t4-t3), as reported by Meseguer et al. (2011). NS, not significant.
Table 3.4: The timing of morphokinetic events and their correlation with blastocyst total cell number, standardized to t2

<table>
<thead>
<tr>
<th>Developmental event</th>
<th>Cell number</th>
<th>Event timing standardized to t2</th>
<th>Correlation to blastocyst cell number</th>
</tr>
</thead>
<tbody>
<tr>
<td>2nd cleavage (t3)</td>
<td>3-cell</td>
<td>24.3 ± 0.3</td>
<td>NS</td>
</tr>
<tr>
<td>t4</td>
<td>4-cell</td>
<td>25.7 ± 0.2</td>
<td>NS</td>
</tr>
<tr>
<td>3rd cleavage (t5)</td>
<td>5-cell</td>
<td>35.9 ± 0.2</td>
<td>P &lt;0.01</td>
</tr>
<tr>
<td>t6</td>
<td>6-cell</td>
<td>36.6 ± 0.2</td>
<td>NS</td>
</tr>
<tr>
<td>Cavitation</td>
<td></td>
<td>62.2 ± 0.4</td>
<td>P &lt;0.05</td>
</tr>
<tr>
<td>Hatching</td>
<td></td>
<td>66.9 ± 0.9</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data is expressed as mean ± SEM. n= 183 embryos in total, five biological replicates. s2 represents the interval between 3-cell and 4-cell (s2= t4-t3), as reported by Meseguer et al. (2011). NS, not significant.
3. Developmental kinetics of the preimplantation embryo

3.4.5. Frequency distribution and variation between biological replicates of embryo 2-cell division timings

As t2 was the earliest event that had a high correlation to blastocyst cell number, even when standardized to syngamy, further analysis was carried out on t2 to investigate the frequency distribution of embryo cleavage timing at the 2-cell stage. The mean t2 is 16.9 h PI, and the median t2 is 16.2 PI. The frequency distribution is positively skewed and demonstrates that slower cleaving 2-cell embryos have a larger range of timings (Fig 3.2a). Quartile values (quartile 1, median and quartile 3) were calculated based on data collected on t2, and embryos cleaving before and inclusive of the value of quartile 1 were categorized as quartile 1 (Q1) embryos, embryos cleaving between Q1 and median were categorized as quartile 2 (Q2), embryos cleaving between median and quartile 3 (Q3) were categorized as quartile 3, and embryos cleaving after Q3 were categorized as quartile 4 (Q4). 2-cell division timings of embryos in Q1 ranges from 13.9 – 15.75 h PI, embryos in Q2 ranges from 15.76 – 16.23 h PI, embryos in Q3 ranges from 16.24 – 16.74 h PI and embryos in Q4 ranges from 16.75 – 20.7 h PI (Fig 3.2b). Over five biological replicates, variation within each quartile for all the four quartiles (Q1-Q4) was observed but this variation was insignificant (Table 3.5).
3. Developmental Kinetics of the Preimplantation Embryo

Figure 3.2: a) Frequency distribution of embryo 2-cell cleavage timings b) Frequency distribution of embryo 2-cell cleavage timings, with illustration of quartile distributions

n =183 embryos in total (Q1 n =46, Q2 n =43, Q3 n= 47, Q4 n =47), five biological replicates. The value of quartile 1 is 15.75 h post insemination (PI), the median time of 2-cell cleavage time is 16.30 h PI and the value of quartile 3 is 16.74 h PI.
3. Developmental kinetics of the preimplantation embryo

a)

b)
### Table 3.5: Variation in quartile timings between each biological replicate

<table>
<thead>
<tr>
<th>Biological Rep</th>
<th>Quartile 1 (h PI)</th>
<th>Quartile 2 (h PI)</th>
<th>Quartile 3 (h PI)</th>
<th>Quartile 4 (h PI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15.0 ± 0.1</td>
<td>15.9 ± 0.1</td>
<td>16.3 ± 0.1</td>
<td>17.7 ± 0.3</td>
</tr>
<tr>
<td>2</td>
<td>15.2 ± 0.1</td>
<td>15.9 ± 0.1</td>
<td>16.1 ± 0.1</td>
<td>19.1 ± 1.6</td>
</tr>
<tr>
<td>3</td>
<td>15.5 ± 0.1</td>
<td>15.8 ± 0.1</td>
<td>16.2 ± 0.1</td>
<td>17.7 ± 0.8</td>
</tr>
<tr>
<td>4</td>
<td>14.9 ± 0.2</td>
<td>15.9 ± 0.04</td>
<td>16.4 ± 0.04</td>
<td>18.3 ± 0.4</td>
</tr>
<tr>
<td>5</td>
<td>15.5 ± 0.1</td>
<td>15.9 ± 0.03</td>
<td>16.2 ± 0.03</td>
<td>17.4 ± 0.1</td>
</tr>
</tbody>
</table>

Data is expressed as mean ± SEM. n= 183 embryos in total, five biological replicates. h PI; hours post insemination.
3.4.6. Correlation of quartile timings to blastocyst total cell number

The morphokinetic parameters t2, t5 and cavitation were divided into four groups based on their quartiles and correlated to their blastocyst cell number on day 5 (Fig 3.3). A similar relationship was observed across all parameters, in which faster quartiles have a significantly higher average blastocyst cell number as compared to the slowest quartile (Fig 3.3).
3. Developmental Kinetics of the Preimplantation Embryo

Figure 3.3: Correlation of quartile timings to blastocyst total cell number

a) Cell division timings of t2 correlated with blastocyst total cell number. b) Cell division timings of t5 correlated with blastocyst total cell number. c) Cell division timings of cavitation correlated with blastocyst total cell number. a, b, c $P <0.05$; further significance not shown on graph: a) Quartile 2 vs. 4, $P <0.001$, b) Quartile 1 vs. 3, $P <0.01$, Quartile 1 vs. 4, $P <0.001$, c) Quartile 1 vs. 4, $P <0.001$.

a, b, c Different letters represent significant differences between groups. Data is expressed as mean ± SEM. 183 embryos in total. (Q1 n=46, Q2 n=43, Q3 n= 47, Q4 n =47).
3. Developmental kinetics of the preimplantation embryo

\[ a \]

![Bar chart for cell numbers at t2 in quartiles](image1)

\[ b \]

![Bar chart for cell numbers at t5 in quartiles](image2)

\[ c \]

![Bar chart for cell numbers at cavitation time in quartiles](image3)
3.5. Discussion

Continuous assessment of embryo morphology and development, using time-lapse microscopy, revealed that morphokinetics positively correlate to day 5 blastocyst total cell number. Data from the present study demonstrate that timing of syngamy, duration between syngamy and t2 (syn-t2), timing of first cleavage (t2), third cleavage (t5), and cavitation are significantly correlated to blastocyst total cell number. These morphokinetic parameters were identified using data standardized to the timing of pronuclear syngamy, to eliminate the effect of the time taken for fertilization to occur. Following the data transformation, only t2, t5, t6 and cavitation time remained significantly correlated to blastocyst cell number. There was a loss of statistical significance in the correlation between t3 and t4 and blastocyst cell number, as well as a reduced P value across remaining significantly correlated events (t2, t5, t6 and cavitation), although significance was maintained. These results suggest that the time of fertilization resulted in asynchrony of pronuclear syngamy, and only developmental events t2, t5, t6 or cavitation timings may be correlated to blastocyst total cell number. To account for the significant duration taken for t2 to occur, which may result in a subsequent cumulative delay in t5, t6 and cavitation, the data was standardized to t2 and it was found that only t5 and cavitation remained significantly correlated to blastocyst cell number. There is a tighter distribution of cleavage times during earlier pre-compaction developmental events as compared to post-compaction. Timing of morphokinetic events did not follow a normal distribution, and when t2, t5 and cavitation division timings were sorted into quartiles, there is a tighter distribution of cleavage times for quartiles 1, 2 and 3 as compared to quartile 4. Blastocyst total cell numbers of quartile 4 from t2, t5 and cavitation times were significantly lower compared to other quartiles.

Previous 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 form a blastocyst (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). These studies observed developmental arrest at the pre-compaction stages and recorded blastocyst
developmental rates with later cleavage division timings (Arav et al. 2008; 78.7% blastocyst development, Pribenszky et al, 2010; 83.5 % blastocyst development). By comparison, the present experiment, embryos showed consistently high blastocyst developmental rates, which were unable to be correlated reliably with morphokinetics. Furthermore, embryos were derived from in vitro fertilization to control for duration of fertilization, as opposed to embryos flushed from mated mice (Arav, et al., 2008, Pribenszky, et al., 2010). The present data correlating cleavage stage morphokinetics to blastocyst total cell number is consistent with studies by Arav et al. (2008) and Pribenszky et al. (2010). Varying strains of mice (C57BL/6J x BALB/c; Arav et al., NMRI x ICR; Pribenszky et al.) may also have contributed to the differences in blastocyst developmental rates between these studies. Of note, Arav et al. (2008) and Pribenszky et al. (2010) cultured embryos under atmospheric oxygen (20 %), which in another study has been reported to delay t2 by an average of 0.45 h and t5 by an average of 3.17 h (Wale and Gardner, 2010), and negatively affect blastocyst development (Quinn and Harlow, 1978).

Arav et al. (2008) categorized mouse embryos into three groups; (i) before, (ii) within and (iii) after the ‘shortest-half’ time, which was defined as the duration in which 50 % of the embryo population cleaved. By using this categorization, only 11 % of embryos were classified as ‘fast cleaving embryos’, 53 % were within the shortest-half time, 36 % were classified as ‘slow cleaving embryos’ and it was found that the proportion of embryos that progressed to the blastocyst stage was significantly lower in the slow cleaving group as compared to the fast cleaving and shortest-half groups. Despite no developmental differences between the fast cleaving and shortest-half groups, the study suggested that selection of embryos for transfer from the shortest-half group is advantageous compared to the fast cleaving group, as earlier cleavage in human embryos has been linked to chromosomal abnormalities (Magli, et al., 2007). However, the incidence of chromosomal abnormalities is more prevalent in human embryos obtained via IVF (>50 %) and recent studies have identified that aneuploid embryos are more likely to be delayed, rather than premature (Campbell, et al., 2013a, Vera-Rodriguez, et al., 2015). In addition, the shortest-half analysis limited the number of embryos classified as fast or slow-cleaving, with majority of the embryos being classified as (ii) within the ‘shortest-half’ time, hence, to divide embryos equally, the
present study categorized morphokinetic parameters into quartiles, as previously published by Meseguer et al. (2011).

Data from the present morphokinetic experiments can be further related to previous reports (Arav, et al., 2008, Grisart, et al., 1994, Herrero, et al., 2013) showing that early divisions were closely synchronized in embryos and that later developmental events occurred 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 to non-implanting embryos, where timings 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 of unknown viability. The distribution observed in this study had a long tail extending to later cleavage division timing, similar to the pattern observed in human embryos (Meseguer, et al., 2011). Several morphokinetic parameters, including hierarchical selection models and algorithms have been proposed to be predictive of viability (Meseguer, et al., 2011, Wong, et al., 2010).

Correlation has been reported between morphokinetics and pregnancy in human embryos (Meseguer, et al., 2011, Wong, et al., 2010). Analysing data from frozen embryos that were thawed and cultured over 5 days, Wong et al. (2010) combined data obtained from time-lapse microscopy and gene expression analysis and determined that duration of first cytokinesis (duration of the last step in mitosis, from the appearance of the furrow to the physical separation of the two daughter cells), cc2 and s2 were predictive of blastocyst formation. Of significance, a study by Meseguer et al. (2011) reported that t2 and t5 is correlated to implantation potential (Meseguer, et al., 2011). These morphokinetic markers are consistent with data from the current experiment. Interestingly, the same group proposed that embryo cell divisions might occur prematurely, as the percentage of implanting embryos in the first quartile was lower than the two central quartiles for all cell divisions (Meseguer, et al., 2011). This was not observed in the present study, whereby the first two quartiles of t2, t5 and cavitation were correlated to a higher cell number. Statistically, Meseguer’s research group only
Developmental kinetics of the preimplantation embryo

found a difference for t3 and t5, and in addition, human embryos exhibit premature cell cycle divisions (such as cleavage from 2- to 3-cell in > 5 h; Rubio, et al. 2012), which have not been observed in mouse embryos. To assist in the embryo selection process, the research group created a classification tree based on critical morphological analysis (level of fragmentation, size of blastomeres, multi-nucleation) and of the optimal time range of t5, s2 and cc2 parameters. Ten categories were determined with a range of implantation rates of 66 % (highest) vs. 8 % (lowest) of the ranked embryos.

Several groups have extended the search for morphokinetic markers and numerous parameters were identified, such as time of fertilization to t5, s2 (Cruz, et al., 2012), t4, t5, t6, t7, t8 (Dal Canto, et al., 2012) and duration of t5 to t8 (Hashimoto, et al., 2012). Identification of parameters negatively correlated with pregnancy outcomes were also identified, such as premature pronuclear syngamy (Azzarello, et al., 2012), extremely short cc2 (> 5 h vs. 10-12 h)(Rubio, et al., 2012), and time to expanded blastocyst (Storr, et al., 2015). Subsequently, prospective RCT studies confirmed previously identified morphokinetic parameters negatively correlated with blastocyst development (Conaghan, et al., 2013, Kirkegaard, et al., 2013b), but not implantation potential and pregnancy rates (Kirkegaard, et al., 2013b). However, a later prospective RCT study reported higher ongoing pregnancy rates compared to standard incubation method, using Meseguer et al. (2011)’s hierarchical model (Rubio, et al., 2014). Despite promising data, morphokinetic parameters remains variable between IVF clinics (Meseguer, et al., 2012), and may be related to culture conditions such as oxygen levels (Kirkegaard, et al., 2013a, Wale and Gardner, 2010), oocyte (Escrich, et al., 2010), sperm quality and chromosomal abnormalities (Kirkegaard, et al., 2012), type of culture media (Van Langendonckt, et al., 2001) and day of embryo transfer (Kirkegaard, et al., 2013b). Of note, in all mammalian species studies to date, the asynchronous spatial transfer of pre-compaction embryos into the uterus has been shown to compromise fetal development (Barnes, 2000, Walker, et al., 2015).

In the current study, blastocyst cell number differences (>16 cells) were observed between the fastest and the slowest quartiles at times t2, t5 and cavitation, and further studies are required to determine if, similar to human embryos, variation in
3. Developmental Kinetics of the Preimplantation Embryo

morphokinetic parameters results in differences in implantation and fetal development potential. Additionally, the majority of time-lapse studies thus far have investigated human embryos, whilst the present study focused on mouse embryos. As EGA begins at 2-cell stage in mice and at 4-cell stage in humans, this may result in different interpretations of morphokinetics data and disparity between studies. Furthermore, different timing of EGA may have resulted in the range of t2 timings. Of the data available on human and mouse embryo morphokinetics, it appears t5 is a common marker linked to embryo viability.

Time-lapse analysis offers the possibility to continuously monitor embryo development without disturbance, and identify dynamic embryo morphology events that were previously not possible. As a non-invasive method, it can potentially be used to predict embryo development and quality, as shown in data presented in this chapter, where earlier occurrence of morphokinetic events (syngamy, t2, t5 and cavitation) are correlated with higher blastocyst cell number. Additionally, morphokinetic parameters time ranges identified by the laboratory can also be used as a means of quality control. Despite this, data from various time-lapse studies have not succeeded in determining defined ranges for embryo selection that can be applied across IVF clinics worldwide. Limitations of proposed algorithms work within quartiles or time ranges, which creates exact limits and may not be correct in categorizing embryos. Data from this experiment provided evidence that t2 is highly correlated to blastocyst cell number, and is a clear, observable event, as opposed to t5 and cavitation where overlapping of blastomeres and camera focus may prevent accurate observations. Furthermore, t2 as a marker of embryo viability provides information for clinics that selects to transfer embryos on day 3 and/or cryopreserve embryos at the pre-compaction stage. As such, t2 is the chosen morphokinetic marker for subsequent studies in this thesis. However, it is acknowledged that the use of t2 as the morphokinetic marker as opposed to other markers (e.g. t5) or a combination of morphokinetic markers is a limitation of the study. Although the use of a combination of morphokinetic markers may provide higher accuracy in determining embryo viability, there were experimental constraints. However, even with this in mind, in the initial analysis, t2 gave the strongest correlation with blastocyst cell number, and therefore represent a viable morphokinetic parameter to utilize experimentally.
4. Blastocyst carbohydrate and amino acid metabolism of the kinetically different groups of cleavage stage embryos selected with time-lapse imaging

Part of this chapter has been published as:

4. Blastocyst carbohydrate and amino acid metabolism of the kinetically different groups of cleavage stage embryos selected with time-lapse imaging

4.1. Introduction

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, cow 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 (Gott, et al., 1990, Leese and Barton, 1984). 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 day 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. 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 to arrested embryos. Brison et al. (2004) further revealed that the turnover of aspartate, glycine and leucine in human pronucleate oocytes was significantly correlated with higher viability following transfer on day 2. Analysis of amino acids would seem prudent given that they play several functions in regulating embryo development, serving as osmolytes (Dawson and Baltz, 1997, Lawitts and Biggers, 1992), intracellular pH regulators (Edwards, et al., 1998a), 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, 2012a), has also been implicated in the regulation of carbohydrate metabolism, as it appears to be the rate-limiting factor in the MAS (Lane and Gardner, 2005a). Consequently,
relationships between carbohydrate and amino acid utilization need to be further characterized to better understand their relationship with post transfer viability.

Over thirty years ago it was recognised by Robert Edwards and colleagues that earlier cleavage was a strong predictor of embryo quality as transfer of early cleaving embryos results in higher implantation and pregnancy rates (Edwards, et al., 1984), an observation subsequently repeated several times (Lundin, et al., 2001, Shoukir, et al., 1997, Van Montfoort, et al., 2004). Integration of a high-frequency imaging system within an incubator allowed continuous monitoring of embryo development without the disturbance of embryo culture conditions, and prompted renewed interest in quantifying developmental kinetics of embryos as a biomarker of viability (Arav, et al., 2008, Meseguer, et al., 2011, Pribenszky, et al., 2010, Wale and Gardner, 2010). Studies using such technologies have shown that 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 (Meseguer, et al., 2011, Wong, et al., 2010). In mice, cleavage division timings of 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), an observation confirmed by data discussed in Chapter 3. Although morphokinetics of mouse blastocyst have been correlated with blastocyst cell numbers (Chapter 3), correlation of morphokinetics to subsequent fetal quality and subsequent viability post transfer remains to be elucidated.

To date, metabolism (carbohydrate and amino acid) and morphokinetics as viability markers have been used independently as predictive markers of embryo viability. However, the correlation between them has not been investigated. Of note, correlations are based on continuous data as opposed to distinct categories amongst embryos, which leads to the risk of selecting against embryos that may still have the potential to create a successful pregnancy. For example, a retrospective study utilized a time-lapse blastocyst prediction model to determine potential viability of blastocysts and correlated it to pregnancy. Although the model would have increased implantation rate by 30 %, more importantly, 50.6 % of embryos that resulted in pregnancy were categorized as non-usable (Kirkegaard, et al., 2014). Therefore, the use of multiple viability markers,
4. Blastocyst carbohydrate and amino acid metabolism of the kinetically different groups of cleavage stage embryos selected with time-lapse imaging

as opposed to a single measurement, may plausibly increase the accuracy of embryo selection and relationship between viability markers must first be identified. However, relationship between viability markers must first be identified. Consequently, the aim of this study was to investigate the relationship between cleavage stage embryo morphokinetics, blastocyst carbohydrate and amino acid metabolism and to determine whether there were correlations with embryo quality and subsequent viability post-transfer.
4.2. Experimental design

4.2.1. Oocyte and embryo collection

Oocytes were collected from 3 to 4 week old female virgin F1 (C57BL/6 x CBA) mice stimulated with 5 IU PMSG at 5 pm, followed by hCG 49 h later at 6 pm. Cumulus-oocyte complexes were collected 15 h post-hCG (8 am) in equilibrated fertilization medium and transferred to 45 µl drops of fertilization medium and cultured under Ovoil in 6% CO\textsubscript{2}, 5% O\textsubscript{2} and 89% N\textsubscript{2} at 37 °C. Sperm were collected from 8 to 12 week old F1 hybrid male mice (C57BL/6 x CBA), as per described in Section 2.2.3.

Pronucleate oocytes were subsequently cultured individually in 2 µl drops of G1 with HSA under Ovoil in 6% CO\textsubscript{2}, 5% O\textsubscript{2} and 89% N\textsubscript{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 with HSA for a further 48 h to the end of the culture period. Time-lapse images of individual embryos were generated every 15 min throughout the 5 days of culture. The timings of the first, second and third cleavage division were recorded and embryos sorted into quartiles based on 2-cell cleavage time, as previously described by Meseguer et al. (2011) and described in Chapter 3, with the fastest (1\textsuperscript{st}) quartile designated ‘fast’ embryos and the slowest (4\textsuperscript{th}) quartile designated ‘slow’ embryos.

4.2.2. Assessment of embryo cell lineage and numbers

Blastocysts were differentially stained to determine 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). Trophoderm nuclei were labelled with propidium iodide after permeabilising the cell membrane using a complement reaction leaving the ICM nuclei remained unlabelled. Blastocysts were then stained with bisbenzimide in 10% ethanol to stain all nuclei. Blastocysts were subsequently mounted in glycerol and photographed under fluorescence microscope and cell numbers counted.
4. Blastocyst carbohydrate and amino acid metabolism of the kinetically different groups of cleavage stage embryos selected with time-lapse imaging

4.2.3. Analysis of morphokinetics relationship with carbohydrate and amino acid metabolism

The timings of the first cleavage division were recorded and embryos sorted into quartiles, with the fastest (1st) quartile designated ‘fast’ embryos and the slowest (4th) quartile designated ‘slow’ embryos. To quantitate amino acid utilization during a 24 h period between day 4 and 5 of culture, at 72 h post insemination (PI), 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. At the end of culture, blastocysts were either analyzed for carbohydrate metabolism and dual stained for ICM and TE number, assessed for implantation potential using an in vitro outgrowth model, or transferred to a recipient female to determine actual implantation rate and fetal development.

Carbohydrate levels were assayed by quantitative ultramicrofluorimetry (Gardner, 2007, Leese and Barton, 1984) using coupled enzyme-based reactions linked to pyridine nucleotides. 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 calculated on the basis that 2 mol lactate is produced per mol of glucose consumed 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 derivatisation-labelling reagent 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (Aqc) was then added, vortexed immediately and then warmed on a heating
4. Blastocyst carbohydrate and amino acid metabolism of the kinetically different groups of cleavage stage embryos selected with time-lapse imaging

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 quantitated by comparison against a standard calibration curve (Boughton, et al., 2011). Final values of amino acid turnover of embryos were evaluated by standardising 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, 2012a).

4.2.4. Blastocyst outgrowths

Blastocyst outgrowth experiments were carried out at 96 h PI, as previously described (Hannan, et al., 2011). Flat-bottomed 96-well tissue culture dishes were rinsed with sterile PBS and coated with a solution of 10 µg/ml fibronectin). Coated wells were rinsed with sterile PBS and incubated for 2 h with a solution of PBS containing 4 mg/ml BSA. Wells were then rinsed with PBS and subsequently filled with 150 µl of G2 with HSA, overlayed with 120 µl of Ovoil and the plate equilibrated under 6% CO2, 5% O2 and 89% N2 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 (Ti-U eclipse, Nikon) at x10 magnification at time points 48 h, 66 h and 72 h. Images were obtained using NIS Elements BR 3.00, SP7 Laboratory Imaging software (Nikon) and outgrowth area was measured per image using Image J software (Image J).

4.2.5. Embryo transfers

Swiss female mice between 8 to 14 weeks of age were mated with vasectomized males to induce pseudopregnancy. Mating was confirmed by the presence of a vaginal plug. Blastocysts were then transferred 96-97 h PI to the recipient female, at day 3.5 of pseudopregnancy. Recipient female mice were anesthetized with an intraperitoneal
4. Blastocyst carbohydrate and amino acid metabolism of the kinetically different groups of cleavage stage embryos selected with time-lapse imaging

Injection of ketamine (75 ml/kg Ketalar) and medetomidate (1 mg/kg, Domitor). 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 postoperative recovery with an intraperitoneal injection of atipamezole (1 mg/kg, Antisedan) 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).
4.3. Statistics

All data was assessed for Gaussian distribution with Shapiro-Wilk normality test. Data that did not meet assumption of Gaussian distribution underwent nonparametric analysis using the Mann-Whitney test. Data that were normally distributed were statistically analyzed 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 analyzed using Spearman’s correlation coefficient. Cell number differences in quartiles of morphokinetic data were analyzed by analysis of variance and between quartile differences determined using Bonferroni’s multiple comparison test. Embryo transfer results were analyzed using the Chi-square test.
4. Blastocyst carbohydrate and amino acid metabolism of the kinetically different groups of cleavage stage embryos selected with time-lapse imaging

4.4. Results

4.4.1. Morphokinetics (2-cell division timing)

As t2 showed a significant correlation to blastocyst cell number (Chapter 3) and is the earlier and most reliable developmental event compared with t5 and cavitation time, time-lapse images of individual embryos were analyzed t2 was recorded. Embryos were then sorted into quartiles based on their respective t2 timings, as previously described by Meseguer et al. (2011), and in Chapter 3. The fastest (1st) quartile was designated as ‘fast’ embryos and the slowest (4th) quartile designated ‘slow’ embryos. Average timings of the fastest and slowest quartiles were summarized in Table 4.1. On average, ‘fast’ embryos were 2.4 h ahead of ‘slow’ embryos, and there was a higher SEM of ‘slow’ embryos, indicating a larger distribution of timings, as opposed to the tight regulation of timings in ‘fast’ embryos (Table 4.1). Blastocysts developed from ‘fast’ and ‘slow’ cleaving groups of embryos were denoted as ‘fast’ and ‘slow’ blastocysts.

4.4.2. Effect of morphokinetics on cell lineage allocation

Following differential staining for ICM and TE cells in blastocysts that were measured for carbohydrate metabolism, a significant difference in total cell number was seen between ‘fast’ and ‘slow’ blastocysts (Fig 4.1). This was attributable to ‘fast’ blastocysts having a significantly larger ICM compared to ‘slow’ blastocysts (Fig 4.1). There was a significant difference in ICM: TE ratio between ‘fast’ and ‘slow’ blastocysts (‘fast’ 0.18 ± 0.11 vs. ‘slow’ 0.10 ± 0.03, P <0.05).
Table 4.1: Summary of morphokinetics of embryos

<table>
<thead>
<tr>
<th>Hours post insemination (h)</th>
<th>‘Fast’ 1st quartile</th>
<th>2nd quartile</th>
<th>3rd quartile</th>
<th>‘Slow’ 4th quartile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrate metabolism</td>
<td>15.53 ± 0.04</td>
<td>-</td>
<td>-</td>
<td>18.17 ± 0.19 *</td>
</tr>
<tr>
<td>Amino acid metabolism</td>
<td>15.82 ± 0.04</td>
<td>-</td>
<td>-</td>
<td>17.70 ± 0.1 *</td>
</tr>
<tr>
<td>Outgrowths</td>
<td>15.85 ± 0.02</td>
<td>16.20 ± 0.02</td>
<td>16.56 ± 0.02</td>
<td>17.12 ± 0.13 *</td>
</tr>
<tr>
<td>Embryo transfers</td>
<td>15.90 ± 0.05</td>
<td>-</td>
<td>-</td>
<td>17.72 ± 0.07 *</td>
</tr>
</tbody>
</table>

Data is expressed as mean ± SEM. ¹n = 61 embryos in total, ²n = 156 embryos in total, ³n = 129 embryos in total, ⁴n = 129 embryos in total. *P < 0.001 when compared to ‘fast’ cleaving embryos.
4. Blastocyst carbohydrate and amino acid metabolism of the kinetically different groups of cleavage stage embryos selected with time-lapse imaging

Figure 4.1: Correlation of morphokinetics and cell lineage allocation in the blastocyst

n = 23 embryos for ‘Fast’, 24 embryos for ‘Slow’, over 10 biological replicates, """"P <0.001, when compared to ‘fast’ group. Data is expressed as mean ± SEM. Dark portions of the bars represent the average number of TE cells and light portion the average number of ICM cells.
Blastocyst carbohydrate and amino acid metabolism of the kinetically different groups of cleavage stage embryos selected with time-lapse imaging
4. Blastocyst carbohydrate and amino acid metabolism of the kinetically different groups of cleavage stage embryos selected with time-lapse imaging

4.4.3. Effect of morphokinetics on blastocyst carbohydrate metabolism

The rate of glucose consumption by ‘fast’ blastocysts was significantly higher than ‘slow’ blastocysts 98 h PI, (Fig 4.2). Similarly, rate of lactate production by ‘fast’ blastocysts was significantly higher than ‘slow’ blastocysts (Fig 4.2). When expressed as a glycolytic rate (Gardner, et al., 1996), ‘fast’ blastocysts exhibited a significantly lower percentage when compared to ‘slow’ blastocysts (Fig 4.2). When the rates of glucose consumption and lactate production were normalized for cell number, there was no significant difference between fast and slow blastocysts (Table 4.2).

4.4.4. Effect of morphokinetics on blastocyst amino acid metabolism

There were significant differences in the amino acid metabolism profiles between ‘fast’ and ‘slow’ blastocysts (Fig 4.3a). Of the twenty amino acids 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 to those designated as ‘slow’ (Fig 4.3a) and the variation in production of these amino acids from ‘slow’ blastocysts appeared larger. ‘Fast’ blastocysts consumed significantly more aspartate compared to ‘slow’ blastocysts (Fig 4.3a). ‘Fast’ blastocysts produced little or no glutamate compared to ‘slow’ blastocysts (Fig 4.3a). 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 (Fig 4.3b).
Table 4.2: Correlation between morphokinetics and blastocyst carbohydrate metabolism per cell

<table>
<thead>
<tr>
<th></th>
<th>Glucose consumption (pmol/h/cell)</th>
<th>Lactate production (pmol/h/cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>‘Fast’</td>
<td>0.23 ± 0.02</td>
<td>0.23 ± 0.02</td>
</tr>
<tr>
<td>‘Slow’</td>
<td>0.23 ± 0.02</td>
<td>0.26 ± 0.02</td>
</tr>
</tbody>
</table>

Data is expressed as mean ± SEM. n≥ 30 embryos per group, 12 biological replicates.
4. Blastocyst carbohydrate and amino acid metabolism of the kinetically different groups of cleavage stage embryos selected with time-lapse imaging

**Figure 4.2:** Correlation between morphokinetics and blastocyst carbohydrate metabolism

(a) Glucose consumption and lactate production, picomoles per embryo per hour. (b) Glycolytic rate of blastocysts, % glucose converted to lactate.

n ≥ 30 embryos per group, 12 biological replicates, *P < 0.05, ***P < 0.001 when compared to ‘fast’ group. Blue bars represent ‘fast’ cleaving embryos and red bars represent ‘slow’ cleaving embryos.
4. Blastocyst carbohydrate and amino acid metabolism of the kinetically different groups of cleavage stage embryos selected with time-lapse imaging

![Graph showing glucose consumption and lactate production](image)

a) Glucose consumption and lactate production in fast and slow groups.

b) Glycolytic rate (%) comparison between fast and slow groups.

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* denotes statistical significance compared to the fast group.
4. Blastocyst carbohydrate and amino acid metabolism of the kinetically different groups of cleavage stage embryos selected with time-lapse imaging

**Figure 4.3:** 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. 13 biological replicates, "P <0.05, ""P <0.01, white bars represent ‘fast’ blastocysts and dark bars represent ‘slow’ blastocysts.
4. Blastocyst carbohydrate and amino acid metabolism of the kinetically different groups of cleavage stage embryos selected with time-lapse imaging
4. Blastocyst carbohydrate and amino acid metabolism of the kinetically different groups of cleavage stage embryos selected with time-lapse imaging

4.4.5. Effect of morphokinetics on blastocyst outgrowths

‘Fast’ blastocysts from the 1st quartile had a significantly larger outgrowth area after 72 h compared to ‘slow’ blastocysts from the 4th quartile (Fig 4.4a). A subset of these blastocysts were monitored for outgrowth area at 48 h, 66 h and 72 h post blastocyst transfer into outgrowth plate, and it can be seen that at 48 h after transfer into outgrowth plate, ‘slow’ blastocysts from the 4th quartile had a lower outgrowth area at all observation time points (Fig 4.4b). Embryos from the 4th quartile appeared to have a higher percentage change in outgrowth area between time points as compared to the 1st quartile (Table 4.3).

4.4.6. Effect of morphokinetics on pregnancy

Following transfer of blastocysts to pseudopregnant 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 4.4). Of those blastocysts that implanted, 69.6 % of ‘fast’ blastocysts formed fetuses compared to 40.4 % of implanted ‘slow’ blastocysts (Table 4.4). Fetuses that had developed successfully were assessed using a morphological grading (Table 2.2)(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 4.4), however, all parameters, with the exception of eye morphological grade, were lower on average in fetuses from ‘slow’ blastocysts compared to fetuses from ‘fast’ blastocysts. In addition, the sex of one of the fetus developed from ‘slow’ blastocyst group was too young to be sexed, and another fetus was exencephalic (Table 4.4).
**Table 4.3:** Percentage change of outgrowths at different observation points

<table>
<thead>
<tr>
<th>Quartiles</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>From 48 h to 66 h</td>
<td>51.1</td>
<td>44.4</td>
<td>48.7</td>
<td>54.3</td>
</tr>
<tr>
<td>From 66 h to 72 h</td>
<td>29.6</td>
<td>18.6</td>
<td>32.0</td>
<td>39.2</td>
</tr>
</tbody>
</table>

Data is expressed as percentage change since previous observation timepoint (48 h and 66 h). n= 54 embryos across all quartiles, 3 biological replicates.
Figure 4.4: Correlation of morphokinetics and blastocyst outgrowth

Data is expressed as mean ± SEM. (a) Outgrowth area of all quartiles at the end of outgrowth period. n= 129 embryos across all quartiles, 5 biological replications. a b P <0.05, different letters represent significant differences between quartiles. (b) Outgrowth area of all quartiles at 48, 66 and 72 h post blastocyst transfer into outgrowth plate. *Represent significant differences between quartile 1 and 4, *P <0.05. n= 54 embryos across all quartiles, 3 biological replicates.
4. Blastocyst carbohydrate and amino acid metabolism of the kinetically different groups of cleavage stage embryos selected with time-lapse imaging
4. Blastocyst carbohydrate and amino acid metabolism of the kinetically different groups of cleavage stage embryos selected with time-lapse imaging

Table 4.4: Correlation of morphokinetics and pregnancy

<table>
<thead>
<tr>
<th>Parameter</th>
<th>‘Fast’</th>
<th>‘Slow’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Implantation</td>
<td>62.2 ± 6.8%</td>
<td>63.3 ± 8.4%</td>
</tr>
<tr>
<td>Fetal development per implantation</td>
<td>69.6%</td>
<td>40.4%*</td>
</tr>
<tr>
<td>Fetal weight (mg)</td>
<td>128.3 ± 2.4</td>
<td>125.0 ± 3.5</td>
</tr>
<tr>
<td>Placental weight (mg)</td>
<td>96.8 ± 2.9</td>
<td>92.3 ± 3.3</td>
</tr>
<tr>
<td>Fetal:placenta weight ratio</td>
<td>0.76 ± 0.02</td>
<td>0.74 ± 0.03</td>
</tr>
<tr>
<td>Crown-rump length (mm)</td>
<td>10.0 ± 0.08</td>
<td>10.0 ± 0.13</td>
</tr>
<tr>
<td>Limb morphological grade</td>
<td>14.2 ± 0.08</td>
<td>14.1 ± 0.14</td>
</tr>
<tr>
<td>Eye morphological grade</td>
<td>14.7 ± 0.07</td>
<td>14.4 ± 0.15</td>
</tr>
<tr>
<td>Sex of fetus</td>
<td>53.8% F, 46.2% M</td>
<td>47.8% F, 43.5% M</td>
</tr>
<tr>
<td></td>
<td>4.3% n/a, 4.3% Ex</td>
<td></td>
</tr>
</tbody>
</table>

Data is expressed as % mean ± SEM. n= 90 blastocysts transferred per group in 18 mouse recipients. F, female; M, male; n/a, undetermined; Ex, Exencephalic. *P <0.01
4. Blastocyst carbohydrate and amino acid metabolism of the kinetically different groups of cleavage stage embryos selected with time-lapse imaging

4.5. Discussion

This is the first study to incorporate time-lapse and metabolic analysis on the same embryo, where it was determined that there was a correlation between morphokinetics, blastocyst ICM, total cell number and metabolic measures of blastocyst quality, both carbohydrate and amino acid metabolism. Timing of the first cleavage division (t2) was chosen as the strongest morphokinetic marker to identify ‘fast’ and ‘slow’ cleaving embryos, and subsequently, ‘fast’ blastocysts had significantly higher cell numbers and consumed significantly more glucose compared to ‘slow’ blastocysts (Fig 4.5). Of physiological significance, ‘fast’ blastocysts had a significantly lower glycolytic rate compared to ‘slow’ blastocysts. This lower glycolytic rate is not a function of embryo cell number, but rather reflects the fundamentally different metabolic activity of these ‘fast’ blastocysts. Hence, on a per cell basis, cells from ‘fast’ blastocysts are functionally different compared to cells from ‘slow’ blastocysts, and this therefore explains the higher viability of the ‘fast’ blastocysts (Lane and Gardner, 1996). Blastocyst amino acid metabolism also differed significantly between ‘fast’ and ‘slow’ blastocysts, with ‘fast’ blastocysts consuming significantly more aspartate, and less glutamine, alanine and glutamate (Fig 4.5). 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 by ‘slow’ embryos. Furthermore, of the fetuses developed from ‘slow’ blastocysts, one was determined to be exencephalic, and one was significantly premature (Day 12-13). Overall, it has been demonstrated that embryos with different morphokinetics develop to blastocysts with significantly different metabolic profiles, cell lineage allocation and subsequent viability (Fig 4.5).

Blastocysts that developed from ‘fast’ embryos had significantly higher rates of glucose uptake compared to 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, et al., 1996, Gardner and Leese, 1987, Gardner, et al., 2011). Similarly, analysis of carbohydrate metabolism revealed that ‘fast’
4. Blastocyst carbohydrate and amino acid metabolism of the kinetically different groups of cleavage stage embryos selected with time-lapse imaging

Figure 4.5: Illustration summarizing the results of ‘fast’ embryos with respect to morphokinetic and metabolic markers

4. Blastocyst carbohydrate and amino acid metabolism of the kinetically different groups of cleavage stage embryos selected with time-lapse imaging

- **Fast embryos**
  - ↑ Blastocyst cell numbers
  - ↑ Larger outgrowth area
  - ↑ Fetal development per implantation

- **Developmental kinetics**

- **Carbohydrate metabolism**
  - ↑ ICM
  - ↑ Glucose consumption
  - ↓ Glycolytic rate
  - Aspartate has been shown to regulate carbohydrate metabolism

- **Amino acid metabolism**
  - ↑ Aspartate consumption
  - Glutamate consumption instead of production
  - ↓ Breakdown of Ala-Gln in culture medium
4. Blastocyst carbohydrate and amino acid metabolism of the kinetically different groups of cleavage stage embryos selected with time-lapse imaging

blastocysts had significantly higher rates of lactate production when compared to ‘slow’ blastocysts, albeit a smaller change when compared to glucose uptake. At the blastocyst stage, glucose is the preferred substrate for metabolism and it has been shown that approximately half of the glucose consumed is converted to lactate (Gardner and Leese, 1990), and the rest of the glucose is used via different pathways, such as for the energy production via the Krebs cycle, as well as the PPP (O’Fallon and Wright, 1986) and hexosamine biosynthetic pathway (Pantaleon, et al., 2008, Sutton-McDowall, et al., 2006).

It is imperative to maintain the appropriate activities of energy generation pathways, for example, the amount of glucose converted to lactate (glycolytic activity). In 1996, Lane and Gardner determined that F2 blastocysts with glycolytic activity corresponding to that observed in vivo (~50 %) were shown to be highly viable (80 % fetal development) as compared to randomly selected blastocysts that had a 20 % fetal development rate. In the incubation medium used for analysis of carbohydrate metabolism by ultramicrofluorescence, glucose was the sole carbohydrate for the source of energy. As such, lactate produced is derived from metabolism of this available glucose, and therefore, glycolytic rate of the embryo can be determined. Hence, blastocysts exhibiting an increased glycolytic rate (more lactate produced than glucose consumed, are presumed to be utilizing endogenous energy reserves, such as glycogen, for lactate production (Lane and Gardner, 1996). However, this was not observed in the present studies, as glycolytic rate percentages remained below 100 % for both ‘fast’ and ‘slow’ blastocysts. With regards to the increased production of lactate from ‘fast’ blastocysts, it has recently been proposed that increased lactate production may have important roles, by providing a low pH environment that facilitates the implantation process via uterine tissue breakdown and increased angiogenesis for fetal-maternal blood supply, and in immune modulation to prevent maternal rejection (Gardner, 2015b).

Analysis of both glucose uptake and lactate production in this study revealed that ‘fast’ blastocysts exhibit a significantly lower percentage of glucose converted to lactate, as compared to ‘slow’ blastocysts. This result was observed due to ‘fast’ blastocysts
Blastocyst carbohydrate and amino acid metabolism of the kinetically different groups of cleavage stage embryos selected with time-lapse imaging

consuming 1.5 times more glucose than ‘slow’ blastocysts, as opposed to producing 1.2 times more lactate than ‘slow’ blastocysts. Previous investigations have demonstrated that increased rates of fetal development are correlated to higher levels of glucose consumption at the blastocyst stage (Gardner, et al., 2001, Gardner and Leese, 1987, Gardner, et al., 2011, Renard, et al., 1980). Similarly, higher rates of glucose consumption was observed in ‘fast’ blastocysts, which have significantly higher ICM numbers, higher ICM is to TE ratio, developed a larger outgrowth area and underwent higher levels of fetal development per implantation when compared to ‘slow’ blastocysts. These findings support previous research that development of the ICM is positively correlated with viability, while increased glycolytic rate is negatively correlated with subsequent viability (Lane and Gardner, 1996, Lane and Gardner, 1997a). Of interest, uptake of glucose has been shown to be greater in ICM compared to TE cells and significantly, all the glucose consumed by ICM cells can be accounted for by lactate formation, whereas in TE cells, lactate formation accounts for half of the glucose consumed (Hewitson and Leese, 1993). Therefore, different numbers of cells in either ICM or TE lineage, as observed in the present study, can result in significant differences in glycolysis of the overall blastocyst.

Interestingly, differences in glucose consumption between male and female mouse embryos have been previously reported (Gardner, et al., 2010, Gardner and Leese, 1987, Lane and Gardner, 1996). During preimplantation embryo development, there is a period between embryonic genome activation and X chromosome inactivation, where both X chromosomes of the female embryo is active (Epstein, et al., 1978). The enzyme, glucose-6-phosphate dehydrogenase (G6PDH) is located on the X chromosome and involved in glucose metabolism, specifically, the PPP. Therefore, previous studies have shown that mouse female embryos have a 27 % increase in glucose consumption as compared to the male embryos (Lane and Gardner, 1996). However, a significant difference in the sexes of the fetuses following embryo transfer was not found in this experiment, indicating that the glycolytic activity reported is not skewed by sex of the embryo.
Analysis of amino acid metabolism showed that kinetically different embryos have different amino acid metabolic profiles. The production of glutamine and alanine, plausibly from the breakdown of alanyl-L-glutamine, was elevated in ‘slow’ blastocysts compared to ‘fast’ blastocysts and may therefore reflect differences in the ability of kinetically different blastocysts to process alanyl-L-glutamine, as well as to regulate alanine and glutamine pools, considering that this substrate is 10 times higher in concentration in G-media compared to all other amino acids (Table 2.1). Total amino acid turnover was found to be significantly lower in ‘fast’ blastocysts compared to ‘slow’ blastocysts and this difference can be attributed primarily to the larger production of glutamine and alanine by ‘slow’ blastocysts. In clinical IVF media, alanyl-L-glutamine is commonly used in replacement of glutamine, which would otherwise break down to form toxic ammonium in the culture medium (Lane and Gardner, 1994, Vickery, et al., 1935, 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 consumes more glutamate and subsequently displayed a significantly lower amino acid turnover compared to embryos cultured in 5 % oxygen (Wale and Gardner, 2012a). Interestingly, the same authors previously showed that embryos compromised by culture in atmospheric oxygen (20 %) 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 developed into blastocysts that consumed less glutamate compared to faster cleaving embryos.

Brison et al. (2004) reported that human pronucleate oocytes with higher viability also have a lower amino acid turnover (Brison, et al., 2004). However, that study employed atmospheric oxygen conditions, which have been shown to affect amino acid utilization (Wale and Gardner, 2012a; 2013) and negatively impact blastocyst and fetal development, as well as resultant blastocyst gene expression and proteome (Gardner and Lane, 2005, Harlow and Quinn, 1979, Katz-Jaffe, et al., 2006b). Of interest, Booth et al. (2007) conducted multiple observations on porcine embryos for cleavage time, and analyzed these cleavage stage embryos for amino acid turnover. They found that
faster cleaving embryos have a higher consumption of methionine, asparagine and arginine and a higher turnover rate compared to slower cleaving embryos (Booth, et al., 2007). As their study was on cleavage stage porcine embryos grown in media with different concentrations of amino acids compared to 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 (Gardner, 1998, Lamb and Leese, 1994).

Aspartate, the most highly consumed amino acid by the mouse blastocyst, has been shown to regulate carbohydrate metabolism in the embryo via its own metabolism through the MAS (Lane and Gardner, 2005a). 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 greater glucose utilization through the Embden-Meyeroff pathway. In turn, this would mean that less pyruvate would need to be converted to lactate in order to generate the cytosolic NAD$^+$ required to maintain glucose flux. Consequently, this helps to explain 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 to ‘slow’ blastocysts. Interestingly, in analysis of outgrowth areas between ‘fast’ and ‘slow’ quartiles, there was a significantly higher percentage change in outgrowth area of the slowest quartile, suggesting a compensatory response to delayed cleavage timing. In support of this, of the ‘slow’ blastocysts that developed to fetuses, growth and normality appears unaffected, although an exencephalic fetus was developed from a ‘slow’ blastocyst. Furthermore, there remains a higher fetal loss following implantation from transfer of ‘slow’ blastocysts, indicating that delayed cleavage timing may predispose the blastocyst to developmental arrest after implantation, or that ICM cells in ‘slow’ blastocysts were insufficient to form a complete fetus, as the ICM is to TE ratio is halved in the ‘slow’ blastocysts. 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.

In embryo culture, there is accumulation of toxic ammonium due to spontaneous breakdown and metabolism of amino acids by the embryo (Gardner and Lane, 1993), and several pathways may be involved in ammonium sequestration in the early embryo. It has been suggested that alanine, produced via transamination from pyruvate, may be potentially used as an ammonium sink, thereby preventing the build-up of ammonium ions in the culture medium (Partridge and Leese, 1996, Wale and Gardner, 2013). In cow blastocysts, it was found that there was a significant decrease in alanine production when blastocysts were cultured in the absence of pyruvate (Partridge and Leese, 1996), but there was no significant dose-response increase in alanine production with increasing concentrations of ammonium. However, when post-compaction mouse embryos were exposed to 300 μM ammonium for 48 h, there was a significant increase in glutamine and alanine production (Wale and Gardner, 2013), which was also observed in the present study. Wale and Gardner (2013) found that aspartate consumption was significantly increased in the presence of 150 μM ammonium, and suggested that aspartate may be involved in ammonium sequestration via its conversion to asparagine, or via alternate pathways such as aspartate and citrulline to produce arginine and fumarate for ammonium sequestration. The ability of the embryo to utilize alanine and aspartate for ammonium sequestration may affect embryo development and viability, and the present study observed that ‘slow’ blastocysts have a significantly higher alanine production, and ‘fast’ blastocysts have higher aspartate consumption. Furthermore, an exencephalic fetus developed following transfer of ‘slow’ blastocysts, and Lane and Gardner (1994) reported that ammonium in culture medium may induce exencephaly in a dose-dependent manner (Lane and Gardner, 1994). The relationship of amino acid utilization for ammonium sequestration may result in variation in morphokinetic of the embryo, and warrants further investigation.
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 (Fig 4.5). 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 show that there is a relationship between embryo 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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5. Gene expression analysis of kinetically different groups of blastocysts and fetuses
5. Gene expression analysis of kinetically different groups of blastocysts and fetuses

5.1. Introduction

Controlled embryo metabolism is important to provide sufficient energy, at the right time, for the molecular processes that regulate ongoing development. As shown in Chapter 4, which has subsequently been published (Lee, et al., 2015), morphokinetics and metabolism are closely associated. Carbohydrate metabolism, specifically glucose uptake, by the human blastocyst is positively related to viability. Glucose consumption and lactate production, expressed a ratio as glycolytic rate, have also been related to embryo viability in the mouse (Lane and Gardner, 1996). Results from Chapter 4 show that embryos with an earlier 2-cell cleavage (i.e. 1st quartile, ‘fast’ embryos) develop into blastocysts with significantly higher glucose consumption and lower glycolytic rate as compared to embryos that cleaved later (i.e. 4th quartile, ‘slow’ embryos). In addition to quantifying carbohydrate metabolism of mouse blastocysts, Lee et al. (2005) analyzed global amino acid profiling of the same cohort of embryos analyzed for carbohydrate metabolism. Data revealed that aspartate consumption, which is the most highly consumed amino acid in the mouse (Lamb and Leese, 1994, Wale and Gardner, 2012a), is significantly higher in groups of kinetically faster embryos compared to slower embryos. The study revealed for the first time that kinetically different cleavage stage embryos develop into blastocysts with significantly different metabolic profiles that is correlated with subsequent embryo viability post transfer and demonstrated that there is a relationship between the embryo biomarkers, morphokinetics, carbohydrate and amino acid metabolism (Lee, et al., 2015).

As the relationship between these embryo selection biomarkers and viability becomes better established, a more detailed understanding of transcriptional regulation level is required. Wong et al (2010) show that aberrant cytokinesis observed from morphokinetic data of an arrested 2-cell stage human embryo were correlated with reduced expression of key cytokinesis genes (ANLN, CFL1, DIAPH1, DIAPH2, DNM2, ECT2, MKLP2, MYLC2, PHOA) compared with developmentally normal embryos at the same stage (Wong, et al., 2010). However, an arrested 4-cell stage embryo that underwent prolonged cytokinesis during its first division shown reduced expression of
only ANLN and ECT2 genes (Wong, et al., 2010). Based on subsequent results obtained from analyzing expression of different groups of genes (e.g. maternal factors and embryonic genome activation markers), Wong et al. (2010) concluded that prediction of embryo development using morphokinetics is reliant on inheritance of maternal transcripts, which were expressed at altered levels in abnormal embryos. Other factors may include inherited genetic mutations, aneuploidy, environmental insults to germ cells and events during fertilization and sperm-related factors (Wong, et al., 2010).

To date, gene expression analysis of kinetically different mouse embryos to understand the genetic basis of timing of development has not specifically been investigated. As results from previous data (Chapter 4) revealed a relationship between morphokinetics, carbohydrate, and amino acid metabolism with embryo viability, the molecular characterization of kinetically different blastocysts may reveal differential transcription of genes related to cell division, the metabolic pathways described and the implantation process. Consequently, the aim of this study was to quantitate the mRNA levels of genes involved in aspartate and carbohydrate metabolism, control of development and control of implantation in blastocysts that were kinetically different from each other based on their t2. As transfer of these kinetically different blastocysts resulted in higher loss of fetal development post implantation (Chapter 4), this study was also aimed to determine if the mRNA levels are significantly different in placenta and fetal tissue retrieved from fetuses that developed from kinetically different blastocysts.
5. Gene expression analysis of kinetically different groups of blastocysts and fetuses

5.2. Experimental design

5.2.1. Oocyte and embryo collection

Oocytes were collected from 3 to 4 week old female virgin F1 (C57BL/6 x CBA) mice stimulated with 5 IU PMSG at 5 pm, followed by hCG 49 h later at 6 pm. Cumulus-oocyte complexes were collected 15 h post-hCG (8 am) in equilibrated fertilization medium and transferred to 45 µl drops of fertilization medium and cultured under Ovoil 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 x CBA), as per described in Section 2.2.3.

Pronucleate oocytes were cultured individually in 2 µl drops of G1 with HSA under Ovoil 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 with HSA 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 timings of the first cleavage division were recorded and embryos sorted into quartiles based on 2-cell cleavage time, as previously described by Meseguer et al. (2011) and by Lee et al. (2015), with the fastest (1st) quartile designated ‘fast’ embryos and the slowest (4th) quartile designated ‘slow’ embryos. Blastocysts from the 1st and 4th quartiles were snap frozen in liquid nitrogen for gene expression analysis.

5.2.2. Embryo transfers, placental and fetal liver tissue collection

Swiss female mice between 8 to 14 weeks of age were mated with vasectomized males to induce pseudopregnancy. Mating was confirmed by the presence of a vaginal plug. Embryos were then transferred 96-97 h PI to the recipient female, at day 3.5 of pseudopregnancy. Recipient female mice were anesthetized with an intraperitoneal injection of ketamine (75 ml/kg Ketalar) and medetomidate (1 mg/kg, Domitor). Ten blastocysts were transferred into each recipient female through a dorsal incision, with a
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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 postoperative recovery with an intraperitoneal injection of atipamezole (1 mg/kg, Antisedan) to reverse the effects of the medetomidate, while the analgesic effects of ketamine remained. Pregnant recipients were killed on E13.5 and placental and fetal liver tissue was collected into individual eppendorf tubes. Following collection, eppendorf tubes were snap frozen in lysis buffer with β-Mercaptoethanol (0.7 µl β-Mercaptoethanol to 100 µl lysis buffer, 2 µl of Lysis Buffer- β-Mercaptoethanol mixture per blastocyst) in liquid nitrogen until analysis.

5.2.3. RT-PCR and qRT-PCR

5.2.3.1. RNA extraction from day 5 blastocysts

Total RNA from Day 5 blastocysts was extracted using Absolutely RNA Nanoprep Kit (#400753, Agilent Technologies, USA), as per manufacturer’s instructions. Day 5 blastocysts were snap-frozen in lysis buffer with β-Mercaptoethanol (0.7 µl β-Mercaptoethanol to 100 µl lysis buffer, 2 µl of Lysis Buffer- β-Mercaptoethanol mixture per blastocyst). For RNA extraction, the blastocyst lysates were thawed and supplemented with an equal volume of 70% ethanol, then mixed thoroughly by vortexing for 5 seconds. The mixtures were then transferred to an RNA-binding nanospin cup and centrifuged for 1 min (12, 000G). This was followed by DNase treatment and subsequent washes for DNA-free total RNA. RNA concentration was quantified using a Nanodrop 2000 spectrophotometer (Thermo Scientific). As limited amount of RNA can be extracted per embryo, 36 embryos were grouped together for a sample analysis. Four biological samples of 36 embryos each were collected for each of the ‘fast’ and ‘slow’ group of blastocysts.
5. Gene expression analysis of kinetically different groups of blastocysts and fetuses

5.2.3.2. RNA extraction from placenta and fetal liver tissues

Frozen fetal liver and placenta samples from 2.8.1 were thawed on ice. For RNA extraction, 600 µl of Trizol (Life Technologies) was added to individual samples and a pestle was used to grind the tissue until it had formed a homogenous suspension. Following this, 120 µl of chloroform (Chem Supply, Australia) was added and tubes, which were then inverted for 4 min and chilled on ice for a further 5 min. The tubes were centrifuged at 4 °C at 13 000G for 15 min and 300 µl of the supernatant was extracted and added to a fresh tube containing 300 µl of isopropanol. These tubes were chilled on ice for 10 min before centrifugation at 18 000G for 10 min. Supernatant was then removed and discarded and 400 µl of 70% ethanol was added followed by centrifugation at 18 000G for 1 min. Again, supernatant was removed and discarded. DNAse treatment was carried out using a DNAse treatment kit (Ambion, Life Technologies). The RNA pellet centrifuged from the extraction step was resuspended in 20 µl of water and incubated in a heating block at 65 °C for 5 min. The mixture was then incubated with 2 µl of 10x DNase buffer and 1 µl of DNase at 37 °C for 45 min, followed by addition of 2 µl of inactivating reagent. This was left at room temperature for 2 min before being manually flicked twice. Tubes were then centrifuged at 18 000G for 1 min and supernatant (with RNA) was removed and stored in a fresh tube at -80 °C before cDNA synthesis.

5.2.3.3. cDNA synthesis

The M-MLV reverse transcriptase kit (#M1701, Promega, Australia) was used to synthesize cDNA. Water was added to RNA extracted as outlined in 2.9.1 and 2.9.2, to make up a total volume of 12 µl. To this mixture, 1 µl of Oligo-dt (Geneworks, SA, Australia) was added, and the tube was incubated at 70 °C for 5 mins, then cooled to 4 °C. The following was then added to each incubation tube: 4 µl M-MLV 5x buffer, 0.5 µl RNase inhibitor, 1 µl dNTP, 0.4 µl M-MLV reverse transcriptase and 1.1 µl water. This mixture was then incubated at 37 °C for a further 90 min and stored at 4 °C. A
control tube without M-MLV reverse transcriptase (RT) was also set up in parallel with test samples, and cDNA was diluted 1 in 3 by the addition of 40 µl of water before use in subsequent PCR reactions.

5.2.3.4. qRT-PCR

5x HOT FIREPol EvaGreen qPCR Mix Plus (ROX) (Solis BioDyne, Estonia) was used for qRT-PCR reactions. The qPCR reaction mix per sample included: 2 µl 5x HOT FIREPol EvaGreen qPCR Mix Plus, 0.5 µl forward primer, 0.5 µl reverse primer, 6 µl water, 1 µl cDNA (as outlined in 2.9.2). Triple technical repeats were tested for. Samples were amplified using ViiA 7 Real Time PCR System (Life Technologies). Briefly, the samples were initially heated to 95 °C for 15 min followed by 40 cycles of the following conditions: 95 °C for 15 sec, 60 °C for 15 sec and 72 °C for 30 sec. The cycle threshold (Ct) values for GAPDH, β-actin and 18s Ribosomal RNA were each used to normalise against the sample results, and raw Ct values were analyzed using Q-Gene software package (Simon, 2003).
5. Gene expression analysis of kinetically different groups of blastocysts and fetuses

5.2.3.5. Selected genes for gene expression analysis

The list of genes selected is listed below (Table 5.1). Genes involved in aspartate and carbohydrate metabolism, control of development and control of implantation in blastocysts were chosen.

5.3. Statistics

All data was assessed with the Shapiro-Wilk test for normality. Differences in expression levels of genes between ‘fast’ and ‘slow’ blastocysts, placenta and fetal liver tissue samples were statistically analyzed with Student’s unpaired t-test.
5. Gene expression analysis of kinetically different groups of blastocysts and fetuses

**Table 5.1:** List of genes analyzed for gene expression analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gapdh</td>
<td>Reference genes</td>
</tr>
<tr>
<td>18S</td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td></td>
</tr>
<tr>
<td>Wnt3a</td>
<td>Blastocyst development</td>
</tr>
<tr>
<td>Eomes</td>
<td>Trophectoderm differentiation</td>
</tr>
<tr>
<td>Cdx2</td>
<td></td>
</tr>
<tr>
<td>Slc2a1</td>
<td>Transport of glucose</td>
</tr>
<tr>
<td>Slc2a3</td>
<td></td>
</tr>
<tr>
<td>Ped</td>
<td>Regulates timing of development</td>
</tr>
<tr>
<td>Igf2r</td>
<td>Cell proliferation</td>
</tr>
<tr>
<td>B3gnt</td>
<td>Cell differentiation and adhesion</td>
</tr>
<tr>
<td>Got1</td>
<td>Enzyme involved in MAS (also known as aspartate aminotransferase)</td>
</tr>
<tr>
<td>Pkm2</td>
<td>Regulatory enzyme promoting aerobic glycolysis</td>
</tr>
</tbody>
</table>

**Table 5.1:** List of genes analyzed for gene expression analysis.
5. Gene expression analysis of kinetically different groups of blastocysts and fetuses

5.4. Results

5.4.1. Gene expression levels of kinetically different blastocysts

The expression of genes in Table 5.1 was quantified in ‘fast’ (1st quartile) and ‘slow’ (4th quartile) groups of blastocysts, with the exception of Cdx2, which was omitted due to data collection error. The gene expression levels of ‘slow’ blastocysts were expressed relative to ‘fast’ blastocysts, where ‘fast’ blastocysts have a value of 1.0, with the expression of B3gnt5, Slc2a1, Slc2a3, Got1 and Pkm2 found to be significantly lower in ‘slow’ blastocysts (Fig 5.1). Overall, average expression of all genes in ‘slow’ blastocysts appeared lower compared to ‘fast’ blastocysts (Fig 5.1). Of interest, Slc2a1, Slc2a3, Got1 and Pkm2 are genes implicated in the regulation of carbohydrate and aspartate metabolism. To further illustrate the differences between ‘fast’ and ‘slow’ blastocysts of significantly different genes, differences between ‘fast’ and ‘slow’ blastocysts were plotted for each these genes independently (Fig 5.2).

5.4.2. Gene expression levels of placental tissue between fetuses derived from embryos chosen using morphokinetics

Following in vitro culture, ‘fast’ and ‘slow’ blastocysts were transferred into pseudopregnant recipients. Recipients were killed on day 13.5 and placentae were collected from fetuses and analyzed for gene expression levels of genes listed in Table 5.1, with the exception of Eomes, which was omitted due to sampling error. Fetuses were categorized as ‘fast’ or ‘slow’ fetuses depending on development from transferred ‘fast’ or ‘slow’ blastocysts. The gene expression levels of placentae from ‘slow’ fetuses were expressed relative to those belonging to ‘fast’ fetuses. The genes B3gnt5, Cdx2, Ped and Igf2r were on average higher than levels expressed in placenta tissue, while the remaining genes were expressed at lower levels in placenta tissues retrieved from ‘slow’ blastocysts (Fig 5.3). Of interest, genes implicated in aspartate and carbohydrate metabolism, Slc2a1, Got1 and Pkm2, were significantly lower in placental tissue.
Gene expression analysis of kinetically different groups of blastocysts and fetuses obtained from ‘slow’ fetuses as compared to ‘fast’ fetuses, illustrated individually in Fig 5.4.

5.4.3. Gene expression levels of fetal liver tissue between fetuses derived from embryos chosen using morphokinetics

Fetal liver tissue was also collected and analyzed for gene expression levels of the genes listed in Table 5.1, with the exception of Eomes, which was omitted due to sampling error. The gene expression levels of liver tissue from ‘slow’ fetuses were expressed relative to liver tissue obtained from ‘fast’ fetuses. With the exception of Wnt3a and Slc2a1, all other genes were on average expressed at a higher level in ‘slow’ fetuses as compared to ‘fast’ fetuses (Fig 5.5). Genes implicated in aspartate metabolism, Got1 and Pkm2, are significantly different in liver tissue obtained from ‘slow’ fetuses, with significantly higher levels expressed in liver tissue of ‘slow’ fetuses as compared to liver tissue obtained from ‘fast’ fetuses (Fig 5.6).
5. Gene expression analysis of kinetically different groups of blastocysts and fetuses

Figure 5.1: Summary of gene expression levels referenced to gene 18S of ‘slow’ blastocysts relative to ‘fast’ blastocysts

n= 4 biological samples per group, where 1 sample consist of 36 blastocysts in total, collected over 12 culture weeks. #; significantly different genes. Data is expressed as mean ± SEM.
5. Gene expression analysis of kinetically different groups of blastocysts and fetuses

[Bar graph showing relative fold change to fast blastocysts for various genes, with some genes marked with # symbols.]

- B3gnt5
- Eomes
- Wnt3a
- Slc2a1
- Slc2a3
- Got1
- Ped
- Igf2r
- Pkm2
5. Gene expression analysis of kinetically different groups of blastocysts and fetuses

**Figure 5.2:** Levels of gene expression of ‘slow’ blastocysts relative to ‘fast’ blastocysts referenced to gene 18S

a) B3gnt5, b) Slc2a1, c) Slc2a3, d) Pkm2, e) Got1. n= 4 biological samples per group, where 1 sample consist of 36 blastocysts in total, collected over 12 culture weeks. *P <0.05, **P <0.01 when compared to ‘fast’ blastocysts. Data is expressed as mean ± SEM.
5. Gene expression analysis of kinetically different groups of blastocysts and fetuses
5. Gene expression analysis of kinetically different groups of blastocysts and fetuses

**Figure 5.3:** Summary of gene expression levels referenced to gene *18S* of placenta tissue obtained from fetuses developed from ‘slow’ blastocysts relative to placenta tissue obtained from fetuses developed from ‘fast’ blastocysts.

n =10 biological samples per group. #; significantly different genes. Data is expressed as mean ± SEM.
5. Gene expression analysis of kinetically different groups of blastocysts and fetuses
5. Gene expression analysis of kinetically different groups of blastocysts and fetuses

Figure 5.4: Levels of gene expression from placenta tissue obtained from ‘slow’ and ‘fast’ fetuses referenced to gene 18S

a) Slc2a1, b) Got1, c) Pkm2, n =10 biological samples per group. *P <0.05 when compared to placenta tissue obtained from ‘fast’ fetuses. Data is expressed as mean ± SEM.
5. Gene expression analysis of kinetically different groups of blastocysts and fetuses

![Bar charts showing relative fold change for different groups.]

- **a)**
  - Fast: Relative fold change = 1.0
  - Slow: Relative fold change = 0.2

- **b)**
  - Fast: Relative fold change = 1.0
  - Slow: Relative fold change = 0.5

- **c)**
  - Fast: Relative fold change = 1.0
  - Slow: Relative fold change = 0.6

* denotes a significant difference.
5. Gene expression analysis of kinetically different groups of blastocysts and fetuses

**Figure 5.5:** Summary of gene expression levels referenced to gene 18S of liver tissue obtained from fetuses developed from ‘slow’ blastocysts relative to liver tissue obtained from fetuses developed from ‘fast’ blastocysts.

n= 10 biological samples per group. #; significantly different genes. Data is expressed as mean ± SEM.
5. Gene expression analysis of kinetically different groups of blastocysts and fetuses
5. Gene expression analysis of kinetically different groups of blastocysts and fetuses

Figure 5.6: Levels of gene expression from liver tissue obtained from ‘slow’ and ‘fast’ fetuses referenced to gene 18S

a) Got1, b) Pkm2, n= 10 biological samples per group. *P < 0.05 when compared to liver tissue obtained from ‘fast’ fetuses. Data is expressed as mean ± SEM.
5. Gene expression analysis of kinetically different groups of blastocysts and fetuses

![Bar chart a) showing relative fold change for fast and slow groups.](image)

![Bar chart b) showing relative fold change for fast and slow groups.](image)
5. Gene expression analysis of kinetically different groups of blastocysts and fetuses

5.5. Discussion

The present experiment analyzed gene expression of kinetically different mouse blastocysts, specifically investigating levels of mRNA of genes involved in cytokinesis, embryo development, implantation process, carbohydrate and aspartate metabolism. Results confirm the relationship between morphokinetics, carbohydrate and amino acid metabolism, with changes in expression levels of genes implicated in these biomarkers. The timing of the first cleavage division (t2) was used to classify embryos as ‘fast’ (1\textsuperscript{st} quartile) or ‘slow’ (4\textsuperscript{th}) embryos, and day 5 blastocysts that developed from ‘fast’ embryos expressed higher levels of all genes analyzed (Table 5.1). Genes implicated in glucose transport and aspartate metabolism was expressed at significantly higher levels in faster blastocysts and placental tissue from fetuses that developed from ‘fast’ blastocysts. Fetuses developed from ‘fast’ and ‘slow’ cleaving groups of embryos were denoted as ‘fast’ and ‘slow’ fetuses. Results from placental tissue were in line with data from kinetically different blastocysts, with genes implicated in glucose transport, glucose and aspartate metabolism significantly up regulated in placental tissue from ‘fast’ fetuses. Fetal tissue sampled in this experiment belonged to the liver, which consist mainly of hepatocytes that are highly metabolically active. Interestingly, genes implicated in glucose and aspartate metabolism was expressed at significantly lower levels in ‘slow’ blastocysts exhibited a higher expression in liver tissue from ‘slow’ fetuses, suggesting a compensatory response in the slower fetuses.

Data from Chapter 4 have shown that there are similar implantation rates between ‘fast’ and ‘slow’ blastocysts, however there is a decreased loss of pregnancy following implantation in ‘fast’ blastocysts, and therefore an increased viability in this group of embryos. Levels of $B3gnt5$, implicated in cell differentiation and adhesion, are significantly higher in ‘fast’ blastocysts. In a different strain of mice (BDF1), Parks et al. (2011) have observed decreased levels of $B3gnt5$ in blastocysts that failed to implant. $B3gnt5$, the Lc3-synthase gene, is responsible for the initiation of formation of the lactoseries glycosphingolipids (GSL), which are integral components of mammalian cell membranes that participate in cell adhesion during embryogenesis and cell
differentiation (Yamashita, et al., 1999). Significantly, disruption of the B3gnt5 gene leads to preimplantation lethality due to disruption in GSL biosynthesis (Biellmann, et al., 2008). Hence, the decreased levels of B3gnt5 observed in slower blastocysts in the present study may have contributed to the observed loss of pregnancy following implantation.

Furthermore, genes coding for channel proteins involved in glucose transport across the plasma membrane, Slc2a1 and Slc2a3, also known as the glucose transporter (GLUT) genes, were significantly higher in ‘fast’ blastocysts. Higher levels of these transporter genes aligns with previous data (Chapter 4) that glucose consumption is significantly higher in faster blastocysts as compared to ‘slow’ blastocysts. Slc2a1 has previously been shown to be expressed throughout the period of preimplantation development (Hogan, et al., 1991), while Slc2a3 is the primary glucose transporter of trophectoderm cells of the blastocyst (Pantaleon, et al., 1997). The same group later determined that the activation and transcript of Slc2a3 supports the hexosamine biosynthesis pathway in mouse blastocysts (Pantaleon, et al., 2008), one of the alternative pathways used by the mouse blastocyst for glucose metabolism. Of interest, significant differences were only observed in transcriptional levels of Slc2a1, and not Slc2a3, in placental tissue of ‘fast’ and ‘slow’ fetuses, which may indicate that increased levels of glucose transported into placental tissue of ‘fast’ fetuses were probably not metabolized via the hexosamine biosynthesis pathway.

In addition to genes implicated in the transport of glucose, levels of Pkm2 were significantly higher in ‘fast’ blastocysts and placental tissue attached to subsequently derived ‘fast’ fetuses. Pkm2 is an isoenzyme of the enzyme pyruvate kinase, which is a regulatory enzyme in the last step of glycolysis that converts glucose to pyruvate. The specific isoform, Pkm2, has been shown to promote aerobic glycolysis (Christofk, et al., 2008) and is also present in proliferating cells and in cancer cells (Mazurek, 2007, Vander Heiden, et al., 2011). Ironically, the isoform Pkm2 encourages glucose carbons to be metabolized into pyruvate and lactate, which does not yield the highest possible amount of ATP required for increased cell proliferation. This is in contrast to the
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isoform Pkm1, which is expressed in tissues with high ATP production requirements including skeletal muscle, heart and brain (Imamura and Tanaka, 1982, Mazurek, 2011). However, it has recently been reported that Pkm1 expression in mice fibroblast cells impairs nucleotide production, DNA synthesis and progression through the cell cycle (Lunt, et al., 2015). The lower levels of Pkm2 mRNA in slow blastocysts may indicate an imbalance in the control of glycolysis, resulting in increased generation of ATP through the Krebs cycle, as well as increased conversion of exogenous pyruvate to lactate, which may explain the increase in lactate production in ‘slow’ blastocysts (Chapter 3). Indeed, lower levels of glycolysis, and subsequent decreased viability, were observed in ‘slow’ blastocysts as compared to ‘fast’ blastocysts in Chapter 3. Generation of ATP through the Krebs cycle unnecessarily may have impacted on embryo development and viability, such as by generation of ROS or increased apoptosis.

Got1 codes for glutamate oxaloacetate transaminase 1, also known as aspartate aminotransferase, a key enzyme in the malate-aspartate shuttle (MAS). The MAS shuttle produces NAD+ via a series of reaction beginning with Got1 responsible for the conversion of aspartate to oxaloacetate (Fig 1.3). Higher levels of aspartate consumption by ‘fast’ blastocysts (Chapter 4) and higher mRNA levels of Got1 in ‘fast’ blastocysts and placental tissue from ‘fast’ fetuses suggest that ‘fast’ blastocysts transport higher levels of NAD+ from the mitochondria to the cytoplasm via the MAS. NAD+ is required for glucose metabolism, and an alternative pathway for regeneration is via the conversion of glucose to lactate (aerobic glycolysis). Therefore, higher levels of NAD+ provided by the MAS in ‘fast’ blastocysts may reduce the amount of glucose converted to lactate, as supported by decreased lactate production in ‘fast’ blastocysts as compared to ‘slow’ blastocysts (Chapter 4). Interestingly, levels of Pkm2 and Got1 are significantly higher in ‘slow’ fetuses, with fetal growth parameters being no different to ‘fast’ fetuses. The high mRNA levels of these genes suggest a compensatory response in the ‘slow’ fetuses to maintain fetal quality. However, it is unknown why levels of Pkm2 and Got1 were increased in ‘slow’ fetuses, and warrants further investigation to determine if these F2 generation mice have different levels of metabolism as compared to mice developed from ‘fast’ fetuses.
The *Ped* (preimplantation embryonic development) gene, has been shown to regulate embryo development and subsequent embryo survival, and in particular, is involved in control of cytokinesis during preimplantation embryo development (Goldbard and Warner, 1982, Warner, et al., 1993). However, data from the present experiment demonstrated that *Ped* was not significantly different between fast and slow blastocysts and fetuses. Warner et al. (1987) reported that the expression of the *Ped* gene is as early as the first cleavage division stage (t2) in mice embryos, and linked to the H-2 complex, the major histocompatibility complex of the mouse (Warner, et al., 1987). *Ped* transcribes for the Qa-2 protein, which has been reported to be produced at higher levels in embryos with a faster developmental rate (calculated based on embryonic cell number PI) compared to embryos with little or no expression (Warner and Brenner, 2001). Furthermore, the same gene has been shown to influence cell lineage allocation in the blastocysts, where significantly higher numbers of inner cell mass was observed in a mice strain (BALB/cJ) expressing Qa-2 compared to a strain that does not express Qa-2 protein (BALB/xByJ) (Roudebush, 1998). To date, studies on the *Ped* gene have observed the influence of *Ped* on different strains of mice, or mouse embryos generated from the same strain but of two genotypes (carrying either fast alleles and slow alleles).

In mice, *Igf2r*, insulin-like growth factor 2 receptor, belongs to the insulin family of proteins that regulates the insulin growth factor pathway, which plays a critical role in differentiation, cell growth and apoptosis of different cell types, such as rat hepatoma cells (Hari, et al., 1987), and human myoblasts (Shimizu, et al., 1986). Specifically, there is evidence that *Igf2r* is implicated in lysosomal enzyme sorting and endocytosis (Lobel, et al., 1989), protein signaling and stimulation of cell division (Rechler, et al., 1980). In mouse embryos, the receptor is imprinted and maternally expressed from the early post-implantation stage (Lerchner and Barlow, 1997). Further, it was suggested that epigenetic change of *Igf2r* may be linked to large offspring syndrome (Young, et al., 2001), and a reduced expression of *Igf2r* was observed in cow blastocysts derived from slower cleaving embryos (Sugimura, et al., 2012). In the same family of insulin proteins and receptors, *Igf1*, insulin-like growth factor 1 and its receptor has been shown to stimulate growth of mouse embryos *in vitro*, increasing blastocyst development and subsequent ICM numbers (Harvey and Kaye, 1992), and expression is up-regulated
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peri-implantation in the uterus (Kapur, et al., 1992). Inactivating mutations of Igf2r may cause embryonic overgrowth and perinatal lethality (Lau, et al., 1994), however levels of Igf2r are not significantly different between kinetically different embryos, placental and liver tissue of kinetically different fetuses, suggesting that transcriptional regulation of this gene is not altered by morphokinetics of embryos.

Wnt signal transduction regulates the balance between proliferation and differentiation throughout embryogenesis (Logan and Nusse, 2004), and Wnt pathway activity through β-catenin has been detected in several tissue types, such as brain and central nervous system, as well as throughout mouse development (Maretto, et al., 2003). Importantly, Wnt/β-catenin signaling is essential for normal blastocyst function and implantation (Xie, et al., 2008) and Wnt3a has been shown to activate the Wnt/β-catenin signaling pathway and actively transcribed during the blastocyst stage (Kemp, et al., 2005). In particular, inactivation of Wnt3a results in intial implantation but subsequent loss of pregnancy (Luis and Staal, 2009), which was observed in embryo transfers of ‘slow’ blastocysts in Chapter 4. Gene expression analysis of BDF1 blastocysts determined that blastocysts that resulted in successful implantation and healthy fetal development exhibited increased Wnt3a mRNA levels (Parks, et al., 2011), a result that was not observed in the present experiment, suggesting that the Wnt/β-catenin signaling pathway was not affected by morphokinetics of the early embryo.

TE differentiation at the blastocyst stage is crucial for implantation, and Eomes (Eomesodermin) and Cdx2 (caudal-related homeodomain protein), are both key regulators of trophectoderm differentiation. Eomes is a T-box transcription factor, and disruption in gene expression of Eomes in mouse blastocysts results in failure of blastocysts to attach or form trophoblast outgrowths (Strumpf, et al., 2005). Blastocysts from Cdx2-null mutant mice die around the time of implantation due to failure to maintain the blastocoel cavity following loss of TE epithelial integrity (Strumpf, et al., 2005). In the present study, attempts to quantify expression of both Eomes and Cdx2 of kinetically different blastocysts and fetuses failed, due to a data collection error of Cdx2 in blastocyst samples and a sampling error of Eomes in placental and liver tissue.
samples. As implantation rates were observed to be similar between ‘fast’ and ‘slow’ blastocysts (Chapter 4), it is predicted that levels of *Eomes* and *Cdx2* will not be different between kinetically different groups of blastocysts and fetuses. Interestingly, *Cdx2* has been proposed to be a biomarker for intestinal cancer, for the diagnosis of intestinal adenocarcinomas (Bayrak, et al., 2012, Liu, et al., 2007).

In summary, data presented in this chapter study reveal that morphokinetics and the metabolism of carbohydrates and amino acids are related at the level of gene expression. This data reinforces the findings of the previous chapters, which shows a functional relationship between developmental kinetics, embryo metabolism and viability. Collectively, both findings support the reported success of using morphokinetics as a biomarker. At the transcriptional level, faster cleaving embryos develop into blastocysts with significantly higher levels of glucose transporter genes, as well as genes implicated in energy production via the MAS. Subsequently, significantly higher levels of viability are observed in this group of blastocysts, suggesting that the regulation of these pathways impacts viability. Data from the present study also demonstrated that the potential regulation of the MAS and subsequent energy production is critical for carbohydrate metabolism. Importantly, the transcriptional changes seen in the blastocyst are maintained in the developing fetus and placental tissue after transfer. In liver samples obtained from fetuses developed from ‘fast’ blastocysts, expression of genes implicated in aerobic glycolysis and MAS were significantly down regulated compared to ‘slow’ blastocysts, which suggest that the impact of morphokinetic and metabolic changes can have a delayed or prolonged effect during gestation, and may subsequently affect the regulation of metabolism in the next generation.
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6. Analysis of aspartate uptake in relation to glucose, and as a novel biomarker assay
6. Analysis of aspartate uptake in relation to glucose, and as a novel biomarker assay

6.1. Introduction

Amino acids are vital components of culture media, and enhance preimplantation embryo development of all mammalian species studied to date (Bavister and Arlotto, 1990, Devreker and Hardy, 1997, Gardner and Lane, 1993, Gardner, et al., 1994, Takahashi and First, 1992). Supplementation of amino acids to culture media has been shown to significantly increase cleavage rate, percentage blastocyst formation, mitoses and viability (Bavister and Arlotto, 1990, Gardner and Lane, 1993, Lane and Gardner, 1997a, McKiernan, et al., 1995). Oviduct fluid is characterized by relatively high levels of amino acids; alanine, glutamate, glycine, serine, threonine and taurine (Schultz et al., 1981, Gardner and Leese, 1990). Subsequent analysis of the composition of Eagle’s medium revealed a striking homology between the non-essential amino acids group and those abundant in oviduct fluid (Eagle, 1959, Gardner and Lane, 1993). Lane and Gardner (1994, 1997) demonstrated that non-essential and essential amino acids both regulate development but act at different stages with respect to pre- and post-compaction development of the embryo.

Specifically, non-essential amino acids and glutamine significantly increase development of mouse and bovine embryos during pre-compaction stage, whereas no benefits were observed when pre-compaction embryos were cultured in the presence of essential amino acids in either species (Gardner and Lane, 1993, Steeves and Gardner, 1999). Rather, a reduction was observed in the cell number of blastocysts from pre-compaction embryos cultured in the presence of essential amino acids (Gardner and Lane, 1993, Lane and Gardner, 1997a, Steeves and Gardner, 1999). However, when essential amino acids were added post-compaction, ICM numbers increased and subsequent fetal development were improved. Non-essential amino acids were beneficial post-compaction by promoting blastocyst development and hatching (Gardner and Lane, 1993, Lane and Gardner, 1997a). Hence, it was observed that supplementation of non-essential amino acids to culture media during early cleavage up to the 8-cell stage, followed by all amino acids from 8-cell to blastocyst stage is found to provide the highest rates of development and viability after embryo transfer (Lane
and Gardner, 1997a). However, further work on the role of individual essential amino acids during the cleavage stages is warranted.

The non-essential amino acid, aspartate is the most highly consumed in the mouse, cow and pig blastocyst (Humpherson, et al., 2005, Lamb and Leese, 1994, Lee, et al., 2015, Orsi and Leese, 2004, Wale and Gardner, 2012a), and is a key substrate of the malate-aspartate shuttle (MAS)(Fig 1.3). This may be due to its biosynthetic roles, but cytoplasmic levels of aspartate have mainly been suggested to be the rate limiting factor in the activity of the MAS (Kovacevic, 1972). Lane and Gardner (2005) therefore investigated the role of aspartate on mouse embryos and determined that the addition of high concentration of exogenous aspartate (10 mM) increased both lactate uptake and oxygen consumption of zygotes (Lane and Gardner, 2005a). Furthermore, increased extracellular levels of aspartate enable zygotes to utilize lactate, rather than pyruvate, as the sole carbohydrate for development to the blastocyst stage, and resulting in viable pregnancies after transfer (Lane and Gardner, 2005a). Presumably, higher extracellular levels of aspartate increased activity of the MAS and in turn, increased the supply of NAD+, which allows for lactate to be converted to pyruvate for aerobic respiration. Therefore, the MAS have been shown to play an important role in regulating the availability of NAD+ (and hence NADH) levels between the cytosol and the mitochondria in the embryo (Lane and Gardner, 2005).

It has been determined that the activity of the MAS is important for embryo development, as zygotes cultured in the presence of increasing concentrations of the mitochondrial and MAS inhibitor, aminooxyacetate acid (AOA), exhibit impairments in glycolysis, Krebs cycle activity, oxygen consumption and subsequent ATP production, with an associated decrease in blastocyst formation and cell number (Wakefield, et al., 2011). Disruption of the MAS using AOA at the 8-cell stage in mouse embryos also caused a decrease in blastocyst ICM and TE cell numbers and glycolytic rate, with increased lactate production, however blastocyst development itself was unchanged (Mitchell, et al., 2009). Transfer of AOA treated blastocysts resulted in decreased implantation and fetal developmental rates compared to untreated controls, and resultant fetuses had a decreased fetal to placental weight ratio (Mitchell, et al., 2009). Hence, the
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consequence of inhibiting MAS activity during preimplantation embryo development extends beyond implantation and fetal developmental rates. It was shown that low level AOA treatment resulted in significantly altered gene expression in Day 18 fetal brain tissue, affecting gene expression pathways associated with carbohydrate metabolism, neurological development, cellular proliferation, cell death, DNA replication and repair (Fullston, et al., 2011). Therefore, regulation of MAS activity in the embryo is not only related to ongoing pregnancy, but plays early roles in predisposing the fetus to developmental problems during gestation.

In Chapter 4, it was demonstrated that aspartate and glutamate consumption differs between kinetically different groups of blastocysts, with blastocysts derived from ‘fast’ embryos (1st quartile; based on time of 2-cell cleavage division) consuming significantly more aspartate and glutamate than ‘slow’ embryos, (4th quartile). In addition, ‘slow’ blastocysts consumed less glucose. To date, methods used to determine amino acid metabolism have included HPLC or LC-MS, which allows determination of the consumption of multiple amino acids simultaneously, requiring long incubation times (> 24 h) (Brison, et al., 2004, Houghton, et al., 2002b, Sturmey, et al., 2010, Wale and Gardner, 2012a). Low levels of amino acid consumption by mouse embryos have also required the grouping of blastocysts in order to determine levels of amino acids consumed or pooling of embryos (e.g., from 3 blastocysts; Wale and Gardner, 2012, to >30 embryos; Lamb and Leese 1994). Currently, the rate of aspartate consumption measured from individual blastocysts and its relation to glucose and lactate metabolism remains uncharacterized.

Based on the significance of aspartate in embryo metabolism as described, the aim of this experiment was therefore to develop and validate an alternative enzymatic assay using the ultramicrofluorescence method to measure the rate of aspartate consumption by individual mouse blastocysts. The same assay system has already been used to measure single embryo turnover of metabolites such as glucose, lactate, pyruvate and ammonium (Gardner and Lane, 1993, Gardner and Leese, 1986, Gardner and Leese, 1987). To further understand consumption of aspartate and its impact on carbohydrate
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metabolism, the dose-dependent influence of aspartate concentration on aspartate consumption, glucose consumption and lactate production were investigated, as well as relationships to embryo morphokinetics.
6. Analysis of aspartate uptake in relation to glucose, and as a novel biomarker assay

6.2. Experimental design

6.2.1. Development and validation of aspartate assay

Reaction concentrations are listed in Appendix E. The following enzymatic assay was used to measure aspartate consumption (Passonneau and Lowry, 1993):

\[
\text{Aspartate} + \alpha\text{-ketoglutarate} \xrightarrow{\text{Glutamic oxaloacetic transaminase}} \text{Oxaloacetate} + \text{Glutamate} \\
\text{Oxaloacetate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{Malate dehydrogenase}} \text{Malate} + \text{NAD}^+
\]

In the development of this assay, the availability and consumption rate of aspartate and glucose were considered. Previous studies on global amino acid utilization of mouse embryos show approximate rates of aspartate consumption to be around 1.5 to 2.0 pmol/h/embryo at the blastocyst stage (Lamb and Leese, 1994, Wale and Gardner, 2012a). To replicate in vitro culture conditions, the concentration of aspartate in the incubation medium was similar to the concentration found in G2 culture media (100 μM). However, ultramicrofluorescence requires small volumes of incubation medium (100 nl vs. culture drop 2 μl) to facilitate detectability of substrate depletion. Additionally, Passonneau and Lowry (1993) have suggested an excess of ~50 % of substrate availability. Hence, based on the average embryo consumption rate of 1.5 to 2.0 pmol/h, with measurements taken hourly over 3 hours, individual embryos were estimated to have a maximum consumption of 6 pmol. A 50 % excess of substrate availability thus requires 12 pmol of aspartate to be present in the incubation medium; hence the volume of incubation medium is calculated to be 120 nl, with an aspartate concentration of 100 μM.

The rate of glucose consumption was also taken into consideration, as the blastocyst is incubated in a small volume of medium, and is at risk of depleting glucose via glucose metabolism. From data in chapter 4, average glucose consumption of individually cultured day 5 IVF-derived blastocysts was approximately 15.0 pmol/h/embryo. In the enzymatic assay, the concentration of glucose in G2-MOPS medium is 0.5 mM,
therefore to ensure glucose availability, volume of incubation drop was increased to 150 nl to provide 75 pmol of glucose for consumption over 3 h.

Firstly, the concentrations of substrates required for the conversion of oxaloacetate to malate, the 2nd part of the two-part equation, were determined. Concentrations proposed by Passonneau and Lowry (1993) had to be modified to measure single embryo consumption. To determine concentrations of NADH and malate dehydrogenase, a standard of known concentrations (62.5 μM, 125 μM, 250 μM, 400 μM, 500 μM and 600 μM) of oxaloacetate was made in medium G2 with HSA media, but with 0 mM aspartate. Experimental concentrations of NADH and malate dehydrogenase were used to measure the concentration of oxaloacetate of each standard and values were plotted against change in fluorescence to calculate a line of regression. Concentrations of substrates were deemed acceptable when $R^2 > 0.99$ (Fig 6.1). Following multiple rounds of optimization, it was determined that 150 μM of NADH and 3.5 units/ml of malate dehydrogenase was required for full conversion of oxaloacetate to malate and NAD$^+$. 
6. Analysis of aspartate uptake in relation to glucose, and as a novel biomarker assay

**Figure 6.1:** Standard curve of change in fluorescence of NADH against known concentrations of oxaloacetate

Levels of fluorescence produced by NADH were measured before and after the addition of the standard at different concentrations of aspartate (62.5, 125, 250, 400, 500 and 600 μM) to quantitate the change in fluorescence, which was plotted against concentrations of aspartate. Aspartate is converted to oxaloacetate, which will subsequently react with the NADH accordingly, causing a decrease in fluorescence as the concentration of aspartate increases.
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\[ R^2 = 0.9928 \]
Next, the concentrations of substrates required for the conversion of aspartate to oxaloacetate and glutamate, the 1st reaction of the assay, were determined. Similarly, a standard of 62.5 μM, 125 μM, 250 μM, 400 μM, 500 μM and 600 μM aspartate were made in aspartate free G2 medium with HSA and concentrations of substrates were determined as previously described. Following optimization, it was determined that the levels of α-ketoglutarate and glutamic oxaloacetic transaminase required for full conversion of aspartate to oxaloacetate needed to be 120 μM of α-ketoglutarate and 20 units/ml, respectively. The concentrations of substrates required for the aspartate assay reagent mixture are summarized below (Table 6.1). The imidazole buffer was made with 30 mM imidazole and 20 mM imidazole-HCL and the pH of the imidazole buffer was adjusted to a value of 7.0, using a pH meter (Orion Versa Star, Thermo Scientific). Reaction conditions and concentrations of stock solutions are detailed in Appendix E.

To validate the enzymatic assay, a standard curve was run using concentrations of aspartate (62.5 μM, 125 μM, 250 μM, 400 μM, 500 μM and 600 μM). Known concentrations of aspartate were made up and measured as samples. Concentration of samples was subsequently determined using regression line obtained from the standard, and the value of samples was compared to its original value.
6. Analysis of aspartate uptake in relation to glucose, and as a novel biomarker assay

Table 6.1: Media components for aspartate enzyme cocktail

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH</td>
<td>150 μM</td>
</tr>
<tr>
<td>α-ketoglutarate</td>
<td>120 μM</td>
</tr>
<tr>
<td>Glutamic oxaloacetic transaminase (GOT)</td>
<td>20 units/ml</td>
</tr>
<tr>
<td>Malate dehydrogenase</td>
<td>3.5 units/ml</td>
</tr>
<tr>
<td>Imidazole buffer</td>
<td>30 mM imidazole, 20 mM imidazole-HCL</td>
</tr>
</tbody>
</table>

Adapted from Passonneau and Lowry (1993), Enzymatic analysis, a practical guide.
6. Analysis of aspartate uptake in relation to glucose, and as a novel biomarker assay

6.2.2. Relationship of aspartate concentration on aspartate, glucose consumption and lactate production of in vivo blastocysts

6.2.2.1. Embryo collection

3 to 4 old female virgin F1 (C57BL/6 x CBA) mice were stimulated with an intraperitoneal injection of 5 IU PMSG, followed by 5 IU hCG 48 h later. Females were mated with F1 hybrid male mice (C57BL/6 x CBA) overnight, following hCG administration. The presence of a vaginal plug the following morning (Day 0.5) was used as an indicator of successful mating. Successfully mated females were separated and returned to cages for 3 days. On the morning of Day 4, females were sacrificed and the uterine horns dissected out in warmed G-MOPS medium (37 °C). Using a 1 ml syringe and a 19-G needle pre-loaded with warmed G-MOPS medium, uterine horns were flushed three times for in vivo blastocysts in an organ well dish. In vivo blastocysts were collected immediately and placed in respective treatment G2-MOPS wash drops (outlined below) and subsequently washed three times before placement into a defined volume of G2-MOPS (143.7 nl) incubation drop for analysis using ultramicrofluorescence. In vivo blastocysts were used as opposed to cultured embryos so as to eliminate possible effects of culture conditions on blastocyst metabolism of aspartate and carbohydrates.

6.2.2.2. Measurement of aspartate consumption over increasing aspartate concentrations

To investigate the effect of aspartate concentration on subsequent aspartate consumption, blastocysts were incubated in G2-MOPS (37 °C) under paraffin oil (Ovoil, Vitrolife) with one of the following aspartate concentrations: 0.1 mM, 0.25 mM, 0.5 mM, 1 mM or 2 mM. Aspartate levels in incubation drops (143.7 nl) were assayed by ultramicrofluorimetry using coupled enzyme-based reactions (Chapter 6.2.1). Serial 1 nl
samples of media were taken every hour over a three hour period. Aspartate consumption was expressed per blastocyst and following analysis, blastocysts were individually incubated at 37 °C in a solution composed of G-MOPS medium, 10% ethanol and 0.1 mg/ml bisbenzimide for 1 h, followed by rinsing in G-MOPS medium for 10 min for the determination of total cell number. Stained blastocysts were then mounted in glycerol on glass microscope slides and photographed using a fluorescence microscope.

6.2.2.3. Measurement of glucose consumption and lactate production with increasing aspartate concentrations

To determine the effect of aspartate concentration on glucose and lactate metabolism, blastocysts were incubated in warmed G2-MOPS (37 °C) under paraffin oil (Ovoil) with one of the following aspartate concentrations: 0 mM, 0.1 mM, 1.0 mM, 10.0 mM. This range of values was selected as previous research has demonstrated an increased activity of the MAS and ability to use lactate as the sole substrate, when aspartate in the medium is at the level of 10.0 mM (Lane and Gardner, 2005a). Glucose and lactate levels of incubation drops (143.7 nl) were assayed by ultramicrofluorimetry using similar enzyme-based reactions as described. Serial 1 nl samples of media were taken every hour over a three hour period. Glucose consumption and lactate production were expressed per embryo and glycolytic rate was calculated on the basis that 2 mol lactate was produced per mol of glucose consumed by the embryo (Gardner and Leese, 1990), as described in Chapter 4. Following analysis, blastocysts were individually stained for total cell number and rate of glucose consumption and lactate production were further expressed per embryo per cell.

6.2.3. Measurement of aspartate and glucose uptake of kinetically different groups of embryos produced through IVF
6. Analysis of aspartate uptake in relation to glucose, and as a novel biomarker assay

6.2.3.1. Oocyte collection and embryo culture

Oocytes were collected from 3 to 4 week old female virgin F1 (C57BL/6 x CBA) mice stimulated with 5 IU PMSG at 5 pm, followed by hCG 49 h later at 6 pm. Cumulus-oocyte complexes were collected 15 h post-hCG in equilibrated fertilization medium and transferred to 45 µl drops of fertilization medium and cultured under paraffin oil (Ovoil, Vitrolife) in 6% CO₂, 5% O₂ and 89% N₂ at 37 °C. Sperm were collected from 8 to 12 week old F1 hybrid male mice (C57BL/6 x CBA), as per described in Section 2.2.3.

Pronucleate oocytes were cultured individually in 2 µl drops of G1 with HSA under paraffin oil (Ovoil, Vitrolife) in 6% CO₂, 5% O₂ and 89% N₂ 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 with HSA for a further 48 h to the end of the culture period. Time-lapse images of individual embryos were generated every 10 min throughout culture. The timings of the first cleavage division were recorded and embryos sorted into quartiles as previously described by Meseguer et al. (2011) and by Lee et al. (2015), with the fastest (1st) quartile designated ‘fast’ embryos and the slowest (4th) quartile designated ‘slow’ embryos. Following the end of culture, “fast” or “slow” blastocysts were analyzed for aspartate and glucose consumption.

6.2.3.2. Measurement of aspartate and glucose uptake of kinetically different groups of blastocysts created through IVF

To investigate the relationship of embryo cleavage kinetics on aspartate and glucose uptake, ‘fast’ or ‘slow’ blastocysts were incubated in warmed G2-MOPS (37 °C) under paraffin oil (Ovoil) with aspartate concentration at 0.1 mM, similar to culture condition. Aspartate and glucose levels of incubation drops (143.7 nl) were assayed by ultramicrofluorimetry. Serial 1 nl samples of media were taken every hour over a three
hour period. Aspartate and glucose uptake were expressed per embryo. Following analysis, blastocysts were individually stained for total cell number and rate of aspartate and glucose consumption were further expressed per embryo per cell.

6.3. Statistics

All data were assessed for normal distribution with the Shapiro-Wilk normality test and are normally distributed. Correlation of aspartate consumption over increasing aspartate concentrations was determined using Pearson’s correlation. One-way ANOVA with post-hoc Bonferroni’s test were used to analyze the relationship of increasing aspartate concentrations to glucose consumption and lactate production. Glycolytic rate percentages were arc-sine transformed and compared using Student’s unpaired t-test. The same test was used to analyse aspartate and glucose consumption values from kinetically different blastocysts.

All analyses were performed using GraphPad Prism version 5.04 for Windows (GraphPad Software).
6. Analysis of aspartate uptake in relation to glucose, and as a novel biomarker assay

6.4. Results

6.4.1. Effect of aspartate concentration on subsequent aspartate consumption

The rate of aspartate consumption in \textit{in vivo} blastocysts increased significantly with aspartate concentration (Fig 6.2). Significant differences were observed when blastocysts were incubated in G2-MOPS with 1 mM and 2 mM of aspartate, a 10-fold and 20-fold increase in aspartate concentration compared to normal media concentration of aspartate (0.1 mM), respectively. Similarly, when the rate of aspartate consumption was further expressed per blastocyst total cell number, a positive relationship was observed between increasing aspartate consumption and increasing aspartate concentrations (Fig 6.2). Cell numbers of the flushed blastocysts varied between groups and it was necessary to normalize for this difference in cell number (Table 6.2).
6. Analysis of aspartate uptake in relation to glucose, and as a novel biomarker assay

<table>
<thead>
<tr>
<th>Aspartate concentration (mM)</th>
<th>0.1</th>
<th>0.25</th>
<th>0.5</th>
<th>1.0</th>
<th>2.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blastocyst cell number</td>
<td>54.3 ± 2.4</td>
<td>57.3 ± 4.0</td>
<td>56.7 ± 3.4</td>
<td>58.9 ± 3.9</td>
<td>41.1 ± 3.9</td>
</tr>
</tbody>
</table>

n = 8 blastocysts per group, 5 biological replicates. Data is expressed as mean ± SEM.
6. Analysis of aspartate uptake in relation to glucose, and as a novel biomarker assay

**Figure 6.2:** Effect of aspartate concentration on subsequent aspartate consumption

a) Uptake per embryo, b) Uptake per cell.

n =8 blastocysts per group, 5 biological replicates. Data is expressed as mean ± SEM. \( a, b, c P <0.01 \). \( a, b, c \) Different letters represent significant differences between groups.
6. Analysis of aspartate uptake in relation to glucose, and as a novel biomarker assay

(a) and (b) show the relationship between aspartate concentration (mM) and uptake rate (pmol/embryo/h or pmol/cell/h). The graphs illustrate significant differences at certain concentrations, indicated by different letters (a, b, c) above the bars.
6. Analysis of aspartate uptake in relation to glucose, and as a novel biomarker assay

6.4.2. Effect of aspartate concentration on subsequent glucose consumption and lactate production of *in vivo* blastocysts

The rate of glucose consumption and lactate production per blastocyst was not significantly different when *in vivo* blastocysts were incubated in G2-MOPS with increasing aspartate concentrations (Fig 6.3a and Fig 6.3c). Total cell numbers of *in vivo* blastocysts were not significantly different between increasing concentrations of aspartate (Table 6.3). However, when further expressed per blastocyst cell number (Fig 6.3b and Fig 6.3d), the rate of glucose consumption per cell significantly increases relative to aspartate concentration (Fig 6.3b). The amount of lactate production per cell was however not significantly different (Fig 6.3d). When expressed as a glycolytic rate, percentages were also not significantly different amongst blastocysts incubated with different concentrations of aspartate (Fig 6.3e). Hence, the increase of exogenous aspartate concentration in culture media caused an increase in glucose consumption per cell; however, the percentage of glucose converted to lactate was not affected.
Table 6.3: Effect of increased dose-dependent aspartate concentration on day 4 blastocyst total cell numbers

<table>
<thead>
<tr>
<th>Aspartate concentration (mM)</th>
<th>0.0</th>
<th>0.1</th>
<th>1.0</th>
<th>10.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blastocyst cell number</td>
<td>58.1 ± 3.4</td>
<td>62.1 ± 3.4</td>
<td>62.2 ± 3.8</td>
<td>54.1 ± 3.7</td>
</tr>
</tbody>
</table>

n ≥12 blastocysts per group, 47 blastocysts in total, 7 biological replicates. Data is expressed as mean ± SEM.
6. Analysis of aspartate uptake in relation to glucose, and as a novel biomarker assay

**Figure 6.3:** Effect of aspartate concentration on subsequent glucose consumption and lactate production

- a) Glucose consumption per blastocyst.
- b) Glucose consumption per cell.
- c) Lactate production per blastocyst.
- d) Lactate production per cell.
- e) Glycolytic rate.

n ≥12 blastocysts per group, 47 blastocysts in total, 7 biological replicates. Data is expressed as mean ± SEM. \(^{b}P < 0.05, \quad ^{c}P < 0.01\) \(^{a},^{b},^{c}\) Different letters represent significant differences between groups. Blue bars represent glucose consumption, orange bars represent lactate production and green bars represent glycolytic rates of embryos.
6. Analysis of aspartate uptake in relation to glucose, and as a novel biomarker assay
6. Analysis of aspartate uptake in relation to glucose, and as a novel biomarker assay

6.4.3. Measurement of aspartate and glucose uptake of kinetically different embryos produced by IVF

Embryos derived from IVF were identified to be ‘fast’ or ‘slow’ cleaving embryos and ‘fast’ embryos were determined to cleave to 2-cell stage ~1.9 h earlier than ‘slow’ embryos (Table 6.4). Although no significant differences were observed with total cell numbers, “fast” embryos developed into blastocysts with an average ~14.9 more cells than ‘slow’ blastocysts (Table 6.4). The rate of aspartate and glucose consumption was significantly higher in ‘fast’ blastocysts as compared to ‘slow’ blastocysts, with ‘fast’ blastocysts consuming over double the amount of aspartate compared to (Fig 6.4a). When the rate of aspartate and glucose consumption was further expressed per cell, there was no significant difference observed (Fig 6.4b).
6. Analysis of aspartate uptake in relation to glucose, and as a novel biomarker assay

Table 6.4: Total cell number and 2-cell cleavage division timings of kinetically different embryos

<table>
<thead>
<tr>
<th></th>
<th>Total cell number</th>
<th>2-cell cleavage time PI (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fast</td>
<td>71.5 ± 6.6</td>
<td>15.0 ± 0.2^a</td>
</tr>
<tr>
<td>Slow</td>
<td>56.6 ± 3.8</td>
<td>16.9 ± 0.2^b</td>
</tr>
</tbody>
</table>

n =15 blastocysts per group, 4 biological replicates. Data is expressed as mean ± SEM. ^a,b P <0.05. ^a,b Different letters represent significant differences between groups. PI; post insemination
6. Analysis of aspartate uptake in relation to glucose, and as a novel biomarker assay

Figure 6.4: Aspartate and glucose consumption of kinetically different embryos
(a) Uptake per blastocyst. (b) Uptake per cell

n ≥15 blastocysts per group, 31 blastocysts in total, 4 biological replicates. Data is expressed as mean ± SEM. *P <0.05, Blue bars represent ‘fast’ cleaving embryos, red bars represent ‘slow’ cleaving embryos
6. Analysis of aspartate uptake in relation to glucose, and as a novel biomarker assay

a)

b)
6. Analysis of aspartate uptake in relation to glucose, and as a novel biomarker assay

6.5. Discussion

The data presented in this chapter describe successful novel validation of an ultramicrofluorescence enzymatic assay to measure, the rate of aspartate consumption of individual blastocysts. In vivo flushed blastocysts were used for the validation of the assay, to remove any variables associated with in vitro embryo culture. Using this embryo metabolic assay, the influence of exogenous aspartate in culture media was found to significantly affect the rate of consumption of aspartate by in vivo blastocysts. Increase of aspartate concentration in culture media is also associated with a significant increase in glucose consumption by in vivo blastocysts. Interestingly, lactate production was not increased, despite higher glucose consumption, indicating that the additional glucose consumed was not used for aerobic glycolysis, and may be metabolized through other pathways, such as the Krebs cycle. Similar to in vivo blastocysts, glucose consumption is positively correlated to aspartate consumption in in vitro blastocysts. The data also confirmed results from Chapter 4 that kinetically faster embryos at the 2-cell stage develop into blastocysts that consume significantly more aspartate compared to blastocysts developed from kinetically slower embryos.

Aspartate plays a key metabolic role via regulation of the MAS, which subsequently affects carbohydrate metabolism (Lane and Gardner, 2005a). Previously, it was believed that pyruvate was the sole substrate capable of supporting pronucleate oocytes to develop past the 2-cell stage (Brinster, 1965a) and in addition to pyruvate, lactate has been shown to support embryo development from the 2-cell stage and acts synergistically with pyruvate (Cross and Brinster, 1973). As pyruvate can be formed from lactate through the enzyme LDH, it is interesting that formation of pyruvate in this way was not possible in pronucleate oocytes as they are unable to develop in the absence of exogenous pyruvate. The relative ratios of pyruvate to lactate have been shown to directly affect the NADH to NAD⁺ ratio, which subsequently affects the pathway in which glucose is metabolized (Lane and Gardner, 2000, Rieger, 1992). In support of this, it was shown that a greater proportion of 2-cell embryos develop when
both pyruvate and lactate are present as nutrient sources as compared to either source alone (Brinster, 1965b).

When exogenous aspartate in high concentrations (10 mM) was added to the culture medium, it was found that pronucleate oocytes could develop normally past the 2-cell stage even in the absence of pyruvate (Lane and Gardner, 2005a). With the increase of MAS activity driven by exogenous aspartate, the cytoplasmic NADH to NAD$^+$ ratio and the subsequent redox state of the pronucleate oocyte was affected, in that study. Specifically, increased levels of NAD$^+$ enable the fertilized oocyte to convert lactate to pyruvate and develop normally past the 2-cell stage. The effect of exogenous aspartate on blastocysts was previously unknown, and the data from this chapter confirms that increasing exogenous levels of aspartate positively affect the level of aspartate consumption in in vivo blastocysts, similar to pronucleate oocytes. The rate of aspartate consumption was not significantly affected when the concentration of aspartate was increased from 0.1 to 0.5 mM, but was significantly increased when concentrations of aspartate reached 1.0 and 2.0 mM. Hence, to alter aspartate consumption by the blastocyst, concentrations of exogenous aspartate must be 1.0 mM or higher.

The activity of the MAS is dependent on the affinity of the enzyme, glutamic oxaloacetate transaminase to binding aspartate, measured as $K_m$ (Michaelis constant), defined as the substrate concentration at half the maximum velocity of the reaction. An enzyme with a low $K_m$ has a high affinity for its substrate, and therefore a lower concentration of substrate is required to achieve a maximum reaction rate. It has been shown that the $K_m$ of cytoplasmic and mitochondrial glutamic oxaloacetate transaminase at the in vitro pronucleate oocyte stage is ~2.0 mM (Lane and Gardner, 2005a). In the present study, the concentrations of exogenous aspartate must be 1.0 mM or higher to alter aspartate consumption at the in vivo blastocyst stage, suggesting that the $K_m$ of glutamic oxaloacetate transaminase is relatively low, although this remains to be quantitated. This is in contrast to some tumour cells, in which the $K_m$ of glutamic oxaloacetate transaminase can be as high as 5 mM, requiring much higher levels of aspartate to achieve maximal reaction rates (Kovacevic, 1972).
Aerobic glycolysis ensures substrate and co-factor availability for proliferation, and the regeneration of NAD$^+$ that is required for glucose metabolism (Gardner, 2015b). Results demonstrated that increase of exogenous aspartate resulted in an increased glucose consumption of *in vivo* blastocysts. Interestingly, lactate production was not altered with the increase of glucose consumption, suggesting that the increase in glucose consumed was not metabolized to lactate via aerobic glycolysis. A high MAS activity would generate more intracellular NAD$^+$, and less pyruvate would be converted to lactate in order to generate the NAD$^+$ required to metabolize glucose, supporting the observation that lactate production was not increased following the increase of glucose consumption. This also agrees with observations that inhibition of the MAS causses increased lactate production (Mitchell, et al., 2009). Of note, aspartate was not the sole amino acid in the incubation medium, as compared to glucose being the sole carbohydrate in the same medium, which is glucose. Aspartate may be produced from oxaloacetate by transamination in the Krebs cycle, and is part of the oxaloacetate/aspartate family, which includes lysine, asparagine, methionine, threonine and isoleucine. These amino acids were present in the incubation media and thus, may have affected the metabolism of aspartate.

One of the possible pathways through which glucose may have been metabolized, is the stimulation of the Krebs cycle by higher levels of aspartate in the cytoplasm. As the activity of the MAS increases with increased aspartate, there is presumably an increase in the reduced NADH that is transferred via the MAS into the mitochondria. Reduced NADH can then transfer electrons to the electron transport chain and facilitate energy production by the Krebs cycle, resulting in increased ATP production. Furthermore, glutamic oxaloacetate transaminase can catalyze the conversion of aspartate to oxaloacetate, which is a key intermediate in the Krebs cycle. Alternatively, the increase in glucose consumption may be due to its conversion to glucose-6-phosphate for the pentose phosphate pathway (PPP) (O'Fallon and Wright, 1986). In support of this, stimulation of the PPP in 8- to 16-cell cow embryos increased glucose metabolism without affecting lactate production (De La Torre-Sanchez, et al., 2006). The conversion of glucose to fructose-6-phosphate can also provide substrates for the hexosamine biosynthesis pathway, associated with synthesis of glycolipids and
6. Analysis of aspartate uptake in relation to glucose, and as a novel biomarker assay

proteoglycans (Wells, et al., 2001). Increased consumption of glucose, driven by increased aspartate uptake, may also stimulate protein synthesis, as protein synthesis is ATP and glucose dependent (Bolton, et al., 1984). Furthermore, aspartate is well known in somatic cells for its roles as a precursor to protein biosynthesis. When the supply of glucose is in excess of the requirements of the developing embryo, it may also be stored as glycogen, and broken down to regenerate glucose-6-phosphate when required (Ozias and Stern, 1973, Stern and Biggers, 1968).

At the pre-compaction stage, consumption of aspartate was not significantly increased compared to other amino acids (Booth, et al., 2005, Wale and Gardner, 2012a). Consumption of aspartate is reliant on the expression of amino acid transport proteins, such as the glutamate aspartate transporter (Glast), also known as solute carrier family 1 (Slc1a3), or excitatory amino acid transporter 1 (EAAT1). In the mouse embryo, Glast is sodium dependent, and highly expressed only at the blastocyst stage, which explains the increased consumption of aspartate at the blastocyst stage (Van Winkle, et al., 1991). Similarly, the increase of the transporter protein occurs during blastocyst development in the pig (Prather, et al., 1993). Another aspartate transporter protein, volume-sensitive organic osmolyte/anion channels (VSOAC) is present from the pronucleate oocyte stage, but displays low activity in the transport of aspartate (Van Winkle, 2001). The change in expression of amino acid transport systems reflects the differences in embryonic needs between the pre- and post-compaction stages. Although it has previously been shown that human and mouse preimplantation embryos contain the same amino acid transport systems (Hammer, et al., 2000), data on human embryo amino acid metabolism indicates that aspartate is produced, rather than consumed, at both the pre- and post-compaction stages (Brison, et al., 2004, Houghton, et al., 2002b, Krisher, et al., 2015, Picton, et al., 2010, Stokes, et al., 2007, Sturmey, et al., 2010). Embryos from some of these studies, however, were cultured under high oxygen conditions (~20%), and it has been shown that high oxygen concentrations significantly alters the embryo’s amino acid and carbohydrate metabolism (Wale and Gardner, 2012a). Further, some embryos from the above studies were surplus embryos donated for research purposes, and hence may have been of poorer quality with a different physiology, or were cryopreserved prior to culture to the blastocyst stage, again, possibly altering embryo
physiology. Hence, further investigations on human embryo amino acid metabolism under physiological oxygen conditions are warranted.

Developmental kinetics of the embryo have been related to subsequent viability (Meseguer, et al., 2011, Wong, et al., 2010), however, the underlying reason to the variation in morphokinetics have not been identified. In Chapter 4, it was reported that kinetically faster embryos at the 2-cell stage develop into blastocysts that consume significantly more aspartate and glutamate compared to blastocysts that developed from kinetically slower embryos. Results from this chapter confirmed observations that aspartate consumption is higher in kinetically faster embryos as compared to slower embryos and suggests that the higher aspartate and glucose consumption in these kinetically faster embryos lead to a more efficient metabolism of glucose, hence resulting in a kinetically more efficient embryo. The mRNA for the enzymes involved in the shuttle activity have been identified in at all stages of development (Lane and Gardner, 2005a), and gene expression analysis of genes implicated in transport of glucose and MAS activity were significantly lower in ‘slow’ cleaving embryos.

Inhibition of the MAS at the blastocyst stage has been shown to compromise subsequent viability and fetal growth (Mitchell, et al., 2009) and kinetically faster embryos were shown to have a higher developmental potential, as there was reduced post-implantation fetal loss and a higher outgrowth area from ‘fast’ blastocysts compared with ‘slow’ blastocysts (Lee, et al., 2015). It is therefore possible that there is increased MAS activity in ‘fast’ blastocysts, facilitating glucose consumption and subsequent metabolism. In addition to its contribution to the activity of the MAS, aspartate may also be involved in ammonium sequestration via its conversion to asparagine, catalyzed by asparagine synthetase (Wale and Gardner, 2013). Glutamate may also be involved in ammonium sequestration via its conversion to glutamine, catalyzed by glutamine synthetase (He, et al., 2007, Orsi and Leese, 2004), and in Chapter 4, it was reported that ‘fast’ blastocysts consumed glutamate while ‘slow’ blastocysts produces glutamate. Ammonium has been demonstrated to negatively affect fetal growth (Lane and Gardner, 1994) and it is possible that ammonium does so by
down-regulating the metabolism of aspartate, as ammonium has been shown to reduce the oxidative capacity of tissues by disrupting the MAS activity (Lai, et al., 1989).

One significant issue with this experiment is the limited number of blastocysts used. As ultramicrofluorescence requires significant time for measurement of each blastocyst, as well as the enzymatic assay requiring ten minutes to ensure full conversion of aspartate to subsequent substrates, the number of blastocysts analyzed was limited. To investigate the hypothesis that increased glucose consumption was metabolized via other pathways as opposed to conversion to lactate via glycolysis, radiolabelled glucose could be used to trace the metabolic fate of glucose consumed or enzyme inhibitors can be used to limit the metabolism of glucose via a pathway of interest. Bromodeoxyuridine (BrdU) may also be used and incorporated into cellular DNA to detect possible increase of cell proliferation. Future conduct of these types of experiments would be useful in extending the present findings.

In conclusion, the data in the chapter show that uptake of aspartate can be significantly altered by concentration of exogenous aspartate. Furthermore, MAS activity is potentially influenced by aspartate concentration, affecting subsequent blastocyst glucose consumption, but not lactate production. Results of higher aspartate and glucose consumption suggest that kinetically faster embryos have a higher MAS activity and therefore increased embryo development and viability. Of significance, lactate production was not increased despite higher glucose consumption in the presence of increased exogenous aspartate in the media, indicating that the increase in glucose consumption was not used for aerobic glycolysis. While the blastocysts in this experiment were not transferred to assess their viability, the data from this and previous chapters collectively suggest that utilization of glucose uptake assay, as well as the newly optimized aspartate uptake assay, represent two readily applicable quantitative tests of viability at the single blastocyst level.
7. General Discussion
7. General discussion

7.1. Thesis discussion

Identification of quantifiable biomarkers of developmental and implantation competence remains critically important to improve embryo selection of the most viable preimplantation embryo in clinical IVF, in order to improve pregnancy outcomes and reduce complications. Using the mouse model, this research examined the use of morphokinetics as a marker of embryo viability and normality towards this objective. It was demonstrated that kinetically different cleavage stage embryos develop into blastocyst with significantly different metabolic profiles and viability, even at the transcriptional level, and a novel assay was developed to facilitate the measurement of aspartate metabolism in individual blastocysts, revealing its relationship to glucose metabolism.

The results from Chapter 3 provide novel data to support previous reports that cleavage division timings of mouse embryos are tightly regulated (Arav, et al., 2008), and as embryo development proceeds, morphokinetic parameters are less regulated and exhibits a wider range of times (Wale and Gardner, 2010). Assessment of embryo morphokinetics revealed that the earlier occurrence of morphokinetics parameters; timing of syngamy, duration between syngamy and t2 (syn-t2), first cleavage (t2), third cleavage (t5) and cavitation, are positively correlated to day 5 blastocyst total cell number, which is a further assessment of embryo normality as compared to blastocyst development rate in other studies (Arav, et al., 2008, Pribenszky, et al., 2010). Additionally, blastocyst cell number as a measure of embryo development has been positively correlated to subsequent fetal development (Lane and Gardner, 1997b). The identified parameter, t2, is in agreement with the same parameter identified in the mouse (Pribenszky, et al., 2010), as well as t5, which have been identified in humans (Meseguer, et al., 2011). Hence, for clinics that maintain the practice of day 3 embryo transfer, predictive markers of embryo development of the pre-compaction embryo (e.g., t2) are particularly useful (Kirkegaard, et al., 2015a). The kinetic marker can also be utilized to predict developmental competence of an early cleavage stage embryo, in order to assist in embryo selection for transfer, extended culture to the blastocyst stage or for cryopreservation of surplus embryos.
The goal of IVF should be to transfer a single embryo with the highest likelihood of establishing a healthy pregnancy and subsequent delivery, and extended culture of embryos to the blastocyst stage has helped enable assessment of embryo implantation potential (Gardner, et al., 2004, Milki, et al., 2000). However, some concerns have been raised (Maheshwari, et al., 2015) with regards to exposure of embryos for prolonged periods to in vitro culture conditions, thereby potentially altering genomic imprinting and gene expressions (Manipalviratn, et al., 2009, Niemitz and Feinberg, 2004), an increase in preterm deliveries and congenital abnormalities (Dar, et al., 2014, Maheshwari, et al., 2013) and altering sex ratios (Chang, et al., 2009). However, it was raised that negative conclusions by Maheshwari et al. (2015) regarding transfer of blastocysts, drawn conclusions from studies that did not account for oxygen concentrations and/or the increased use of vitrification as a superior embryo freezing technique as compared to slow-freezing (Gardner, 2015a). Furthermore, more recent data reported no negative associations with blastocyst transfer as compared to day 3 transfers (Chambers, et al., 2015, Maxwell, et al., 2015, Oron, et al., 2015). Therefore, optimized culture conditions were chosen to investigate predictive embryo biomarkers in this thesis, in order to reflect more closely embryo physiology in vitro.

Interestingly, data from human studies suggest an optimal period of morphokinetic development, where embryos that cleave too early or too late have significantly lower rates of implantation (Meseguer, et al., 2011), rather than just the delayed embryos. It is well established that cleaving earlier is a suitable indicator of developmental competence when compared to a later cleaving embryo (Lundin, et al., 2001, Van Montfoort, et al., 2004), and previous studies in mice have found that cleavage stage embryos with earlier cell divisions have a higher correlation to blastocyst development (Arav, et al., 2008, Pribenszky, et al., 2010). Similarly, the results of the present study show that the earliest cleaving group of embryos has the highest correlation to blastocyst cell number. When embryos were selected on the basis of t2 (first cleavage division), and were cultured further in an outgrowth model to assess implantation potential, the group with the earliest onset of t2 had a significantly larger outgrowth area compared to all other quartiles (Chapter 4). The differences to the suggested optimal timing window for cleavage division onset may be attributed to the incidence of chromosomal abnormalities, which is prevalent in human embryos (>50 %) (Campbell,
7. General discussion

et al., 2013a), with rates of aneuploidy increasing with maternal age (Ata, et al., 2012, Magli, et al., 2001). Alternatively, the differences may be due to EGA occurring at different cleavage stages in humans (4- to 8-cell stage) (Braude, et al., 1988) as compared to mouse (2-cell stage) (Flach, et al., 1982). To date, no data on mouse chromosomal abnormalities and its relationship developmental kinetics have been reported, however, the incidence of chromosomal abnormalities in IVF mouse embryos of a different strain (C57BL/6 x DBA/2) are relatively low, ~4 % (Carrell, et al., 2007), and mice strain used in the present studies are F1 hybrid mice (C57BL/6 x CBA), which are genetically and phenotypically uniform.

Data presented in this thesis provided novel information about morphokinetic parameters of cleavage stage embryos in relation to their subsequent blastocyst quality in the mouse, identified using an embryos derived from IVF and enhanced culture conditions routinely used in human IVF. Time-lapse analysis provides the ability to continuously monitor dynamic embryo morphology events in real time, and morphokinetic time ranges identified in the laboratory can also be used as a means of quality control. However, no consensus has been reached regarding a ‘universal’ morphokinetic marker(s), with time-lapse studies suggesting different models for embryo selection that have been associated with blastocyst development and/or viability (Campbell, et al., 2013b, Conaghan, et al., 2013, Meseguer, et al., 2011). Furthermore, the retrospective application of one model (Conaghan, et al., 2013), which resulted in identification of ‘non-usuable’ embryos, revealed that 14.2 % these ‘non-usuable’ embryos still resulted in implantation (Kirkegaard, et al., 2014). Furthermore, the more widespread use of multiple types of time-lapse devices across different laboratories may make determination of a universal morphokinetic algorithm far more difficult. Hence, morphokinetics can provide a new way of measuring embryo development, but algorithms remains to be vigorously tested with RCTs and correlated to pregnancy outcomes. In order to further improve embryo selection, the use of morphokinetics combined with other proven biomarkers, such as metabolism, would potentially reduce this variability and further increase accuracy.
Accordingly, the next study in the thesis (Chapter 4) incorporated time-lapse and metabolic analysis on the same embryo to determine relationships between morphokinetics and biomarkers. Data from Chapter 4 demonstrated that kinetically different cleavage stage embryos develop into blastocysts with significantly different metabolic profiles and viability, and a correlation was identified between morphokinetics, blastocyst ICM numbers and blastocyst metabolism. Based on the finding in Chapter 3, that timing of the first cleavage division (t2) was a significant morphokinetic marker to identify ‘fast’ and ‘slow’ groups of embryos, it was subsequently found that blastocysts developed from ‘fast’ embryos (termed ‘fast’ blastocysts), consumed significantly more glucose and produced lactate at the blastocyst stage compared with those derived from ‘slow’ embryos (termed ‘slow’ blastocysts). Significantly, however, ‘fast’ blastocysts exhibited a significantly lower glycolytic rate than ‘slow’ blastocysts.

Interestingly, even in the presence of adequate levels of oxygen for oxidative metabolism of glucose to produce ATP, blastocysts have been shown to exhibit high levels of glycolysis (conversion of glucose to lactate)(Gardner and Leese, 1990). This phenomenon is termed ‘aerobic glycolysis’ (Warburg, 1956), and has been recently reviewed by Gardner (2015)(Gardner, 2015b). Aerobic glycolysis ensures substrate and co-factor availability for glucose metabolism via the Krebs cycle (and subsequently ETC), PPP, hexosamine biosynthesis pathway and the polyol pathway (Gardner, 1998), which enables cell proliferation and synthesis of cellular building blocks. Importantly, aerobic glycolysis, which can be measured via glycolytic rate, is one mechanism that regenerates NAD+, which is required for the breakdown of glucose and its subsequent intermediates in the cytoplasm (Fig 7.1). Mouse blastocysts displaying a higher glucose uptake and a lower glycolytic rate, similar to the metabolic profile of in vivo blastocysts, have been correlated with higher embryo viability (Lane and Gardner, 1996). The results of Chapter 4 are in agreement with that pivotal study, showing a clear correlation between lower glycolytic rates, early onset of cleavage stage morphokinetics and higher embryo viability. Further, lower glycolytic rates observed in ‘fast’ blastocyst also indicates that on a per cell basis, there was significantly different levels of aerobic glycolysis between ‘fast’ and ‘slow’ blastocysts. Of note, there were no differences in blastocyst morphologies, highlighting the need for metabolic markers to derive more
Figure 7.1: Schematic diagram of glucose metabolism by the blastocyst

Glucose is transported across the plasma membrane and is converted into pyruvate via the Embden-Meyerhof pathway. This requires a supply of NAD$^+$ for the pathway to proceed, which can be regenerated via conversion of pyruvate to lactate. Activity of the MAS also regenerates NAD$^+$, via the conversion of aspartate to malate for the malate-aspartate shuttle located in the mitochondria and cytosol. NAD$^+$ consumption and production are depicted in red and green, respectively. Conversely, NADH is also regenerated, which drives oxidative phosphorylation in mitochondria.
7. General discussion
information regarding embryo viability and normality. Subsequent implantation rates between ‘fast’ and ‘slow’ blastocysts were found to be similar, however, it was determined that ‘fast’ blastocysts had a significantly larger outgrowth area in vitro and significantly higher fetal development per implantation in vivo. Results indicated that there was ~30% more post-implantation fetal loss and resorption associated with ‘slow’ blastocysts, with only 23 of 57 implantations surviving to day 14. Furthermore, of the ‘slow’ fetuses that survived in utero, one was determined to be exencephalic, and another with premature growth resembling day 12-13 fetal development, indicating that fetuses developed from ‘slow’ blastocysts may be predisposed to abnormal fetal development.

Profiling of amino acids revealed that their metabolism differs significantly between ‘fast’ and ‘slow’ blastocysts, with ‘fast’ embryos consuming significantly more aspartate than ‘slow’ blastocysts. Results from the present study confirmed that aspartate is the most highly consumed amino acid by the mouse blastocyst (Lamb and Leese, 1994, Wale and Gardner, 2012a). Of physiological significance, aspartate is a rate limiting substrate in the MAS, which has been shown to regulate carbohydrate metabolism in the embryo (Lane and Gardner, 2005a). Higher consumption of aspartate by ‘fast’ blastocysts may therefore reflect an increase in MAS activity, another means of generating more cytosolic NAD$^+$ to facilitate 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 is consistent with the observation that ‘fast’ blastocysts have a lower glycolytic rate than ‘slow’ blastocysts as they also exhibit higher aspartate consumption. In bovine studies, Sugimura et al. (2012) observed that decreased oxygen consumption by kinetically slower embryos was associated with increased blastomere fragmentation, reduced ability of the blastocyst to hatch and decreased pregnancy rates. Given that decreased oxygen consumption may be an indication of abnormal mitochondrial function and metabolism (Wakefield, et al., 2011), and the mammalian blastocyst exhibits the highest oxygen consumption of the preimplantation stages (Houghton, et al., 1996), further investigation is warranted to identify if kinetically different mouse embryos have different rates of oxygen consumption.
To determine if the transcriptome of kinetically different embryos are altered, the study in Chapter 5 analyzed the transcriptional patterns of cytokinetic, metabolism and implantation genes (Table 5.1) of blastocysts, liver and placenta tissue derived from kinetically different groups of embryos. Data revealed that transcriptional levels of the analyzed genes (Table 5.1), appeared higher in ‘fast’ blastocysts compared to ‘slow’ blastocysts. However, this was not statistically significant, and further experiments would be required to confirm that ‘fast’ blastocysts do indeed have higher levels of transcription. Significantly, levels of B3gnt5, implicated in cell adhesion and differentiation during embryogenesis (Yamashita, et al., 1999), were higher in ‘fast’ blastocysts. Disruption of B3gnt5 has been shown to result in preimplantation lethality (Biellmann, et al., 2008), however results from Chapter 4 demonstrated that implantation rates were similar between ‘fast’ and ‘slow’ blastocysts. However, there was a loss of pregnancy following implantation of ‘slow’ blastocysts, as well as a higher incidence of fetal encephaly, which may be due to the involvement of B3gnt5 in neurogenesis and brain development during gastrulation (Henion, et al., 2001).

The relationship between regulation of glucose flux and aspartate utilization was further established by the differences in transcript levels of glucose transport genes, Slc2a1, Slc2a3, glycolytic enzyme pyruvate kinase, Pkm2, and glutamate oxaloacetate transaminase 1, Got1 (data from Chapter 5). Increased transcripts of glucose transport genes in ‘fast’ blastocysts aligns with data from Chapter 4, where consumption of glucose was significantly higher in ‘fast’ blastocysts compared to ‘slow’ blastocysts. At the blastocyst stage, the need for glucose is increased as embryos become increasingly transcriptionally active (Telford, et al., 1990), and glucose metabolism ensures substrate availability for synthesis of nucleic acids (Reitzer, et al., 1980) and protein (Frei, et al., 1989) and blastocoel formation (Watson, et al., 2004). Therefore, lower levels of glucose transport genes suggest that the ‘slow’ blastocysts are limited in glucose consumption for cellular proliferation processes, which may explain the lower cell numbers, as well as delayed time of cavitation as observed in Chapter 3.

As discussed earlier (Fig 7.1), a supply of NAD\(^+\) is necessary for the Embden-Meyerhof pathway, regenerated through aerobic glycolysis. ‘Fast’ blastocysts have a higher level
of \textit{Pkm2}, a particular isoform of the key regulatory enzyme of aerobic glycolysis, pyruvate kinase. The protein encoded by \textit{Pkm2} has been shown to be necessary for regulation of aerobic glycolysis, with a conversion to the M1 isoform reversing aerobic glycolysis and resulting in decreased lactate production in cancer cells (Christofk, et al., 2008). Therefore, higher levels of \textit{Pkm2} in ‘fast’ blastocysts may directly implicate the process of glycolysis, causing the higher levels of lactate production observed in Chapter 4. Conversely, lower levels of \textit{Pkm2} in ‘slow’ blastocysts may result in dysregulated glucose metabolism observed in Chapter 4. Recently, it has been proposed that in addition to aerobic glycolysis providing necessary biosynthetic precursors to ensure further cellular processes, the production of lactate also plays a role in facilitating embryo implantation, by creating a low pH environment to encourage disaggregation of uterine tissues for trophoblast invasion, promoting angiogenesis and modulating local immune response during the invasion process (Gardner, 2015b).

‘Fast’ blastocysts further exhibited higher transcript levels of \textit{Got1}, which is a key enzyme in the MAS. These results suggest increased MAS activity, which in turn will increase levels of NAD$^+$ supply for the Embden-Meyerhof pathway, and thus increase energy production of the blastocyst. Collectively, the results to date suggest a hypothetical mechanism explaining how kinetically faster embryos are more energetically efficient in metabolism and subsequently develop into blastocysts with higher viability (Figure 7.2).

Following on from the findings that genes implicated in glucose and aspartate metabolism were altered at the blastocyst stage of kinetically different embryos, transfer of blastocysts to recipient females were carried out prospectively to obtain Day 14 fetuses and corresponding placental tissues. Gen expression analysis of placental tissues are consistent with the blastocyst gene expression data, where the genes \textit{Slc2a1}, \textit{Got1} and \textit{Pkm2} are also expressed at significantly higher levels in fetuses developed from kinetically faster embryos as compared to the slower embryos. In contrast, levels of \textit{Pkm2} and \textit{Got1} were significantly lower in the liver tissue of the day 14 fetuses developed from ‘fast’ blastocysts, which does not follow previous patterns of expression in the day 5 blastocyst and placental tissues.
These results suggest that in the liver tissue obtained from fetuses developed from ‘slow’ blastocysts, there was a compensatory response to produce higher levels of transcripts to up-regulate or maintain required levels of metabolism for fetal developmental. With data previously determining that there is a lower ICM in ‘slow’ blastocysts (Chapter 4), the allocation of cells to the different lineages, TE and ICM, may have resulted in significant differences in the metabolism of the embryo, as supported by differences in levels of gene transcripts from cell types placental tissue (originating from TE) and liver tissue (originating from ICM). This may be related to previous findings that glycolysis rate differs between TE cells and ICM, with TE cells converting half of the glucose consumed into lactate, whereas the ICM exclusively undergoes aerobic glycolysis (Hewitson and Leese, 1993). Furthermore, studies have demonstrated that mitochondria perturbations plays a role in fetal programming and downstream effects are seen following embryo transfer (Doherty, et al., 2000, Ecker, et al., 2004). Normal mitochondrial function is vital for embryo survival and metabolic control, and defective ATP production may cause a cascade of events leading to disrupted cellular processes, epigenetic dysregulation and compromised fetal development (Gardner and Lane, 2005, Lane and Gardner, 2005b).

Results from the current study provide strong evidence that aspartate is implicated in glucose metabolism of the embryo, and therefore aspartate is proposed to be a potential biomarker for embryo viability. However, conventional techniques used for measurement of amino acids (HPLC, LC-MS) do not have the sensitivity to measure consumption in individual blastocysts. Therefore, Chapter 6 describes the successful adaptation and validation of the ultramicrofluorescence method (used in earlier chapters to measure glucose and lactate levels) to measure the rate of aspartate consumption of individual blastocysts. Subsequently, the increase of exogenous aspartate concentration in the medium was found to significantly increase aspartate and glucose consumption by in vivo blastocysts, in line with data from Chapter 4 and 5 that aspartate is implicated in the regulation of glucose metabolism. Interestingly, lactate production was not increased despite higher glucose consumption, indicating that the increase in glucose consumption was not used for aerobic glycolysis, and that the tested blastocysts maintained glycolysis levels in line with viability.
7. General discussion

**Figure 7.2:** Summary of results of chapter 4 and 5 in a schematic diagram of metabolism of a kinetically faster blastocyst

Firstly, glucose consumption of the blastocyst is increased, along with higher transcript levels of glucose transporter genes. Higher levels of pyruvate kinase isoform M2 (Pkm2) drives the conversion of pyruvate to lactate, resulting in increased lactate production and higher NAD\(^+\) regeneration. Aspartate consumption is increased, and there are also higher levels of glutamate oxaloacetate transaminase (Got1), required for the conversion of aspartate to malate for the malate-aspartate shuttle activity. More conversion of aspartate to malate increases NAD\(^+\) regeneration, which drives the conversion of glucose to pyruvate via the Embden-Meyerhof pathway. Promotion of the Embden-Meyerhof pathway will increase Krebs cycle activity and energy production via the electron transport chain, as well as co-factor availability for other pathways (pentose phosphate pathway, hexosamine biosynthetic pathway, polyol pathway; not shown here), which are responsible for cellular proliferation processes. Data here supports a model of energetically efficient metabolism for kinetically faster blastocysts, which were found to have a significantly higher viability post implantation. MAS; Malate aspartate shuttle.
7. General discussion
7. General discussion

Several pathways for glucose metabolism have been discussed earlier (Fig 7.1), and more experiments are necessary to determine the metabolic fate of increased glucose consumption.

Currently, the present concentration of aspartate (0.1 mM) in commercial IVF media is based on Eagle’s Minimal Essential Medium (Eagle, 1959). Given the influence of exogenous aspartate on aspartate and glucose uptake and its subsequent link to embryo viability, future experiments should investigate the influence of aspartate concentrations on embryo morphokinetics and viability, and the quantification of aspartate consumption as a biomarker for embryo viability warrants further investigation. This will also contribute to further media optimization, to better support normal blastocyst development and improve viability.

With the relationship of aspartate, glucose and lactate identified using the newly validated assay on in vivo blastocysts, the same methods were then utilized to determine the aspartate and glucose uptake of kinetically different embryos created through IVF. Data from individual blastocysts confirmed results from LC-MS, in which embryos were grouped, that kinetically faster embryos at the 2-cell stage develop into blastocysts that consume significantly more aspartate compared to slower embryos. These results are consistent with previously published mass spectrometry data (Lee, et al., 2015), thereby validating the use of targeted single metabolic analysis, which is a faster, cheaper and more accurate method over mass spectrometry. Furthermore, targeted metabolic analysis is a more sensitive and precise method suited to embryo selection as compared to broader metabolomics approaches using NIR or proton nuclear magnetic resonance spectroscopy platforms, which have failed to show a correlation to live birth rate (Vergouw, et al., 2014). Therefore, the data presented in this chapter support the move to a more targeted approach to analysing embryonic metabolism, if it is to be used for the selection of the most viable embryos within a cohort.
7.2. Conclusions

This thesis has examined the use of morphokinetics as a marker of mouse embryo development, and identified key developmental events significantly correlated to subsequent blastocyst cell number. The time of cleavage division to the 2-cell stage was subsequently used to categorize embryos into kinetically different groups ('fast' and 'slow' embryos) and the blastocyst metabolism of these groups of embryos was determined. The studies demonstrated that kinetically different cleavage stage embryos develop into blastocysts with significantly different metabolic profiles and viability. Of significance, kinetically faster embryos exhibited higher glucose and aspartate consumption at the blastocyst stage, and a lower glycolytic rate, and transfer of these blastocysts resulted in a ~30 % higher fetal survival post-implantation compared with kinetically slower embryos. Additionally, the transcriptome of kinetically slower embryos was altered, with a decrease in mRNA expression levels of genes associated with glucose and aspartate metabolism in the blastocyst and placental tissue, providing further evidence that aspartate is implicated in glucose metabolism, and is a potential biomarker for embryo viability.

7.3. Clinical relevance and significance

This research provides a deeper understanding of the developmental kinetics of mouse embryos produced using IVF and builds on the current knowledge of carbohydrate and amino acid metabolism of the embryo. Although independent studies of carbohydrate; amino acid metabolism and morphokinetics as biomarkers have been associated with viability, these biomarkers do not provide absolute certainties of the prospective viability of the embryo, which can only really be determined by embryo transfer. However, the data strongly suggest that glucose and aspartate metabolism, in combination with morphokinetics of an embryo are intimately linked and collectively reflect embryo viability. As such, the use of multiple viability biomarkers, as opposed to a single parameter, is likely to increase the accuracy of embryo selection. Within the ‘fast’ group of embryos, for example, there was variability in the glycolytic rate of
resultant blastocysts, indicating that not all ‘fast’ blastocysts have an equivalent metabolism that will be supportive of embryo viability. Additionally, the use of the newly validated aspartate assay can be incorporated to ensure high aspartate consumption of the embryo, in order to maintain NAD⁺, which will drive subsequent glucose metabolism. Hence, the selection of an early cleaving embryo with a blastocyst metabolism of high glucose consumption and lactate production, with a low glycolytic rate and an increased aspartate consumption may result in the highest success rates of predicting viability and normality of an embryo (Fig 7.2).

Although the studies presented in this thesis have provided increased knowledge of embryo metabolism and its relation to morphokinetics and subsequent viability, it has also raised several new possibilities. In particular, the fact that aspartate concentration appears to drive glucose consumption, and the unknown fate of the increased levels of consumed glucose. This relates to the concentrations of amino acids, in particular aspartate, used in culture media for clinical IVF, and reinforces the importance of optimized embryo culture on embryo physiology, viability and normality. It is envisaged that data from this thesis will assist in the generation of improved culture media and conditions to support the development of better quality blastocysts, as well as superior embryo selection diagnostics to increase accuracy of determining single pre- or post-compaction embryo viability. Both approaches are likely to reduce both the number of embryos transferred and the number of IVF attempts needed to achieve the delivery of a healthy baby.
Appendices
Appendix A: Mouse embryo assay

The mouse embryo assay (MEA) was used as a quality control test of consumables, plastic ware, chemical reagents and media solutions prior to experimental embryo culture. For the assay, mouse pronucleate oocytes were collected from superovulated pre-pubescent F1 hybrid female mice (C57BL/6 x CBA) that underwent mating with F1 hybrid male mice overnight. Cumulus-oocyte complexes were collected at 9 am the next morning and denuded in GMOPS with HSA for no more than 5 min. The time in the hyaluronidase was reduced by the gentle pipetting of the pronucleate oocytes to remove cumulus cells. After rinsing, oocytes were then cultured in simple G1.2 supplemented with 5 % HSA, in either control (previously approved) or test (newly made) media groups and cultured to the blastocyst stage on day 5. Embryo development was assessed at specific time points to determine on-time development and at the end of the culture period (Day 4), blastocyst cell number was determined. The test item was determined to be ‘embryo safe’ if it meets specific criteria, listed below.

To test culture dishes, embryos were cultured on the actual test dish and compared to control dishes that have previously passed MEA. For other plastic ware such as test tubes, media were placed in the tubes for 1 week at 4 °C, and embryos were then grown in this media. Pronucleate oocytes were cultured in simple G1.2 supplemented with 5% HSA for 19 h and subsequently transferred to simple G1.2 without supplementation of HSA, as albumin has been shown to better maintain embryo physiology and metabolism in vitro and may act as chelators and mask any inhibitory effects of the test item (George, et al., 1989). The embryos were scored on Day 4, 4pm and Day 5, 9am (74 h and 91 h of culture respectively). A minimum of 20 embryos were cultured under each test condition, and to pass the assay, the following criteria must be achieved:

- Blastocyst on day 4, ≥ 50%
- Expanded blastocyst on day 5, ≥ 80%
- Total cell number, ≥ 60
To test culture media, pronucleate oocytes were cultured in the new batch of simple G1.2 supplemented with 5% HSA for 5 days and compared to pronucleate oocytes cultured in control (passed) simple G1.2 supplemented with 5% HSA. To pass the assay, the following criteria must be achieved:

- Compacting on day 3, \( \geq 60\% \)
- Blastocyst on day 4, \( \geq 50\% \)
- Expanded blastocyst on day 5, \( \geq 80\% \)
- Total cell number, \( \geq 60 \)
### Appendix B: Media Stock solutions

**Table B1: Media stock solutions for IVF fertilization media**

<table>
<thead>
<tr>
<th>Stock A</th>
<th>Molecular weight</th>
<th>Weight (g) for 50 ml</th>
<th>Final concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium chloride</td>
<td>58.44</td>
<td>2.7936</td>
<td>95.6</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>74.56</td>
<td>0.2050</td>
<td>5.5</td>
</tr>
<tr>
<td>Sodium phosphate</td>
<td>119.98</td>
<td>0.0300</td>
<td>9.5</td>
</tr>
<tr>
<td>Magnesium sulphate heptahydrate</td>
<td>246.47</td>
<td>0.1232</td>
<td>1.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Stock B</th>
<th>Molecular weight</th>
<th>Weight (g) for 10 ml</th>
<th>Final concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium chloride</td>
<td>147.02</td>
<td>0.2646</td>
<td>1.8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Stock C</th>
<th>Molecular weight</th>
<th>Weight (g) for 10 ml</th>
<th>Final concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>180.16</td>
<td>0.5675</td>
<td>3.15</td>
</tr>
<tr>
<td>Sodium Lactate (L-isomer)</td>
<td>112.06</td>
<td>1.1766</td>
<td>10.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Stock Alanyl-glutamine</th>
<th>Molecular weight</th>
<th>Weight (g) for 10 ml</th>
<th>Final concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanyl-glutamine</td>
<td>217.2</td>
<td>0.1086</td>
<td>0.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Stock Non-essential amino acids (NEAAs)</th>
<th>Molecular weight</th>
<th>Weight (g) for 10 ml</th>
<th>Final concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>89.09</td>
<td>0.0089</td>
<td>0.1</td>
</tr>
<tr>
<td>Aspartate</td>
<td>133.1</td>
<td>0.1330</td>
<td>0.1</td>
</tr>
<tr>
<td>Asparagine</td>
<td>150.14</td>
<td>0.1501</td>
<td>0.1</td>
</tr>
<tr>
<td>Glutamate</td>
<td>169.1</td>
<td>0.1691</td>
<td>0.1</td>
</tr>
<tr>
<td>Glycine</td>
<td>75.07</td>
<td>0.0751</td>
<td>0.1</td>
</tr>
<tr>
<td>Proline</td>
<td>115.13</td>
<td>0.1151</td>
<td>0.1</td>
</tr>
<tr>
<td>Serine</td>
<td>105.09</td>
<td>0.1051</td>
<td>0.1</td>
</tr>
</tbody>
</table>
### Appendix B

#### Table B2: Preparation of IVF fertilization media from stock solutions

<table>
<thead>
<tr>
<th>Component</th>
<th>IVF fertilization media (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MilliQ-H₂O</td>
<td>6.4</td>
</tr>
<tr>
<td>Stock A</td>
<td>1</td>
</tr>
<tr>
<td>Stock Bicarb</td>
<td>1</td>
</tr>
<tr>
<td>Stock B</td>
<td>0.1</td>
</tr>
<tr>
<td>Stock C</td>
<td>0.1</td>
</tr>
<tr>
<td>Stock Ala-gln</td>
<td>0.1</td>
</tr>
<tr>
<td>Stock NEAAs</td>
<td>0.1</td>
</tr>
<tr>
<td>Stock Taurine</td>
<td>0.1</td>
</tr>
<tr>
<td>Stock pyruvate</td>
<td>0.1</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>2.5 µl</td>
</tr>
<tr>
<td><strong>Total (filtered)</strong></td>
<td><strong>9</strong></td>
</tr>
<tr>
<td>pH (ungassed)</td>
<td>7.6</td>
</tr>
<tr>
<td>Osomolality</td>
<td>290 ± 5</td>
</tr>
<tr>
<td>Aliquot and add 0.2ml HSA</td>
<td>1.8</td>
</tr>
</tbody>
</table>
**Table B3**: Media stock solutions for culture media

<table>
<thead>
<tr>
<th>Stock A</th>
<th>Molecular weight</th>
<th>Weight (g) for 100 ml</th>
<th>Final concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium chloride</td>
<td>58.44</td>
<td>5.2643</td>
<td>90.08</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>74.56</td>
<td>0.4108</td>
<td>5.5</td>
</tr>
<tr>
<td>Sodium phosphate</td>
<td>119.98</td>
<td>0.0300</td>
<td>0.25</td>
</tr>
<tr>
<td>Magnesium sulphate heptahydrate</td>
<td>246.47</td>
<td>0.2465</td>
<td>1.0</td>
</tr>
<tr>
<td>Penicillin</td>
<td>0.0600</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Stock B</th>
<th>Molecular weight</th>
<th>Weight (g) for 100 ml</th>
<th>Final concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium bicarbonate</td>
<td>84.01</td>
<td>2.1003</td>
<td>25.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Stock C</th>
<th>Molecular weight</th>
<th>Weight (g) for 10 ml</th>
<th>Final concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium chloride</td>
<td>147.02</td>
<td>0.2646</td>
<td>1.8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Stock D</th>
<th>Molecular weight</th>
<th>Weight (g) for 10 ml</th>
<th>Final concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>180.16</td>
<td>0.0901</td>
<td>0.5</td>
</tr>
<tr>
<td>Sodium Lactate (L-isomer)</td>
<td>112.06</td>
<td>1.1766</td>
<td>10.5</td>
</tr>
<tr>
<td>Sodium pyruvate</td>
<td>110.04</td>
<td>0.0352</td>
<td>0.32</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Stock E</th>
<th>Molecular weight</th>
<th>Weight (g) for 10 ml</th>
<th>Final concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>180.16</td>
<td>0.5675</td>
<td>3.15</td>
</tr>
<tr>
<td>Sodium Lactate (L-isomer)</td>
<td>112.06</td>
<td>0.6578</td>
<td>5.87</td>
</tr>
<tr>
<td>Sodium pyruvate</td>
<td>110.04</td>
<td>0.0110</td>
<td>0.1</td>
</tr>
</tbody>
</table>
### Appendix B

#### Stock F

<table>
<thead>
<tr>
<th>Stock F</th>
<th>Molecular weight</th>
<th>Weight (g) for 100 ml</th>
<th>Final concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA</td>
<td>372.24</td>
<td>0.0037</td>
<td>0.01</td>
</tr>
</tbody>
</table>

#### Stock Non-essential amino acids (NEAAs)

<table>
<thead>
<tr>
<th>Stock Non-essential amino acids (NEAAs)</th>
<th>Molecular weight</th>
<th>Weight (g) for 10 ml</th>
<th>Final concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>89.09</td>
<td>0.0089</td>
<td>0.1</td>
</tr>
<tr>
<td>Aspartate</td>
<td>133.1</td>
<td>0.1330</td>
<td>0.1</td>
</tr>
<tr>
<td>Asparagine</td>
<td>150.14</td>
<td>0.1501</td>
<td>0.1</td>
</tr>
<tr>
<td>Glutamate</td>
<td>169.1</td>
<td>0.1691</td>
<td>0.1</td>
</tr>
<tr>
<td>Glycine</td>
<td>75.07</td>
<td>0.0751</td>
<td>0.1</td>
</tr>
<tr>
<td>Proline</td>
<td>115.13</td>
<td>0.1151</td>
<td>0.1</td>
</tr>
<tr>
<td>Serine</td>
<td>105.09</td>
<td>0.1051</td>
<td>0.1</td>
</tr>
</tbody>
</table>

#### Stock Alanyl-glutamine

<table>
<thead>
<tr>
<th>Stock Alanyl-glutamine</th>
<th>Molecular weight</th>
<th>Weight (g) for 10 ml</th>
<th>Final concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanyl-glutamine</td>
<td>217.2</td>
<td>0.1086</td>
<td>0.5</td>
</tr>
</tbody>
</table>

#### Stock Taurine

<table>
<thead>
<tr>
<th>Stock Taurine</th>
<th>Molecular weight</th>
<th>Weight (g) for 10 ml</th>
<th>Final concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taurine</td>
<td>125.14</td>
<td>0.01251</td>
<td>0.1</td>
</tr>
</tbody>
</table>

#### Stock Vitamins

<table>
<thead>
<tr>
<th>Stock Vitamins</th>
<th>Molecular weight</th>
<th>Weight (g) for 100 ml</th>
<th>Final concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Ca Pantothenate</td>
<td>238.3</td>
<td>0.001</td>
<td>0.0042</td>
</tr>
<tr>
<td>Pyidoal-HCL</td>
<td>203.62</td>
<td>0.001</td>
<td>0.0049</td>
</tr>
<tr>
<td>Thiamine-HCL</td>
<td>367.37</td>
<td>0.0001</td>
<td>0.00027</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>337.27</td>
<td>0.001</td>
<td>0.00296</td>
</tr>
</tbody>
</table>

#### Stock MOPS

<table>
<thead>
<tr>
<th>Stock MOPS</th>
<th>Molecular weight</th>
<th>Weight (g) for 100 ml</th>
<th>Final concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOPS (pH = 7.35)</td>
<td>209.3</td>
<td>4.814</td>
<td>23.0</td>
</tr>
</tbody>
</table>
**Appendix B**

**Table B4**: Preparation of embryo culture media from stock solutions

<table>
<thead>
<tr>
<th>Component</th>
<th>G1.1 (ml)</th>
<th>G2.2 (ml)</th>
<th>Simple G1 (ml)</th>
<th>G2-MOPS (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MilliQ-H₂O</td>
<td>6.7</td>
<td>6.6</td>
<td>7</td>
<td>6.52</td>
</tr>
<tr>
<td>Stock A</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Stock B</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0.08</td>
</tr>
<tr>
<td>Stock C</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Stock D</td>
<td>0.1</td>
<td>-</td>
<td>0.1</td>
<td>-</td>
</tr>
<tr>
<td>Stock E</td>
<td>-</td>
<td>0.1</td>
<td>-</td>
<td>0.1</td>
</tr>
<tr>
<td>Stock F</td>
<td>0.1</td>
<td>-</td>
<td>0.1</td>
<td>-</td>
</tr>
<tr>
<td>Stock NEAAs</td>
<td>0.1</td>
<td>0.1</td>
<td>-</td>
<td>0.1</td>
</tr>
<tr>
<td>Stock Ala-gln</td>
<td>0.1</td>
<td>0.2</td>
<td>-</td>
<td>0.2</td>
</tr>
<tr>
<td>Stock Taurine</td>
<td>0.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Stock EAA</td>
<td>-</td>
<td>0.1</td>
<td>-</td>
<td>0.1</td>
</tr>
<tr>
<td>MOPS</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Stock Vitamins</td>
<td>-</td>
<td>0.1</td>
<td>-</td>
<td>0.1</td>
</tr>
<tr>
<td>Hyaluronan</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>HSA</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>3.75 μl</td>
<td>3.75 μl</td>
<td>3.75 μl</td>
<td>3.75 μl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>pH (ungassed)</td>
<td>8.0</td>
<td>8.0</td>
<td>8.0</td>
<td>2.3</td>
</tr>
<tr>
<td>Osomolality</td>
<td>273 ± 5</td>
<td>268 ± 5</td>
<td>273 ± 5</td>
<td>273 ± 5</td>
</tr>
</tbody>
</table>
Appendix C: Differential nuclear staining solutions and protocol

All chemical products were sourced from Sigma, with the exception of guinea pig serum. Guinea pig serum was supplied by IMVS (Australia).

**Table C1**: Stock solutions for differential nuclear staining of cells

<table>
<thead>
<tr>
<th>Stock</th>
<th>Preparation</th>
<th>Storage conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absolute ethanol</td>
<td>-</td>
<td>25 °C</td>
</tr>
<tr>
<td>Anti-DNP</td>
<td>10 μl</td>
<td>-20 °C</td>
</tr>
<tr>
<td>Bisbenzimide</td>
<td>10 mg/ml</td>
<td>Wrap in foil, -80 °C</td>
</tr>
<tr>
<td>Glycerol</td>
<td>-</td>
<td>25 °C</td>
</tr>
<tr>
<td>Guinea pig serum</td>
<td>50 μl</td>
<td>-80 °C</td>
</tr>
<tr>
<td>Propidium iodide</td>
<td>50 μl of 2mg/100 ml G-MOPS</td>
<td>-20 °C</td>
</tr>
<tr>
<td>Pronase</td>
<td>0.25g/50 ml G-MOPS</td>
<td>-20 °C</td>
</tr>
<tr>
<td>PVP</td>
<td>4 mg/ml PVP to 10 ml simple G1 medium</td>
<td>4 °C</td>
</tr>
<tr>
<td>TNBS</td>
<td>-</td>
<td>25 °C</td>
</tr>
<tr>
<td>Wash medium</td>
<td>G-MOPS medium</td>
<td>4 °C</td>
</tr>
</tbody>
</table>

**Table C2**: Working solutions for differential nuclear staining of cells

<table>
<thead>
<tr>
<th>Solutions</th>
<th>Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-DNP</td>
<td>Add 90 μl of G1-PVP to 10 μl aliquot of Anti-DNP</td>
</tr>
<tr>
<td>Bisbenzimide</td>
<td>Add 10 μl of Bisbenzimide stock to 890 μl G-MOPS medium and 100 μl absolute ethanol.</td>
</tr>
<tr>
<td>TNBS</td>
<td>Add 10 μl of TNBS stock to 90 μl of PVP medium</td>
</tr>
<tr>
<td>Guinea pig serum working solution</td>
<td>Add 50 μl of guinea pig serum to 50 μl of propidium iodide</td>
</tr>
</tbody>
</table>
Appendix D

**Table C3:** Steps and incubation times in differential nuclear staining protocol

<table>
<thead>
<tr>
<th>Solution</th>
<th>Time of incubation (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Pronase</td>
<td>5</td>
</tr>
<tr>
<td>2. Wash</td>
<td>5</td>
</tr>
<tr>
<td>3. TNBS</td>
<td>10</td>
</tr>
<tr>
<td>4. Wash</td>
<td>5</td>
</tr>
<tr>
<td>5. Anti-DNP</td>
<td>10</td>
</tr>
<tr>
<td>6. Wash</td>
<td>5</td>
</tr>
<tr>
<td>7. Guinea pig serum working solution</td>
<td>5</td>
</tr>
<tr>
<td>8. Bisbenzimide solution</td>
<td>Up to 20</td>
</tr>
<tr>
<td>9. Wash</td>
<td>5</td>
</tr>
</tbody>
</table>

Embryos were incubated in sequential order and time shown in Table C3. 10 μl of each solution per 10-20 embryos were prepared in clockwise order and overlayed with paraffin oil (Sigma). Following the last step of wash, glycerol was added onto a glass slide (~ 10 μl/ drop) and embryos were transferred into the drops of glycerol using a glass pipette. A coverslip is applied to the glass slide and the slide is stored in the dark at 4 °C until reading and imaging.
Appendix D: Dish Set up

Figure D1: Dish set up for IVF and embryo culture

a) IVF fertilization dish. b) Time-lapse dishes for individual embryo culture c) Culture dishes for group embryo culture
Appendix D

a)

45 µl IVF fertilization
wash drop

45 µl IVF fertilization
media drops

9 ml Ovoil overlay

b)

10 µl G1.2/ G2.2 wash drop

2 µl G1.2/ G2.2 culture media drops

2 µl G1.2/ G2.2 ‘blank’ culture media drops, for amino acid analysis

5 ml Ovoil overlay

c)

20 µl G1.2/ G2.2
media wash drop

20 µl G1.2/ G2.2
media drops
Appendix E: Ultramicrofluorescence conditions

For each assay, reagent solutions were prepared that contained a buffer and all of the cofactors and enzymes needed for the reaction. Each media sample was added to a 0.2 ml thin-wall PCR tube (Thermowell GOLD, Axygen), which contained reagents specifically linked to either glucose, lactate or aspartate. Conditions of the assays were set to force the reactions to completion, ensuring the total conversion of the substrate being analyzed. All samples and cocktails were mixed and 10 min allowed for the reaction to go to completion at room temperature.
### Table E1: Reagents for glucose assay

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Final concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgSO$_4$·7H$_2$O</td>
<td>3.7</td>
</tr>
<tr>
<td>NADP$^+$</td>
<td>0.6</td>
</tr>
<tr>
<td>ATP</td>
<td>0.5</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>0.5</td>
</tr>
<tr>
<td>Hexokinase (U/ml)</td>
<td>12 U</td>
</tr>
<tr>
<td>G6DPH (U/ml)</td>
<td>6 U</td>
</tr>
<tr>
<td>Epps buffer (pH 8.0)</td>
<td>-</td>
</tr>
</tbody>
</table>

Make up in 150 ml water, pH to 8.0 with 1 M NaOH. Dilute to 200 ml.

### Stock solutions

- 5 mM Dithiothreitol: 7.72 mg in 10 ml water
- 37 mM MgSO$_4$·7H$_2$O: 91.2 mg in 10 ml water
- 10 mM ATP: 30.3 mg in 5 ml water
- 10 mM NADP$^+$: 39.4 mg in 5 ml water

### Reagents for cocktail

<table>
<thead>
<tr>
<th>Reagents for cocktail</th>
<th>Volume (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>37 mM MgSO$_4$·7H$_2$O</td>
<td>100</td>
</tr>
<tr>
<td>10 mM NADP$^+$</td>
<td>50</td>
</tr>
<tr>
<td>10 mM ATP</td>
<td>50</td>
</tr>
<tr>
<td>5 mM Dithiothreitol</td>
<td>100</td>
</tr>
<tr>
<td>Hexokinase/ G6DPH</td>
<td>50</td>
</tr>
<tr>
<td>Epps buffer (pH 8.0)</td>
<td>750</td>
</tr>
</tbody>
</table>

Freeze in 0.5 ml aliquots

1 mM standard

| Glucose                               | 0.018g in 100 ml water |
### Table E2: Reagents for lactate assay

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Final concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA</td>
<td>2.6</td>
</tr>
<tr>
<td>NAD⁺</td>
<td>4.76</td>
</tr>
<tr>
<td>Lactate dehydrogenase (U/ml)</td>
<td>100 U</td>
</tr>
<tr>
<td>Glycine hydrazine buffer (pH 9.4)</td>
<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Glycine Hydrazine buffer</th>
<th>Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>7.5</td>
</tr>
<tr>
<td>Hydrazine</td>
<td>5.2</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.2</td>
</tr>
<tr>
<td>2 M NaOH</td>
<td>51 ml</td>
</tr>
</tbody>
</table>

Suspend glycine, hydrazine and EDTA in water. Add NaOH. Dilute to 100 ml.

### Stock solutions

| NAD⁺                                          | 40 mg/ml, freeze in 75 μl aliquots |

### Cocktail

<table>
<thead>
<tr>
<th>Glycine</th>
<th>Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>7.5</td>
</tr>
<tr>
<td>Hydrazine</td>
<td>5.2</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.2</td>
</tr>
<tr>
<td>2 M NaOH</td>
<td>51 ml</td>
</tr>
<tr>
<td>NAD⁺ (40 mg/ml)</td>
<td>One 75 μl aliquot</td>
</tr>
</tbody>
</table>

### 1 mM standard

| Lactate                                       | 0.0112g in 100 ml water |
### Table E3: Reagents for aspartate assay

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Final concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-ketoglutarate</td>
<td>0.12</td>
</tr>
<tr>
<td>NADH</td>
<td>0.15</td>
</tr>
<tr>
<td>Malate dehydrogenase (U/ml)</td>
<td>3.5 U</td>
</tr>
<tr>
<td>GOT (U/ml)</td>
<td>20 U</td>
</tr>
<tr>
<td>Imidazole buffer (pH 7.0)</td>
<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Imidazole buffer</th>
<th>Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imidazole</td>
<td>0.2042</td>
</tr>
<tr>
<td>Imidazole-HCL</td>
<td>0.4192</td>
</tr>
</tbody>
</table>

Suspend imidazole, imidazole-HCL in water. pH to 7.0. Dilute to 100 ml.

<table>
<thead>
<tr>
<th>Stock solutions</th>
<th>Weight (g) into 10 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.2 mM α-ketoglutarate</td>
<td>First make 12 mM solution (0.0201 in 10 ml), then 1 into 10 dilution to get 1.2 mM</td>
</tr>
<tr>
<td>1.5 mM NADH</td>
<td>0.0106</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cocktail</th>
<th>Volume (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.2 mM α-ketoglutarate</td>
<td>150</td>
</tr>
<tr>
<td>1.5 mM NADH</td>
<td>100</td>
</tr>
<tr>
<td>Malate dehydrogenase (U/ml)</td>
<td>3.5 U</td>
</tr>
<tr>
<td>GOT (U/ml)</td>
<td>20 U</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>1 mM standard</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartate</td>
<td>0.0133g in 100 ml water</td>
</tr>
</tbody>
</table>


Bibliography


Bibliography


Bibliography


Bibliography


Bibliography


Johnson WH, Loskutoff NM, Plante Y, Betteridge KJ. Production of four identical calves by the separation of blastomeres from an in vitro derived four-cell embryo. *Vet Rec* 1995;137: 15-16.


Lane M, Baltz JM, Bavister BD. Na+/H+ antiporter activity in hamster embryos is activated during fertilization. Dev Biol 1999;208: 244-252.


Lee YS, Thouas GA, Gardner DK. Developmental kinetics of cleavage stage mouse embryos are related to their subsequent carbohydrate and amino acid utilization at the blastocyst stage. *Hum Reprod* 2015;30: 543-552.
Bibliography


Lerchner W, Barlow DP. Paternal repression of the imprinted mouse Igf2r locus occurs during implantation and is stable in all tissues of the post-implantation mouse embryo. *Mech Dev* 1997;61: 141-149.


Bibliography


Milki AA, Hinckley MD, Fisch JD, Dasig D, Behr B. Comparison of blastocyst transfer with day 3 embryo transfer in similar patient populations. Fertil Steril 2000;73: 126-129.


Rechler MM, Zapf J, Nissley SP, Froesch ER, Moses AC, Podskalny JM, Schilling EE, Humbel RE. Interactions of insulin-like growth factors I and II and multiplication-


Bibliography


Van Langendonckt A, Demylle D, Wyns C, Nisolle M, Donnez J. Comparison of G1.2/G2.2 and Sydney IVF cleavage/blasto cyst sequential media for the culture of


Bibliography


Wale PL, Gardner DK. Oxygen Regulates Amino Acid Turnover and Carbohydrate Uptake During the Preimplantation Period of Mouse Embryo Development. *Biol Reprod* 2012b.


Author/s:
Lee, Yee Shan Lisa

Title:
Identifying metabolic determinants of embryo viability and normality

Date:
2015

Persistent Link:
http://hdl.handle.net/11343/92349

File Description:
Identifying Metabolic Determinants of Embryo Viability and Normality