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Declaration

I certify that the work represented in this thesis is the result of original research and has not been presented to any other University for examination. However it was present at meetings and both in abstract form and final reports.

External contributions to the research have been acknowledged in the text.

NAME:

SIGNATURE: DATE:
Acknowledgement

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Abstract

“The role of L-proline in pre-implantation mouse embryo development in vitro”

Amino acids are known to play important roles in pre-implantation embryo development, including regulation of cell volume and metabolism. Inclusion of L-glutamine, glycine and betaine in embryo culture medium has been shown to improve development in vitro by acting as organic osmolytes, thereby regulating cell volume. The purpose of the present study was to determine the role of L-proline in pre-implantation mouse embryo development in vitro. In order to ensure the osmotic effect of L-proline, the osmolality of culture medium was prepared in different ranges (258, 270, 296 and 336 mOsm/kg). One-cell embryos were cultured in amino acid free modified human tubal fluid medium and potassium simplex optimization medium, at low density (1 embryo/100μl) and high density (1 embryo/μl). Amino acids (L-glutamine, L-proline, D-proline, glycine, L-alanine, betaine) were added to the culture medium and development of the embryos was scored every 24h until day 6 of pregnancy (blastocyst stage). Blastocysts were fixed, stained with DAPI and imaged on a confocal microscope. Cell numbers in each blastocyst were then counted. At low density, with 270–336mOsm/kg L-proline increased development at day 6 (p<0.001) to the blastocyst stage, and also increased the proportion of blastocysts that were hatching at day 6 (p<0.01) compared to embryos cultured in the absence of L-proline. However, there was no change in the number of cells in the embryos that reached the blastocyst stage. In contrast, at high density, L-proline had no effect on development compared to the absence of L-proline. L-proline improved development and blastocyst expansion despite when embryos were cultured in upon and sub-optimal (hyper osmolality and low density)conditions.
### Abbreviations

<table>
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<th>Abbreviation</th>
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<tbody>
<tr>
<td>APS</td>
<td>Ammonium persulphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>bet</td>
<td>Betaine (N,N,N-trimethyl glycine)</td>
</tr>
<tr>
<td>BGIT</td>
<td>Betaine transporter</td>
</tr>
<tr>
<td>BL</td>
<td>Blastocyst</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>D-pro</td>
<td>D-proline</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>eIF4B</td>
<td>Eukaryotic translation initiation factor 4B</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>ES cells</td>
<td>Embryonic stem cells</td>
</tr>
<tr>
<td>GFPT</td>
<td>Glutamine-fructose-6-phosphate amidotransferase</td>
</tr>
<tr>
<td>gly</td>
<td>Glycine</td>
</tr>
<tr>
<td>GLYT-1</td>
<td>Glycine transporter-1</td>
</tr>
<tr>
<td>GLUT3</td>
<td>Glucose transporter 3</td>
</tr>
<tr>
<td>hCG</td>
<td>Human chorionic gonadotropin</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid</td>
</tr>
<tr>
<td>ICM</td>
<td>Inner cell mass</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>IU</td>
<td>International units</td>
</tr>
<tr>
<td>JNK/STAT</td>
<td>Janus-activated kinase/Signal transducer and activator of transcription</td>
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KSOM  Pottasium simplex optimised medium
L-ala  L-alanine
LDH  Lactate dehydrogenase
LIF  Leukemia inhibitory factor
L-gln  L-glutamine
L-pro  L-proline
modHTF  Modified human tubal fluid
MAPK  Mitogen-activated protein kinase
MAS  Malate aspartate shuttle
MCT  Monoacryboxylic transporter
mOsm/kg  mili osmolar per kilogram
mMTF  modified mouse tubal fluid
mTOR  Mammalian target of rapamycin
NaCl  Sodium Chloride
NAD+  Nicotinamide adenine dinucleotid
No a.a.  No amino acids
NS  Not significant
PAF  Platelet activating factor
PBS  Phosphate buffered saline
PFA  Paraformaldehyde
PI3K  Phosphatidylinositol 3-kinase
PMS  Pregnant mare’s serum gonadotropin
PMSF  phenylmethylsulfonyl fluoride
PVA  Polyvinyl alcohol
QS  Quackenbush swiss
<table>
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<tbody>
<tr>
<td>SAT</td>
<td>System A amino acid transporter</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate poly-acrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SMIT</td>
<td>Na(^+)/myo-inositol transporter</td>
</tr>
<tr>
<td>SOM</td>
<td>Simplex optimised medium</td>
</tr>
<tr>
<td>TAUT</td>
<td>Taurine/(\beta) amino acid transporter</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris buffered saline-tween</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acid</td>
</tr>
<tr>
<td>TE</td>
<td>Trophectoderm cells</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TEP</td>
<td>Trophectodermal projections</td>
</tr>
<tr>
<td>TGF-(\beta)</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>VSOAC</td>
<td>Volume sensitive organic-osmolyte anionic channel</td>
</tr>
<tr>
<td>ZP</td>
<td>Zona pelucida</td>
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CHAPTER ONE

INTRODUCTION
1.1 PRE-IMPLANTATION DEVELOPMENT

Pre-implantation embryo development in the mouse, begins with fertilisation of the oocyte, which takes place in the ampullary region in vivo. Fertilisation of the oocyte occurs when the haploid sperm penetrates through the extracellular glycoprotein coat, known as the zona pellucida that surrounds the oocyte to inseminate the oocyte and result in formation of the zygote. Pre-implantation development takes place over the first 4.5 days in vivo after the oocyte is fertilised and includes repeated cleavage of the zygote, compaction and differentiation to form a blastocyst (Cockburn and Rossant, 2010) (Fig.1.1).

The fertilised one-cell embryo (day 1 of pre-implantation development) has two pronuclei and one or more polar bodies and is surrounded by the zona pellucida, which will surround the embryo until the blastocyst stage. Between embryonic day (E) 1.5 and 2.5 the one-cell embryo will undergo cell divisions to multiply the number of cells within the embryo. By day E3.0 the embryo will have reached the sixteen-cell stage. Between the sixteen and thirty-two cell stage (morula stage, day E4), the embryo will undergo compaction which blastomeres become tightly joined and form gap junctions to allow the exchange of ions and small molecules to pass from one cell to other. Compaction results in the first differentiation event of the embryo as distinct outer and an inner layer of cells are formed. Cavitation also begins and a fluid filled region is formed, known as the blastocoel. By day E4.5 the embryo is known as a blastocyst which consists of an outer layer of polarised cells known as trophectoderm (TE) cells and a pocket of pluripotent inner cell mass (ICM) cells lying immediately beneath one pole of the TE.
Fig. 1.1 Overview of the pre-implantation embryonic development stages of the mouse embryo. Images were taken by S. Ozsoy (using an Olympus IX 51 Microscope) every 24 hours after culture (40X magnification).

1.1.1 Cleavage of the Fertilised Zygote

The fertilised zygote has two pronuclei, one from the sperm and one from the oocyte. These pronuclei fuse within the fertilised egg to form the diploid zygote, which includes determination of polar bodies. The polar bodies are formed during ovulation and fertilisation. The first polar body is segregated in meiosis 1 while the second polar body is extruded in meiosis 2, in the late stages of oogenesis. The first cleavage stage, from one-cell to two-cells, is under control of maternal mRNA already present in the oocyte. At this stage, genomic transcription is suppressed. However, embryonic genome transcription begins to occur during G2 phase of the cell cycle at the late two-cell stage, at a time when maternal mRNA is being degraded (Ciemerych and Sicinski, 2005).

During the four and eight-cell stages, new transcription is essential as it is required for the increase in rate of protein synthesis that occurs during the morula/blastocyst (Sakkas and Vassalli, 2003).

Compaction is the first morphological change to occur in the embryo and prepares the embryo for the first differentiation event. Between the sixteen to thirty two-cell stages
the cells change their morphological and functional phenotype into a polarized form in conjunction with formation of a subset of apolar cells. Generally, a sixteen-cell compacted embryo consists of nine outer polarised cells, and seven inner apolar cells (Johnson and Ziomek, 1981). The basal migration of the nucleus and the apical aggregation of actin, clathrin, endosomes and microvilli highlight the polarization of the outer cells (Sakkas and Vassalli 2003). The polar, outer cells develop into TE cells, and are smaller and flatter compared to the apolar, inner cells which form the ICM. During compaction tight junctions form between the outer cells and these are important for blastocoel formation to form a blastocyst. The TE segregates the ICM from the external uterine environment.

1.1.2 Blastocyst Formation

At the late morula-early blastocyst stage the ICM and TE cells differ in the variety and complexity of their intercellular junctions. In mouse embryos the TE cells are adhered by tight junctions, desmosomes and gap junctions (Moriwaki et al., 2007). Whereas ICM cells are more rounded and grouped tightly, therefore less extensive junctions are required and only gap junctions have been observed (Ducibella et al., 1975; Moriwaki et al., 2007).

Once the tight junctions have formed between the TE cells they now engage in transport activity and ions such as Na⁺, Cl⁻, K⁺, Ca²⁺ and Mg²⁺ are transported across the TE to the inside of the morula (Fig. 1.2). This establishes an ionic gradient that contributes to water influx. This movement of water, results in formation of the blastocoel (Leese et
al., 2001; Moriwaki et al., 2007) (Fig. 1.2). The blastocoel determines the embryonic and abembryonic axis, with the ICM present at the embryonic pole (Tesarik, 1988).

The blastocyst consists of ICM, TE and the blastocoel. ICM cells are the founder pluripotent cells of the embryo: They will give rise to all of the cell types of the developing embryo and adult as well as several different extra-embryonic tissues. The TE will give rise to the placenta and extra-embryonic membranes (Hardy and Spanos, 2002). On day 4.5, the zona pellucida is digested by proteolitic activity so that the blastocyst adheres to the wall of the uterus, and begins to burrow into it; this marks the start of the implantation phase of embryo development, which includes gastrulation, early organogenesis, and organogenesis/growth (Fig. 1.3).
Fig. 1.3 Stages of development after fertilisation. The zygote will become a blastocyst which upon hatching adheres to the uterine wall and implants. After implantation gastrulation occurs to form three germ layers known as ectoderm, mesoderm and endoderm. Gastrulation is followed by organogenesis in which organs develop in the germ layers resulting in development of the fetus before birth.

1.2 CULTURING PRE-IMPLANTATION MOUSE EMBRYOS

Pre-implantation embryo development can also occur *in vitro* in simple chemically defined media (Summers and Biggers, 2003). However, it is slower and embryos are not as viable as those that develop *in vivo*. This shows that oviductal fluid which bathes the pre-implantation embryo provides a more suitable environment for pre-implantation
embryo development than *in vitro* culture media. Therefore improving *in vitro* embryo development depends on culturing zygotes to the blastocyst stage in culture media with the optimal components including salts, pH buffer(s), energy sources and certain amino acids.

### 1.2.1 Discovery of Embryo Culture

Culture of pre-implantation embryos was first performed in Germany in 1941. Embryos were cultured on a blood clot with an extract of oviduct tissue (Kuhl and Kuhl, 1941). Later, it was shown that eight-cell mouse embryos develop into blastocysts in a simple chemically defined medium containing glucose (Whitten and Biggers, 1968); however, embryos cultured from the one-cell stage in this medium would not develop past the two-cell stage.

Advances in the improvement of development of pre-implantation embryos *in vitro* then depended largely on including or omitting specific medium components and/or altering their concentration (Biggers, 2002). The embryos obtained from mice developed successfully when they were collected and handled in Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer instead of a bicarbonate buffer, suggesting the importance of a stable pH during the manipulation of embryos outside the incubator (Hogan et al., 1986).

In early culture experiments with mouse embryos, developmental arrest often occurred at the two-cell stage. This was referred to as the ‘two-cell block’. The two-cell block occurred when embryos were cultured from the one-cell embryo stage, whereas when
embryos were cultured after the two-cell stage they could develop to the blastocyst stage (Whitten, 1957). Further optimisation of the culture medium showed that modification of osmolality, addition of EDTA and glutamine enabled development \textit{in vitro} from the zygote through to the blastocyst stage (Biggers, 1998). These modifications will be discussed in subsequent sections.

1.2.2 Important Components in Simple Chemically Defined Medium

1.2.2.1 Energy Sources

At the early stages of development the embryo mainly uses the carboxylic acids, lactate and pyruvate as sources of energy (Fig. 1.4) and is the preferred energy source instead of glucose until compaction (Brinster, 1973; Leese and Barton, 1984).
Fig. 1.4 The uptake of pyruvate, lactate and glucose in pre-implantation stages. Pyruvate is preferred as an energy source in the first two stages and is transported by monocarboxylic transporter (MCT). Additionally pyruvate is needed after the two-cell stage until compaction in order to convert into lactate so that the coenzyme nicotinamide adenine dinucleotid (NAD\(^+\)) is regenerated for cell division in subsequent stages. Also the conversion of pyruvate to lactate enables NADH production. Malate-aspartate shuttles (MAS) have a role in transporting to inner mitochondria from cytoplasm NADH for oxidation, ATP production and the regeneration of NAD\(^+\). After compaction until the end of pre-implantation development glucose is the main energy provider. It is transported by glucose transporter 3 (GLUT 3) to facilitate blastocyst formation. Images by S. Ozsoy.

In order to assess the importance of pyruvate α-cyano-4-hydroxycinnamate, an inhibitor of pyruvate, uptake in erythrocytes and across the inner mitochondrial membrane of liver mitochondria was added in the incubation. It also inhibited the implantation development in mouse embryos. In the absence of α-cyano-4-hydroxycinnamate pyruvate was carried throughout embryonic development (Gardner and Leese, 1988). The monocarboxylic acid transporter (MCT) is responsible for lactate and pyruvate transport into the embryo, particularly at the unfertilised oocyte to
two-cell stage and also plays an important role in the control of cytosolic pH ($pH_i$) (Gibb et al., 1997; Harding et al., 1999). After the two-cell stage, lactate has a role as an energy source until compaction (Fig. 1.4). Lactate is readily oxidised by one and two-cell mouse embryos, however is not adequate to overcome the two-cell block (Lane and Gardner, 2000). Lactate is first converted to pyruvate by lactate dehydrogenase (LDH) in the mitochondria and then into nicotinamide adenine dinucleotide (NAD\(^+\)) and thus NADH is produced (Lane and Gardner, 2005). NADH is important for oxidation, ATP production and also for regeneration of NAD\(^+\). However, mitochondria are impermeable to NADH and so movement of NADH across the mitochondrial membrane is thought to be mediated by malate-aspartate shuttles (MAS). As a result pyruvate and lactate work in cooperation with MAS shuttles in order to regenerate NAD\(^+\) so that the cell can continue using lactate and glucose as an energy source in further divisions (Lane and Gardner 2005).

However, when pyruvate and lactate alone are added to the culture media embryos can not grow to the blastocyst stage unless there was supplementation with glucose. In the presence of pyruvate, lactate and glucose ~95% of 1-cell embryos develop to the blastocyst stage (Brown and Whittingham, 1991). This indicates that glucose improves development from the morula to the blastocyst stage (Fig. 1.4). The uptake of glucose in the mouse embryo is via glucose transporter isoform 3 (SLC2A3, GLUT3) or which is expressed from the time of compaction (Pantaleon et al., 1997). Glucose is required to provide ATP for the high energy requirements of the developing blastocyst (Pantaleon and Kaye, 1998). Glucose also acts as a signal to activate embryonic gene expression, differentiation and development through hexosamine biosynthesis (Pantaleon et al., 2008). Deletion of the $Slc2a3$ gene in mice results in embryo lethality.
Chapter 1- Introduction

at day E7.5 due to increased apoptosis from E6.5. Morula and blastocysts develop normally in Slc2a3⁻/⁻ mice (Schmidt et al., 2009).

1.2.2.2 Ethylenediaminetetraacetic acid (EDTA)

As mentioned above, embryos of some mouse strains, eg. outbred strains (CD-1, CF-1 or CFLP) arrest at the two-cell stage in chemically defined medium (Biggers 1998). In contrast, embryos from inbred strains such as C57BL/6, DBA/2 can develop to blastocysts even if cultured in the same medium (Biggers, 1998; Goddard and Pratt, 1983; Scott and Whittingham, 1996). One and two-cell embryo divisions are largely under the control of maternal mRNA with the majority of the embryonic genome being transcribed at the end of the one-cell stage in mouse embryos (Cockburn and Rossant 2010). It is hypothesised that in outbred strains protein synthesis is inadequate at the cleavage of the two-cell stage. If there is a loss of a factor in the cytoplasm there can be changes in transcriptional system or abnormal activation of the embryonic genome can occur that will cause the inadequate levels of protein synthesis (Goddard and Pratt, 1983; Robl et al., 1988; Sakkas et al., 1993; Zanoni et al., 2009). In addition the reciprocal cross between the outbred female and inbred male blocks the development at the two-cell stage that is caused by the female genes. (Goddard and Pratt 1983; Zanoni et al. 2009).

The two-cell block is relieved by adding ethylenediaminetetraacetic acid (EDTA) to the culture medium (Gardner and Lane, 1996; Matsukawa et al., 2002; Suzuki et al., 1988). EDTA is a cell membrane-impermeable metal-ion chelator. By chelating transition metal ions it can prevent the ions from participating in chemical reactions that generate
harmful oxygen radicals (Matsukawa, Ikeda et al. 2002). It was known that EDTA overcame the two-cell block however was only beneficial in one-cell embryos and because it inhibited glycolysis activity embryos could not develop to blastocyst stage (Gardner et al., 2000). EDTA is included in all currently popular mouse embryo culture media. The first recognized medium suitable for overcoming the two-cell block and allowing embryos develop to blastocyst stage was CZB (Table 1.1). This medium contained EDTA and glutamine so that one-cell embryos developed to blastocyst stage (Gardner and Lane 1996).
Table 1.1 The components and concentration (mM) of culture media used for pre-implantation embryo development (modified from Biggers 1998).

<table>
<thead>
<tr>
<th>Compound</th>
<th>CZB</th>
<th>MTF</th>
<th>KSOM</th>
<th>modHTF</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>81.62</td>
<td>94.7</td>
<td>95</td>
<td>101.6</td>
</tr>
<tr>
<td>KCl</td>
<td>4.8</td>
<td>4.78</td>
<td>2.5</td>
<td>4.69</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>1.18</td>
<td>1.19</td>
<td>0.35</td>
<td>0.37</td>
</tr>
<tr>
<td>MgSO$_4$</td>
<td>1.18</td>
<td>1.19</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>1.7</td>
<td>1.71</td>
<td>1.71</td>
<td>2.04</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>25.12</td>
<td>25</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Lactate</td>
<td>31.3</td>
<td>4.79</td>
<td>10</td>
<td>21.4</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>0.27</td>
<td>0.37</td>
<td>0.2</td>
<td>0.33</td>
</tr>
<tr>
<td>Glucose</td>
<td>-</td>
<td>3.4</td>
<td>0.2</td>
<td>2.78</td>
</tr>
<tr>
<td>Glutamine</td>
<td>1</td>
<td>-</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.11</td>
<td>-</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>BSA (mg/ml)</td>
<td>5</td>
<td>4</td>
<td>1</td>
<td>3</td>
</tr>
</tbody>
</table>

1.2.2.3 Sodium Chloride (NaCl)

Apart from the strain dependence of the two-cell block, osmolality is another factor that can cause development to arrest at the two-cell stage in vitro. One-cell embryos do not develop past the two-cell stage in vitro when the osmolality of the culture medium is at the osmolality of the oviduct fluid, which is estimated to be 310–360 mOsm/kg (Collins and Baltz, 1999). By reducing the concentration of NaCl so that the medium osmolality is below 300 mOsm/kg embryos can develop into blastocysts. Thus by adjusting NaCl,
osmolality differs for each culture medium, for instance modified human tubal fluid (modHTF; Table 1.1) medium has an osmolality of between 275 and 295 mOsm/kg that enhances development of mouse embryos to blastocyst stage. However, approximately 250 mOsm/kg potassium simplex optimised medium (KSOM; Table 1.1) is appropriate to culture embryos to blastocyst stage (Baltz and Tartia, 2010; Lawitts and Biggers, 1992; Whitten and Biggers, 1968).

1.2.2.4 Amino Acids

Amino acids are molecules containing a carboxylic group, an amide group and a variable side chain. There are twenty standard amino acids that are grouped in two by essential and non-essential amino acids. In pre-implantation embryo development non-essential amino acids are generally used in the cleavage stages until morula stage and essential amino acids are metabolised after eight cell stage (Van Winkle, 2001).

Although pre-implantation embryos will develop in amino acid and protein free medium (Biggers et al., 2000) it is well recognised that development is improved by the addition of amino acids. Twenty standard amino acids were utilised in vitro in order to improve development (Brinster, 1965; Cholewa and Whitten, 1970; Gwatkin, 1966; Spindle and Pedersen, 1973). In 1993 Gardner and Lane used modified mouse tubal fluid (mMTF) medium (Table 1.1) either with amino acids or without amino acids for pre-implantation embryo development and found that both treatments gave the same percentage of development, but the embryos cultured in mMTF with amino acids developed faster. Also, medium containing amino acids resulted in increased blastocysts and cell numbers compared to the medium without amino acids (Gardner and Lane, 1993). Edwards et.
al. 1998 have demonstrated the benefit of adding amino acids in the culture medium to regulate intracellular pH up to four-cell stage mouse embryos (Edwards et al., 1998). Non-essential amino acids and glutamine had the ability to buffer intracellular pH against an acid load in early embryo development (Edwards et al., 1998).

Glutamine is a non-essential amino acid that improves development of one-cell mouse embryos to blastocyst stage and increases cell numbers (Rezk et al., 2004). It has been shown that glutamine also functions as an energy source along with glucose. It donates carbon to refill the citric acid cycle and has a role in protein synthesis in early embryo development (Chatot et al., 1990).

Glutamine acts as an amide donor to the hexosamine pathway and activates glutamine-fructose-6-phosphate amidotransferase (GFPT). The activation of GFPT allows fructose-6-phosphate to metabolise to glucosamine-6-phosphate which can also be activated by glucosamine, a substrate that enters the pathway downstream. Thus, between one and two-cell stage glucosamine could be substituted for glucose and support GLUT3 activation on hexosamine pathway and blastocyst formation (Pantalone et al., 2008).

According to the investigations, an improved culture medium is when energy sources such as lactate, pyruvate and glucose are supplied in the medium; however, it is important that the culture medium contains amino acids such as non-essential amino acids and glutamine, to allow embryos develop at the cleavage stage and overcome the two-cell block also to improve later stages in pre-implantation (Lane and Gardner, 2007; Nagy, 2003).
1.3 AMINO ACID TRANSPORTERS

An amino acid transporter is a membrane transport protein that transports amino acids. These amino acid transporters in embryos are of the solute carrier (SLC) family. The group members of the SLC family include both charged and uncharged organic molecules as well as inorganic ions. The regulation of cellular proliferation and differentiation by amino acids is due to the transportation by SLC family amino acid transporters in different pre-implantation stages (Van Winkle 2001). These amino acid transporters were identified according to Na⁺ co-transport or the transporters substrate binding site structure (Christensen, 1990). The difference in substrates, kinetics or regulations of amino acids makes the variety of the systems (Table 1.2).
Table 1.2 Amino acid transport systems and their expression in the preimplantation embryo.

<table>
<thead>
<tr>
<th>Embryo transporter system</th>
<th>Amino acid substrates</th>
<th>Gene</th>
<th>Stages highly expressed</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMINO</td>
<td>Proline, Betaine</td>
<td>SLC6A20</td>
<td>1-2 cell</td>
<td>Anas et. al., 2007</td>
</tr>
<tr>
<td>Gly</td>
<td>Glycine</td>
<td>SLC6A9</td>
<td>1-8 cell</td>
<td>Van Winkle, 1988</td>
</tr>
<tr>
<td>b&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Arginine</td>
<td>SLC7A1, SLC7A2, SLC7A3</td>
<td>1-2 cell, 8 cell</td>
<td>Van Winkle, 2001</td>
</tr>
<tr>
<td>System N</td>
<td>Glutamine, Asparagine, Histidine</td>
<td>SLC38A5</td>
<td>4-8 cell</td>
<td>Van Winkle and Dickinson, 1995</td>
</tr>
<tr>
<td>X&lt;sub&gt;AG&lt;/sub&gt;</td>
<td>Glutamate, Asparagine</td>
<td>SLC1A1, SLC1A2, SLC1A3</td>
<td>Blastocyst</td>
<td>Kanai and Hediger, 2004</td>
</tr>
<tr>
<td>X&lt;sub&gt;A&lt;/sub&gt;</td>
<td>Asparagine</td>
<td>SLC1A1, SLC1A2, SLC1A3</td>
<td>Blastocyst</td>
<td>Kanai and Hediger, 2004</td>
</tr>
<tr>
<td>B&lt;sup&gt;0.1&lt;/sup&gt;</td>
<td>Leucine, Arginine</td>
<td>SLC6A14</td>
<td>Blastocyst</td>
<td>Van Winkle, 2001</td>
</tr>
<tr>
<td>System A</td>
<td>Glycine, Proline</td>
<td>SLC38A1, SLC38A2, SLC38A4</td>
<td>Blastocyst, Inner cell mass</td>
<td>Mackenzie and Erickson, 2004</td>
</tr>
<tr>
<td>β</td>
<td>Taurine</td>
<td>SLC6A6</td>
<td>All</td>
<td>Van Winkle, 1994</td>
</tr>
<tr>
<td>System L</td>
<td>Leucine, Valine, Isoleucine</td>
<td>SLC7A5, SLC7A8</td>
<td>All</td>
<td>Van Winkle, 1990</td>
</tr>
<tr>
<td>System T</td>
<td>Tryptophan, Tyrosine</td>
<td>SLC16A10</td>
<td>All</td>
<td>Van Winkle, 2001</td>
</tr>
</tbody>
</table>
Amino acids grouped as non-essential and essential amino acids activate transporters at different times of development. Non-essential amino acids are known to be sufficient for improving development in cleavage stages whereas essential amino acids are transported after eight-cell stage (Van Winkle 2001).

Gly, IMINO, B⁰,⁺ and β are the systems that are in the sodium and chloride dependent neurotransmitter transporter family (SLC6’s) (Chen et al., 2004). GLYT1 the transporter of Gly system is one of the most known transporter in pre-implantation embryos (Steeves et al., 2003). It is active through 1-8 cell stage and transports glycine (Dawson and Baltz, 1997; Van Winkle et al., 1990b) and is inhibited by sarcosine (Hobbs and Kaye, 1986). SIT1 is the transporter of the IMINO system and is active in 1-2 cell stage embryos (Anas et al., 2007). It transports imino acids such as betaine and proline. High-affinity glutamate and neutral amino acid transporter family (SLC1’s) (Kanai and Hediger, 2004) contains essential amino-acid transporters that are $X_{AG}^+$, $X_{A}^-$ systems. These systems are activated by L-glu, L-asp and D-asp accompanied by co-transport of Na⁺, H⁺ and counter-transport of K⁺ at blastocyst stage. Cationic amino acids, large neutral L-amino acids and neutral L-amino acids are transported via the Cationic amino acid transporter/glycoprotein-associated family (SLC7’s) (Verrey et al., 2004). The transporter is cationic amino acid transporter (CAT) for cationic amino acids and LAT1 and 2 for neutral amino acids. Co-transport of Na⁺ is only via SLC7A8 family, system L transporter (LAT2) when neutral L-amino acids are transported. SLC16 gene family consists of monocarboxylic amino acid transporters (MCT) and aromatic amino acid transporters (Halestrap and Meredith, 2004). T-type amino acid transporter 1 (TAT1) is the transporter that is different to MCTs by transporting aromatic amino acids without co-transport of Na⁺. Another transporter described in pre-
implantation embryos is from the SLC38 gene family, sodium-coupled neutral amino acid transporters (Mackenzie and Erickson, 2004; Van Winkle and Dickinson, 1995). These are the system A/N transporters that correspond in functional properties and patterns of regulation. System A transports aliphatic amino acids and is pH sensitive. While system A transporters are expressed at the blastocyst stage and is only cotransport of Na⁺ system N transporter is expressed at the four-eight cell stage and is co-transport of Na⁺ and counter-transport of H⁺.

1.4 THE ROLES OF AMINO ACIDS IN PRE-IMPLANATION MOUSE EMBRYO DEVELOPMENT

Amino acids have many functions in cells. The most important role of amino acids is to link together and generate proteins. Amino acids are nutrients and function in metabolism, pH regulation, signalling and also in hyper and hypo osmotic conditions they act as organic osmolytes.

1.4.1 Action of amino acids as organic osmolytes

Organic osmolytes include amino acids such as glycine, glutamate, betaine, proline, taurine, isoleucine, valine, aspartate, beta-alanine and alanine (Burg and Ferraris, 2008; Wehner et al., 2003). These amino acids regulate cell volume in different osmolalities (Baltz and Tartia 2010) (Fig. 1.5). Organic osmolytes are used instead because of the decrease in cell volume that inorganic ions cause. In hyper osmotic conditions the import of inorganic ions will be detrimental for embryo viability and the cell size will decrease (Richards et al., 2010).
Hyper osmolality ≥300mOsm/kg

Fig. 1.5 The control of cell volume in hyper osmotic conditions. Cells of the embryo shrink because of water efflux when the concentration of NaCl and/or raffinose is increased in the culture medium. However, when amino acids are added to the medium they are transported into the cells and normal cell volume is restored (modified from Baltz and Tartia 2010).

Hyper osmotic conditions in vitro do not allow embryos enhance development. However the addition of amino acids to the culture medium improves embryo development. In pre-implantation embryos betaine and its substrate proline are transported through one-two cell stage via SLC6A20 (Anas et al., 2007). It has been shown that glycine accumulation as an organic osmolyte is ~2.5X10^{-8} mmol/embryo at 310 or 340 mOsm/kg whereas less at 250 mOsm/kg through one-eight cell stage (Dawson et al., 1998). Therefore glycine protects the embryo in hyper osmotic conditions from one to eight cell stage additionally it has been shown that the transport is via SLC6A9 transporter (Van Winkle et al., 1988) however in post-compaction stages it is thought to be transported by a different transporter, SLC6A14 (Richards et al., 2010). Glutamine is another osmolyte that rescues embryos from two-cell to blastocyst...
stage. It has been demonstrated that glutamine uptake is similar to glycine uptake in two-cell embryos (Lewis and Kaye, 1992). Therefore it is thought to be transported via SLC6A9 at the cleavage stages and via SLC6A14 after compaction (Richards et al., 2010). Taurine which is transported in pre-implantation embryo development has been shown to taken via the SLC6A6 transporter more in hyper osmotic conditions than hypo osmotic conditions in one-cell embryos. However at blastocyst stage taurine uptake is greater in hypo osmotic conditions comparing with hyper osmotic conditions (Van Winkle et al., 1994). Indeed it has been shown that instead of taurine in hyper osmotic conditions β-alanine is transported via the SLC6A6 transporter in order to protect the embryo against the efflux (Hammer and Baltz, 2003).

Furthermore the tonicity of oviductal fluid has been shown to be in a high range such as 310–360 mOsm/kg however in vitro culture conditions embryos are able to develop with a range of 290–300 mOsm/kg which has been shown as iso osmotic conditions (Collins and Baltz, 1999). Additionally it has been shown that amino acids are present in the oviductal fluid thus in hyper osmotic culture conditions the presence of osmolyte amino acids to culture medium would allow embryos develop to blastocyst stage (Aguilar and Reley, 2005; Collins and Baltz, 1999). These organic osmolyte amino acids in the oviductal fluid are known as glycine in a high amount and effective osmolyte (Dawson and Baltz, 1997; Van Winkle et al., 1990b) and alanine following glycine. Glutamine and proline also have significant amounts as α-amino acids in the oviductal fluid. There are β-amino acids that also have been studied and taurine, hypotaurine and β-alanine are the highest amounts in the oviductal fluid (Baltz, 2001; Guerin and Menezo, 1995; Guerin et al., 1995).
1.4.2 Amino acids in metabolism

Metabolism of amino acids in the cell is complex because there are twenty different amino acids that most degrade into several pathways (Zubay, 1983). As an energy source amino acids are precursors of lipids, carbohydrates and nucleic acids.

The catabolic metabolism of amino acids is the removal of amino group from each sequence. Glutamate is a key molecule in cell metabolism. The transamination of glutamate is $\alpha$-ketoglutarate which functions in citric acid cycle (Zubay, 1983).

In pre-implantation embryos despite normal development to the blastocyst stage under certain *in vitro* conditions embryo viability and fetal development may be abnormal. Embryo viability depends on the density of embryo culture (i.e. volume of medium per embryo) and the components of the culture medium. Amino acids are one of the components that have been shown to improve embryo viability. For example it has been shown that when glutamine is added to the medium there is no block to development by glucose (Chatot et al., 1989) and there is an increase in cleavage rates (Rezk et al., 2004). However the nutritional requirements of the embryo changes in every stage as it develops. Thus it has been shown that glutamine also prevents embryo development because of ammonium breakdown (Gardner and Lane, 1993; Zander et al., 2006). Therefore the embryo environment is reproduced according to the maternal reproductive tract so that the culture medium with the components described above is prepared to meet the requirements of the developing embryo with better viability.
1.4.3 Signalling pathways activated by amino acids

Amino acids are transported in the cell in order to activate intracellular signalling pathways that involve mammalian target of rapamycin (mTOR) (Cardenas et al., 1999; Lynch, 2001; Nobukuni et al., 2005). Amino acids indeed activate Ras homolog enriched in brain (Rheb), an upstream regulator of mTOR (Hanrahan and Blenis, 2006). However Tuberous sclerosis 1/2 (TSC1/2) complex inactivates Rheb unless growth factors are transported in the cell. Therefore the activated amount of Rheb depends on the inhibition of TSC1/2 complex which is the downstream of PI3K/AKT signalling pathway (Garami et al., 2003) (Fig. 1.6).
Fig. 1.6 Mammalian target of rapamycin (mTOR) signalling pathway is regulated by amino acids through the amino acid transporter. After the amino acid enters the cell it will activate Ras homolog enrich in brain (Rheb), which is an upstream molecule of mTOR so that mTOR can phosphorylate S6K and 4eBP1 proteins to start mRNA translation (Van Winkle et al., 2006). However activation of Rheb is PI3K/AKT signalling pathway dependent. Tuberous sclerosis 1/2 (TSC1/2) complex inhibits Rheb unless AKT is phosphorylated via PI3K pathway (Garami et al., 2003).
mTOR is a serine-threonine kinase that contains 2549 amino acids and belongs to the PI3K protein family. It regulates cell growth, cell proliferation, cell motility, cell survival, protein synthesis and transcription (Hay and Sonenberg, 2004). mTOR generates two functionally distinct multiprotein complexes: mTORC1 and mTORC2. mTORC1 is rapamycin-sensitive whereas mTORC2 is insensitive to rapamycin and growth-factor-responsive (Robitaille and Hall, 2008). These mechanisms provide the accumulation of cellular mass and therefore regulate cell size (Murakami et al., 2004; Robitaille and Hall, 2008). mTORC1 activates translation initiation and elongation (Robitaille and Hall, 2008).

Amino acids activate mTOR by stimulating other signalling pathways so that deprivation of amino acids transported in the cell will cause inhibitory effect on mRNA translation regulated via mTOR (Kimball and Jefferson, 2006; Wang and Proud, 2006). In pre-implantation embryos leucine stimulates mTOR signalling pathway after it is transported via SLC6A14 transporter (Van Winkle et al., 2006). mTOR phosphorylates PHAS/4EBP proteins hence releases eIF4E selectively and phosphorylates S6K to start the translation of mRNAs encoding proteins (Van Winkle et al., 2006) (Fig. 1.6). In ES cells it has been shown that other than leucine, L-proline activates mTOR signalling pathway (Washington et al., 2010). However leucine does not have any effect in ES cell differentiation whereas L-proline does.

Knockouts of mTOR are lethal at embryonic day E5.5 to 6.5 (Gangloff et al., 2004; Murakami et al., 2004; Yang and Guan, 2007). Homozygous knockout blastocysts have a normal shape and size. However, when embryos are cultured in vitro ICM and TE fail to proliferate normally (Murakami, Ichisaka et al. 2004). Rapamycin is known as the
inhibitor of mTOR and associates with its intracellular receptor FKBP12. The complex of FKBP12-rapamycin binds directly to the FKBP12-rapamycin binding (FRB) domain of mTOR. In blastocysts, rapamycin inhibits trophoblast outgrowth, thus imitating the effect of mTOR deletion. In contrast rapamycin does not affect the proliferation of ICM (Martin and Sutherland, 2001; Murakami et al., 2004). Also by inhibiting mTOR signalling p70S6K, downstream of the pathway will be inhibited. Observations in one-cell embryos have shown that S6K1 is active throughout the cell cycle. However rapamycin decreases the activity of M-phase promoting factor so that stops development in one-cell stage (Xu et al., 2009).

1.4.4 Trophic factors in pre-implantation embryo development in vitro

Trophic factors play important roles in embryo development \textit{in vivo}. Trophic factors are secreted by both the reproductive tract and the embryo itself and thereby have both paracrine and autocrine actions on pre-implantation development (Diaz-Cueto and Gerton, 2001). It has been shown that platelet activating factor (PAF), tumor necrosis factor (TNF), transforming growth factor (TGF), epidermal growth factor (EGF), insulin-like growth factor (IGF), leukemia inhibitory factor (LIF) improve embryo development (Hardy and Spanos 2002). And also EGF, TGF-\(\alpha\), heparin binding-epidermal growth factor (HB-EGF), IGF-I, PDGF-B, LIF and granulocyte-macrophage colony stimulating factor (GM-CSF) are the trophic factors that have roles in reproductive tract receptivity (Hardy and Spanos 2002). Insulin is another trophic factor that is involved in pre-implantation embryo development in order to be one of the regulators of embryonic development (Kaye et al., 1992). These trophic factors in the
embryonic development decrease cell death, increase cell survival, proliferation, metabolism and protein synthesis (Hardy and Spanos 2002).

Trophic factors bind to specific receptors on the plasma membrane of the embryonic blastomeres and thereby activate intracellular signalling pathways that regulate cell proliferation and cell survival. Generally these pathways are thought to involve the MAPK and PI3K signalling pathways. MAPK is a serine-threonine-specific protein kinase that functions in regulation of gene expression, mitosis, differentiation, proliferation and cell survival/apoptosis (Pearson et al., 2001).

In pre-implantation embryo development, the MAPK pathway can be activated by a number of trophic factors, including fibroblast growth factor (FGF), TGF-α or EGF. Binding of these factors to their receptors activates signal transduction through growth factor receptor bound protein-2 (GRB2), Ras, Raf, MEK1/2, and extracellular signal-regulated kinases 1/2 (ERK 1/2) (Eswarakumar et al., 2005) (Fig. 1.7).
Fig. 1.7 EGF and IGF stimulate MAPK and PI3K signaling pathways respectively in order to enhance embryo development.

Additionally IGF is thought to stimulate embryo survival and development via PI3K/AKT signalling pathway (Riley and Moley, 2006). The pathway involves the phosphorylation of PI3K, which converts PIP2 to PIP3. PDK1, which is recruited to the PIP3 in the plasma is then phosphorylated and this in turn results in phosphorylation of AKT, which has a role in embryo survival and development (Fig. 1.7). There are three AKT family members; Akt1, Akt2 and Akt3. Akt1 has a role in cell survival by
phosphorylating and inactivating apoptotic processes, such as BAD, Caspase 9 and forkhead factors (Brunet et al., 1999; Downward, 2004; Yamaguchi and Wang, 2001).

Thus it is important to consider the action of trophic factors during in vitro culture. Maternally-derived trophic factors are absent during in vitro culture of pre-implantation embryos. In addition culture of embryos in large volumes of medium or individually (i.e. at low density) dilution of autocrine factors that are required for optimal embryo development and viability (Lane and Gardner, 1992; O'Neill, 1997; Vutyavanich et al., 2010).

1.4 AIMS AND HYPOTHESES

There is strong evidence that amino acids have a role in pre-implantation development in vitro. In vivo oviductal fluid contains amino acids. Thus amino acids improve growth, metabolism, proliferation and differentiation in embryonic development. When chemically defined medium is supplemented with amino acids, there is an increase in the development to blastocyst stage and an improvement in the number of cells and ICM in blastocysts. Generally amino acids added in groups in the culture medium increase cell numbers and ICM, however supplementation by a single amino acid has not been thoroughly investigated.

As ES cell are derived from the ICM in embryos they are commonly used as a model for pre-implantation embryo development. Differentiation and signal transduction has been induced in embryonic stem (ES) cells by L-proline (Golos et al., 2010; Washington et al., 2010). Therefore L-proline is one of the amino acids that may
enhance development of embryos to the blastocyst stage *in vitro*. Furthermore little is known about L-proline stimulating proliferation and differentiation via specific pathways such as mTOR and MAPK in the embryo.

Thus the main aim of this study is to investigate whether the addition of L-proline alone will improve development to blastocyst stage at low density (1 embryo/100 µl) because of the dilution of growth factors that are secreted by the embryo. I hypothesise that there will be an increase in cell numbers and ICM numbers in blastocysts cultured with L-proline at low density comparing to high density (1embryo/µl) in iso osmotic conditions.

Another aim is to culture embryos in a range of high and low osmolality in order to investigate whether L-proline acts as an organic osmolyte, in order to understand which transporters transit L-proline into the cell to regulate the cell volume. I hypothesise that at hyper osmotic conditions ($\geq 300$ mOsm/kg) L-proline will act as an organic osmolyte such as betaine and will be transited in the cell via the betaine transporter.

Last aim of this study is in order to investigate whether L-proline stimulates mTOR and MAPK signalling pathways. Also I hypothesise that L-proline will stimulate differentiation by MAPK signalling pathway.
CHAPTER TWO

MATERIALS AND METHODS
2.1 MEDIA FOR EMBRYO COLLECTION AND MANIPULATION

2.1.1 Medium for embryo collection

All sterile and non-sterile plastics used for media and chemical preparation were from Sarstedt (Nümbert, Germany) unless otherwise stated. 1000µl plugged pipette tips were from BioPointe Scientific (San Diego, CA, USA). Zygotes were isolated into Hepes (N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid) buffered modified HTF (Hepes-modHTF) (O'Neill, 1997) prepared from the stock solutions shown in Table 2.1. The composition of Hepes modHTF is shown in Table 2.2. Milli-Q grade water (Millipore) was used to dissolve measured components as well as prepare media. The pH of the medium was adjusted using a pH meter (901 - PH, Brisbane, Australia) to between 7.4 and 7.45 using 2 M NaOH and the osmolality was measured using a cryoscopic osmometer (Gonotec, GmbH, Berlin, Germany) to confirm that it was in the correct range. 0.3 mg/ml bovine serum albumin (BSA; Sigma-Aldrich; St. Louis, MO, USA) was then added to the media. The media was filtered with a sterile 0.22 µm filter (Milipore; Billerica, MA, USA) and stored at 4°C for two weeks.

Generally, BSA at a concentration of 1-4 mg/ml is added to mouse embryo culture medium in order to prevent the embryos sticking to the Petrie dish during isolating and culture. The concentration of BSA used in this thesis was reduced to 0.3 mg/ml to minimise any affect of contaminants present in the BSA (O'Neill, 1997).
Table 2.1. Stock solutions used for preparing media for embryo collection and culture

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Component</th>
<th>Molecular Weight</th>
<th>Amount/volume H₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 M</td>
<td>NaCl</td>
<td>58.44</td>
<td>2.92 g / 50 ml</td>
</tr>
<tr>
<td>0.1 M</td>
<td>KCl</td>
<td>74.55</td>
<td>0.37 g / 50 ml</td>
</tr>
<tr>
<td>0.1 M</td>
<td>KH₂PO₄</td>
<td>136.09</td>
<td>0.1361 g / 10 ml</td>
</tr>
<tr>
<td>0.1 M</td>
<td>MgSO₄ . 7H₂O</td>
<td>246.47</td>
<td>0.2465 g / 10 ml</td>
</tr>
<tr>
<td>1 M</td>
<td>H₂O</td>
<td>84.01</td>
<td>0.4201 g / 5 ml</td>
</tr>
<tr>
<td>0.001 M</td>
<td>Na₂EDTA</td>
<td>372.24</td>
<td>0.0186 g / 50 ml</td>
</tr>
<tr>
<td>0.1 M</td>
<td>Na Pyruvate</td>
<td>110.00</td>
<td>0.055 g / 5 ml</td>
</tr>
<tr>
<td>0.1 M</td>
<td>Glucose</td>
<td>180.16</td>
<td>0.0901 g / 5 ml</td>
</tr>
<tr>
<td>6 mg/ml</td>
<td>Penicillin</td>
<td></td>
<td>60 mg / 10 ml</td>
</tr>
<tr>
<td>0.17 M</td>
<td>CaCl₂ . 2H₂O</td>
<td>147.02</td>
<td>0.2514 g / 10 ml</td>
</tr>
<tr>
<td>0.154 M</td>
<td>HEPES</td>
<td>238.3</td>
<td>1.835 g / 50 ml</td>
</tr>
</tbody>
</table>

* Prepared for one week use only.

# Prepared for one month use.

* The pH of HEPES was adjusted to 7.4 by addition of 2 M NaOH.
Table 2.2. Composition of Hepes buffered modHTF with adjusted NaCl

<table>
<thead>
<tr>
<th>Component</th>
<th>Hepes-modHTF 258 mOsm/kg</th>
<th>Hepes-modHTF 270 mOsm/kg</th>
<th>Hepes-modHTF 296 mOsm/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Final conc (mM)</td>
<td>Volume of stock (ml/50ml)</td>
<td>Final conc (mM)</td>
</tr>
<tr>
<td>NaCl</td>
<td>95</td>
<td>4.75</td>
<td>85</td>
</tr>
<tr>
<td>KCl</td>
<td>4.6</td>
<td>2.30</td>
<td>4.6</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.4</td>
<td>0.20</td>
<td>0.4</td>
</tr>
<tr>
<td>MgSO₄ . 7H₂O</td>
<td>0.2</td>
<td>0.10</td>
<td>0.2</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>4</td>
<td>0.20</td>
<td>4</td>
</tr>
<tr>
<td>Lactic acid (60 %)</td>
<td>21.4</td>
<td>0.20g</td>
<td>21.4</td>
</tr>
<tr>
<td>Na Pyruvate</td>
<td>0.4</td>
<td>0.20</td>
<td>0.4</td>
</tr>
<tr>
<td>Glucose</td>
<td>2.8</td>
<td>1.4</td>
<td>2.8</td>
</tr>
<tr>
<td>Phenol Red</td>
<td>0.04%</td>
<td>0.02</td>
<td>0.04 %</td>
</tr>
<tr>
<td>Penicillin</td>
<td>0.06 mg/ml</td>
<td>0.50</td>
<td>0.06 mg/ml</td>
</tr>
<tr>
<td>H₂O</td>
<td></td>
<td></td>
<td>20.00</td>
</tr>
<tr>
<td>CaCl₂ . 2H₂O</td>
<td>2.04</td>
<td>0.60</td>
<td>2.04</td>
</tr>
<tr>
<td>HEPES</td>
<td>21</td>
<td>6.82</td>
<td>21</td>
</tr>
<tr>
<td>H₂O</td>
<td></td>
<td></td>
<td>12.71</td>
</tr>
</tbody>
</table>

Note: The NaCl concentration is adjusted to achieve the targeted osmolality for each modHTF solution.
2.1.2 Culture media

Isolated one-cell mouse embryos were washed and cultured in modified human tubal fluid (modHTF) or potassium simplex optimized medium (KSOM). The stock solutions used to prepare the media are listed in Table 2.1. The components of media are listed on Table 2.3 for modHTF and Table 2.4 for KSOM. Because some of the culture experiments were based on osmotic effect, modHTF medium has three versions with adjusted NaCl and lactate. The pH of the medium was adjusted between 7.4 and 7.45 using 1 M HCl. The osmolality of the media was measured to confirm it was in the correct range. After measuring the osmolality, 0.3 mg / ml BSA was added to the media and filter sterilised with a 0.22 µm filter (Millipore). Media were stored at 4°C for up to two weeks.

The hypothesis was that amino acids increase blastocyst development when they were supplemented in the culture medium singularly, therefore even L-glutamine was present in the recipe of O’Neill, was not added in our study as a component in culture medium. It was added on day 1 of the experiment in order to observe the effect of L-glutamine. In addition, BSA was reduced from 3 mg/ml to 0.3 mg/ml as mentioned earlier.
Table 2.3. The composition of modHTF media used for embryo culture in high osmolality experiments.

<table>
<thead>
<tr>
<th>Component</th>
<th>mod HTF 270 mOsm/kg</th>
<th>mod HTF 296 mOsm/kg</th>
<th>mod HTF 336 mOsm/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Final conc (mM)</td>
<td>Volume of stock (ml/50ml)</td>
<td>Final conc (mM)</td>
</tr>
<tr>
<td>NaCl</td>
<td>85</td>
<td>4.25</td>
<td>102</td>
</tr>
<tr>
<td>KCl</td>
<td>4.6</td>
<td>2.30</td>
<td>4.6</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.4</td>
<td>0.20</td>
<td>0.4</td>
</tr>
<tr>
<td>MgSO₄ . 7H₂O</td>
<td>0.2</td>
<td>0.10</td>
<td>0.2</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>25</td>
<td>1.25</td>
<td>25</td>
</tr>
<tr>
<td>Na₂EDTA</td>
<td>0.11</td>
<td>5.50</td>
<td>0.11</td>
</tr>
<tr>
<td>Lactic acid (60 %)</td>
<td>21.4</td>
<td>0.20g</td>
<td>21.4</td>
</tr>
<tr>
<td>Na Pyruvate</td>
<td>0.4</td>
<td>0.20</td>
<td>0.4</td>
</tr>
<tr>
<td>Glucose</td>
<td>2.8</td>
<td>1.4</td>
<td>2.8</td>
</tr>
<tr>
<td>Phenol Red</td>
<td>0.04 %</td>
<td>0.02</td>
<td>0.04 %</td>
</tr>
<tr>
<td>Penicillin</td>
<td>0.06 mg/ml</td>
<td>0.50</td>
<td>0.06 mg/ml</td>
</tr>
<tr>
<td>H₂O</td>
<td>20.00</td>
<td></td>
<td>20.00</td>
</tr>
<tr>
<td>CaCl₂ . 2H₂O</td>
<td>2.04</td>
<td>0.60</td>
<td>2.04</td>
</tr>
<tr>
<td>H₂O</td>
<td>13.48</td>
<td></td>
<td>12.63</td>
</tr>
<tr>
<td>**Raffinose</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

** Added and dissolved before adjusting the pH to 7.4 – 7.45.
Table 2.4. The composition of modHTF and KSOM medium used for embryo culture in low osmolality experiments.

<table>
<thead>
<tr>
<th>Component</th>
<th>modHTF 251 mOsm/kg</th>
<th>KSOM 258 mOsm/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Final conc (mM)</td>
<td>Volume of stock (ml/50ml)</td>
</tr>
<tr>
<td>NaCl</td>
<td>85</td>
<td>4.25</td>
</tr>
<tr>
<td>KCl</td>
<td>4.6</td>
<td>2.30</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>0.4</td>
<td>0.20</td>
</tr>
<tr>
<td>MgSO$_4$ . 7H$_2$O</td>
<td>0.2</td>
<td>0.10</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>25</td>
<td>1.25</td>
</tr>
<tr>
<td>Na$_2$EDTA</td>
<td>0.11</td>
<td>5.50</td>
</tr>
<tr>
<td>Lactic acid (60 %)</td>
<td>10</td>
<td>0.0935 g</td>
</tr>
<tr>
<td>Na Pyruvate</td>
<td>0.4</td>
<td>0.20</td>
</tr>
<tr>
<td>Glucose</td>
<td>2.8</td>
<td>1.4</td>
</tr>
<tr>
<td>Phenol Red</td>
<td>0.04 %</td>
<td>0.02</td>
</tr>
<tr>
<td>Penicillin</td>
<td>0.06 mg/ml</td>
<td>0.50</td>
</tr>
<tr>
<td>H$_2$O</td>
<td>20.00</td>
<td>20.00</td>
</tr>
<tr>
<td>CaCl$_2$ . 2H$_2$O</td>
<td>2.04</td>
<td>0.60</td>
</tr>
<tr>
<td>H$_2$O</td>
<td>13.08</td>
<td>20.65</td>
</tr>
</tbody>
</table>
2.1.3 Amino acids

Four different non-essential amino acids were used in this study. The amino acids were prepared as stock solutions in MilliQ water at the following concentrations 0.1 M L-proline, 0.1 M D-proline, 0.1 M glycine, 0.25 M L-glutamine (Sigma). The amino acid analogue betaine (N,N,N trimethylglycine; Sigma) was also used and prepared as a 0.25 M stock in Milli-Q water. Stocks were filtered using a sterile 0.22 µm filter, aliquoted (100 µl/tube) and stored at -20ºC.

2.1.4 Hyaluronidase

Hyaluronidase is utilized in vivo by the spermatozoa to penetrate the cumulus mass surrounding the oocytes by breaking down the β-N-acetyl-hexosamine glycosidic bonds in hyaluronic acid (Alberts, 2007). Thus, hyaluronidase (Type II from sheep testes, Sigma-Aldrich) was used to separate zygotes from any remaining cumulus cells. Hyaluronidase was prepared as a 1mg/ml stock in Hepes buffered modHTF + 0.3 mg/ml BSA, filter sterilised, aliquoted and stored at -20ºC.

2.2 MICE

Outbred Quackenbush Swiss (QS) mice (Animal Resource Centre, Perth) were used in accordance with the Australian Code of Practice for Use of Animals in Research and were approved by the University of Sydney Animal Care and Ethics committee. Female mice, between 4-10 weeks old and 10-30 weeks old male mice were used in these
Chapter 2 – Materials and Methods

studies. Mice were housed under a 12-hour light and dark cycle (lights on at 6 AM) at 20-22ºC and food and water was available.

2.3 EMBRYO MANIPULATION

2.3.1 Superovulation of mice and isolation of one-cell embryos

In order to induce ovulation, female mice were superovulated by intraperitoneal injection of 10 International Units (I.U.) of pregnant mares’ serum gonadotropin (PMS; Intervet, Sydney, Australia) and after 48 ± 2 hours by 10 I.U. of human chorionic gonadotropin (hCG; Intervet). Female mice were then paired with males overnight for mating. The presence of a vaginal plug the next day indicated successful mating and this was designated Day 1 of pregnancy.

The mice were euthanased by cervical dislocation and their abdomens soaked with 100% ethanol to reduce the risk of contamination. A small cut was made in the skin along the mid-line of the abdomen. Pinching the skin along two sides of the cut and tearing towards the head and tail revealed the abdominal peritoneum. To reveal the intestine the peritoneum was cut with surgical scissors. Fine forceps were used to push the intestine to one side before separating the oviduct from the ovary and uterus. Fat and mesometrium were dissected from the uterus with fine forceps and the oviduct, which contained zygotes, and a small section of uterus was removed and placed into a Petri dish lid that was filled with PBS. After washing the oviducts with PBS they were transferred into Hepes modHTF + 0.3 mg/ml BSA for isolating the embryos.
The ampulla of the oviduct was torn using fine forceps, to release the cumulus mass inside the ampullary region. Hyaluronidase (approximately 100 µl of 1 mg/ml) was poured into Hepes modHTF + 0.3 mg/ml BSA containing the zygotes surrounded by cumulus mass and incubated at 37°C in CO₂ incubator (MCO-17AIC, Sanyo, Japan) for 1-2 minutes until the cumulus cells separated from the zygotes.

Zygotes were transferred to a new 35 mm dish (Corning, New York) containing Hepes modHTF + 0.3 mg/ml BSA with a pulled pasteur pipette that was attached to rubber tubing and a mouthpiece. Zygotes were washed thoroughly and transferred to a 55 mm (Corning, New York) Petrie dish containing drops of modHTF + 0.3 mg/ml BSA and overlayed with mineral oil (Sigma). The dish was set up at least 1 hour before the experiment and placed in the CO₂ incubator to allow the medium to warm to 37°C and equilibrate with the 5% CO₂. Only zygotes with two pronuclei were used in the experiments.

2.3.2 Embryo culture

One-cell embryos obtained from QS female mice were cultured for six days. For low density culture, embryos were cultured individually in 100 µl in the wells of a 96-well plate (Corning, New York). Whereas for high density culture embryos were cultured in groups of 10-20 embryos at a density of 1 embryo/µl in drops of medium in a Petrie dish overlayed with oil. Embryos were incubated at 37°C in 5% CO₂ in air. Embryos were scored every 24 h after for development to the appropriate stage, namely 2-cell, 4-8-cell, compact 8-cell-morula, blastocyst and hatching blastocyst at 48, 72, 96, 120, and 144 h post-HCG injection, respectively. Experiments were repeated at least three times.
2.3.3 Nuclear staining of blastocysts and cell counting

On day six (144 h post HCG), blastocysts were fixed in 4% paraformaldehyde (PFA) in PBS + 1 mg/ml polyvinyl alcohol (PBS + PVA) for 15 minutes and then washed briefly in PBS + PVA and transferred to PBS + PVA + 0.3% triton-X100 for 10 minutes. In order to stop blastocysts collapsing during the mounting process, they were then washed through serial dilutions of glycerol (25, 50 and 75%) in PBS + PVA for 10 minutes in each dilution. To mount blastocysts for confocal imaging, coverslips were first coated with 5 µl of 0.01 % poly L-lysine and allowed to dry in air. Vaseline was then placed around the edges of the coverslip and a 5 µl drop of Vectashield containing 1.5µg/ml DAPI (Vector Laboratories, Burlingame, CA, USA) was placed in the centre. The blastocysts were transferred into the Vectashield and a glass slide (Livingstone, Rosebury, NSW, Australia) was placed on top, being sealed with the vaseline, therefore preventing the blastocysts from getting squashed.

Confocal microscopy was used to visualise the nuclei of each blastocyst (Bosch Institute, Advanced Microscopy Facility). The 405 nm laser of a Zeiss LSM 510 Meta Confocal microscope (Carl Zeiss, Jena, Germany) was used with the 40X objective. A Z-series optical section was taken from the top to the bottom of each blastocyst at 2.5 μm intervals to enable the visualisation of nuclei within each cell of the blastocyst. Cells were counted using LSM Image Browser software (Carl Zeiss) on a computer screen.
2.4 WESTERN BLOT ANALYSIS

2.4.1 Preparation of protein lysates from embryos

Embryos, collected at the 2-cell stage, were washed in PBS + 1 mg/ml PVA, placed in a 1.5 ml microcentrifuge tube in minimal volume and stored at -80°C until needed. 10 µl Cell Lysis Buffer (Cell Signaling Technology, Danvers, MA, USA) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) was added to the tube containing the embryos. 2 µl of 6X SDS loading buffer, which contains 0.35 M Tris Cl (pH 6.8), 10.28 % (v/v) SDS, 36 % (v/v) glycerol, 0.6 M DTT and bromophenol blue were then added. Protein samples were heated to 95°C for 5 minutes and then spun at 14.000 rpm for 5 minutes before running on an SDS-polyacrylamide gel.

2.4.2 SDS-Polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE was performed using the mini-Protean II apparatus (Bio-Rad). First, a 10% resolving gel was prepared, with the composition shown in Table 2.5, and poured to the required height between a big glass plate with 0.75 mm spacers and a small plate which are attached to a casting stand. The resolving gel was then overlaid with Milli-Q water while the gel polymerised. When the gel had set, the water was removed and the stacking gel (Table 2.5) was poured on top. A comb with 10 wells of 0.5 cm depth was placed in the stacking gel and left to set for approximately 20 minutes. After it was set, the comb was removed gently and the gel placed in the electrophoresis apparatus. The upper and lower chambers were filled with 1 x Running Buffer (Table 2.5) and the wells were washed.
A pre-stained protein marker was loaded alongside the embryo protein lysates and co-electrophoresed as size standards (180, 116, 90, 58, 48.5, 36.5 and 26.6 kDa proteins; Sigma-Aldrich). The samples have run through the stacking gel at 80 V until the dye reached the resolving gel and the voltage was increased to 120 V until the dye ran off the gel. This process took approximately 90 minutes.
### Table 2.5. Composition of solutions used for Western blotting

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% Resolving Gel</td>
<td>10% (v/v) Acrylamide: Bis-acrylamide (29:1)</td>
</tr>
<tr>
<td></td>
<td>375 mM Tris, pH 8.8</td>
</tr>
<tr>
<td></td>
<td>0.1 % (w/v) SDS</td>
</tr>
<tr>
<td></td>
<td>0.1 % (w/v) APS</td>
</tr>
<tr>
<td></td>
<td>0.04% (v/v) TEMED</td>
</tr>
<tr>
<td>5% Stacking Gel</td>
<td>5% (v/v) Acrylamide: Bis-acrylamide (29:1)</td>
</tr>
<tr>
<td></td>
<td>125 mM Tris, pH 8.8</td>
</tr>
<tr>
<td></td>
<td>1 % (w/v) SDS</td>
</tr>
<tr>
<td></td>
<td>1 % (w/v) APS</td>
</tr>
<tr>
<td></td>
<td>1% (v/v) TEMED</td>
</tr>
<tr>
<td>5X Running Buffer</td>
<td>12.5 mM Tris</td>
</tr>
<tr>
<td></td>
<td>125.2 mM Glycine</td>
</tr>
<tr>
<td></td>
<td>1.7 mM SDS</td>
</tr>
<tr>
<td>10X Transfer Buffer</td>
<td>47.9 mM Tris</td>
</tr>
<tr>
<td></td>
<td>38.6 mM Glycine</td>
</tr>
<tr>
<td></td>
<td>1.3 mM SDS</td>
</tr>
<tr>
<td></td>
<td>20% (v/v) Methanol</td>
</tr>
<tr>
<td>TBS</td>
<td>10 mM Tris-HCl, pH 7.6</td>
</tr>
<tr>
<td></td>
<td>150 mM NaCl</td>
</tr>
<tr>
<td>TBST</td>
<td>10 mM Tris-HCl, pH 7.6</td>
</tr>
<tr>
<td></td>
<td>150 mM NaCl</td>
</tr>
<tr>
<td></td>
<td>0.1% (v/v) Tween-20</td>
</tr>
</tbody>
</table>
2.4.3 Protein transfer to nitrocellulose membrane

The stacking gel was discarded using a blade and a blotting sandwich was prepared for transferring the proteins in the gel to a nitrocellulose membrane. A nitrocellulose transfer membrane (Hy-BLOT, Australia) and two pieces of Whatman 3mm paper were cut to the same size as the gel. The gel, two pads that come with the electrophoresis apparatus (Bio-Rad), the membrane and the Whatman papers were soaked in 1X transfer buffer, chilled to 4 ºC (Table 2.5), for 20 minutes. The sandwich was prepared as described in the manufacturer’s instructions. The tank was filled with 1X transfer buffer, chilled to 4ºC and the frozen Bio-ice cooling unit was added at the back of the apparatus. The proteins were transferred at 100V for 2 hours. When the transfer was completed the membrane was removed from the blotting apparatus and the corner of the membrane was cut to enable its orientation. The membrane was stained with 0.1% (w/v) Ponceau S stain for 5-10 minutes so that the transferred proteins could be visualised. Ponceau S stain was rinsed from the membrane by TBST (Table 2.5).

2.4.4 Incubation with antibodies and fluorescent detection

Before incubating the membrane, first step was to block non specific binding of antibodies to the membrane with a blocking buffer (Odyssey®; Li-cor Biosciences, Lincoln, NE, USA) for 1 hour at room temperature or overnight at 4 ºC with gentle shaking. The primary and secondary antibodies were diluted in the blocking buffer containing 0.1% Tween-20 as shown in Table 2.5. The membrane was incubated with the primary antibody overnight at 4ºC with gentle shaking. The following day, the membrane was rinsed three times for 10 minutes with TBST while gently shaking. The
membrane was incubated with secondary antibodies at room temperature for 1 hour with shaking, in the dark to protect the fluorescent dyes from fading. Again the membrane was rinsed 3 times for 10 minutes at room temperature with shaking in the dark. The membrane was rinsed with TBS (Table 2.4) for 5-10 minutes at room temperature and then visualised using the Odyssey® imager (Li-cor Biosciences), according to manufacturer’s instructions.
Table 2.6. Antibody dilutions used for western blotting

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Primary antibody dilution</th>
<th>Secondary antibody</th>
<th>Secondary antibody dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-ERK 1/2 (Cell signalling*, 9101)</td>
<td>1:500</td>
<td>Donkey anti-Rabbit IR Dye (Odyssey ®; Li-cor Biosciences)</td>
<td>1:5000</td>
</tr>
<tr>
<td>p-AKT (Cell signalling, 4058)</td>
<td>1:10,000</td>
<td>Donkey anti-Rabbit IR Dye (Odyssey ®; Li-cor Biosciences, 926-32213)</td>
<td>1:10,000</td>
</tr>
<tr>
<td>total-AKT (Cell signalling, 4691)</td>
<td>1:2000</td>
<td>Donkey anti-Rabbit IR Dye (Odyssey ®; Li-cor Biosciences)</td>
<td>1:10,000</td>
</tr>
<tr>
<td>α-tubulin (Sigma, T 9026)</td>
<td>1:500</td>
<td>Donkey anti-Mouse IR Dye (Odyssey ®; Li-cor Biosciences, 926-32222)</td>
<td>1:5000, 1:10,000</td>
</tr>
</tbody>
</table>

* Cell Signaling Technologies (Danvers, MA, USA)

### 2.5 IMMUNOFLOURESCENCE OF EMBRYOS

#### 2.5.1 Fixing and blocking non-specific binding

Two cell stage embryos were fixed in 4% paraformaldehyde in PBS containing 1 mg/ml PVA (PBS + PVA) for 15 minutes and then washed in PBS + PVA. Embryos were then permeabilised in PBS + PVA + 0.3% Triton-X100 for 15-30 minutes and then washed in PBS + PVA. Non-specific antibody binding was blocked by incubating the embryos in PBS + PVA + 0.1% Tween-20 + 0.7% BSA for 15-30 minutes. The whole process was completed at room temperature in 96-well plates.
2.5.2 Incubation with primary and secondary antibody and fluorescent detection

The primary and secondary antibodies were diluted in PBS + 1 mg / ml PVA + 0.1 % Tween- 20 + 0.7 % BSA as shown in Table 2.7. Embryos were incubated in the primary antibody overnight at 4 ºC. They were then washed in PBS + PVA + 0.1 % Tween- 20 + 0.7 % BSA twice briefly and a third time for 10-30 minutes. Embryos were then incubated in the secondary antibody at room temperature for 2 hours in the dark. Embryos were then washed three times with the final wash being 10-30 min long. Embryos were then transferred to coverslips in the centre of which a 3 µl drop of Vectashield containing DAPI had been placed. Vaseline was placed around the edges of the coverslip and a glass slide was placed over the top of the coverslip. Nuclei and secondary antibody fluorescence were visualised using the Zeiss LSM 510 Meta confocal microscope (Bosch Institute, Advanced Microscopy Facility) with the 405 nm and Argon (488 nm) lasers with a 40X objective.

Table 2.7. Antibody conditions utilized in Immunofluorescence

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Primary antibody dilution</th>
<th>Secondary antibody</th>
<th>Secondary antibody dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>p- ERK 1/2</td>
<td>1:200</td>
<td>Alexa Fluor 488 goat anti-rabbit IgG</td>
<td>1:200</td>
</tr>
<tr>
<td>(Cell signalling, 9101)</td>
<td></td>
<td>(Molecular Probes ®*, A-11008)</td>
<td></td>
</tr>
<tr>
<td>p- AKT</td>
<td>1:50</td>
<td>Alexa Fluor 488 goat anti-rabbit IgG</td>
<td>1:200</td>
</tr>
<tr>
<td>(Cell signalling, 4058)</td>
<td></td>
<td>(Molecular Probes ®*, A-11008)</td>
<td></td>
</tr>
</tbody>
</table>

* Molecular Probes ® (Invitrogen)
2.6 STATISTICAL ANALYSIS

The data are expressed as the mean ± SEM with the number of experiments (n) in parentheses, and were compared using either a paired or unpaired T-Test depending on the data collected. Proportions were compared using the chi-square test.
CHAPTER THREE

RESULTS
3.1 EFFECTS OF AMINO ACIDS ON PRE-IMPLANTATION DEVELOPMENT OF MOUSE EMBRYOS

Amino acids are one of the most important regulators of pre-implantation embryo development. When added into the culture medium, amino acids improve development and stimulate differentiation (Baltz, 2001; Dawson and Baltz, 1997; Devreker et al., 2001; Gardner and Lane, 1993; Lee et al., 2004; Van Winkle, 2001). They also increase the developmental potential *in vivo* (Gardner and Lane, 1997). Previously, the effect of amino acids on development *in vitro* was examined using groups of amino acids (such as non-essential amino acids or all 20 standard amino acids) (Lane and Gardner, 1997). In addition, experiments have investigated the role of amino acids as organic osmolytes regulating cell volume in early embryos. It has been shown that L-glutamine, glycine, betaine and L-proline play a role in regulating cell volume under hyper osmolality culture conditions (≥300 mOsm/kg) (Anas et al., 2008; Baltz, 2001). Therefore, in order to examine the role of L-proline in development of mouse pre-implantation embryos in the present study, any affect L-proline had as an organic osmolyte needed to be taken into consideration.
3.2 THE EFFECT OF AMINO ACIDS ON EMBRYO DEVELOPMENT IN ISO
OSMOTIC CULTURE MEDIA

3.2.1 Effect of L-proline and L-glutamine on development in 270 mOsm/kg modHTF at low density

The tonicity of oviductal fluid has been estimated to be 290–300 mOsm/kg. Furthermore, it has been shown that medium with an osmolality of 290–300 mOsm/kg has no effect on the volume of zygotes (Collins and Baltz 1999). Thus, in order to investigate the effect of L-proline on development under isotonic conditions, zygotes were cultured in medium with an osmolality of 270 mOsm/kg. L-glutamine was omitted from the control medium (modHTF) and used as the positive control since it has previously been shown to improve development (Rezk et al., 2004). Embryos were extracted at the zygote stage and cultured in vitro through to the blastocyst stage (for 5 days) at low density (1 embryo/100 µL). Low density was used to minimise the effect of autocrine growth factor signalling. Media containing no amino acids, 1 mM L-glutamine, 400 µM L-proline, 400 µM D-proline, 400 µM glycine, 400 µM L-alanine or 1 mM betaine were used. D-proline was used as a negative control because it is not transported by the majority of proline transporters (Metzner et al., 2006). There was a significant increase in blastocyst development (Fig. 3.1a) and hatching of blastocysts (Fig. 3.1b) when embryos were cultured in medium containing L-proline compared with no amino acids. Addition of L-glutamine also significantly increased development of blastocysts and blastocyst hatching. However, addition of D-proline or other amino acids had no effect on blastocyst development, although L-alanine increased hatching.
Despite the improvement observed in development, neither L-glutamine nor L-proline had an affect on the number of cells in the blastocysts (Fig. 3.1c).

In order to determine the developmental stage at which L-proline was exerting an effect, embryos were scored every 24 h. Addition of 400 µM L-proline or 1 mM L-glutamine significantly increased the rate of development to the five to eight-cell, morula and blastocyst stages (Fig. 3.2) compared to embryos grown in the absence of amino acids. This effect on embryo development was not observed in the presence of 400 µM D-proline, 400 µM glycine, 400 µM L-alanine or 1 mM betaine (Fig. 3.2).
Fig. 3.1. Influence of amino acids on development of mouse embryos cultured in 270 mOsm/kg modHTF at low density. Zygotes were cultured to the blastocyst stage (for 5 days) in modHTF containing either no amino acids (no a.a.), 1 mM L-glutamine (L-gln), 400 µM L-proline (L-pro), 400 µM D-proline (D-pro), 400 µM glycine (gly), 400 µM L-alanine (L-ala) or 1 mM betaine (bet). (a) Percentage of zygotes that developed to the blastocyst stage, (b) percentage of blastocysts that hatched, and (c) cell numbers in blastocysts. Values in (c) are mean ± SEM. The number of embryos analysed from 9 separate experiments is given parentheses. * $P < 0.05$ and *** $P < 0.001$ compared to no amino acids.
Fig. 3.2 The effect of amino acids on embryo development to the 2 cell, 5–8 cell, morula and blastocyst (BL) stages. Embryos were cultured in 270 mOsm/kg modHTF from the zygote to blastocyst stage at low density and development was assessed at 48, 72, 96 and 144 h post HCG following the addition of 1 mM L-glutamine (L-gln) or 400 µM L-proline (L-pro), 400 µM glycine (gly), 400 µM L-alanine (L-ala), 1 mM betaine (bet) or 400 µM D-proline (D-pro). The number of embryos analysed from 4 separate experiments is given in parenthesis. * $P < 0.05$ compared to no amino acids.
3.3 EFFECT OF CULTURE DENSITY ON THE L-PROLINE INDUCED IMPROVEMENT IN EMBRYO DEVELOPMENT IN 270 mOsm/kg MODHTF

Embryos cultured at low density have a poor rate of development compared with those cultured at high density (O’Neill 1997). At low density, embryotrophic factors are diluted in the high volume of culture medium; therefore, the effect of amino acids can be examined with minimal interference from these trophic factors. In order to investigate any interaction between the effect of L-proline and signalling mediated by embryotrophic factors, the effect of culture density on embryo development was investigated.

In order to confirm that high density culture improves development compared to low density, embryos were cultured in 270 mOsm/kg modHTF without amino acids in groups of 10–20 at a density of 1 embryo/µL and compared with embryos cultured individually at the low density (1 embryo / 100 µL). At high density, more embryos developed to the blastocyst stage, and these blastocysts had greater cell numbers, compared to those cultured at low density (Fig. 3.3).
Fig. 3.3. Effect of culture density on the development of mouse zygotes to the blastocyst stage. Embryos were cultured in 270 mOsm/kg modHTF and development was assessed for 5 days. (a) Percentage of embryos developed to blastocyst stage, (b) number of cells in blastocysts. Values in (b) are mean ± SEM. The number of embryos analysed from 9 separate experiments is given in the bar. * $P < 0.05$; *** $P < 0.001$ compared to no amino acids.
The effect of L-proline on embryo development at high density was examined. Embryos were cultured in 270 mOsm/kg modHTF containing 1 mM L-glutamine, 400 μM L-proline, 400 μM D-proline, 400 μM glycine, 400 μM L-alanine or 1 mM betaine and compared with development of embryos cultured in the absence of amino acids for 5 days at high density (1 embryo/μl). There was no significant difference in development to the blastocyst stage with the addition of L-glutamine, L-proline or D-proline whereas there was decreased development when glycine, L-alanine or betaine was added (Fig. 3.4a). Similarly, for cell numbers, there was no effect with L-glutamine, L-proline, D-proline or L-alanine but there was decrease in cell numbers in the presence of glycine or betaine (Fig. 3.4c). However, addition of L-glutamine and L-proline significantly increased the proportion of hatching blastocysts whereas glycine and betaine decreased blastocyst hatching (Fig. 3.4b).
Fig. 3.4 Effect of amino acids on mouse embryos cultured in 270 mOsm/kg modHTF for 5 days at high density. Zygotes were cultured to the blastocyst stage in modHTF supplemented with 400 µM L-proline (L-pro), 400 µM D-proline (D-pro), 1 mM L-glutamine (L-gln) or without amino acids (no a.a.). (a) Percentage of zygotes that developed to the blastocyst stage, (b) hatching blastocysts, and (c) cell number in blastocysts. Values in (c) are mean ± SEM. The number of embryos analysed from 9 separate experiments is given in parentheses. ** \( P < 0.01 \) and *** \( P < 0.001 \) compared to no amino acids.
3.4 THE EFFECT OF L-PROLINE ON BLASTOCYST DEVELOPMENT IN HYPO AND HYPER OSMOTIC CULTURE MEDIA

Since L-proline can act as an organic osmolyte in pre-implantation embryos (Anas et al., 2007), 400 µM L-proline was added to media at different osmolalities (251, 258, 270, 296, and 336 mOsm/kg).

3.4.1 Effect of L-proline and L-glutamine on blastocyst development in 251 mOsm/kg modHTF

The effects of L-proline were assessed in 251 mOsm/kg modHTF at low density. 251 mOsm/kg modHTF was prepared by reducing the concentration of lactate to 10 mM from its value of 21.4 mM in 270 mOsm/kg modHTF. In order to study whether addition of L-proline to 251 mOsm/kg modHTF had any effect on development, embryos were cultured for 5 days either without amino acids or with 1 mM L-glutamine or 400 µM L-proline. There was no significant difference in the development of blastocysts after culture in either L-glutamine or L-proline (Fig. 3.5a). There was, however, a significant increase in the proportion of hatching blastocysts after culture in L-glutamine, whereas L-proline had no effect on blastocyst hatching when compared with embryos cultured in the absence of amino acids (Fig. 3.5b). In addition, there was no significant difference in cell numbers in blastocysts on day 6 in any of the treatment groups (Fig. 3.5c).
Fig. 3.5 Effect of L-proline and L-glutamine on development of mouse embryos cultured in 251 mOsm/kg modHTF for 5 days at low density. Zygotes were cultured to the blastocyst stage in modHTF supplemented with 400 µM L-proline (L-pro), 1 mM L-glutamine (L-gln) or without amino acids (no a.a.). (a) Percentage of zygotes that developed to the blastocyst stage, (b) percentage of hatching blastocysts, and (c) cell number in blastocysts. Values in (c) are mean ± SEM. The number of embryos analysed from 9 separate experiments is given in parentheses. * $P < 0.05$ compared to no amino acids.
3.5 EFFECTS OF L-PROLINE AND L-GLUTAMINE ON THE DEVELOPMENT TO BLASTOCYST STAGE IN 258 mOsm/kg KSOM

Potassium simplex optimized medium (KSOM) has been identified by many investigators to enhance blastocyst development and increase blastocyst cell numbers (Baltz and Tartia, 2010; Biggers, 2005).

The effect of amino acids on the development of zygotes to blastocysts in 258 mOsm/kg KSOM was examined under low density culture conditions (1 embryo/100µL). There was no significant difference in blastocyst development when L-proline, D-proline, glycine, L-alanine or betaine was present whereas L-glutamine significantly increased blastocyst development when compared to medium containing no amino acids (Fig. 3.6a). Despite the augmentation of development when L-glutamine was present there was no effect on the proportion of hatching blastocysts. However, there was a decrease in the proportion of hatching blastocysts when betaine was added (Fig. 3.6b). There was also a significant decrease in cell numbers in blastocysts when L-proline, glycine or L-alanine was present, while no significant difference occurred with the other 3 amino acids when compared with no amino acids in culture medium (Fig. 3.6c). Addition of 400 µM L-proline or 1 mM L-glutamine to KSOM had no effect on embryo development when embryos were scored every 24 hours (Fig. 3.7).
Fig. 3.6 Effect of amino acids on mouse embryos cultured for 5 days in 258 mOsm/kg KSOM at low density. Zygotes were cultured to the blastocyst stage in KSOM containing either no amino acids (no a.a.), 1 mM L-glutamine (L-gln), 400 µM L-proline (L-pro), 400 µM D-proline (D-pro), 400 µM glycine (gly), 400 µM L-alanine (L-ala) or 1 mM betaine (bet) (a) Percentage of zygotes that developed to the blastocyst stage, (b) percentage of hatching blastocysts, and (c) cell number in blastocysts. Values in (c) are mean ± SEM. The number of embryos analysed from 7 separate experiments is given in parentheses. * $P < 0.05$ compared to no amino acids.
Fig. 3.7 Effect of 400 µM L-proline (L-pro) and 1 mM L-glutamine (L-gln) on development to 2-cell, 5–8 cell, morula and blastocyst (BL). Embryos were cultured in 258 mOsm/kg KSOM at low density from one-cell stage to the blastocyst stage and the development was assessed at 48, 72, 96 and 144 h post HCG. The number of embryos analysed from 4 separate experiments is given in parentheses.

The addition of 400 µM L-proline to 258 mOsm/kg KSOM had no effect on development at low density. However, in other studies was shown that low osmolality KSOM enhanced development comparing to CZB and M16 culture media (Baltz and Tartia, 2010). When the L-proline concentration in KSOM was increased to 1 mM and embryos cultured at low density there was still no significant difference in development (Fig. 3.8a) or hatching (Fig. 3.8b) compared to the absence of amino acids.
3.6 EFFECT OF L-PROLINE ON DEVELOPMENT IN 258 mOsm/kg KSOM FOR EMBRYOS CULTURED AT HIGH DENSITY

L-proline did not effect embryo development in modHTF at high density in iso osmotic conditions (270 mOsm/kg). Therefore, embryos were cultured in KSOM with and without amino acids in order to observe the effect of amino acids on development in hypo osmotic conditions at high density. 1 mM L-glutamine, 400μM L-proline, 400μM D-proline, 400μM glycine, 400μM L-alanine or 1 mM betaine was added to the culture medium and embryos were scored every 24 h post HCG for 5 days. There was no significant difference in blastocyst development with any of the added amino acids except glycine, which showed reduced development (Fig. 3.9a). However, there was a
significant decrease in the number of cells in blastocysts following culture in L-glutamine, glycine and betaine (Fig. 3.9c), and there was a significant decrease in blastocyst hatching when D-proline, glycine, L-alanine or betaine was added (Fig. 3.9b).
Fig. 3.9 Influence of amino acids on mouse embryos cultured for 5 days in 258 mOsm/kg KSOM at high density. One-cell embryos were cultured to the blastocyst stage in KSOM (no amino acids) supplemented with 1 mM L-glutamine (L-gln), 400 µM L-proline (L-pro), 400 µM D-proline (D-pro), 400 µM glycine (gly), 400 µM L-alanine (L-ala) or 1 mM betaine (bet). (a) Percentage of embryos developed to blastocyst stage, (b) percentage of hatching blastocysts, and (c) cell number in blastocysts. Values in (c) are mean ± SEM. The number of embryos analysed from 6 separate experiments is given in parentheses. * \( P < 0.05 \); ** \( P < 0.01 \) and *** \( P < 0.001 \) compared to no amino acids.
In pre-implantation development, the differentiation of inner cell mass (ICM) and trophectoderm (TE) cells by the blastocoele, identifies the first event of morphogenic and cellular differentiation. Proliferation and apoptosis are the processes that are involved in morphogenesis of organs (Abud, 2004). Cavitation can consist at the time of differentiation. However the mechanisms are not known in pre-implantation embryos as much as in embryonic stem (ES) cells. As ES cells are derived from pre-implantation embryos the mechanisms that are involved in differentiation and cell proliferation could be the analogous to the mechanisms in pre-implantation embryos. The intracellular signalling pathways that have a role in differentiation, cell survival and proliferation are mitogen activated protein kinase (MAPK=ERK1/2), Phosphoinositide 3-kinase (PI3K) and mammalian target of rapamycin (mTOR) signalling pathways respectively. In ES cells mTOR signalling pathway has been shown to be activated by amino acids such as L-proline (Washington et al., 2010). Therefore it is hypothesised that L-proline might have a role in differentiation and cell proliferation by activating ERK1/2 and mTOR signalling pathways in pre-implantation mouse embryos. AKT pathway was observed in order to study mTOR pathway because AKT phosphorylates mTOR by raptor complex.

Zygotes were cultured for 24 h individually at low density to the two-cell stage in 270 mOsm/kg modHTF culture medium with and without 400 µM L-proline. The results analysed by western blot and immunostaining are present below.
3.7.1 The effect of L-proline on ERK1/2 and AKT signalling pathways analysed by Western blot

The effect of L-proline on phosphorylation of AKT and ERK1/2 after culture of 1-cell embryos for 24 h in the presence or absence of L-proline was examined by Western blotting. α-tubulin was used as a loading control. There were no bands showing pERK activation by L-proline in two-cell embryos. α-tubulin and BSA, which is added in the culture medium, was detected. Even embryos were washed well in PBS / PVA after fixing, BSA could not be removed, and thus it has appeared. With the same blot, two-cell embryos were probed with 1 : 10,000 pAKT. The bands appeared, however there was no significant difference between the two treatments when the expression of pAKT was normalised to α-tubulin (Fig. 3.10).
Fig. 3.10 Western blot results. 1-cell embryos (24 h post HCG) were cultured for a further 24 h in 270 mOsm/kg modHTF containing either no amino acids (no a.a.) or 400 μM L-proline (L-pro). (a) Scan of the bands on the blot from two-cell embryos incubated in pERK and pAKT fluorescence detection (b) the plot of two treatments of pAKT expression. 

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3.7.2 The effect of L-proline on ERK1/2 and AKT signalling pathways analysed by confocal immunofluorescence microscopy

The effect of L-proline on intracellular signalling pathways in pre-implantation embryos was further investigated using immunostaining.

Zygotes were cultured in 270 mOsm/kg modHTF in the presence or absence of 400 μM L-proline for 24 h at low density. The resulting 2-cell embryos were fixed and stained with anti-pERK and anti-pAKT antibodies. Embryos were co-stained with DAPI to detect the nucleus. Culture of embryos in L-proline resulted in a significant increase in the nuclear localisation of both pERK and pAKT (Fig. 3.11).
Fig. 3.11 Effect of L-proline on localisation of pERK and pAKT. Zygotes were cultured for 24 h in 270 mOsm/kg modHTF without amino acids (no a.a.) or with the addition of 400 µM L-proline (L-pro) at low density. 2-cell embryos immunostained for (a) pERK and (b) pAKT. Blue indicates DAPI staining of the chromosomes. Percentage of blastomeres with nuclear staining for (c) pERK and (d) pAKT. The number of embryos analysed from 3 separate experiments is given in parentheses.

* $P < 0.05$; ** $P < 0.01$ compared to no amino acids
3.7.3 The effect of L-proline on apoptosis

In pre-implantation embryos, apoptosis occurs between the morula and blastocyst stages (Betts and King, 2001). The elimination of ICM cells in blastocysts is less when embryos are grown in amino-acid supplemented culture media *in vitro* (Devreker et al., 2001).

1-cell embryos were cultured to the blastocyst stage in 270 mOsm/kg modHTF with and without 400 µM L-proline for 5 days at low density. Blastocysts were fixed in 4 % PFA and mounted in Vectashield containing DAPI for fluorescence visualisation. Then, the number of apoptotic/fragmented nuclei in blastocysts was counted manually and the average number of dead cells calculated for each blastocyst. There was a significant decrease in the number of apoptotic cells in blastocysts cultured in L-proline (Fig. 3.12).
Fig. 3.12 Effect of L-proline on apoptosis in blastocysts. Zygotes were cultured to the blastocyst stage in 270 mOsm/kg modHTF for 5 days at low density. (a) Fluorescence images of blastocysts stained with DAPI (blue). The arrows indicate the fragmented DNA in apoptotic cells. (b) Average numbers of apoptotic cells per embryo. Values are mean ± SEM. The number of embryos analysed from 3 separate experiments is given in parentheses. * P < 0.05 compared to no amino acids.
3.8 THE EFFECTS OF L-PROLINE ON IN MEDIUM OF VARIOUS OSMOLALITIES

Even though the addition of 400 µM glycine, 400 µM L-alanine or 1 mM betaine had no effect at 270 mOsm/kg (Fig. 3.13). The presence of these amino acids enhanced the development by increasing NaCl from 85 mM to 102 mM. Also D-proline significantly improved blastocyst development at 336 mOsm/kg, however in lower osmolalities there was no significant difference compared to no amino acids (Fig. 3.13a). In contrast L-proline and glycine significantly decreased cell number in blastocysts in 258 mOsm/kg KSOM. In 296 mOsm/kg modHTF there was an increase in cell number when L-proline and L-alanine were added however in 336 mOsm/kg modHTF neither L-proline nor L-alanine had an effect on cell numbers. Betaine enhanced cell number in 336 mOsm/kg modHTF whereas in other osmotic conditions betaine had no effect on cell number in blastocysts (Fig. 3.13b).
Fig. 3.13. The role of L-proline as an organic osmolyte in mouse embryos in different osmolality modHTF at low density. Embryos were cultured for 5 days in low (258 ± 0.33 mOsm/kg KSOM), iso (270 ± 1.11 mOsm/kg modHTF) or high (296 ± 8.5 mOsm/kg , 336 ± 2.84 mOsm/kg modHTF) osmotic conditions containing either no amino acids, 400 µM L-proline, 400 µM D-proline, 400 µM glycine, 400 µM L-alanine or 1 mM betaine. a) Percentage of zygotes that developed to blastocyst stage. b) Number of cells in blastocysts cultured in different osmolalities. a- $P < 0.05$; b- $P < 0.001$ compared to no amino acids.
3.9 THE ROLE OF GLYCINE AND BETAINE TRANSPORTERS ON THE INFLUX OF L-PROLINE

In hyper osmotic conditions it is thought that L-proline is transported by a betaine transporter, because L-proline and betaine have been shown to share similar transport mechanisms (Anas et al., 2007). In previous studies it was hypothesized that proline and betaine could be the substrates of an osmolyte transporter in hyper osmotic conditions (Anas et al., 2007). Also, it was discovered that betaine was not transported by the glycine transporter and the osmolyte betaine transporter was not present in early embryo development (Anas et al., 2007). In the present study, the development of embryos in iso osmotic conditions at low density in medium containing L-proline in the presence of either 5 mM glycine or 5 mM betaine was examined in order to investigate which transporter L-proline will be transported by. Development to the blastocyst stage was significantly decreased when glycine was added into L-proline containing medium whereas there was no significant difference in the presence of betaine. There was no effect on the number of cells in blastocysts when betaine was added into L-proline-containing medium. Whereas addition of glycine significantly decreased cell numbers (Fig. 3.14).
Fig. 3.14 Competition of L-proline and glycine. Zygotes were cultured in 270 mOsm/kg modHTF for 5 days containing no amino acids (no a.a.), 400 µM L-proline (L-pro), 400 µM L-proline + 5 mM glycine (L-pro + gly) or 400 µM L-proline + 5 mM betaine (L-pro + bet) at low density. (a) Percentage of blastocyst development, and (b) number of cells per blastocyst. Values in (b) are mean ± SEM. The number of embryos analysed from 2 experiments is given in parentheses. * P < 0.05; ** P < 0.01; NS=Not Significant compared to L-proline.
CHAPTER FOUR

DISCUSSION
4.1 ROLE OF L-PROLINE IN PRE-IMPLANTATION DEVELOPMENT OF MOUSE EMBRYOS

Amino acids are known to play important roles in embryo development and are present in the mouse oviductal and uterine fluids *in vivo* (Leese et al., 2001; Menezo and Guerin, 1997). In addition, inclusion of amino acids in the culture medium improves development of pre-implantation embryos *in vitro* (Collins and Baltz 1999). There are a number of mechanisms by which amino acids can enhance development including their action as organic osmolytes and by regulating intracellular signalling pathways. Studies on embryonic stem (ES) cells have shown that L-proline activates the mammalian target of rapamycin (mTOR) intracellular signalling pathway thereby regulating proliferation and differentiation (Washington et al., 2010). As ES cells are derived from blastocysts they are thought to be good models of early embryo development. For this reason, the present study was carried out with the aim of investigating the role of L-proline in pre-implantation development.

4.2 L-PROLINE AND L-GLUTAMINE IMPROVE BLASTOCYST DEVELOPMENT

Embryos cultured in 270 mOsm/kg modified human tubal fluid (modHTF) medium with and without amino acids grew to the blastocyst stage at low density. Culturing zygotes in modHTF with the addition of L-proline or L-glutamine improved blastocyst development and the proportion of hatching blastocysts. However the resultant blastocysts had no difference in cell numbers. Embryo development was enhanced at 5–8 cell stage with the addition of L-proline or L-glutamine and this persisted until the
blastocyst stage. The presence of glycine, L-alanine or betaine had no effect on embryo development at the 5–8 cell, morula or blastocyst stages, suggesting that the effect on development is specific to only selected amino acids. There have been no previous studies that have examined the effect of L-proline alone on development under low density conditions. Previous studies, however, have shown that the addition of all 20 standard amino acids to either KSOM (Ho et al., 1995) or mMTF (Gardner and Lane 1993) improved blastocyst formation. When only non-essential amino acids (including L-proline) were added to modHTF an increase in blastocyst formation was still observed suggesting that L-proline may be responsible for the improvement seen in these previous studies.

Little is known about the mechanisms by which amino acids improve development, differentiation or cellular proliferation. Amino acids may either be metabolised or activate intracellular signalling pathways and thereby act in a trophic-factor like way (Fig. 4.1). Since autocrine trophic factors remain dilute during low density embryo culture (O’Neill 1997), addition of specific amino acids to the culture media may improve development by acting in a trophic-factor like way.
L-proline might be acting by a trophic factor-like mechanism, might have a role in metabolism and/or stimulating intracellular signalling pathways by acting as trophic factor-like way.

An interesting observation from the present study was that there was no increase in cell numbers despite improvement in blastocyst formation in the presence of L-proline or L-glutamine. The breakdown of L-glutamine increases ammonium concentration in the medium and this has been shown to be detrimental for the embryo in terms of cell numbers (Gardner and Lane, 1993; Lane et al., 2003). In the present study, embryos were cultured in the same medium containing L-glutamine for five days. Thus the breakdown of L-glutamine over this period may have resulted in an increase in ammonium that may have prevented an increase in cell numbers. L-proline is metabolised in mitochondria and there is no ammonium build-up due to its breakdown (Casalino et al., 2011; Mitsubuchi et al., 2008).

The addition of L-proline and L-glutamine to modHTF increased hatching of blastocysts even though there was no increase in cell numbers in the blastocysts. Before hatching occurs, the zona pellucida (ZP) thins because of the pressure of the expanding blastocyst and the elasticity of the zona glycoproteins (Gardner, 2001). Zona lysins
secreted by the embryo also thin the ZP (Gardner 2001). Hatching might be regulated by dynamic cellular components such as actin-based trophectodermal projections (TEP), which make the first contact with the uterine epithelium so that the blastocyst can attach and implant (Gonzales et al., 1996). These TEPs may have a role in the delivery to the blastocyst of hatching regulators, such as autocrine and paracrine growth factors (i.e. LIF, TGF-α, HB-EGF, INF-τ) or cytokines, which then regulate secretion of proteases (Gardner, 2001; Seshagiri et al., 2009). As mentioned previously, L-proline and L-glutamine may be acting in a trophic factor-like way thereby stimulating the same pathways as those activated during hatching, causing secretion of proteases and an increase in hatching. Alternatively, L-glutamine and L-proline might be accelerating blastocyst hatching simply because the rate of development is increased.

Addition of D-proline to 270 mOsm/kg modHTF had no effect on embryo development. Although experiments using high osmolality medium showed that D-proline is transported into pre-implantation embryos (see later for discussion). There are no studies that have shown D-proline activating mTOR signalling pathway therefore D-proline does not activate trophic factor pathways that trophic factors stimulate.

4.3 THE EFFECT OF L-PROLINE ON DEVELOPMENT IS DEPENDENT ON CULTURE DENSITY

Blastocyst development and the numbers of cells in the blastocysts were increased in embryos cultured in 270 mOsm/kg modHTF without amino acids at high density (1embryo/μl) when compared to development of embryos cultured at low density (1embryo/100μl). This result confirms observations from other studies which show
improvement in blastocyst development by culture of embryos in groups or at high density (Bagis and Odaman Mercan, 2004; Canseco et al., 1992; Kato and Tsunoda, 1994; Lane and Gardner, 1992; O'Neill, 1997; Paria and Dey, 1990) due to the action of autocrine trophic factors (O'Neill, 2008). The presence of neither L-proline nor L-glutamine improved blastocyst development further at high density, despite the improvement seen at low density. This suggests that amino acids are acting in a similar way to embryo trophic factors (Fig. 4.2). The addition of non-essential and essential amino acids to medium M16 (270 mOsm/kg) has been shown to increase blastocyst development at high density (Lamb and Leese, 1994). However, no previous studies have examined the effect of L-proline or L-glutamine alone in 270 mOsm/kg modHTF, or equivalent iso osmotic conditions, at high density.
Fig. 4.2 High density versus low density with the presence of L-proline. In high density (1 embryo/µl) culture conditions (a), embryos are cultured in groups and secreted embryo trophic factors (eg. PAF) can reach mechanistically relevant concentrations. Thus, trophic factors might be improving embryo development bypassing the need for L-proline or L-glutamine. However, at low density (b) (1 embryo/100µl) trophic factors are diluted and development is poor. (c) Addition of L-proline or L-glutamine to the culture medium improves blastocyst development to a similar level to that seen in high density culture.

Despite the lack of improvement in blastocyst development there was an increase in blastocyst hatching with the addition of L-proline or L-glutamine. If the amino acids are acting in a trophic factor-like way and thereby mediating the effect on hatching this action is independent of the growth factors present at high density. This suggests that these amino acids may be stimulating pathways normally activated by maternally
derived growth factors (e.g. leukaemia inhibitory factor (LIF)) which are absent during *in vitro* culture. In ES cells, L-proline stimulates directed differentiation and is involved in the activation of a number of signalling pathways already stimulated by LIF (Washington et al., 2010). One of the roles of LIF in early embryonic development is to regulate protease activity required for hatching of the mouse blastocyst (Harvey et al., 1995).

**4.4 L-PROLINE DOES NOT IMPROVE DEVELOPMENT IN HYPO OSMOTIC CONDITIONS**

In the present study, the osmolality of modHTF was lowered to 251 mOsm/kg by reducing lactic acid (from 21.4 to 10 mmol/L) in order to observe the effect of L-proline in hypo osmotic conditions at low density. Under these conditions, 1-cell embryos developed poorly to the blastocyst stage. However, previous studies have shown improved blastocyst development in KSOM which has the same osmolality (Lawitts and Biggers, 1993; Perin et al., 2008). Therefore, we also cultured zygotes in 258 mOsm/kg KSOM rather than low osmolality modHTF.

The addition of either 400 µM or 1 mM L-proline to 258 mOsm/kg KSOM did not improve blastocyst development at low density. In contrast, in previous studies, addition of all 20 amino acids to KSOM supported high rates of blastocyst development (Biggers et al., 2000; Wiemer et al., 2002). It has been shown that zygotes initially swell when placed in 250 mOsm/kg KSOM (Collins and Baltz 1999). The swelling activates the Volume-Sensitive Organic Osmolyte-Anion Channel (VSOAC) (Baltz 2001) and organic osmolytes, including L-proline, efflux from the cell so that the cell
shrinks back to its normal size (Fig. 4.3). Thus, even though L-proline is transported into the embryo some of it might be effluxed and the remaining amount of L-proline inside the cell might not be sufficient for it to exert the effect on development as seen under iso osmotic conditions.
Fig. 4.3 The effect of L-proline in blastocyst hatching at hypo osmotic conditions. Even though L-proline (shown as yellow pentagon) is transported in under hypo osmotic conditions (≤258 mOsm/kg) the cell swells because of the hypo osmoticity. However, swelling activates the Volume Sensitive Organic-Osmolyte Anionic Channel (VSOAC) resulting in efflux of organic osmolytes such as L-proline, allowing the cell shrink to its normal size.

The addition of L-glutamine to 258 mOsm/kg KSOM improved blastocyst development, as observed previously by others (Biggers, 1998; Lawitts and Biggers, 1993). L-glutamine efflux via VSOAC in embryos has not been studied, although in other cells there is evidence that it is not conducted by VSOAC (Basarsky et al., 1999; Pasantes-Morales et al., 1994; Rutledge et al., 1998). Thus, L-glutamine is transported
into the embryo under low osmolality conditions, and since there may be no VSOAC-mediated efflux it may be able to reach sufficient concentration within the cell to exert an effect on development.

### 4.5 THE EFFECT OF L-PROLINE AT HIGH DENSITY IS NOT IMPROVED BY CULTURE IN KSOM MEDIUM

In the present study, the effect of L-proline, L-glutamine and D-proline was also examined in hypo osmotic conditions at high density. Amino acids had no effect on blastocyst development in KSOM. No studies have been reported on the effect on blastocyst development of L-proline added alone at high density. Previous studies have also shown that addition of L-glutamine alone had no effect on blastocyst development (Devreker and Hardy, 1997) however addition of all 20 amino acids (Biggers and Racowsky, 2002; Perin et al., 2008) to KSOM increased zygote development to the blastocyst stage. Therefore in hypo osmotic and high density *in vitro* culture L-proline alone is not sufficient and may only improve development in the presence of L-glutamine when KSOM medium is used.

Blastocyst cell numbers were also examined after culture in 258 mOsm/kg KSOM at high density and there was no effect after the addition of either L-proline or D-proline, whereas addition of L-glutamine decreased blastocyst cell numbers. As mentioned before, the metabolism of L-glutamine increases ammonium production (Lane and Gardner 1993). Therefore, it is possible that the increase in ammonium in the culture medium reduced cell proliferation. Evidence to confirm this could be obtained by transferring embryos to new medium after 48 h (Lane and Gardner 2003) or by
replacing L-glutamine with glycine-glutamine (Summers et al., 2005). In contrast at low density there is no decrease in blastocyst cell numbers because embryos are not adequate to metabolise L-glutamine.

The presence of L-proline and L-glutamine did not have any effect on hatching blastocysts, whereas D-proline reduced the proportion of blastocysts hatching. The decrease may be due to the efflux of D-proline and also L-proline. Since L-proline is secreted by the embryo it is already accumulated and since it is a regulator of cell volume it might be effluxed via VSOAC. Thus deprivation of L-proline and D-proline might inhibit cells develop so that even zygotes reach the blastocyst stage they might not be able to hatch (Fig. 4.4).
Fig. 4.4 The effect of D-proline in blastocyst hatching in hypo osmotic conditions. In hypo osmotic conditions VSOACs might be activated in order to regulate cell volume. D-proline (shown as pink triangles) is transported into the cells of the embryo, which would cause further cell swelling. Since VSOACs are active L-proline (shown as yellow pentagons) as well as D-proline and other organic osmolytes may efflux through VSOAC. The loss of these amino acids/osmolytes returns the cell volume to normal, so that blastocyst development can still occur, but reduces the ability of the blastocyst to hatch.

4.6 THE EFFECTS OF L-PROLINE ON INTRACELLULAR SIGNALLING PATHWAYS

Cell growth and cell proliferation are controlled by three main intracellular signalling pathways, namely the mammalian target of rapamycin (mTOR), mitogen activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) signalling pathways (Fingar and Blenis, 2004). Amino acids and trophic factors can stimulate these signalling pathways in multicellular organisms. It has been shown in embryonic stem (ES) cells that L-proline activates mTOR signalling pathways (Tan et al., 2011;
Washington et al., 2010). Since ES cells are derived from blastocysts there is a possibility that mTOR related signalling pathways could be activated by L-proline in early embryo development.

In the present study, to assess whether there is an effect of L-proline on the signalling pathways mentioned above, Western blot and confocal immunostaining techniques were used. However there were no detection of pERK and pAKT with 126 embryos cultured negative control and 111 embryos cultured in L-proline. The activity of these kinases in 2-cell embryos was then examined by immunostaining. Treatment of embryos for 24 h with L-proline at 270 mOsm/kg modHTF at low density caused an increase in the nuclear staining of pERK1/2 and pAKT in 2-cell embryos thus observation supports L-proline as having a trophic factor-like action. Even though there was no significant improvement in development to the 2-cell stage, the increase in nuclear localisation of pERK1/2 and pAKT suggests that these pathways might be cooperating with mTOR or some other signalling pathways that are not expressed until the 5–8 cell stage when the improvement in development is seen (Fig. 4.5). It has been shown in human cervical carcinoma HeLa cells that ERK1/2, PI3K/AKT/mTOR signalling pathways converge to phosphorylate the eukaryotic translation initiation factor 4B (eIF4B) for mRNA translation (Shahbazian et al., 2006). Furthermore, in mouse oocytes it has been shown that pAKT is required for resumption of meiosis. AKT phosphorylation via the PI3K signalling pathway is important for oocyte maturation and both its phosphorylated residues (Thr308 and Ser473) are associated with spindle function (Hoshino and Sato, 2008; Hoshino et al., 2004; Kalous et al., 2006).
Fig. 4.5 L-proline might be acting as trophic factor-like way thereby stimulating same pathways of trophic factors.

In blastocysts, the number of apoptotic cells decreased with the addition of L-proline to 270 mOsm/kg modHTF. Apoptosis was reduced in both trophectoderm cells (TE) and
inner cell mass (ICM) by L-proline. This supports a role for AKT in the signalling pathway activated by L-proline. No previous studies have examined the effect of L-proline alone on apoptosis during pre-implantation development. However, adding all amino acids reduces apoptosis in embryos cultured in KSOM at low density (Brison and Schultz, 1997). Despite the decrease in apoptotic cells seen in blastocysts we observed no significant increase in total blastocyst cell number following culture in L-proline, probably because of the small number of apoptotic cells.

4.7 L-PROLINE IMPROVES DEVELOPMENT IN THE ABSENCE OF ANY ACTION AS AN ORGANIC OSMOLYTE

As mentioned in previous sections, the addition of L-proline to 270 mOsm/kg modHTF increased blastocyst development whereas the other organic osmolytes tested namely glycine, betaine and L-alanine had no effect on development. This suggests that L-proline was not acting as an organic osmolyte but was acting in a trophic factor-like way. Glycine, L-alanine and betaine also have roles in metabolism but these have not been examined in embryonic development. Betaine is involved in methylation reactions and improves digestibility, growth performance and feed conversion in poultry (Metzler-Zebeli et al., 2009). Additionally, betaine is utilised to maintain liver, heart and kidney health in humans (Craig, 2004). Glycine has roles in the nervous system, control of gluconeogenesis and is used in the synthesis of haemoglobin, glutathione, DNA and RNA (Springboard, 2004). L-alanine derived from protein breakdown is converted to pyruvate and can then be utilised to synthesise glucose via gluconeogenesis in the liver (Lehninger, 2005).
In hyper osmotic conditions, the presence of L-proline, glycine, betaine, L-alanine or D-proline increased blastocyst development. This is consistent with previous studies showing that glycine, betaine, alanine and proline act as organic osmolytes to regulate cell volume in early embryonic development (Anbari and Schultz, 1993; Baltz and Tartia, 2010; Van Winkle et al., 1990a). It has been shown that ≥300 mOsm/kg represents hyper osmotic condition for mouse embryo culture in vitro (Collins and Baltz 1999). The osmolality of oviductal fluid is measured at 360 mOsm/kg (Van Winkle, Haghghat et al. 1990) conditions in which embryos do not survive in vitro unless osmolytes are added to the culture medium (Baltz and Tartia 2010). Similarly, in vivo, embryos survive the hyper osmotic conditions because the oviductal fluid is rich in high concentrations of osmolytes such as glycine (Dawson and Baltz 1997), alanine, glutamine, proline, taurine, hypotaurine and β-alanine (Guerin and Menezo, 1995; Guerin et al., 1995).

In hyper osmotic conditions, we observed that L-proline and L-alanine increased cell numbers in 296 mOsm/kg culture conditions and betaine increased cell numbers in 336 mOsm/kg culture conditions. In chick embryo fibroblasts, betaine and proline improve cell proliferation in hyper osmotic conditions(Petronini et al., 1992). That previous study has not mentioned about the hyper osmotic conditions therefore the addition of betaine to the culture medium may be improving proliferation ≥300 mOsm/kg whereas L-proline may be effective ≥295 mOsm/kg in mouse pre-implantation embryos.

4.8 TRANSPORT SYSTEMS IN PRE-IMPLANTATION EMBRYOS

Amino-acid transporters previously identified in pre-implantation embryo development are categorised either as symport or antiport transporters and belong to the solute carrier
gene family (SLCs). There are several Na\(^+\)-dependent and independent amino-acid transport systems which play a role in early embryo development including System β, System A, IMINO, Gly, B\(^{0,+}\), and Imino (Hyde et al., 2003; Van Winkle, 2007) (Table 4.1).

Table 4.1. Amino acid transport systems in pre-implantation mouse embryos (Modified from Hyde et al., 2003 and Van Winkle, 2007)

<table>
<thead>
<tr>
<th>Embryo transporter system</th>
<th>Protein</th>
<th>Amino acid substrates</th>
<th>Gene</th>
<th>Time of Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>β</td>
<td>TAUT</td>
<td>Taurine</td>
<td>SLC6A6</td>
<td>All stages</td>
</tr>
<tr>
<td></td>
<td>SAT1</td>
<td>Glutamine, Glycine, L-proline</td>
<td>SLC38A1</td>
<td>4-8 cell stage</td>
</tr>
<tr>
<td></td>
<td>SAT2</td>
<td></td>
<td>SLC38A2</td>
<td>Blastocyst inner cell mass</td>
</tr>
<tr>
<td></td>
<td>SAT3</td>
<td></td>
<td>SLC38A4</td>
<td></td>
</tr>
<tr>
<td>IMINO</td>
<td>SIT1</td>
<td>L-proline, Betaine</td>
<td>SLC6A20</td>
<td>1-2 cell stage</td>
</tr>
<tr>
<td>B(^{0,+})</td>
<td>B(^{0})AT</td>
<td>Leucine, Arginine, Glycine</td>
<td>SLC6A14</td>
<td>Blastocyst stage</td>
</tr>
<tr>
<td>Gly</td>
<td>GLYT1</td>
<td>Glycine, L-proline</td>
<td>SLC6A9</td>
<td>1 through 8-cell stage</td>
</tr>
<tr>
<td>Imino</td>
<td>PAT1</td>
<td>L,D-Proline, Glycine</td>
<td>SLC36A1</td>
<td>Not studied in embryos</td>
</tr>
</tbody>
</table>

Amino-acid competition experiments were performed to identify the L-proline transporter. Under iso osmotic conditions, glycine prevented L-proline-mediated improvement in development whereas betaine had no effect. This suggests that glycine and L-proline might be transported via the same transporter. Other studies have shown
that in 1-2 cell embryos, L-proline and betaine are substrates of a Na\(^+\)-dependent transporter called SIT1 (Anas et al., 2007). It is known that betaine and glycine are not transported by the same transporter in embryos (Dawson and Baltz, 1997; Hammer and Baltz, 2002; Steeves and Baltz, 2005; Steeves et al., 2003). Since there is competition between L-proline and glycine, the uptake of L-proline might be via GLYT1 or SAT1/2/3, which are all known to transport both L-proline and glycine. In ES cells, L-proline is transported by SAT2 (Tan et al., 2011) and its ability to stimulate ES-cell differentiation can be inhibited by millimolar concentrations of Gly (Washington et al., 2010), presumably by preventing cellular uptake via this transporter.

D-proline also acts as an organic osmolyte in hyper osmotic conditions suggesting that possibly due to the influx of D-proline. Other studies have shown the uptake of D-proline is via PAT1 and PROT in epithelial and brain cells respectively (Foltz et al., 2004; Metzner et al., 2006).

### 4.9 CONCLUSIONS AND FUTURE DIRECTIONS

The present study has demonstrated that L-proline and L-glutamine increases blastocyst development possibly due to acting as the trophic factor-like way in iso osmotic conditions of modHTF medium at low density. There was no effect of L-proline or other amino acids added into 270 mOsm/kg modHTF at high density. Decreasing the osmolality of culture medium did not enhance the effect of L-proline in 258 mOsm/kg KSOM at low or high density possibly due to the efflux of L-proline via VSOAC. L-proline enhanced blastocyst development in hyper osmotic (296 and 336 mOsm/kg) modHTF at low density consistent with L-proline acting as an organic osmolyte in
hyper osmotic conditions. However L-proline may be stimulating a number of signalling pathways other than acting as an osmolyte. Despite the fact that other studies have shown that proline and betaine are transported via same transporter in one-two cell embryos they were not in a competition for the same transporter in blastocysts. However a competition between glycine and L-proline as a substrate to enhance blastocyst development and cell numbers was observed. L-proline could also activate signalling pathways in order to regulate differentiation. There is decrease in total dead cells with the addition of L-poline suggesting that L-proline might be inhibiting apoptosis. Additionally phosphorylation of ERK and AKT was increased by addition of L-proline to 270 mOsm/kg modHTF suggesting that these signalling pathways might be stimulated by L-proline.

Overall another way to observe the improvement of L-proline may be to culture zygotes that are in vitro fertilised. Also the activation of L-proline on differentiation and proliferation needs to be assessed with downstream pathways of ERK 1/2 and AKT signalling pathways. L-glutamine could be replaced with glycineglutamine to prevent the detrimental effect of ammonium build-up and increase the cell numbers in blastocysts cultured in low osmolality conditions.
CHAPTER FIVE

REFERENCES


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