

**Proline Transporters in the
Mouse Oocyte and Embryo
and Ethics of Embryo Modification**

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Statement of Authentication

This thesis is submitted to The University of Sydney in fulfilment of the requirements for the degree of Doctor of Philosophy in Medicine.

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. All assistance is acknowledged in the appropriate location within the text.

I, hereby declare that I have not submitted this material, either in full or in part, for a degree at this or any other institution.

Thesis attribution statement

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I co-designed the research experiments and prepared the manuscript with co-authors. Ph.D. student Madeleine Hardy, honours student Michelle Guttman-Jones and I performed the experiments. Madeleine Hardy and I analysed the data and prepared the figures. All authors read and agreed to published version of the manuscript.

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Christine Critchley and Diane Nicol conceived the study. Gordana Bruce and Jarrod Walshe contributed to the design of the survey and collected data. Co-authors and myself made a substantial contribution toward the development of the final manuscript approved for publication.

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List of commonly used abbreviations

AA	Amino acid
AAT	Amino acid transporter
Ala	Alanine
ART	Assisted reproductive technology
ATP	Adenosine triphosphate
Bet	Betaine
cAMP	Cyclic adenosine monophosphate
COC	Cumulus oocyte complex
CRISPR-Cas9	Clustered regularly interspaced short palindromic repeats -associated protein-9 nuclease
Cys	Cysteine
EAA	Essential amino acid
ESC	Embryonic stem cells
ERK	Extracellular-regulated kinase
FSH	Follicle stimulating hormone
GJC	Gap junction communication
GV	Germinal vesicle
Gln	Glutamine
Gly-Gln	Glycyl-L-glutamine
Gly	Glycine
HCG	Human chorionic gonadotrophin
His	Histidine
mTOR	Mammalian target of rapamycin
NEAA	Non-essential amino acids
IGF	Insulin-like growth factor
ICM	Inner cell mass
IVF	In vitro fertilisation
ICSI	Intra cytoplasmic sperm injection
LH	Luteinising hormone
MeAIB	(Methylamino)isobutyric acid
MI	Metaphase one
MII	Metaphase two
KO	Knockout

Orn	Ornithine
Pro	L-Proline
PA	L-pipecolic-acid
PRODH	Proline oxidase
P5C	Pyrroline-5-carboxylic acid
ROS	Reactive Oxygen Species
Sar	Sarcosine
SLC	Solute linked carrier
VOC	Volatile organic compound

Abstract

Uptake of amino acids (AA) into the oocyte and embryo occurs via specific AA transporters (AAT) located on the plasma membrane of the cell. Expression of AAT rely not only on genetic regulation but also the accumulation of intracellular AA, either by synthesis or by uptake from the reproductive fluid bathing the embryo. Embryos cultured *in vitro* therefore critically rely on the composition of the embryo culture media, including the AA component, for optimal embryo development. The amino acid L-proline (Pro) acts in a growth-factor-like manner to stimulate preimplantation embryo development and has the ability to scavenge intracellular reactive oxygen species (ROS). It is hypothesised that Pro is taken up into the oocyte and embryo via AAT to support normal embryonic development via Pro-mediated mechanisms.

The present study aimed to identify transporters of Pro in the oocyte and preimplantation embryo. To investigate the effect of Pro on preimplantation development *in vitro* fertilisation (IVF) of oocytes was performed in the presence (and absence) of individual AA. Embryos fertilised in medium containing Pro and/or its analogue pipecolic acid (PA) had increased blastocyst formation and inner cell mass cell numbers compared to embryos fertilised in medium containing no AA, betaine, glycine or histidine. Expression of Pro transporters in the oocyte was identified by measuring the rate of uptake of radiolabelled Pro in the presence of a molar excess of unlabelled AAs. Three transporters novel to the oocyte were identified, one that was sodium-dependent, PROT (SLC6A7), and two others that were sodium-independent, PAT1 (SLC36A1) and PAT2 (SLC36A2). Immunofluorescent staining showed localisation of PROT in intracellular vesicles and limited expression on the plasma membrane, while PAT1 and PAT2 were both expressed on the plasma membrane. The presence of Pro and/or PA during

IVF also significantly reduced mitochondrial activity and ROS levels in oocytes suggesting that these AAs act as a scavenger of ROS in the oocyte.

The next aim of this thesis was to determine the specific stages of preimplantation embryo development when Pro uptake is required, and if transporters of Pro are expressed at this stage. Addition of Pro to embryo culture medium from the zygote stage improved preimplantation embryo development *in vitro*. In wild-type embryos Pro increased development to the cavitation stage (day 4). In B⁰AT1 knockout embryos (*Slc6a19*^{-/-}) Pro induced improvement at the 4-8 cell stage (day 3) and blastocyst stage (day 6) but not at cavitation (day 4), suggesting B⁰AT1 is the main contributor to Pro uptake on day 4 of development. Immunofluorescence staining revealed B⁰AT1 and its accessory protein ACE2 to be present in the preimplantation embryo and in the cumulus cells of cumulus-oocyte complexes. In oocytes, and at the 4-8 cell stage in *Slc6a19*^{-/-} mice, unlabelled Pro failed to prevent radiolabelled Pro uptake suggesting B⁰AT1 is normally expressed at these stages in wild-type mice. Compensation for the loss of an individual Pro transporter by upregulation of another transporter of Pro indicates the critical role of Pro in normal embryo development. These data for the first time show that Pro transporters are present in the oocyte and at specific stages of preimplantation development and findings from this study highlight the essential role that Pro has in supporting preimplantation development *in vitro*.

Parthenogenetically activated oocytes are a source of pluripotent stem cells that have therapeutic purposes in regenerative medicine. Human oocytes that were discarded as a product of IVF were matured *in vitro* and activated by chemical means. Activated human oocytes did not develop to the blastocyst stage in the commercial media used in this study. Parthenogenetic activated mouse oocytes cultured in a simplified medium containing Pro showed increased development to the blastocyst stage compared to parthenotes cultured in medium with no AAs

or groups of AA. This study demonstrates a simplified medium can be beneficial to maintain development of parthenotes to blastocyst stage.

Intentionally applying technology to embryos to cause developmental and heritable changes raises ethical considerations. The law in Australia allows genetic modification of animals for research purposes but prohibits making heritable changes in human embryos regardless of whether the treated embryo is discarded thereafter. An aim of this study is to determine the opinion of at least a segment of the Australian public on the topic of embryo modification. Present legislation does not allow researchers to apply germline editing technology to human embryos for research purposes however initial reports indicate that the public would support this. To begin to understand public attitude in Australia about this matter a questionnaire was given to an audience attending a Q and A panel event during Sydney Science Week. The majority of respondents were supportive in allowing heritable changes in human embryos for clinical and research purposes. This is the first study of its kind in Australia to collect public opinion on gene editing of the human embryo.

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Chapter 1

Introduction

1.1 Oocyte and preimplantation embryo development

Each species of living organism has a unique embryonic development process that transforms a single cell into a complex multicellular organism beginning with the fertilised egg. The processes of cell growth and differentiation that give rise to live offspring comes from both the genetic material inside the oocyte and from the external environment in which the embryo develops. The complexity of factors needed for development from oocyte to blastocyst are still unknown, however the transport of amino acids (AA) is a critically defining factor.

The intrinsic quality of the oocyte is a major determinant of female fertility and subsequent embryo development potential (Hendriksen et al., 2000). Nuclear, cytoplasmic and epigenetic maturation are primary physiological processes that contribute to oocyte developmental competence (Picton et al., 1998, He et al., 2021). Nuclear maturation occurs with a surge of luteinising hormone (LH) which triggers the breakdown of the germinal vesicle (GV) membrane so that chromosomes can line up on the spindle near the cell membrane for the first meiotic division to occur (Van Winkle, 1988), (Picton et al., 1998). Epigenetic alterations on maternal genes, which have been acquired by genetic and environmental influence, are removed at this stage of development via chromatin remodelling (Reik et al., 2001, Schultz, 2005). Epigenetic and cytoplasmic maturation involves the processes of de novo DNA methylation and reassembly of cell cycle machinery respectively which are necessary for the oocyte to progress from metaphase I (MI) to metaphase II (MII) (Reik et al., 2001, He et al., 2021). The mature MII oocyte within the preovulatory follicle is surrounded by cumulus cells which form the cumulus oocyte complex (COC) (Diaz et al., 2007). Cumulus cells play a central role in transporting AA into the oocyte via gap junction communication (GJC) (Appeltant et al., 2015). The AAs glycine (Gly), alanine (Ala), L-proline (Pro) and histidine (His), as well as pyruvate, are taken up at a high rate by mouse oocytes within the COC, to

support oocyte maturation (Eppig, 1982, Colonna et al., 1983, Haghghat et al., 1990). Shortly after ovulation the connection between the COC begins to break down and AA can no longer be transported to the oocyte via this mechanism but directly via transporters expressed on the plasma membrane of the oocyte (Eppig, 1982, Van Winkle, 1988, Su et al., 2009). Movement of ions (i.e. K^+ , Cl^- , Ca^{2+}) is required for oocyte activation and fertilisation, and this occurs via AA transporters (AAT), or through hydrophilic pores in the plasma membrane such as Ca^{2+} channels, Cl^- channels or K^+ channels (Van Winkle, 1988, Kline et al., 1994, Day et al., 2000). Cytoplasmic maturation is completed by a series of intracellular Ca^{2+} oscillations that are initiated at the site of sperm-egg fusion (or via parthenogenetic activation) and continue for several hours following sperm entry (Day et al., 2000, Jones, 2005, Nomikos et al., 2013). The increase in Ca^{2+} required to maintain these oscillations, is due to intracellular Ca^{2+} release, and Ca^{2+} influx through voltage-gated channels (Day et al., 2000). Expression of calcium activated chloride channels, which conduct Cl^- , lactate and pyruvate, induce changes in the membrane potential which is critical for fertilisation success (Dalati, 2016). AAs also act as electron carriers, and so the ionic environment is maintained by the expression of transporters and ion channels on the oocyte plasma membrane. Fertilisation is complete when sperm and oocyte haploid chromosomes join to form a diploid zygote, indicated by extrusion of a second polar body and the presence of two pronuclei.

Preimplantation embryo development involves a complex series of cellular and genetic interactions driven by both intracellular and extracellular factors all within a critical time-sensitive period. After fertilisation the zygote encased within the zona pellucida progresses through a series of cytoplasmic cleavages, surrounded by oviductal fluid as it moves towards the uterine cavity (see Fig. 1.1). In addition to notable morphological events, the early embryo (i.e. 2-cell stage in mice, 4- to 8-cell stage in humans) undergoes extensive embryonic

reprogramming and ceases genetic instruction from maternal mRNA and switches to instructions from the embryonic genome (embryonic mRNA) (Braude et al., 1979). At this stage the embryo has limited capacity to maintain normal cellular function if stress is applied and is particularly vulnerable to epigenetic defects during reprogramming (Lane, 2001, El Hajj et al., 2013). There is also a significant increase in metabolic and energy requirements as the embryo develops from zygote to blastocyst stage. During early development, from fertilisation to the 4-cell stage, the mouse embryo primarily depends on pyruvate as an energy source (Biggers et al., 1967). As the embryo rapidly divides from the 4- to 8-cell stage the energy requirements increase and changes from pyruvate and lactate metabolism to that of glucose via glycolysis (Brinster et al., 1966, Gardner et al., 1988). Mouse embryo culture studies have demonstrated that the uptake of non-essential AAs (NEAA) and glutamine (Gln) is critical to support development during early cleavage stages (Gardner et al., 1993, Lane et al., 1997). After the 8-cell stage, transport of essential AA (EAA) benefits development (see Fig. 1.1) (Gardner et al., 1997, Lane et al., 1997). Blastomeres compact and cavitation separates the blastomeres into two cell lineages, the pluripotent inner cell mass (ICM) and a single layer of outer trophoblast cells of the blastocyst. Glucose transporters and AA transport proteins inserted into the plasma membrane facilitate the uptake of nutrients from the extracellular fluid in response to the increase in nutritional consumption by blastocysts (Gardner et al., 1988, Van Winkle, 1988, Lane et al., 1997). The final stage of preimplantation development is marked by the blastocyst hatching from the zona pellucida and implanting into the uterine epithelium. The uterine fluid contains nutritional components needed for blastocyst implantation to occur.

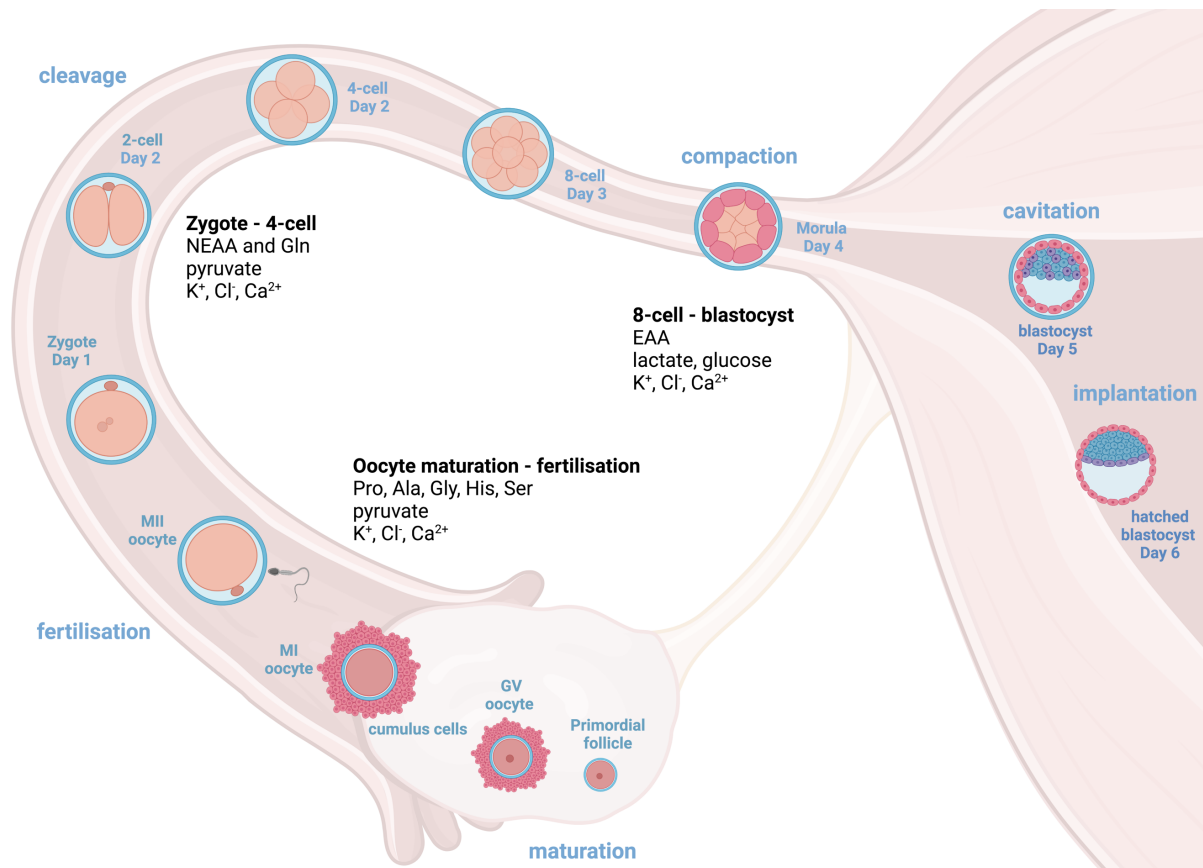


Figure 1.1 Transport of nutrition and ions during preimplantation development. Germinal vesicle (GV) and metaphase I (MI) oocytes surrounded by cumulus cells take up nutrients from follicular fluid for maturation, and metaphase II (MII) oocytes from oviductal fluid to support fertilisation. Non-essential amino acids (NEAA), Gln and pyruvate are the primary nutrients needed to support development from fertilisation to the 4-cell stage. Glucose and essential amino acids (EAA) benefit development from the 8-cell to blastocyst stage. Expression of ion transporters and channels throughout development function to regulate membrane potential and signalling pathways. Images created using BioRender software.

1.1.1 Suboptimal technology - a simplified solution to optimise medium

When removed from the in vivo environment of the ovary, oocyte competency and subsequent embryo viability is immediately effected by multiple environmental factors including temperature, humidity, exposure to light, volatile organic compounds (VOCs) and composition of culture medium (Hall et al., 1998, Summers et al., 2003, Sun et al., 2004, Lv et al., 2019,

Consensus Group 2020). In Australia, approximately 23% of patients can expect a clinical pregnancy following IVF treatment, and only 18.6% will result in a live birth (ANZARD Annual Report, 2020). Low success rates in ART could be attributed to one, or a combination of unfavourable culture conditions together with patient status such as age-related abnormalities in oocytes (Santonocito et al., 2013). The fertilisation medium and embryo culture medium is a critical factor in ART to support fertilisation and maintain embryo development outside of the reproductive tract. The conventional monoculture originally developed by Biggers and associates (Lawitts et al., 1991, Erbach et al., 1994) uses one formulation containing nutrition, buffers and energy components needed for the embryo to develop *in vitro* from fertilisation to blastocyst stage. Essentially allowing the embryo to choose what to take up from the medium. Conversely, sequential culture media have been designed to mimic the *in vivo* conditions of the reproductive tract and uterine cavity reflecting the changing nutritional requirements of the embryo as it develops (Gardner et al., 1997, Gardner, 1998, Gardner et al., 1998, Gardner et al., 2003, Lane et al., 2007, Biggers et al., 2008).

However, the culture medium used in ART remains suboptimal and likely affects embryo development and viability. For example, embryo culture causes increased metabolic stress, alterations to gene expression and increases the incidence of epigenetic disorders in offspring such as Beckwith-Wiedemann and Angelman syndrome (Leese et al., 1998, Summers et al., 2003, Summers et al., 2005, Fernandez-Gonzalez et al., 2007). There is also increasing evidence that culture medium causes epigenetic alterations to the embryonic germline that can be passed to offspring and future generations (Zhu et al., 2021). A simplified culture medium has been shown to improve embryo quality by lowering metabolic activity (Krisher et al., 2015, Ermisch et al., 2020) and this has been hypothesised to produce more viable embryos (Leese et al., 2008). Embryos have an optimal range of metabolic activity during preimplantation

antioxidant levels are hypothesised to be the most viable (Lane et al., 1996, Houghton et al., 2002, Leese et al., 2008). *In vitro* culture of bovine embryos with a combination of monosaccharides, fructose and glucose, increases metabolic activity and produces lower quality blastocysts compared to embryos that are cultured with fructose or glucose alone (Krisher et al., 2015). *In vitro* culture of mouse embryos in medium with a reduced AA content (50% reduction) produces equal quality blastocysts that have lower metabolic activity (Ermisch et al., 2020). These studies suggest that although embryos can develop to the blastocyst stage in medium with high concentrations of AA, or combinations of sugars, these conditions may overwhelm the embryo and cause metabolic distress which is detrimental to the overall viability of the embryo (Ermisch et al., 2020). Despite some improvements to medium formulations embryos that develop *in vitro* overall have less chance of implanting and resulting in a live birth compared to those that are fertilised and developed *in vivo* (Mahdavinezhad et al., 2019). Further optimisation of the embryo culture medium is required.

1.1.2 Effect of amino acids in culture medium and in fertilisation medium

AA and their transport play an important role in regulating metabolism, gene expression and cellular function (Leese et al., 2021). AA are routinely added to culture medium to support oocyte maturation (Hong et al., 2007) and to optimise preimplantation development *in vitro* (Brinster, 1965, Summers et al., 2000, Gardner, 2008, Kane, 2019). Free AA including Pro, Ala, Gly and Gln are present in high concentrations in the mammalian reproductive tract and are accumulated in cytoplasmic pools in the oocyte and early embryo (Guerin et al., 1995, Richards et al., 2010). This suggests a physiological role for AA and their transport in preimplantation development. It is well established that the exogenous addition of essential AA (EAA) and non-essential AA (NEAA) to culture medium benefits preimplantation

development (Gardner et al., 1996, Lane et al., 1997, Gardner et al., 1998, Leese et al., 2021) but their presence benefits different stages (Devreker et al., 2001). Addition of NEAA plus Gln promotes cleavage in mouse embryos to the 8-cell stage (Gardner et al., 1993, Lane et al., 1997) whereas addition of EAA benefits development after the 8-cell stage and increases the number of cells in the ICM of mouse blastocysts (Gardner et al., 1997, Lane et al., 1997). It is possible for mouse embryos to develop in a simple salt medium free of AA (Whitten, 1956, Lawitts et al., 1991) but embryos cultured with AA are more likely to reach the blastocyst stage due to increased cleavage rates, increased number of cells in the ICM and also show increased rates of blastocyst hatching (Gardner, 1998). Embryos cultured in medium containing select individual AA, arginine (Arg), Pro or glutamate (Glu) have lower metabolic activity and are more viable than embryos cultured with groups of AA (Ermisch et al., 2020). Conversely some individual AA including leucine (Leu) and cysteine (Cys) can be detrimental to hamster embryo development (McKiernan et al., 1995) or Gln to mouse embryo development, due to its breakdown in medium to embryo-toxic ammonium (Lane et al., 2001). Thus inclusion (and exclusion) of select AA in medium may increase embryo viability and reduce detrimental effects on oocyte quality (Lane et al., 2001, Ermisch et al., 2020). When Pro or Gln are added to the culture of mouse embryos the percentage of embryos that form blastocysts and hatch increases (Morris et al., 2020). This improvement in development is specific to Pro and not due to its action as an osmolyte, since addition of other osmolytes, Gly or Bet, did not improve embryo development (Morris et al., 2020).

Much less is known about the effect of select AA in the fertilisation medium. The fertilisation medium supports sperm-egg fusion following IVF or ICSI and is critical for optimising oocyte competency. However it is not known which specific AA are taken up by the oocyte to support embryo potential in the fertilisation medium. A systematic review of 22 randomised control trials (Mantikou et al., 2013) comparing commercially available fertilisation medium used in

human ART found no improvements in fertilisation rate or live birth rate between commercial media (Mantikou et al., 2013). A modified KSOM fertilisation medium that contains groups of AA also did not improve mouse fertilisation rates or subsequent embryo development (Summers et al., 2000). Although fertilisation in the presence of groups of AA was found to confer other benefits to embryo development including increased rate of blastocyst hatching, increased number of cells in the ICM, and blastocysts with more organised extra cellular matrix (Summers et al., 2000). The addition of individual AA Gln or glycyl-L-glutamine (GlyGln) to the fertilisation medium has no effect on fertilisation rate or on subsequent development in mouse (Summers et al., 2005) or bovine embryos (Hagemann et al., 1998) however IVF in the presence of GlyGln increases the number of cells in the ICM and decreases the number of dead cells in blastocysts (Summers et al., 2005). Further research is needed to elucidate the effect of select individual AAs in fertilisation medium.

1.2 Functions of proline in mammalian cells

Pro is a small (115.13 g/mol), non-polar conditionally essential AA endogenously synthesised in the mitochondria of cells from Arg, ornithine (Orn) and Gln (Patriarca et al., 2021). Pro is a major contributing AA in the synthesis of collagens in the extracellular matrix of cells (Krane, 2008, Psychogios et al., 2011). The breakdown of collagen in tumour tissue releases free Pro which is catabolised by proline dehydrogenase (PRODH), an enzyme which initiates the Pro catabolic pathway, which provides ATP to the cells (Elia et al., 2017, Pure et al., 2018). Free Pro (3 μ M) is found in the central nervous system (Broer, 2006) where it can be taken up by the high-affinity Pro transporter PROT and converted directly into the neurotransmitter Glu (Freneau et al., 1992). The ability for neurons to take up and metabolise Pro is critical for normal neurological function. Pro acts as a potent neurotoxin when injected into the hippocampus causing neuronal cell death (Nadler et al., 1988). High concentration of Pro in

the brain due to metabolic disorders of PRODH, or mutations to the brain Pro transporter PROT, has been linked to cognitive dysfunction and neural disorders including schizophrenia (Wyse et al., 2011, Nagaoka et al., 2020).

Mouse oviductal and uterine fluid contains up to 350 μ M Pro (Lakhiani, 2017) and accounts for 3% of total AA in reproductive fluid (Guerin et al., 1995) suggesting a physiological role for Pro in mammalian embryo development. Supplementation of Pro in medium increases the content of Pro in oocytes (Lakhiani, 2017) with Pro accumulation being 10 times higher in oocytes cultured with Pro compared to freshly isolated oocytes, 200 mM or 20 mM respectively (Lakhiani, 2017). Uptake of Pro increases when cumulus cells are attached to oocytes and GJC is present (Colonna et al., 1983).

1.2.1 Proline transport regulates cell volume

Embryos are sensitive to the osmolarity of the extracellular environment (Baltz, 2001, Baltz et al., 2010, Morris et al., 2020). The osmolarity of oviductal fluid is relatively high (300 – 330 mOsm/kg) and organic osmolytes are taken up by embryos to maintain cell volume and prevent cell shrinkage from water moving out of cell (Richards et al., 2010). Several AA have been identified for their ability to act as organic osmolytes in cleavage stage embryos including Glu, Bet, Ala, Gly and Pro (Richards et al., 2010). Zygotes cultured in hypertonic conditions of the oviduct have reduced blastocyst formation and increased embryo arrest due to osmotic stress and other effects of the culture medium (Baltz, 2001, Baltz et al., 2010, Morris et al., 2020) thus the addition of specific AA the medium is essential to support *in vitro* embryo development. Hypertonic culture conditions stimulate System A transport activity and increased expression of mRNA for the neutral AAT, SNAT2, which leads to an increase of neutral AA accumulation for cell volume recovery (Franchi-Gazzola et al., 2004). When exogenous Pro is added to hyperosmotic medium, cleavage stage embryos accumulate Pro, and

the number of embryos that survive and develop to blastocyst stage is increased (Baltz et al., 2010, Morris et al., 2020) providing an important role for Pro in the recovery of cell volume (see Fig. 1.2). In hypertonic conditions Pro accumulation is due to the upregulation of organic osmolyte transporters on the plasma membrane, including GLYT1 (Van Winkle et al., 1988) or SIT1 (Anas et al., 2008) in oocytes and early embryos respectively, and SNAT2 in human fibroblasts and mouse ESCs (Franchi-Gazzola et al., 2004, Bevilacqua et al., 2005).

1.2.2 Proline transport stimulates growth factor-like signalling pathways

Embryos have a unique ability to autonomously direct their own development. Both oocytes and embryos secrete growth factor signalling proteins that act in a positive feedback loop to regulate cell survival and cleavage rate (O'Neill, 1997). Growth factors are also produced from maternal reproductive tract cells (paracrine), ovaries and glands (endocrine) and act on embryo development (Zamfirescu et al., 2021). In culture medium, where paracrine and endocrine factors are absent, autocrine growth factors are critical for embryo development. Autocrine factors not only provide autologous support but also have an effect on embryos in proximity of each other (Lane et al., 1992, Green et al., 2013). For example, embryos cultured in high density (10 embryos/10 μ l) are more likely to develop to blastocyst stage with increased cell numbers compared to those cultured at low density (1 embryo/100 μ l) (Lane et al., 1992, Green et al., 2013) and this is due to action of growth hormones and cytokines secreted by the embryo into the surrounding medium (O'Neill, 1998, Guzeloglu-Kayisli et al., 2009, Green et al., 2013).

In low density culture, where autocrine support is diluted, exogenous addition of a physiological concentration of Pro (400 μ M) or Gln (1 mM) stimulates embryo development to the blastocyst stage and increases the number of hatching blastocysts (Morris et al., 2020).

These results are similar to the improvement rates seen in embryos cultured in high density culture without AA (Lane et al., 1992, Green et al., 2013, Morris et al., 2020). Pro is not acting solely as an organic osmolyte to improve embryo development as other organic osmolytes Gly and Bet did not improve embryo development (Morris et al., 2020).

Mammalian target of rapamycin complex (mTORC1) regulates protein synthesis and cell death via upstream signalling pathways including extracellular signal-regulated kinase (ERK) and protein kinase B (Akt) (Zamfirescu et al., 2021). Inhibition of Akt causes delayed blastocyst hatching (Riley et al., 2005) and inhibiting ERK causes embryos to arrest at early cleavage stages (Maekawa et al., 2007). Subunits of the ERK/Akt pathway are present throughout preimplantation development and are regulated by AA availability (Murakami et al., 2004, Morris et al., 2020, Zamfirescu et al., 2021). Addition of Pro to culture medium increases nuclear localisation of the phosphorylated proteins ERK and Akt (Zamfirescu et al., 2021) and activation of Akt signalling at the 2- and 4-cell stage and ERK signalling at the 2- to 8-cell stage (Morris et al., 2020). When blastocysts are cultured with rapamycin, to block mTORC1 pathway activity, the Pro mediated improvement in embryo development is prevented (Zamfirescu et al., 2021). Exogenous addition of Pro to culture medium only partially restores mTORC1 signalling in blastocysts (Morris et al., 2020, Zamfirescu et al., 2021). These findings indicate that Pro transport in embryos has a role in the activation of Akt, ERK and mTORC1 signalling pathways (see Fig. 1.2) (Morris et al., 2020).

1.2.3 Proline promotes differentiation of embryonic stem cells

ESCs are cells that are early in developmental lineage and are produced from the ICM of blastocysts. In mice models ESCs have been stimulated to differentiate into cardiac cells (Doetschman et al., 1985), skeletal tissue (Braun et al., 1994), and neurons (Bain et al., 1995), and there are an increasing number of clinical trials using ESCs for the treatment of stroke and

spinal cord injury (Bonn, 1998). In culture ESCs have the unique ability to self-renew and differentiate into several different mature somatic cell types. Therefore ESCs are an excellent model for understanding the cellular mechanisms of the early embryo. ESC metabolism relies on a feedback loop that restricts the biosynthesis of intracellular Pro and allows ESCs to stay in an undifferentiated state (D'Aniello et al., 2015). In culture, however, exogenous addition of Pro can overcome this suppressive mechanism and allow ESCs to differentiate into mesenchymal-like stem cells and neural progenitor cells (Washington et al., 2010, Tan et al., 2011, Comes et al., 2013). This occurs via activation of the mTORC1 signalling pathway (Washington et al., 2010). The effect is unique to Pro as other AA including D-Pro, Gly and Leu do not cause stem cells to differentiate (Washington et al., 2010). Pro induces the conversion of ESCs to mesenchymal-like stem cells via epigenetic reprogramming and histone methylation at H3K9 and H3K36 (Comes et al., 2013).

1.2.4 Redox regulation by proline

Redox homeostasis is the balance between oxidant and antioxidant signalling (He et al., 2017). Pro is metabolised in the mitochondria and the cytoplasm of cells down several pathways. One of the pathways involves conversion of Pro to pyrroline-5-carboxylic acid (P5C) by proline oxidase (PRODH) then to glutamate-semi-aldehyde before being converted to Glu and α -ketoglutarate (see Fig. 1.2) (Phang et al., 2008, Phang, 2019). Overexpression of PRODH causes an increase in cell death (Krishnan et al., 2008) and highlights Pro's essential role in the cell survival pathway. Conversely Pro can also be synthesised endogenously via glutamate-semi-aldehyde conversion to P5C then to Pro via P5C reductase (Pycr1) (D'Aniello et al., 2015). The metabolic pathway of Pro metabolism produces ATP energy via both α -ketoglutarate production and Pro-derived high-energy electrons entering the electron transport system (see Fig. 1.2) (Phang et al., 2008, Tanner et al., 2018).

The metabolism of Pro also produces reactive oxygen species (ROS). ROS and hydrogen peroxide (H_2O_2) are sources of free radicals and are common by-products of metabolic activity. Production of ROS in low levels act as signalling messengers to support cell homeostasis, but at high concentrations ROS can cause oxidative distress in embryos (Guerin et al., 2001, Krishnan et al., 2008). In oocytes, low concentrations of H_2O_2 stimulates resumption of meiosis (Pandey et al., 2014) and low concentration of ROS is involved in redox signalling eustress (Niki 2016). However oocytes exposed to high levels of ROS in vivo have lower fertilisation rates, increased embryo arrest at the 2-cell stage and develop embryos with lower viability (Nasr-Esfahani et al., 1990, Guerin et al., 2001, Das et al., 2006). Cryopreservation is a commonly used technique to store or transport oocytes for ART. However the process of vitrification, which requires rapid freezing and subsequent thawing, increases intracellular levels of ROS making the oocyte susceptible to oxidative stress and mitochondrial damage (Cao et al., 2022), and decreases fertilisation potential (Gupta et al., 2010). Supplementation of vitrification medium with exogenous Pro has been reported to lower ROS production in oocytes and increase oocyte survival (i.e. decrease cell apoptosis) upon thawing (Zhang et al., 2016).

Addition of Pro to medium increases production of antioxidant glutathione (GSH) in mouse oocytes (Liu et al., 2023). GSH is a tripeptide that functions as a redox regulator (Sies, 1999) and molecular marker of cytoplasmic maturation in oocytes (Liu et al., 2023). GSH is needed to maintain normal preimplantation development (Winkler et al., 2011). GSH levels are highest in oocytes compared to subsequent embryonic stages (Gardiner et al., 1994) but the mechanisms by which GSH supports oocyte potential and subsequent development are still unknown.

Together these findings highlight an important role for the transport of Pro in regulating cell volume, activation of growth-factor-like signalling pathways, for redox regulation and protection against oxidative stress in mammalian cells (see Fig. 1.2).

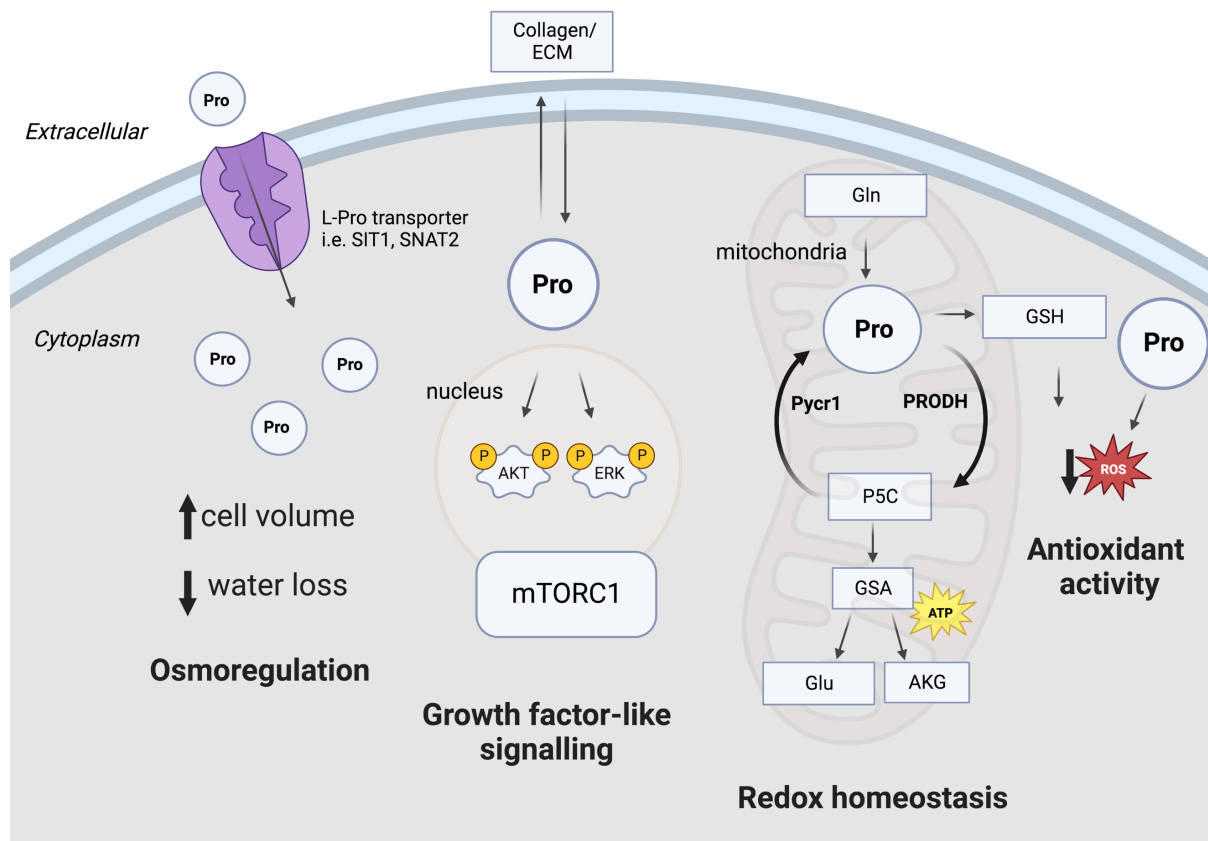


Figure 1.2 Roles of Pro transport in mammalian cells. Pro is accumulated to increase cell volume and activate mTORC1 signalling pathways. Pro is metabolised and synthesised in the mitochondria and provides intermediates for cellular homeostasis. Pro is an important component of collagens in the extra cellular matrix (ECM), and collagen can be catabolised to release free Pro. Metabolism of Pro to GSH, and Pro itself, reduce ROS via antioxidant activity. GSA; glutamate-semi-aldehyde, AKG; α -ketoglutarate. Images created using BioRender software.

1.3 Amino acid transporters

For AA to exert a beneficial effect on oocyte competency and preimplantation development they must be taken up into the cell. Charged molecules and AAs cannot diffuse across the cells phospholipid bilayer so AA transport is achieved by the synthesis and expression of plasma membrane AAT (Van Winkle, 2001). Approximately 5-10% of all genes in the genome encode for transport related proteins (Hediger et al., 2013, Sahoo et al., 2014). The majority of AAT are located on the cell plasma membrane (Hediger et al., 2013). AAT are also located on the membranes of intracellular organelles including vesicles, lysosomes or mitochondria (Broer et al., 2017).

1.3.1 Classification and characteristics of amino acid transporters

The phospholipid bilayer of the cell prevents simple diffusion of AA and ions between the cytoplasm and the extracellular fluid (Hediger et al., 2013). Small uncharged hydrophobic molecules (i.e. Pro, Gly, Ala) can cross the membrane via channel and carrier proteins (see Fig. 1.3). For example the volume-sensitive anion channel enables diffusion of Cl^- and uncharged AAs across the plasma membrane to regulate cell volume and Cl^- concentration in early embryos (Sonoda et al., 2003, Arnaiz et al., 2013). Facilitated transporters move AAs down their electrochemical gradient (Hediger et al., 2004). An example of facilitated transport are the cationic AA transporters (CATs) of the SLC7 family which are present in most cells and tissues (Verrey et al., 2004). AA which are lipophobic or with an electrical charge cannot cross into the cell by facilitative transport but rely on active AA transport (Van Winkle, 2001). Active transporters utilise energy-coupling systems to transport substrates up the electrochemical gradient to areas of higher concentration (i.e. driven by the kinetic energy of concentration gradients) (Hediger et al., 2013). There are two types of active transport systems classified according to their ion coupling (Cl^- , H^+ , K^+ and/or Na^+). Primary active transporters maintain

ion gradients across the membrane and are dependent on the energy of adenosine triphosphate (ATP). For example, the Na^+/K^+ pump (Kandasamy et al., 2018). Secondary active transporters utilise the electrochemical gradient of one molecule to drive another molecule up its electrochemical gradient and the co-transported substrates can move in the same or opposite direction (see Fig. 1.3) (Kandasamy et al., 2018). For example, uniporters can transport one substrate in a specific direction, cotransporters, also known as symporters, simultaneously transports two or more substrates in the same direction and exchangers, also known as antiporters, facilitate the 1:1 exchange of substrates in opposite directions (Fredriksson et al., 2008, Hediger et al., 2013).

A wide variety of AA transport systems have been identified based on the function of their transport proteins. The systems have specific AA substrates that they accept, and may be dependant or independent on co-substrates Na^+ and Cl^- (Van Winkle, 1993). Substrate selectivity is related to the size, structure and charge of the protein side chain being anionic, cationic, zwitterionic or neutral (Fredriksson et al., 2008, Hediger et al., 2013). The suffix (+) or (-) denotes carriage of charged AA substrates (Fredriksson et al., 2008, Hediger et al., 2013). Thus uptake potential is determined by ion coupling, the extracellular environment (i.e. AA availability) and the electrochemical gradient of the AA substrate.

Membrane transport proteins can be further classified by structure with the solute linked carrier (SLC) gene nomenclature system (Hediger et al., 2013). Generally genes are named with SLC prefix followed by a numeral, the letter A and the number of the transporter (Pelland et al., 2009, Hediger et al., 2013). For example the $\text{B}^0\text{AT1}$ transport protein of system B^0 accepts all neutral amino acids (0) and is known by the gene that encodes it *Slc6a19*. There are currently 52 families and 395 transporter genes belonging to the SLC gene family (Kandasamy et al.,

2018). AAT that have been identified in mammalian cells and tissues may also be expressed in the oocyte and preimplantation embryo.

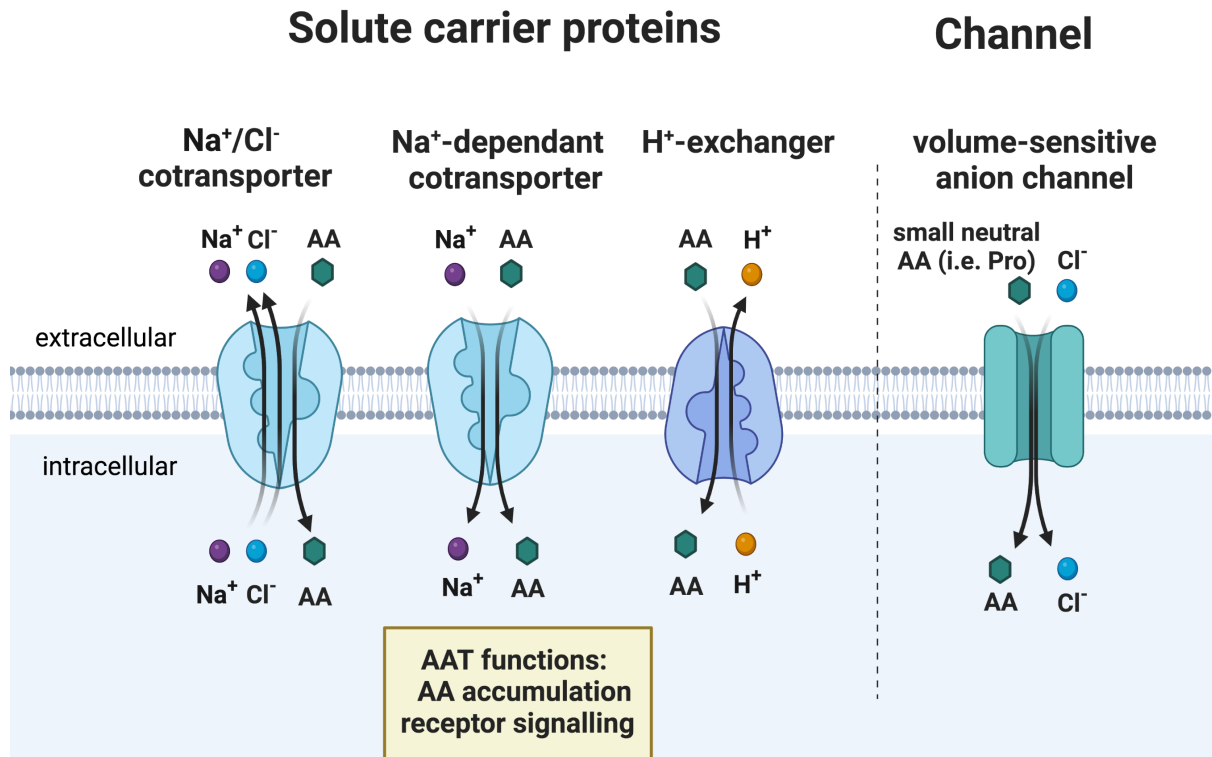


Figure 1.3 Types of amino acid transporters. Solute carrier and channel proteins are located on the cell membrane (i.e. plasma, vesicular, lysosomal, or mitochondrial) and take up specific AA and/or ions from the extracellular environment. AAT function to accumulate amino acids in cytoplasmic pools or function as signalling receptors in preimplantation embryo development. Images created using BioRender software.

1.3.2 Amino acid transport systems present during preimplantation development

Expression of AAT in the oocyte and preimplantation embryo is regulated both developmentally and in response to external signals and stresses (Kriel et al., 2011). For example, the *in vitro* culture of cleavage stage embryos causes upregulation of system b⁺y⁺

in response to suboptimal conditions (i.e hypertonicity or reduced AA availability) (Franchi-Gazzola et al., 2004, Redel et al., 2015). Upregulation of system $b^{+}y^{+}$ causes embryos to accumulate substrates of these systems, including increased transport of the osmolyte Arg, which functions in embryos to stimulate mTORC1 pathways and regulates cell volume (Franchi-Gazzola et al., 2004, Redel et al., 2015).

Competition AA uptake assays are performed to determine if a system or transporter is present in a cell or expressed at a specific time of embryo development. For example, molar excess of a co-transported substrate will competitively inhibit the uptake rate by that transporter.

Fourteen AA transport systems have been identified in oocytes and preimplantation embryos, one or more of these systems may be responsible for the uptake of Pro. Common membrane AA transport systems have been outlined according to their ion dependence, or independence (see Fig. 1.4).

accumulate AA and for protein synthesis (Hediger et al., 2013). System $b^{0,+}$ has been characterised in blastocysts and takes up Leu and Arg to support trophoblast motility through the activation of mTOR1 signalling (Van Winkle, 2021). System L transports large branched chain and benzenoid AA and is present throughout preimplantation embryo development (Van Winkle, 2001). System b^+ and y^+ contain the cationic amino acid transporters (CATs) expressed throughout preimplantation development (Van Winkle, 2021). Transporters of system b^+y^+ have a high affinity for Arg (Redel et al., 2015) and *in vitro* culture increases the expression of AAT of these systems (Redel et al., 2015). **System $B^{0,+}$, β , GLY and IMINO** all belong to the Na^+ and Cl^- dependent neurotransmitter transporter family **SLC6**. Transporters in these systems are highly accumulating as they are coupled to the co-transport of 2-3 Na^+ and one Cl^- (Roux et al., 2000). $B^{0,+}$ accepts both cationic and zwitterionic AA with a high affinity for branched amino acids such as Leu (Van Winkle et al., 1985, Van Winkle, 2021). $B^{0,+}$ is expressed in blastocysts and has an important role in implantation, mTORC1 signalling, and the development of trophoblast motility (Van Winkle et al., 2006). GLY is present in cleavage stage embryos to take up organic osmolytes, preferentially Gly, to counteract the hyperosmotic conditions of the oviduct (Van Winkle, 2020).

In the oocyte, transport system expression differs throughout growth and meiotic maturation. System ASC/asc, $b^{0,+}$ and L is active at all stages including GV, MI and MII. System L decreases (by 40%) in mature MII oocytes (Pelland et al., 2009). GLY, Xc^- and Beta are not active at the GV stage but are expressed in MII oocytes (Pelland et al., 2009). Mammalian membrane transporters and their expression in the oocyte and preimplantation embryo are outlined in Table 1.1.

Table 1.1 Mammalian membrane transporters and expression in preimplantation embryo development.

System	Protein	Gene	Substrates	Inhibitor	Ion dependence	Expression oocyte/ embryos	References
A	SNAT1 SAT1 ATA1 NAT2	<i>Slc38a1</i>	Small aliphatic (Gln, Ala, Asn, Cys, His, Ser),	MeAIB	Cotransporter Na ⁺	system A in ovulated oocyte and blastocysts	(Van Winkle 1988, Broer 2002, Mackenzie et al., 2004, Pelland et al., 2009)
	SNAT2 SAT2 ATA2	<i>Slc38a2</i>	Small aliphatic (Gln, Ala, Asn, Cys, His, Ser, Gly, Pro, Met)	MeAIB	Cotransporter Na ⁺	ICM	(Broer 2002, Mackenzie et al., 2004, Pelland et al., 2009)
	SNAT4 ATA3 NAT3 PAAT	<i>Slc38a4</i>	Small aliphatic (Ala, Asn, Cys, Gly, Ser, Thr)		Cotransporter Na ⁺	unknown	(Mackenzie et al., 2004)
asc	ASC-1	<i>Slc7a10</i>	Small neutral amino acids (Ala, Ser, Cys, Gly, Thr)	Ser, Cys	Antiporter neutral AA	oocytes	(Fukasawa et al., 2000, Pelland et al., 2009)
ASC	ASCT1 SATT	<i>Slc1a4</i>	Ala, Ser, Cys, Thr, Pro	Ser, Cys	Antiporter Na ⁺	ICM	(Pinilla et al., 2001, Pelland et al., 2009, Van Winkle 2021)
	ASCT2 AAAT	<i>Slc1a5</i>	Ala, Ser, Cys, Thr, Gln		Antiporter Na ⁺	blastocyst	(Scalise et al., 2018)
b^{0,+}	b ^{0,+} AT	<i>Slc7a9</i>	Cationic and large neutral (Arg, Ala, Lys, Orn, Cys, Leu)	Ser, Lys	Antiporter (Preferentially extracellular cationic AA against intracellular neutral AA)	MII oocytes, 1-cell to blastocyst	(Broer 2002, Pelland et al., 2009, Van Winkle 2021)
B^{0,+}	ATB ^{0,+}	<i>Slc6a14</i>	Neutral, cationic, bicyclic, BCH, Lys, β-Ala, Pro, Ala	BCH	Cotransporter Na ⁺ /Cl ⁻	cleavage-stage embryos and blastocysts	(Broer 2002, Pelland et al., 2009, Van Winkle 2021)
Beta	Taut	<i>Slc6a6</i>	Taur, b-Ala	β-Ala	Cotransporter Na ⁺ /Cl ⁻	MII oocytes, GV oocytes, 1-cell to blastocyst	(Hammer et al., 2003, Pelland et al., 2009)
y⁺, b⁺	CAT1 ARTC1	<i>Slc7a1</i>	Cationic L-AA (Arg, Lys)	Lys	Facilitated transporter (passive)	1-cell to blastocyst (minor)	(Pelland et al., 2009, Van Winkle 2021)
	CAT2 (A or B splice)	<i>Slc7a2</i>	Cationic L-AA (Arg, Lys)		Facilitated transporter (passive)	1-cell to blastocyst	(Pelland et al., 2009, Van Winkle 2021)

	variants) ARTC2						
GLY	GLYT1	<i>Slc6a9</i>	Gly, Sar	Sar	Cotransporter Na ⁺ /Cl ⁻	MII oocytes, GV oocytes, 1-cell to 8-cell, not active after compaction from morula stage onwards. Not active in blastocysts	(Steeves et al., 2003, Pelland et al., 2009, Van Winkle 2021)
L	LAT1	<i>Slc7a5</i>	Large branched L-AA (Leu, His, Met, Ile, Val, Phe, Tyr, Trp), BCH	BCH	Antiporter EAA	oocytes, increases during maturation. 1-cell to blastocyst	(Van Winkle 2001, Meier et al., 2002, Pelland et al., 2009, Van Winkle 2021)
	LAT2	<i>Slc7a8</i>	Large neutral L-AA (not Pro), BCH	BCH	Antiporter EAA	unknown	(Gandhi et al., 2004)
N	SNAT3	<i>Slc38a3</i>	Gln, His, Asn, Ala		Cotransporter Na ⁺ Antiporter H ⁺	unknown. mRNA in cumulus cells but not in oocytes	(Pelland et al., 2009, Van Winkle 2021)
	SNAT5 JM24 SN2	<i>Slc38a5</i>	Neutral (Gln, Asn, His, Ser, Gly)		Cotransporter Na ⁺ Antiporter H ⁺	4- to 8-cell embryos	(Cubelos et al., 2005)
X_{AG}⁻	EAAT1EAA T2 EAAC1	<i>Slc1a1</i>	Gln, D/L-Asp	Glu	Cotransporter Na ⁺ Antiporter H ⁺	blastocyst	(Broer 2002, Pelland et al., 2009)
X_C⁻	xCT	<i>Slc7a11</i>	Cys (anionic form), Gln		Antiporter Cl ⁻	MII oocytes, GV oocytes increases 5 fold during maturation, UF oocytes, 1-cell embryo	(Broer 2002, Pelland et al., 2009)
IMINO	SIT1 IMINO XT3 Xtrp3	<i>Slc6a20</i>	Pro, Bet, MeAIB, PA, hydroxyl-Pro		Cotransporter Na ⁺ /Cl ⁻	not in oocytes, activated several hours after fertilisation, only expressed in 1-cell to 2-cell stages, SIT1 mRNA only expressed in UF and 1-cell stages	(Takanaga et al., 2005, Anas et al., 2008)
PAT	PAT1 LYAAT1	<i>Slc36A1</i>	Gly, Ala, Pro, GABA, D-Ala, MeAIB, taurine, β-Ala		Cotransporter H ⁺	unknown	(Metzner et al., 2006)

	PAT2	<i>Slc36a2</i>	Gly, Ala, Pro, MeAIB (also transports β -Ala, Sar, Bet, D-Pro)		Cotransporter H ⁺	unknown	(Thwaites et al., 2011)
	PAT3	<i>Slc36a3</i>			Orphan, transporter	unknown	(Boll et al., 2003)
	PAT4	<i>Slc36a4</i>			Orphan, transporter	unknown	(Pillai et al., 2011)
B⁰	B ⁰ AT1	<i>Slc6a19</i>	Neutral AA		Cotransporter Na ⁺	unknown	(Broer 2006)
	B ⁰ AT2	<i>Slc6a15</i>	Neutral AA		Cotransporter Na ⁺	unknown	(Broer et al., 2006, O'Mara et al., 2006)
	PROT	<i>Slc6a7</i>	Pro		Cotransporter Na ⁺ /Cl ⁻	mRNA only expressed in UF and 1-cell stage	(Fremeau et al., 1992, Broer 2006, Anas et al., 2007, Anas et al., 2008)

1.3.3 Expression and function of mammalian proline transporters

Pro transporters have been investigated for their variety of roles in human health and disease. Mutation of AAT genes or dysfunction of AA transport leads to alterations in intracellular AA accumulation and can contribute to the pathogenesis of disease (Zielke et al., 2002, Kleta et al., 2004). For example, Hartnup disorder is a rare condition caused by mutation of the SLC6A9 gene (B⁰AT1). B⁰AT1 is the major transporter of neutral AA in the kidney and intestine, and dysfunction or absence of B⁰AT1 causes an increase of neutral AA in urine (Bohmer et al., 2005, O'Mara et al., 2006). In cancer cells, uptake of exogenous AAs including Pro and Leu, is increased during cell growth (Cormerais et al., 2016, Elia et al., 2017, Pure et al., 2018). Thus blocking AA uptake by inhibiting specific AAT has potential clinical significance for reducing cancer growth (Okano et al., 2020). For example Leu is a source of ATP energy in cells and it has been demonstrated that reducing the uptake of Leu by inhibiting LAT1 causes a reduction of mTORC1 signalling and smaller tumour formation in mice (Cormerais et al., 2016). Characterisation of Pro transporters and their substrates (or pharmacological inhibitors) may also have therapeutic applications in cancer therapies (Zipp et al., 2014).

ASCT1 (SLC1A4) is a Na⁺-dependent antiporter expressed in the small intestine, liver and brain (Pinilla et al., 2001). ASCT1 is a member of the glutamate transporter superfamily expressed on the cell plasma membrane (Broer et al., 2017). SLC1A4 -null mice are reported to have decreased brain volume and function but normal reproductive capacity (Kaplan et al., 2018). ASCT1 has been characterised in the ICM of blastocysts where it likely functions as a signalling transceptor (Formisano et al., 2016).

B⁰AT1 (SLC6A9) is a Na⁺-dependent neutral AA co-transporter expressed in the kidney and intestine (Broer et al., 2004, Bohmer et al., 2005) and is located on the cell plasma membrane

(Broer et al., 2017). Trafficking of B⁰AT1 protein to the plasma membrane involves accessory proteins angiotensin-converting enzyme 2 (ACE2) or collectrin (Danilczyk et al., 2006, Danilczyk et al., 2006, Kowalczyk et al., 2008). Deletion or mutation of B⁰AT1 in mice results in nonlethal effects (Van Winkle, 2021). It is not known if B⁰AT1 is expressed in oocytes or preimplantation embryos.

B⁰AT2 (SLC6A15) is structurally homologous and functionally similar to B⁰AT1, is a Na⁺-dependent cotransporter of all large neutral AA and is highly expressed in the brain, lung and kidney (Broer et al., 2006) and on the cell plasma membrane (Broer et al., 2017). mRNA from SLC6A15 is present in the preimplantation embryo, plasma membrane of oocytes (O'Mara et al., 2006) and in ESC (Tan et al., 2011). It is not known if B⁰AT2 is expressed in oocytes or preimplantation embryos. SLC6A15-null mice have normal reproductive capacity but reduced uptake of Leu and Pro in the brain (Drgonova et al., 2007).

GLYT1 (SLC6A9) is a Na⁺ and Cl⁻-dependent transporter with a high affinity for Gly (Steeves et al., 2003). GLYT1 is expressed on the plasma membrane of intestinal cells and enterocytes and is the major transporter of the neurotransmitter Gly in the brain (Steeves et al., 2003, Broer et al., 2017). GLYT1 is transiently expressed in cleavage stage embryos and functions to regulate cell volume via the transport of organic osmolytes (Adams et al., 1995, Steeves et al., 2003). SLC6A9-null mice have normal fertility and normal embryonic development but acquire severe motor and respiratory complications soon after birth resulting in neonatal death (Gomez et al., 2003).

NTT4 (SLC6A17) is a Na⁺-dependent neutral AA transporter (Zaia et al., 2009). NTT4 takes up the majority of neurotransmitters in the brain including Pro and Leu, and is expressed on the membrane of intracellular vesicles (Zaia et al., 2009). It is not known if NTT4 is expressed in oocytes or preimplantation embryos.

PAT1 (SLC36A1) is a Na^+ -dependent, proton-coupled exchanger highly expressed at the luminal surface of the intestine (Petrovic et al., 2002) and located on the plasma membrane of cells and lysosomes (Broer et al., 2017). PAT1 and PAT2 differ from the majority of mammalian transporter exchangers in that they function as H^+ coupled amino acid symporters (Thwaites et al., 2011). PAT1 can function in an electroneutral mode by co-transporting H^+ and a short chain fatty acid (Thwaites et al., 2011). PAT1 mRNA has been detected in ESCs (Tan et al., 2011). It is not known if PAT1 is present in oocytes or preimplantation embryos.

PAT2 (SLC36A2) is functionally similar to PAT1, is a Na^+ -dependent, proton-coupled exchanger that transports both L- and D- enantiomers of Pro but is more selective for L- enantiomers (Kennedy et al., 2005). PAT2 is expressed in the kidney located in the cell plasma membrane and is involved in reabsorption of AA (Thwaites et al., 2011, Broer et al., 2017). It is not known if PAT2 is expressed in oocytes or preimplantation embryos.

PROT (SLC6A7) is a Na^+ and Cl^- -dependent transporter located on the membrane of vesicles and the intracellular synapses of the brain (Fremeau et al., 1992, Broer, 2006, Broer et al., 2017). SLC6A7-null mice appear to have normal development and fertility but exhibit the endophenotype of mental illness such as reduced locomotor activity and memory (Schulz et al., 2018). High concentration of Pro in the brain due to mutations of SLC6A7 gene has been linked to cognitive dysfunction and neural disorders like schizophrenia (Wyse et al., 2011, Nagaoka et al., 2020). Pharmacological inhibitors of PROT have been reported to be effective in the treatment of cognitive disorders such as Alzheimer's disease (Zipp et al., 2014). It is not known if PROT is expressed in oocytes or the preimplantation embryo, however SLC6A7 mRNA is present in oocytes and 1-cell embryos (Anas et al., 2008).

SIT1 (SLC6A20) is a Na^+ and Cl^- -dependent transporter expressed in the intestinal brush-border (Kowalczyk et al., 2005, Takanaga et al., 2005) and in preimplantation embryos

(Takanaga et al., 2005, Anas et al., 2008) located on the plasma membrane (Broer et al., 2017). SIT1 is activated at fertilisation and is functional until the 2-cell stage (Takanaga et al., 2005, Anas et al., 2008). SIT1 is not expressed in oocytes or embryos after the 2-cell stage however SLC6A20 mRNA is present in oocytes (Anas et al., 2008).

SNAT2 (SLC38A2) is Na^+ -dependant cotransporter of small neutral AA and is widely expressed in mammalian tissues including the brain, intestine and liver (McGivan et al., 1994) located on the cell plasma membrane (Broer et al., 2017). SNAT2 has a dual function as a transceptor initiating cell signalling pathways for pluripotency (Tan et al., 2011) and in accumulating neutral AA to prevent cell shrinkage (Dall'Asta et al., 1994). Additionally, AA depletion causes rapid intracellular transcription and AAT trafficking of Pro transporter SNAT2 to the plasma cell membrane (Franchi-Gazzola et al., 2004, Hundal et al., 2009). SNAT2 has been characterised in ESCs (Tan et al., 2011).

y+LAT2 (SLC7A6) is a Na^+ -dependent antiporter expressed on the cell plasma membrane of skin fibroblasts, placenta, intestine and kidney (Broer et al., 2017, Rotoli et al., 2020). y+LAT1 (SLC7A7) and y+LAT2 is the light subunits of the transport system y+L (Rotoli et al., 2020). y+LAT2 is expressed in cumulus cells surrounding the oocyte and can transport Pro to the oocyte via gap junctions in the COC (Colonna et al., 1983, Corbett et al., 2014).

1.3.4 Substrate profile of mammalian proline transporters

Pro transporters function by allowing specific AA to bind and cross the plasma membrane and can exhibit different levels of affinity for uptake of the same substrate (see Fig. 1.5). During AA depletion a high affinity transporter is typically expressed to allow rapid transport of its AA substrates, and when AA is plentiful a low affinity transporter is expressed to maintain cellular homeostasis (Broer et al., 2017). GLYT1 has a high affinity for Gly, accepts Pro but

does not transport Ala, Leu, lysine (Lys) or glutamic acid (Steeves et al., 2003). PROT has a high affinity for Pro and also transports His and Sar (Anas et al., 2007). Preferred substrates of SIT1 are Pro, Bet, L-pipecolic acid (PA), Sar and to a lesser extent D-Pro (Stevens et al., 1985, Kowalczyk et al., 2005). SNAT2 can transport Gln, Gly, Cys, Ser, MeAIB, Pro and Ala but not Lys (Tan et al., 2011). B⁰AT1 transports all neutral AA with a high affinity for Leu and valine (Val) and to a lesser extent Pro and Gly (Broer, 2006). B⁰AT1 does not transport His however histidine induces an inward current in B⁰AT1 expressing oocytes (Bohmer et al., 2005, Camargo et al., 2005). Preferred substrates of B⁰AT2 are Pro, Leu, Val and methionine (Met) (O'Mara et al., 2006). In mice, B⁰AT2 does not transport Cys (Broer, 2006). PAT1 accepts Pro, Gly and Ala and unlike other transporters PAT1 does not have a preference between L- and D- enantiomers (Munck et al., 1994, Petrovic et al., 2002). Substrates of PAT2 include L- and D-Pro, Gly, Ala, sarcosine (Sar) and Methylamino isobutyric acid (MeAIB) (Thwaites et al., 2011). NTT4 takes up Pro and Leu (Zaia et al., 2009). ASCT1 transports Pro and has a high affinity for Ala, Ser, Cys and Thr (Pinilla et al., 2001, Formisano et al., 2016). y⁺LAT2 transports cationic and neutral AA including Arg, Leu, Lys, Gln and Pro (Rotoli et al., 2020) (see Fig.1.5).

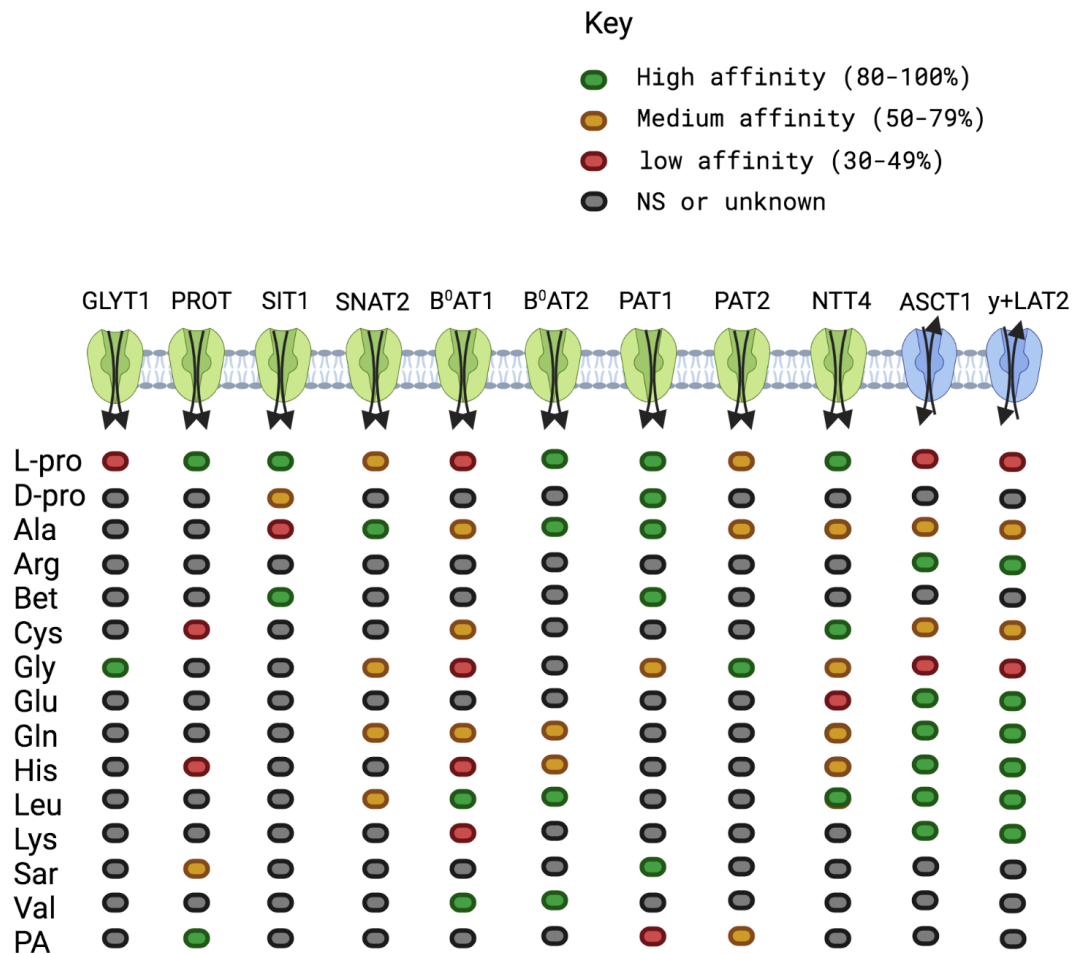


Figure 1.5 Substrate profile of mammalian transporters of proline. Affinity of common amino acids to Pro transporters in somatic cells. All transporters are symporters with exception for ASCT1 and y+LAT2 which are antiporters. NS indicates that the amino acid is not transported or it is unknown if it can be transported. Images created using BioRender software. Figure references (Adams et al., 1995, Broer et al., 2000, Sugawara et al., 2000, Steeves et al., 2003, Broer et al., 2004, Metzner et al., 2005, Broer et al., 2006, Anas et al., 2007, Broer et al., 2017).

1.3.5 Proline transport during preimplantation embryo development

The important role that Pro transporters play in maintaining AA homeostasis in somatic cells suggests that Pro transporters likely have an critical role in maintaining cellular homeostasis in oocyte and preimplantation embryo development. A number of AAT and systems have already

been identified in the oocyte and preimplantation embryo providing a basis for the present study (see Fig. 1.6).

The transporter y+LAT2 is expressed in cumulus cells surrounding the oocyte and can transport Pro to the oocyte via gap junctions in the COC (Colonna et al., 1983, Corbett et al., 2014), however shortly after ovulation the connection between the COC begins to break down and Pro can no longer be transported to the oocyte via this mechanism (Pelland et al., 2009). GLYT1 is expressed in oocytes, cleavage stage embryos and blastocysts and functions to regulate cell volume via the transport of organic osmolytes (Adams et al., 1995, Steeves et al., 2003). Several hours after fertilisation Pro is taken into the 1-cell embryo via the Bet/Pro transporter SIT1 which is functional until the 2-cell stage (Anas et al., 2007). High amounts of intracellular Pro is accumulated in 1- and 2-cell embryos and this can be attributed at least partly to SIT1 (Anas et al., 2007). At least one other unknown transporter that is inhibited by Leu, Cys, Ala and Met but not Bet is also present in the 1-cell embryo (Anas et al., 2007). Molar excess Bet, Gly, Leu and Pro itself inhibits Pro-mediated improvement in cleavage embryos indicating an unknown transporter is expressed after the late 2-cell stage (Morris et al., 2020). The inhibition profile does not fit that of transporters already characterised in embryos (i.e. SIT1 and SNAT2) but fits the substrate profile of the neutral AA transporter B⁰AT1 (SLC6A9) (Morris et al., 2020). SNAT2 transports Pro in ESCs and may also be the transporter responsible for Pro uptake in ICM of blastocysts (Tan et al., 2011). It is not until the blastocyst stage that SNAT2 is expressed in the embryo (Washington et al., 2010, Casalino et al., 2011). The amount of Pro accumulated in oocytes and embryos varies but it is clear that Pro transport has a critical role in development processes.

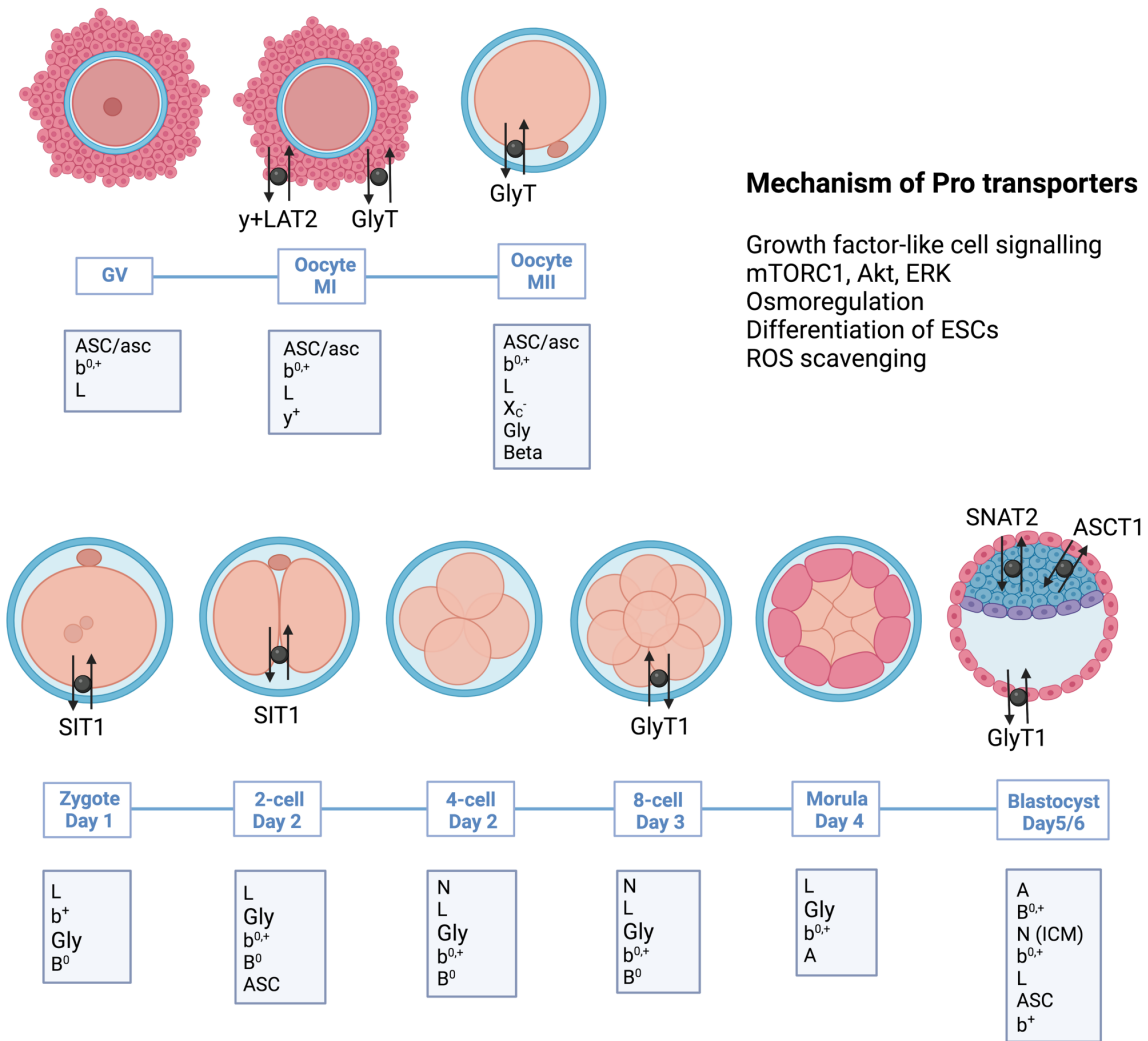


Figure 1.6 Amino acid transport systems and Pro transporters in the oocyte and preimplantation embryo. Gene expression of AAT is regulated both developmentally and in response to external signals and stresses. The transport of Pro into the oocyte and embryo may occur via one or a combination of these systems, or via Pro transporters that have not yet been identified. Pro is taken up by expression of Pro transporters at the oocyte, zygote, 2-cell and blastocyst stages. GLYT1 is transiently expressed throughout preimplantation development. Images created using BioRender software. Figure references (Colonna et al., 1983, Van Winkle, 1988, Haghighat et al., 1990, Adams et al., 1995, Van Winkle, 2001, Steeves et al., 2003, Takanaga et al., 2005, Anas et al., 2008, Pelland et al., 2009, Tan et al., 2011, Corbett et al., 2014, Van Winkle, 2021).

1.4 Embryo modification and ethics

This review will now move from basic science and focus on the ethics of embryo modification. Embryo modification in this context is defined as any method or technology that causes intentional disruption to the physiology and development of the embryo. Ethics is defined as the moral values and the principles of individuals or society. Legislation should be created from the framework of these values and principles.

1.4.1 Animal model in this study

In the first part of this study experiments were conducted that alter the development of mouse embryos to varying degrees (i.e. variation of nutrient availability) in medium, and formation of gene knockout mice. Mice are readily available, inexpensive and their anatomy is well understood. Their small size, large litters and quick reproduction rate are factors for their common use in reproductive research. Intentionally applying technology to embryos that causes developmental and heritable changes raises significant scientific, moral and ethical considerations. In the second part of this introduction the ethics and legislation governing embryo modification research in Australia will be reviewed.

1.4.2 Legislation in Australia

Legislation in Australia allows embryo modification of animals for research purposes but prohibits making heritable changes in human embryos regardless of whether the treated embryo is discarded thereafter. Human embryos are limited in availability for basic research and legal regulations involving human gametes restrict their use. In Australia, the *Prohibition of Human Cloning for Reproduction Act 2002 (PHCR Act)* and the *Research Involving Human Embryos Act 2002 (RIHE Act)* prohibit creating a human embryo purely for research purposes. Current law in this country only allows excess (discarded) embryos produced from IVF procedures

to be used for research but these embryos must be destroyed after 14 days. It is a requirement that approval to use embryos in this manner has been gained by the National Health and Medical Research Committee (NHMRC) Embryo Licensing Committee. The use of animals in research is regulated by the *Australian code for the care and use of animals for scientific purposes* (the Code) to ensure the welfare of animals is protected and their use in research is essential. Each State and Territory has legislation adopted from the Code that promotes the wellbeing of animals used for scientific purposes, in New South Wales this is the *Animal Research Act 1985*. The Australian government recently (2019) announced an amendment to *The Gene Technology Act 2000* to permit the use of gene editing technology in animals and human cell lines with the provision that no novel genetic material is introduced (Mallapaty, 2019). Approval to carry out experiments on animal embryos requires approval of the Animal Ethics Committee and the Office of the Gene Technology Regulator of the institution where the research is to be carried out.

1.4.3 Historical background

The history of human embryo research legislation is interwoven by science and politics. The techniques of IVF and *in vitro* embryo culture were pioneered in animal models almost a century ago from basic research performed on rabbit, hamster and mouse embryos (Bavister, 2002). At the time of these developments some people believed that research on embryos could potentially benefit human reproduction while others believed that scientists were ‘playing god’ (Biggers, 2012). The announcement of the first IVF child born via embryo transfer in 1978 (Steptoe et al., 1978) aroused great public interest, and ethical debate between scientists and policy makers. (Dawson 1987, Biggers, 2012). The debate was focused on the moral status of the human embryo and specific developmental events when this status should first apply (Dawson, 1987, Biggers, 2012). Some claims were that fertilisation marks the beginning of

human life (Dawson, 1987) while others suggested that moral status of an embryo begins when genetic differentiation occurs (Dawson, 1987). The following year in 1979 a 14-day limit for researching human embryos *in vitro* was proposed by the *Ethics Advisory Board of the US Department of Health, Education, and Welfare*. This limit was based on the time it takes for an embryo to develop *in vitro* and before implantation occurs *in vivo*. In 1984 the UK Warnock committee endorsed this limit (Warnock, 1984). Ethical concerns regarding human embryo research were reignited in 1990's when the technique of somatic cell nuclear transfer (SCNT) was applied to create a genetically identical lamb (Willadsen, 1979, Biggers, 2012). Scientists also announced that they had been successful in isolating pluripotent human embryonic stem cells (hESCs) from embryos (Thomson et al., 1998) and could use these for therapeutic cloning. Public fears were that this technology might be applied to clone humans; and this would infringe on 'human dignity' (Caulfield, 2003). In 1998 the World Health Organisation released the *Ethical, Scientific and Social Implications of Cloning in Human Health. Geneva* which addresses the global governance of human cloning. To-date Commonwealth legislation regulating research on human embryos is defined by the moral stand-points of these historical events. The *RIHE Act* prohibits human cloning for reproductive purposes or the development of human-animal hybrids, although the creation of such hybrids is permitted subject to licensing. The 14-day rule is encoded in Australian legislation and is regulated by the NHMRC Licensing Committee which makes it a criminal offence to grow an embryo in the laboratory for more than 14 days after fertilisation. However legislation such as the 14-day limit which was created over 40 years ago might be restricting the advancement of both novel laboratory and clinical research (Cavaliere, 2017). Over a decade ago John Biggers, a pioneer of IVF, highlighted a contradiction of human embryo law when he wrote "*public policy makers like to make the distinction between a procedure done for the benefit to the patient and a procedure done for research... the former is legal while the latter is not*" (Biggers, 2012). Guidelines that

restrict scientists from developing potentially life-saving medical treatments such as those that come from organoid technology is currently (2022) under review by legislators in Australia.

1.4.4 Application and ethics of CRISPR-Cas9 technology

We are now in the midst of another scientific revolution. The discovery of Crispr-CAS9 (clustered regularly interspaced short palindromic repeats -associated protein-9 nuclease), an anti-viral defence system in bacteria, provides a novel tool for researchers and scientists to accurately and effectively manipulate the embryonic genome of humans, plants and animals (Jinek et al., 2012). Although other gene editing methods have existed for many decades (Blelloch et al., 2004) the relative accuracy and improved usability of CRISPR-Cas9 have caused a rapid expansion of genome modification research (Whitworth et al., 2014) including therapeutic applications for the treatment of single gene diseases (Wu et al., 2020). Since the landmark research was published in 2012, policy makers have sought to regulate the use of gene editing technology in human embryos. Currently the *PHCR* and *RIHE* ban any changes to the genome of germline cells (heritable) to be made in human embryos for any reason including research or therapeutic purposes. The 2019 amendment to *The Gene Technology Act 2000* permits the use of gene editing technology in animals and human somatic cell lines with the provision that no novel genetic material is introduced (Mallapaty, 2019). This amendment allows researchers and scientists to make non-heritable modifications which are limited to the treated individual and are not inherited by future generations. For example, a single point mutation of an enzyme-producing gene in humans known as *Piz* causes a severe disease phenotype characterised by liver disease and lung impairment (Bjursell et al., 2018). The CRISPR-Cas9 system has been successfully applied to *Piz* expressing mice to correct the mutated allele and reverse the disease phenotype providing a potential therapeutic application for its use in gene therapy (Bjursell et al., 2018). Technical challenges of using CRISPR-Cas9

in gene therapy are unintended de novo mutations that can occur and off-target activities where the incorrect gene is modified (Zuccaro et al., 2020). Improved gene editing techniques and continued research have been suggested to mitigate these outcomes (Zuccaro et al., 2020) together with whole genome sequencing to detect mutations before administering gene-modified cells to patients (Atkins et al., 2021).

1.4.5 Legislation on gene editing of human embryos

Although there have been considerable advances in the technical application of CRISPR-Cas9, legislation in Australia has not been updated to allow research into the safety and efficacy of CRISPR-Cas9 on human embryos. Some overseas countries have legislation to allow licensed genome editing research to be conducted on donated human embryos but all modified embryos are to be discarded after 14 days. In Europe the *Convention on Human Rights and Biomedicine* allows gene editing research on embryos that are a product of IVF but prohibits the creation of human embryos for its use. Similarly the *Human Fertilization and Embryology Authority* in the UK permits gene editing research on donated human embryos. In China, human germline editing is currently not restricted by legislation and was the first country to have reported the use of CRISPR-Cas9 in human embryos (Liang et al., 2015) a concept that is largely discouraged by the medical research community (Adashi et al., 2020). In the United States, while the use of federal funding for human embryo research is banned, privately funded research on donated embryos is permitted. Australian law does not allow gene editing research on human embryos however a recent public opinion study in Australia reported that the majority of respondents supported gene therapy in general, and human embryonic stem cell research for health-related purposes (Monaghan et al., 2020).

There are already examples where embryo modification has been considered morally, socially and legally acceptable. IVF involves the creation of an embryo via the manipulation of human

gametes and embryos in culture medium. IVF is viewed as socially and morally acceptable and the Australian government provides financial support for infertility treatment. However current legislation restricting human embryo research differs to what activities are permitted in private practise in Australia. The culture composition, for example, can vary greatly between ART clinics and there is minimal standardisation (Dieamant et al., 2017) thus to some extent human embryo experimentation in IVF clinics is permitted. Consequently experimentation on human embryos both in research and the IVF clinic is potentially captured by current legislation.

1.4.6 Public engagement on embryo modification research

Research has just begun on public perception of genome editing in Australia, however, initial indications are that the public are not aware of its potential application in embryo research or for health purposes (McCaughey et al., 2019). A comparative survey of views towards IVF conducted in 2000, of six European countries including Australia and the USA, found fewer than half of respondents considered infertility to be a disease and less than a quarter knew the success rates of having a baby via IVF (Adashi et al., 2000). After participants were told about the high cost of infertility treatment most respondents agreed that IVF should be reimbursed by the government (Adashi et al., 2000). A more recent (2019) survey of 6,000 people reported the majority of respondents agreed that IVF is an ethical technology for the treatment of infertility and agreed IVF should be government funded for the first 2-3 cycles (Fauser et al., 2019). A public engagement event in the US was effective in educating a portion of the lay public on the topic of human gene editing (Rose et al., 2017). A pre- and post-survey was given to participants at a science festival panel discussion titled “*Designer Genes: Should We Be Able to Edit Our Genomes?*”. The authors reported that participants knowledge of gene

editing increased post-event and moral concern over its use was reported to have significantly decreased (Rose et al., 2017).

1.5 Conclusion and Aims of this study

There is considerable evidence that Pro uptake regulates cellular homeostasis and embryo viability. There is a lack of published research on effects of individual AAs in the fertilisation medium, even though it is well known that oocyte quality is a critical determinant of embryo development potential. The first aim of this study is to determine if oocytes take up Pro. I hypothesise that the addition of Pro to fertilisation medium will improve fertilisation rates and subsequent embryo development. The second aim of this study is to identify the preimplantation stage(s) when Pro is needed to improve subsequent embryo development, and to determine if AAT of Pro are expressed in the preimplantation embryo. Pro-mediated improvement in preimplantation development is prevented by molar excess Leu, Gly, and Pro itself (Morris et al., 2020) which are common substrates of B⁰AT1. I hypothesise that Pro is taken up by B⁰AT1 in cleavage-stage embryos to improve development. The third aim of this study is to determine if human oocytes which are unusable for IVF are suitable for parthenogenesis. I hypothesise that a simplified medium with Pro will improve the development of parthenogenetically-activated mouse embryos. The final aim of this study is to determine the opinion of at least a segment of the Australian public on the topic of embryo modification. Present legislation does not allow researchers to apply germline editing technology to human embryos for research purposes however initial reports indicate that the public would support this. I hypothesise that participants of public Q and A expert panel discussion on gene editing will agree to the use of embryo modification technology in research if it is used to benefit human health.

Chapter 2

In vitro Fertilisation of Mouse Oocytes in
L-Proline and L-Pipecolic Acid Improves
Subsequent Development

Article

In Vitro Fertilisation of Mouse Oocytes in L-Proline and L-Pipecolic Acid Improves Subsequent Development

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Abstract: Exposure of oocytes to specific amino acids during in vitro fertilisation (IVF) improves preimplantation embryo development. Embryos fertilised in medium with proline and its homologue pipecolic acid showed increased blastocyst formation and inner cell mass cell numbers compared to embryos fertilised in medium containing no amino acids, betaine, glycine, or histidine. The beneficial effect of proline was prevented by the addition of excess betaine, glycine, and histidine, indicating competitive inhibition of transport-mediated uptake. Expression of transporters of proline in oocytes was investigated by measuring the rate of uptake of radiolabelled proline in the presence of unlabelled amino acids. Three transporters were identified, one that was sodium-dependent, PROT (SLC6A7), and two others that were sodium-independent, PAT1 (SLC36A1) and PAT2 (SLC36A2). Immunofluorescent staining showed localisation of PROT in intracellular vesicles and limited expression in the plasma membrane, while PAT1 and PAT2 were both expressed in the plasma membrane. Proline and pipecolic acid reduced mitochondrial activity and reactive oxygen species in oocytes, and this may be responsible for their beneficial effect. Overall, our results indicate the importance of inclusion of specific amino acids in IVF medium and that consideration should be given to whether the addition of multiple amino acids prevents the action of beneficial amino acids.

Keywords: oocyte; amino acid transporters; proline; pipecolic acid; in vitro fertilisation



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1. Introduction

The presence of individual and groups of amino acids in embryo culture medium impacts preimplantation embryo development [1–8]. Much less is known, however, about the impact of individual amino acids on future development when they are exogenously added to oocytes during in vitro fertilisation (IVF). The addition of specific amino acids or groups of amino acids to the IVF medium can improve various aspects of subsequent development, including the percentage of embryos that reach the blastocyst stage and then hatch [9,10], indicating that amino acids can enter the oocyte in sufficient quantities during the period of fertilisation to exert effects on later embryo development.

The amino acid L-proline (Pro) acts in a growth-factor-like manner to stimulate preimplantation development [7]. When zygotes are cultured at low density (1 embryo/100 μ L, to eliminate the action of embryo-derived growth factors), the addition of Pro increases both the number of embryos reaching the blastocyst stage and their hatching by stimulating development within the late two-cell to eight-cell stages [7]. The addition of Pro also promotes differentiation and neural lineage commitment in embryonic stem cell (ESC) models of post-implantation development [11,12].

The mechanisms by which Pro stimulates pre- and post-implantation development are only partially understood. In embryos cultured at low density, Pro stimulates nuclear translocation of p-Akt^{S473} and p-ERK1/2^{T202/Y204}, and improvement in development is dependent on mTORC1 signalling (rapamycin sensitive) [3]. Activation of these signalling

pathways is also involved in the differentiation of ESCs [11,13,14]. Cellular handling of the production of this conditional amino acid is also involved: ESCs limit the endogenous production of Pro via a mechanism involving the amino acid response pathway, to the extent that differentiation is suppressed and ESC self-renewal maintained [15]. The exogenous addition of Pro overwhelms this suppressive mechanism. Pro also promotes changes to the epigenetic landscape of ESCs via enhanced histone methylation (at H3K9 and H3K36). The mechanism may involve direct or indirect inhibition of the Jumonji domain demethylases, which help control methylation status at these sites [16].

Attention has also turned to Pro's unique metabolism as a source of mechanisms controlling developmental fate [13,14,16–18]. Pro is metabolised via the Pro cycle to pyrroline-5-carboxylic acid (P5C) by proline oxidase (POX), and then to glutamate semi-aldehyde before being converted to glutamate (Glu) and α -ketoglutarate [17,19]. Thus, Pro metabolism can provide a number of intermediates important for cellular function, including the production of ATP via both α -ketoglutarate production and Pro-derived high-energy electrons entering the electron transport system [17].

Pro also has the ability to scavenge intracellular reactive oxygen species (ROS) [20]. Embryos with low metabolism, low glycolytic activity, low amino acid turnover, and high antioxidant levels are hypothesised to be the most viable [21–24], most likely because the embryo expends less energy and has lower production and leakage of superoxide free radicals from mitochondria [21,25,26]. H_2O_2 is another source of ROS, produced by superoxide dismutase conversion of the superoxide radical, especially during the G₂/M phase of cleavage-stage embryos [27–29]. High concentrations of ROS are detrimental to oocyte quality: Oocytes exposed to high levels of ROS *in vivo* have lower fertilisation rates, produce lower quality embryos, and present increased embryo arrest at the two-cell stage in the mouse [28,30].

The transport of Pro into cells occurs via specific amino acid transporters, specifically neutral amino acid transport systems, and are further characterised by their gene sequence homology in the solute carrier transporter (SLC) family. In the mouse oocyte, a number of amino acid transport systems have been identified (L, b⁰⁺, ASC, asc, B⁰, Gly, beta), one or more of which may be responsible for the uptake of Pro into the oocyte [31]. Uptake of Pro into cumulus cells surrounding the oocyte also occurs via y+LAT2 (SLC7A6), and thus Pro is transferred to the oocyte via gap junctions in the cumulus oocyte complex [32,33]. mRNA for the Na⁺-dependent Pro transporter PROT (SLC6A7) and the Na⁺/Cl[−]-dependent betaine/proline transporter SIT1 (SLC6A20) are present in the mouse oocyte, but it is not known if the transporters themselves are expressed and actively take up Pro [34]. After fertilisation, large amounts of Pro are taken up by SIT1, which is active in the zygote and two-cell stages [35]. Pro uptake in zygotes also involves an unidentified Na⁺-dependent betaine-resistant transporter [34,35]. In ESCs, Pro uptake is via the System A Na⁺-dependent SNAT2 transporter (SLC38A2) [14]. System A transporters are also the major transport system in the inner cell mass of blastocysts, but it is not known if SNAT2 is the principal contributor [36]. The different amino acid requirements at specific stages of oocyte and embryonic development reflect the dynamic physiology of the preimplantation embryo, including changes in metabolism and amino acid transporter expression.

L-pipecolic acid (PA) is a product of lysine metabolism and a homologue of Pro, having a six-membered piperidine ring rather than the five-membered pyrrolidine ring of Pro [20]. Both PA and Pro undergo oxidation in mitochondria and promote cell survival during oxidative stress [37]. It is not known if there is a transporter for PA in oocytes, but in somatic cells PA is transported by the high-affinity Pro transporter PROT [38–40] and SIT1 [41,42]. We hypothesised that because PA is structurally similar to Pro it would be taken up into the oocyte and be either metabolised or act as a ROS scavenger in a similar way to Pro, and thereby improve later embryo development [37,43].

This study investigated the effect of the addition of specific amino acids to IVF medium on subsequent preimplantation embryo development, with a particular focus on Pro and its analogue PA. We also examined the effect of molar excess of specific amino acids on uptake

of Pro to determine the transporter(s) responsible for uptake. Finally, we investigated metabolic mechanisms by which Pro and PA may impact oocyte development viability after IVF. Overall, this study shows that Pro and PA can improve preimplantation embryo development when added to IVF medium by reducing mitochondrial activity and ROS in the oocyte.

2. Materials and Methods

2.1. Animals (*Mus Musculus*)

Quackenbush Swiss (QS) mice (Animal Resource Centre, Perth and Lab Animal Services, University of Sydney) were housed under 12 h light:12 h dark conditions as described previously [44]. Experiments were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and approved by the University of Sydney Animal Ethics Committee as required by the NSW Animal Research Act (protocols 5583 and 824).

2.2. Isolation of Oocytes and Zygotes Fertilised In Vivo

Superovulation of female mice aged 4–6 weeks was achieved by intraperitoneal injection with 10 IU pregnant mares' serum gonadotropin (PMS; Intervet, Vic, Australia) followed after 48 ± 2 h by injection with 10 IU human chorionic gonadotropin (hCG; Intervet). To obtain zygotes fertilised in vivo, female mice were paired overnight with individually housed QS male mice (2–8 months of age) immediately following hCG injection. Mating was confirmed the following day by the presence of a vaginal plug. Mice were sacrificed by cervical dislocation at either 13–15 h post-hCG to obtain oocytes or 22 h post-hCG to obtain zygotes. Oocytes and zygotes were isolated into HEPES-buffered modified human tubal fluid (Hepes-modHTF) in which the concentration of NaCl was adjusted to 85 mM to give an osmolality of 270 mOsm/kg [7]. Cumulus cells were removed from zygotes and oocytes by treatment with 1 mg/mL hyaluronidase in HEPES-modHTF and then washed 3 times in HEPES-modHTF to remove remaining cumulus cells.

2.3. In Vitro Fertilisation of Oocytes and Culture of Embryos

In vitro fertilisation was performed in modified Whittingham's medium [45,46] which contained (in mM) NaCl (99.3), KCl (2.7), NaH_2PO_4 (0.36), $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (0.5), NaHCO_3 (25), Na pyruvate (0.5), glucose (5.5), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (1.8), Na lactate (18.7), and 30 mg/mL BSA. At least 4 cumulus masses containing oocytes were added to 500 μL pre-equilibrated (5% CO_2 at 37 °C) Whittingham's medium, containing the appropriate amino acid(s), overlaid with mineral oil (Sigma-Aldrich, NSW, Australia). Treatment groups were (i) no amino acids (no AA), and (ii) 0.4 mM L-proline (Pro) $\pm$ 5 mM His, Gly, betaine (Bet) or PA (as potential competitive inhibitors of the uptake of Pro). The concentration of Pro used in these experiments was based on the physiological concentration of fluid in the reproductive tract [47] and was shown to improve embryo development [7].

Sperm was isolated from the epididymis of a male QS mouse of proven fertility. An incision was made in the efferent ducts between the testis and the epididymis, and at the inferior end of the caudis epididymis. The dissected tissue was immediately washed, and blood vessels drained in PBS. The epididymides were placed into 500 μL Whittingham's medium at 37 °C and 5% CO_2 and the sperm gently squeezed out of the epididymides using forceps and allowed to capacitate for 1.5 h. At 13.5 h post-hCG, 10^6 sperm were added to each drop containing cumulus masses and incubated together for 3 h, after which fertilisation was determined by the presence of a polar body. Zygotes were washed through at least 3 drops of HEPES mod-HTF in the absence of amino acids and then cultured to the blastocyst stage (120 h) at low density (1 embryo/100 μL) in mod-HTF + 0.3 mg/mL BSA, containing no amino acids, at 37 °C in 5% CO_2 under mineral oil [7]. Embryos were scored for their developmental stage every 24 h. In vivo fertilised zygotes were also cultured under these conditions as a control.

2.4. Measurement of L-[³H]-Pro Uptake in Oocytes

Oocytes were incubated in 20 µL assay medium consisting of 1 µM L-[³H]-Pro (L-[2,3,4,5-³H]-proline; 1 mCi/mL; Perkin-Elmer, Vic, Australia, NET483001MC) in HEPES mod-HTF ± molar excess of individual unlabelled amino acids (5 mM for all amino acids except Pro, which was used at 0.4 mM). Unless otherwise stated, the L-isomer was used for all unlabelled amino acids and purchased from Sigma Aldrich, except L-Lys (Chem-Impex, IL, USA). In experiments using Na⁺-free HEPES mod-HTF, the Na⁺ was replaced with N-methyl-D-glucamine (NMDG) at 85 mM. At this low concentration NMDG is not known to affect Pro uptake by Na⁺-independent transporters.

An initial time course experiment was performed in which oocytes were incubated in L-[³H]-Pro for up to 120 min and samples collected at 20-min intervals to show that the uptake of L-[³H]-Pro remained linear over this time course (data not shown). In all subsequent experiments, oocytes in groups of 4 were incubated for 100 min at 37 °C in each treatment and then washed through at least 3 drops of cold (4 °C) HEPES mod-HTF, aspirated, and placed onto a gridded filter mat (Perkin-Elmer, Vic, Australia, #1450-421). The mat was placed inside a plastic sleeve and 4 mL ULTIMA GOLD scintillation fluid (Perkin-Elmer, Vic, Australia, #6013371) was added. The plastic sleeve was placed in a MicroBeta TriLux Plate Counter (Perkin-Elmer, Vic, Australia) and each sample was counted for 30 min. To determine the rate of L-[³H]-Pro uptake (fmol min^{−1} oocyte^{−1}), a standard curve was created for each experiment from standards serially diluted from a stock of 1 µM L-[³H]-Pro in HEPES mod-HTF and fitted by linear regression.

2.5. Immunostaining and Confocal Microscopy

Oocytes were fixed in 4% paraformaldehyde (PFA) for 30 min at room temperature, then washed 3 times in PBS + 1 mg/mL polyvinyl acid (PBS + PVA; Sigma-Aldrich, NSW, Australia), and permeabilised with PBS + PVA + 0.3% Triton X-100 for 30 min at room temperature. The oocytes were blocked by incubation in PBS + PVA + 0.1% Tween-20 + 0.7% BSA for 30 min at room temperature. Antibodies used were rabbit anti-SLC6A7/PROT (GeneTex, CA, USA #GTX51242), rabbit anti-SLC36A1/PAT1 [48], rabbit anti-SLC36A2/PAT2 [48], and Alexa Fluor 488-coupled goat anti-rabbit IgG (Invitrogen, Vic, Australia). Primary antibodies were diluted 1:500, and the secondary antibody 1:200, in PBS + PVA + 0.1% Tween-20 + 0.7% BSA. Samples were incubated in primary antibody for 2 h at room temperature or overnight at 4 °C and then washed 3 times in PBS + PVA + 0.1% Tween-20 + 0.7% BSA. Samples were incubated in the dark in secondary antibody for 1 h at room temperature and then washed 3 times in PBS + PVA + 0.1% Tween-20 + 0.7% BSA before being mounted in 3 µL Vectashield containing 1.5 µg/mL DAPI (Vector Laboratories, CA, USA) to visualise nuclei. Oocytes were imaged using confocal microscopy (LSM Meta 800, Carl Zeiss, Oberkochen, Germany) using 405 nm and 488 nm lasers and a 40× objective. Images were prepared using Fiji by Image J.

For analysis of cell numbers, blastocysts were fixed and permeabilised as described above and mounted in VECTASHIELD containing DAPI. Nuclei were visualised by confocal microscopy using a 405 nm laser. A Z-stack was taken through each blastocyst at 2.5 µm slices to allow for each nucleus to be imaged at least once. Cells in the inner cell mass and trophectoderm were counted manually using Image J. Cell numbers from each blastocyst were averaged.

2.6. Measurement of Mitochondrial Activity and Reactive Oxygen Species

Oocytes were cultured for 3 h in Whittingham's medium containing 0.4 mM Pro ± 5 mM PA Gly, Bet or His. Oocytes were then transferred to Whittingham's medium containing the above amino acid combinations to which 100 nM tetramethylrhodamine methyl ester (TMRM) and 10 µM 2',7'-dichlorofluorescein diacetate (DCFDA) were added. These were incubated for 30 min at 37 °C in 5% CO₂ to allow uptake of the dyes, then washed twice through 2 drops of Whittingham's medium, containing the amino acid combinations above, to remove excess dye. A 35 mm glass-bottomed petri dish was

prepared containing 10 μL drops of medium (under mineral oil) containing each of the treatment conditions $\pm 5 \mu\text{M}$ carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP) as a positive control for mitochondrial activity, or $\pm 60 \mu\text{M}$ hydrogen peroxide (H_2O_2) as a positive control for ROS, pre-equilibrated to 37°C and 5% CO_2 . Oocytes were added to drops and imaged using confocal microscopy (Carl Zeiss, Oberkochen, Germany, LSM Meta 800), with the chamber set to 37°C and 5% CO_2 for live-cell imaging, using a $20\times$ objective and 524 nm and 488 nm lasers. All oocytes were imaged within 4 h of isolation, which was equivalent to the timeline of amino acid treatment in the IVF experiments. Images were analysed using Fiji by Image J to obtain the integrated density of the stain, and the corrected total cell fluorescence was calculated using the following formula: Corrected total cell fluorescence = integrated density—(background fluorescence $\times$ mean area) [49].

2.7. Statistical Analyses

Oocyte/embryo culture experiments were performed a minimum of 3 times with at least 10 oocytes/embryos per treatment group. Development in the different treatment groups was compared by calculating the percentage of embryos that developed to the particular cell stage. Differences between groups were determined using 1-way ANOVA and Tukey's or Dunnett's multiple comparisons test as indicated in the figure legends.

L-[^3H]-Pro uptake experiments were performed at least 3 times with 4 embryos per treatment group. The uptake of L-[^3H]-Pro for each treatment was averaged and the difference between groups compared by 1-way ANOVA and Tukey's multiple comparisons test.

For fluorescence imaging, corrected total cell fluorescence was averaged from 3 independent experiments with a total of 28–71 embryos per treatment, and differences between treatments were determined by one-way ANOVA and Tukey's multiple comparisons test. Data analysis was performed using GraphPad Prism v7.

3. Results

3.1. IVF in the Presence of Pro and PA Improves Subsequent Embryo Development

Oocytes were fertilised for 3 h in the presence or absence of various amino acids, and then subsequently allowed to develop in vitro in the absence of any amino acids. In vitro development was performed at low density (1 embryo/100 μL) and, therefore, in the absence of autocrine support. The proportion of oocytes fertilised was not significantly affected by any amino acid treatment (No AA 45%, Pro 47%, PA 50%, Gly 45%, His 39%, Pro + PA 45%, Pro + Gly 45%, His + Pro 39%). However, addition of 0.4 mM Pro to the medium during IVF improved subsequent development as compared to oocytes fertilised in the absence of amino acids (Figure 1A–D). In particular, the percentage of embryos that had compacted at 72 h and developed to the blastocyst stage at 120 h following IVF in Pro increased compared to oocytes fertilised in the absence of AA. The presence of Pro during IVF also increased the number of ICM cells, with the number reaching levels seen in blastocysts developed from in vivo fertilised oocytes (Figure 1E). IVF in the presence of Pro had no effect on trophectoderm cell number in blastocysts (Figure 1F).

PA is transported by some of the transporters of Pro and may, therefore, compete for and inhibit cellular uptake of Pro by those transporters. Thus, it was hypothesised that the presence of PA in molar excess (5 mM) over Pro (0.4 mM) during IVF would inhibit the effects of Pro. Instead, addition of PA alone to medium during IVF produced results similar to those observed for Pro alone (Figure 1A,E,F), increasing percent development to the compacted and blastocyst stages and increasing ICM cell numbers in blastocysts. The combination of PA with Pro during IVF had no additive or synergistic effects (Figure 1A,E).

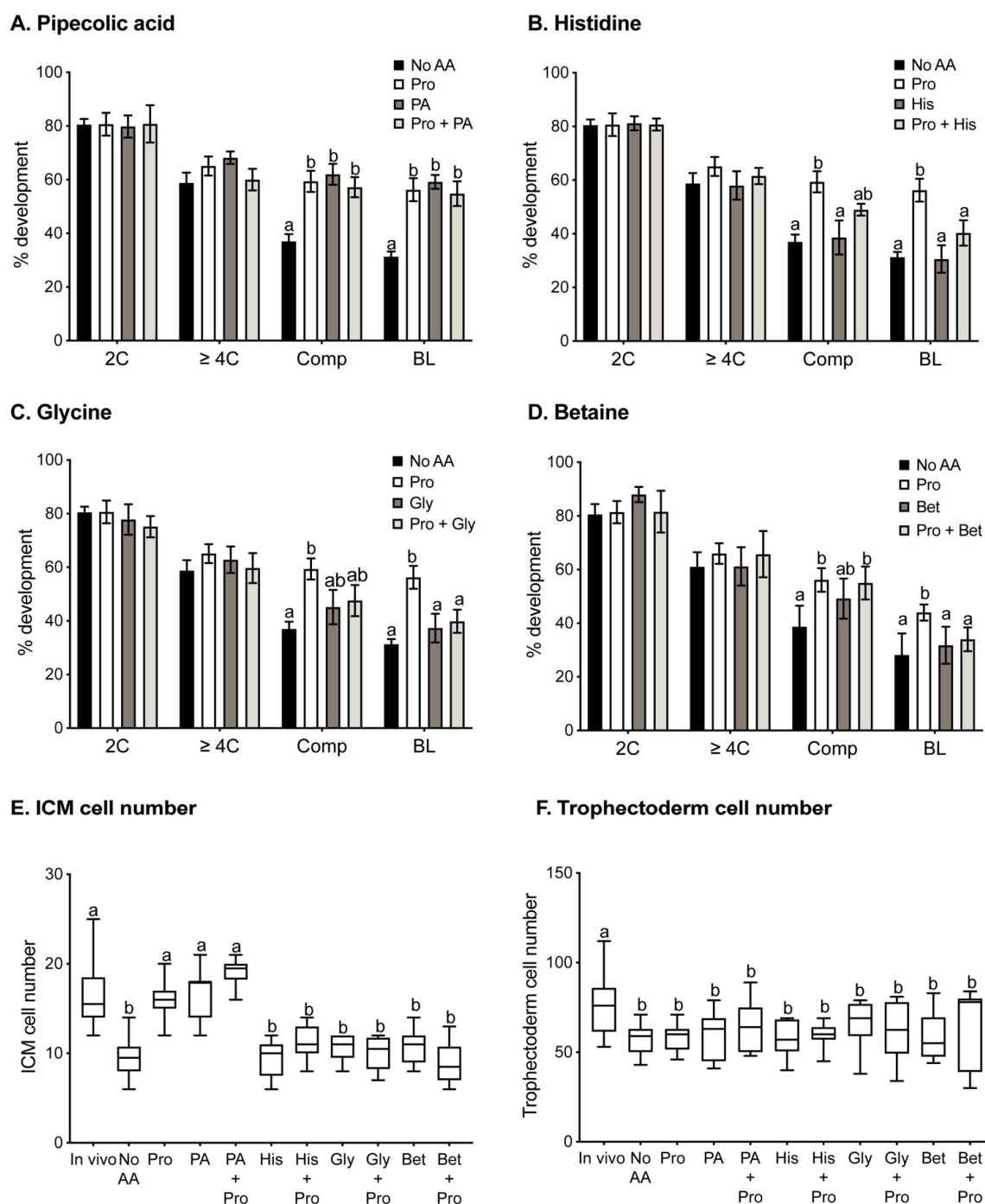


Figure 1. Preimplantation development following IVF in the presence of various amino acids. Fertilisation was performed in the absence of amino acids (No AA) or the presence of 0.4 mM Pro alone or 0.4 mM Pro in the presence of (A) 5 mM PA, (B) 5 mM His, (C) 5 mM Gly, or (D) 5 mM Bet. Following fertilisation, zygotes were cultured in mod-HTF in the absence of added amino acids for 120 h, and the percentages of embryos at the 2-cell (24 h), ≥ 4 -cell (48 h), compacted (72 h), and blastocyst (120 h) stages were recorded. Bars represent mean $\pm$ SEM obtained from 3 independent experiments with a minimum of 10 zygotes per treatment group in each experiment. Blastocysts were fixed and stained with DAPI, and ICM (E) and trophectoderm (F) cell numbers were counted. Whiskers indicate highest and lowest values, and the mean is represented by the centre line in the box. Data were analysed using GraphPad Prism using 1-way ANOVA with Tukey's multiple comparisons test (A–D) or Dunnett's multiple comparison test (E,F). Bars sharing the same letter are not significantly different ($p > 0.05$).

3.2. Gly, Bet and His Inhibit Pro-Mediated Improvement in Development after IVF

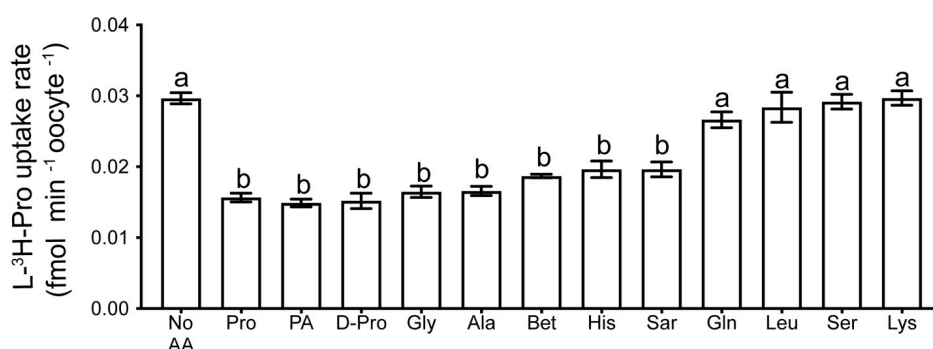
The effect of other potential competitors of Pro uptake during IVF was also examined. When 5 mM Gly, Bet, or His were added individually to the IVF medium, none had any effect on embryo development compared to IVF undertaken in the absence of added amino acids (Figure 1B–F). However, each of these amino acids inhibited the effects observed with Pro (Figure 1B–E), indicating competition for transport of Pro into the oocyte.

3.3. Transport of Pro into Oocytes Has Na^+ -Dependent and Na^+ -Independent Components

A number of amino acid transporters are expressed in the mouse oocyte [31]. To determine which of these transporters is responsible for the transport of Pro, we examined the rate of 1 μM L-[^3H]-Pro uptake in the presence of a range of unlabelled amino acids in molar excess. Excess Pro itself was used to show the proportion of L-[^3H]-Pro uptake that was saturable (i.e., due to a transporter). Common L-amino acids and D-Pro were chosen based on the preferred substrates of known Pro transporters [38]. Trp was chosen as a known inhibitor of specific Na^+ -independent transporters [50,51].

We found uptake of Pro involves both Na^+ -dependent (Figure 2A) and Na^+ -independent (Figure 2B) transport mechanisms. In Na^+ -containing medium, Pro, D-Pro, PA, Gly, Ala, Bet, His, and sarcosine (Sar) all inhibited the uptake of L-[^3H]-Pro ($p < 0.01$). Lys, Leu, Ser, and Gln did not decrease the L-[^3H]-Pro rate of uptake. In Na^+ -free medium, D-Pro, Gly, Trp, Bet, Pro, and PA inhibited the L-[^3H]-Pro rate of uptake ($p < 0.05$), whereas His and Leu had no effect.

A. Na^+ present



B. Na^+ free

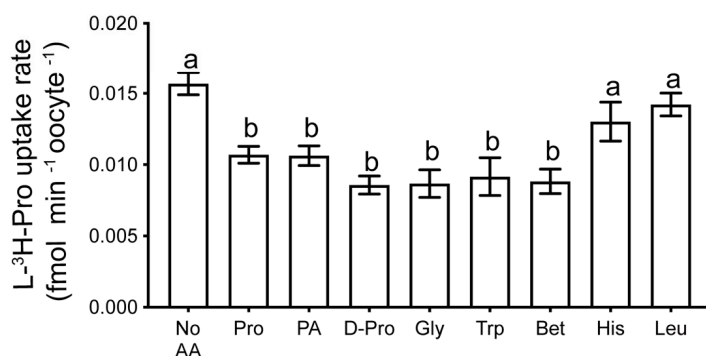


Figure 2. Uptake of L-[^3H]-Pro by oocytes in the presence of other amino acids. Rate of uptake of 1 μM L-[^3H]-Pro in (A) mod-HTF containing Na^+ or (B) Na^+ -free mod-HTF in the presence of unlabelled competing amino acids. The concentration of all competitors was 5 mM, with the exception of Pro (0.4 mM). Each bar represents the mean $\pm$ SEM from at least 3 independent experiments, each with treatments performed in triplicates of 4 oocytes. Data were analysed using 1-way ANOVA with Tukey's post hoc test. Bars with different letters are significantly different ($p < 0.05$). Sar: sarcosine.

3.4. PROT and PAT1/2 Are Expressed in Oocytes

We investigated the expression of three Pro transporters with similar amino acid uptake profiles to what we observed in the oocyte. Expression of PROT, PAT1, and PAT2 was examined by immunofluorescent staining. SLC6A7 (PROT) displayed puncta across the cytoplasm as well as some plasma-membrane staining over the location of the metaphase II spindle (Figure 3). This indicates that PROT is involved in both membrane and vesicular transport of Pro. It is likely that expression of PROT in vesicles contributes to Pro accumulation and/or signalling processes important for oocyte competency. For SLC36A1 (PAT1) and SLC36A2 (PAT2), staining was detected in the plasma membrane as well as in sub-cortical puncta (Figure 3). No staining was detected with the IgG control.

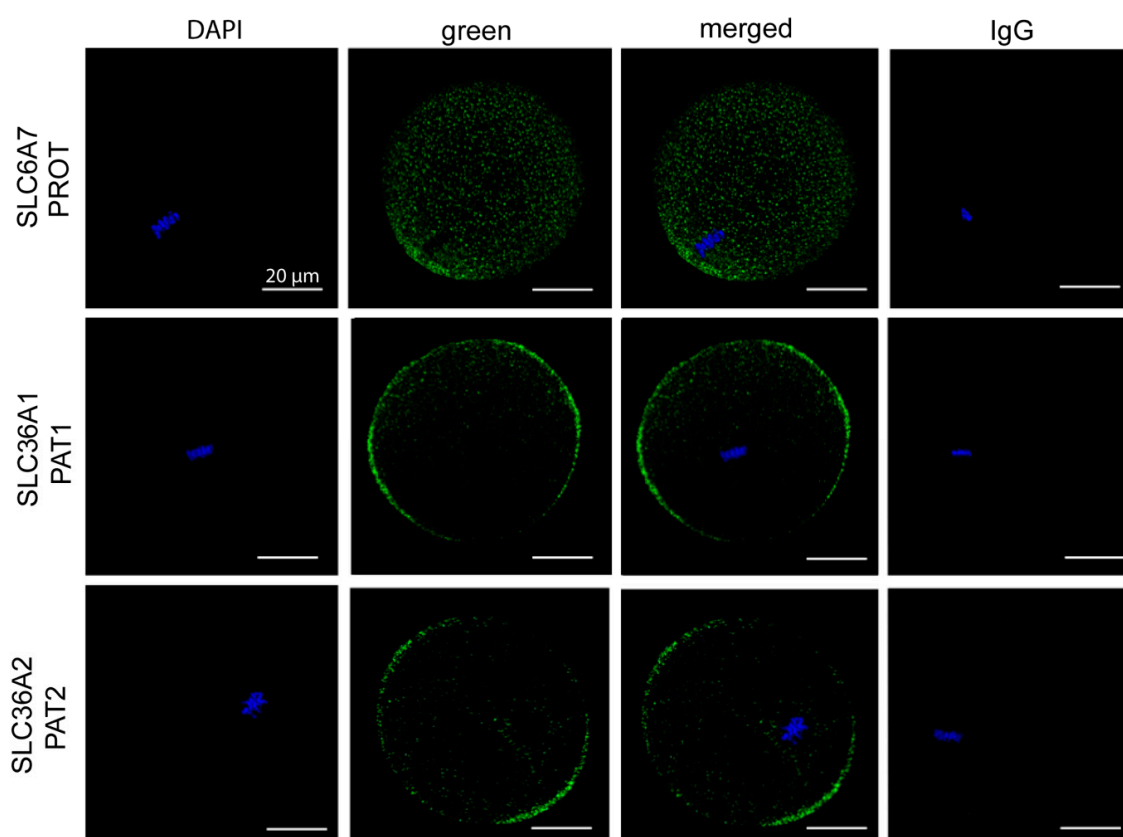


Figure 3. Expression of Pro transporters PROT, PAT1, and PAT2 in mouse oocytes. Oocytes were freshly isolated at 13–15 h post-hCG and exposed to Pro (0.4 mM) for 100 min, fixed, and immunostained for SLC6A7 (PROT), SLC36A1 (PAT1), and SLC36A2 (PAT2) (green). Metaphase chromosomes were counterstained with DAPI. Oocytes were analysed by confocal microscopy using a 40× objective. Images are representative of 6 oocytes taken from 3 independent experiments. A pre-immune IgG equivalent was used as a negative control for the primary antibody. Scale bar = 20 µm for all images.

3.5. Pro and PA Significantly Reduce Mitochondrial Activity in Oocytes

Reduced metabolism has been linked to improved embryo viability [21]. We hypothesised that the presence of Pro or PA during IVF would improve embryo development by decreasing metabolism in the oocyte. Mitochondrial membrane potential is used as a measure of mitochondrial activity and therefore the metabolic activity of cells [21]. Thus, to determine if Pro, PA, or other amino acids were affecting metabolic activity, we examined mitochondrial membrane potential using the potentiometric indicator TMRM in oocytes cultured in medium containing specific amino acids for 3 h. The presence of Pro or PA in the medium reduced TMRM fluorescence compared to the no AA control (Figure 4A,B). The presence of both Pro and PA did not have an additive effect. Gly and His alone had no effect on TMRM fluorescence, while Bet reduced TMRM fluorescence, but not to the same

extent as Pro or PA. Gly, Bet, and His all prevented the Pro-induced decrease in TMRM fluorescence. FCCP uncouples mitochondrial oxidative phosphorylation by interfering with the transport of protons across the mitochondrial membrane. As expected, FCCP decreased TMRM fluorescence and mitochondrial activity (Figure 4D).

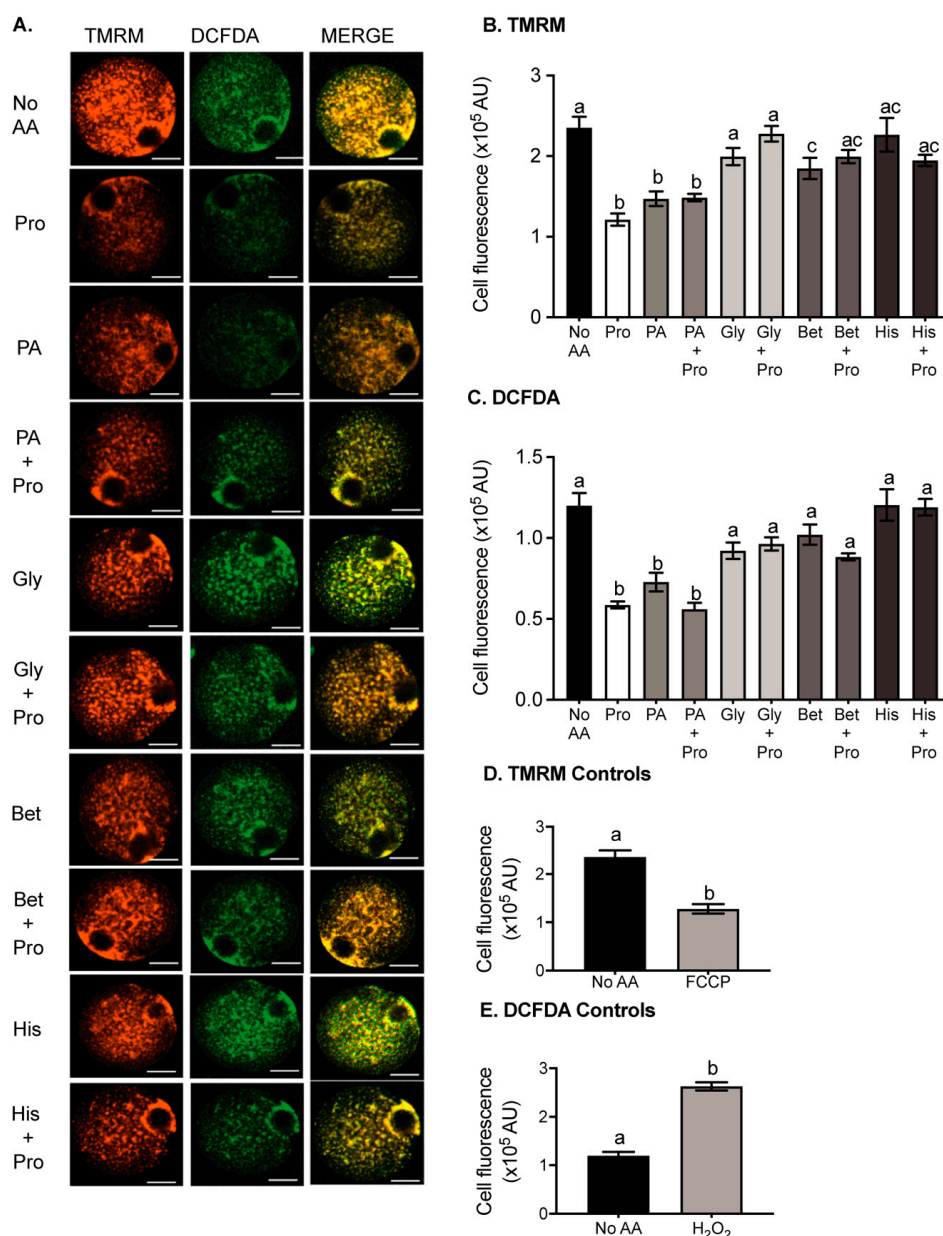


Figure 4. The effect of amino acids on ROS production and mitochondrial activity in oocytes. Oocytes were incubated for 3 h in medium containing either no amino acids (no AA) or 0.4 mM Pro or 0.4 mM PA, Bet, His, or Gly $\pm$ 0.4 mM Pro. Oocytes were then loaded with DCFDA for ROS, and TMRM for mitochondrial activity, and imaged by confocal microscopy. (A) Representative fluorescent images of oocytes in each condition. Quantified cell fluorescence for (B) TMRM and (C) DCFDA. Bars represent mean $\pm$ SEM for 28–71 oocytes, obtained from at least 3 independent experiments. Control oocytes stained with (D) TMRM and (E) DCFDA were transferred to a 10 μ L medium containing 60 μ M H₂O₂ or 5 μ M carbonyl cyanide-p-trifluoromethoxyphenylhydrazine (FCCP) before imaging. Scale bar = 20 μ m for all images. Data were compared by 1-way ANOVA with Tukey's multiple comparisons test. Bars not sharing the same letter are significantly different ($p < 0.05$).

3.6. Pro and PA Significantly Reduce Reactive Oxygen Species in Oocytes

ROS accumulation is detrimental to fertilisation and embryo development [28,30]. Since Pro and PA can act as ROS scavengers [20,37], we investigated whether they were performing this role in the oocyte. ROS were measured in oocytes using DCFDA, which is oxidized by ROS, increasing fluorescence of the dye [52]. The presence of Pro or PA in the medium reduced DCFDA fluorescence in oocytes reflecting a decrease in ROS in comparison to the no AA control (Figure 4A,C). The presence of Gly, Bet, and His alone did not affect DCFDA fluorescence but prevented the Pro-dependent decrease in fluorescence. The positive control, H_2O_2 , caused a large increase in ROS, as expected (Figure 4E).

4. Discussion

In this study, we exposed oocytes to individual amino acids in fertilisation medium to determine if any subsequently improved preimplantation development. In the reproductive tract, oocytes are bathed in oviductal fluid, which contains amino acids and other factors that contribute to normal sperm capacitation, oocyte fertilisation, and preimplantation development. When the oocyte is fertilised outside of its physiological environment, its development potential is significantly impacted by the components of medium [53]. The amino acid composition of commercially available media varies [54]. Although it is known that individual amino acids can benefit embryo development [7,55,56], little is known about how the presence of specific amino acids during fertilisation might impact on later embryo development, and this was therefore the focus of this study.

Fertilisation of oocytes was not significantly affected by supplementing fertilisation medium with any of the amino acids tested (Bet, Gly, His, Pro, and PA). Similarly, the addition of 19 common amino acids together does not increase the proportion of oocytes fertilised when IVF is performed on bovine oocytes [57]. This suggests that amino acids do not impact either the sperm–egg interaction or fertilisation processes occurring after sperm–oocyte fusion. However, the presence of Pro or PA during IVF (followed by their removal) resulted in an increase in the percentage of compacted embryos and blastocysts and inner cell mass cell numbers, suggesting improved embryo viability. IVF in the presence of Bet, Gly, or His did not improve embryo development by these criteria, but they did inhibit the improvements mediated by Pro or PA, suggesting that Bet, Gly, and His compete for uptake of Pro by the same amino acid transporter. These results are consistent with a growing body of evidence showing that competitive inhibition of uptake prevents the beneficial effects that selected amino acids have on various stages of the development process [7,8,11,14,34,35].

In somatic cells there are multiple transporters for Pro including SIT1, PAT1 and 2, PROT, SNAT2, B^0AT1 and 2, NTT4, ASC1, y+LAT2, and GLYT1 [31,34,38,50,58–61]. Of these, SIT1 [34,35], GLYT1 [1,62,63], B^0AT2 [64], PROT [35], and SNAT2 [65] have been described in mouse embryo developmental stages after fertilisation. In oocytes, both Na^+ -dependent (~50% of transport) and Na^+ -independent (~30% of transport) Pro transport were observed. The Bet/Pro transporter, SIT1, can be eliminated as a candidate for Pro uptake since it is not active until after fertilisation [34]. Similarly, y+LAT2 can be eliminated because it is only expressed in cumulus cells [32,33]. Using competitors for L-[3H]-Pro uptake, it was also possible to eliminate a number of other transporters as candidates for Pro uptake in the oocyte: B^0AT1 , B^0AT2 , and NTT4 can be eliminated because Leu did not prevent Na^+ -dependent Pro uptake [14,58,60,61]. We can also eliminate the ASC/asc transporter family and SNAT2 since Ser did not reduce Pro uptake [14,31,66]. GLYT1 is located in the oocyte plasma membrane and actively transports Gly at fertilisation and throughout preimplantation development [1,62,67]. As GLYT1 substrates Gly and Sar both reduced Pro uptake, we cannot rule out the possibility that GLYT1 takes up Pro and PA in oocytes. However, its weak affinity for Pro makes it unlikely to be the main contributor [63]. It should also be noted that some transporters in oocytes and early embryos have characteristics that differ from the corresponding cloned transporters [64,65]. However, based on the available evidence, this leaves PROT, which is Na^+ -dependent,

and PAT1 and PAT2, which are Na⁺-independent, as candidate transporters for Pro in the oocyte [68].

PROT is a good candidate for Pro transport in the oocyte as it is a high-affinity Na⁺-dependent Pro transporter which also transports PA, Sar, and His [69]. Immunostaining for PROT in oocytes showed localisation in cytoplasmic vesicles and limited plasma membrane staining concentrated near the MII chromosomes. In the central nervous system PROT takes up Pro for normal brain function, memory, and locomotor activity [67]. PROT-null mice exhibit brain dysfunction but have no reported fertility or embryological defects suggesting that there is Pro transporter redundancy [70]. It is also likely that compensatory mechanisms are activated when important genes are knocked out that are needed for normal development, as may be the case for PROT. For the Na⁺-independent transporters PAT1 and PAT2, the substrates D-Pro, Gly, and Trp [50,71] all prevented Pro uptake. In addition, immunostaining for both PAT1 and PAT2 showed both are located in the plasma membrane of oocytes. Gly and Ala are found in high concentrations in oviductal fluid which would compete with the uptake of Pro, so it is unlikely that PAT1/2 are the only functional transporters of Pro in vivo [47,72]. Future studies could investigate whether the observed inhibition of Pro transport by the relatively high concentrations of Gly and Ala used in this study was competitive or non-competitive. Our data, therefore, suggest there are multiple transporters of Pro in the oocyte with overlapping substrate specificities. Under in vitro conditions, the relative activity of each of these transporters in oocytes will depend on the amino acid composition of the medium. In particular, the beneficial effects on later embryo development through the exogenous addition of Pro during fertilisation might be mitigated or eliminated through inappropriate addition of other amino acids which reduce its uptake.

Pro and PA may have an immediate impact during IVF by reducing mitochondrial activity and ROS levels in the oocyte. Pro acts as an antioxidant and cryoprotectant by reducing ROS and mitochondrial activity in vitrified mouse oocytes [73]. Amino acids such as Pro which contain a secondary amine group have a lower ionizing potential than those with only a primary amine group, enabling them to easily donate electrons and quench ROS by stabilisation of the free radical [20,74]. The structurally similar PA appears to perform the same functions, acting as a scavenger of ROS and preventing their build-up in the oocyte. Gly, Bet, and His do not have a secondary amine group and were not able to reduce ROS. Instead, all three prevented the Pro-mediated reduction in ROS due to their competitive inhibition of Pro uptake via PAT1/2 and/or GLYT1. This scavenging mechanism is one potential pathway by which Pro and PA reduce ROS in the oocyte.

Both Pro and PA reduced mitochondrial activity in the oocyte. Our data therefore support the theory that metabolically quiet oocytes and embryos have improved viability [21,22]. Metabolism of Pro by proline oxidase produces P5C, which is converted to Glu. Glu can then be either converted to α -ketoglutarate, which enters the TCA cycle, or combine with cysteine and glycine to form the antioxidant glutathione [18,75]. Since the presence of Pro caused a decrease in mitochondrial activity, our data suggest that Pro metabolism does not feed significantly into the TCA cycle. It did not feed high-energy electrons into the ETC, but instead increased glutathione (GSH) production. Pro-mediated increases in GSH would also contribute to reduced ROS [20,76], as has been shown in boar sperm [76] and in-vitro cultured mouse embryos (unpublished data). Alternately, the Pro-induced reduction in mitochondrial activity indicates reduced ETC activity, which may be the mechanism by which Pro reduces ROS. Although the exact mechanism by which Pro reduces mitochondrial activity has not yet been identified, a Pro-induced simultaneous reduction of mitochondrial activity and ROS levels has been observed in other cells [77].

PA is metabolised similarly to Pro, whereby it is broken down by pipecolate oxidase to piperideine-6-carboxylate (P6C) and then to α -amino adipic semialdehyde [37], which can result in increased production of glutamate and thus potentially GSH [78]. As Pro and PA both improve embryo development after IVF and decrease ROS and mitochondrial

activity, we suggest that increased GSH due to Pro and PA metabolism may be one of the mechanisms responsible for their beneficial effect on later embryo development.

There are, however, important differences in development when Pro is added at the time of fertilisation compared to when it is added to already fertilised oocytes [7]. In the latter case, Pro (i) first improves embryo development during cleavage stages (late two-cell to eight-cell) instead of from compaction, and (ii) has no effect on blastocyst cell numbers [7]. This implies that the Pro-mediated mechanisms required to ‘set up’ improvement in later development are importantly different between these two scenarios and are dependent on the timing of the exogenous addition of this amino acid. Nevertheless, the molecular mechanisms at play when Pro is added during fertilisation may well also include one or more of those established for Pro in related work [7,11,15,16,79]. Finally, Pro added during fertilisation in vitro increased the number of inner cell mass cells in blastocysts to the level seen for blastocysts derived from cultured zygotes fertilised in vivo. However, trophoblast cell numbers were not similarly increased. This is consistent with mouse studies showing that IVF reduces trophoblast cell numbers and affects subsequent placental development due to alterations in gene expression including the downregulation of SLC genes [80,81]. This indicates that one or more factors are still missing in the in vitro fertilisation environment.

In conclusion, Pro and PA are beneficial to preimplantation embryo development when added to the fertilisation medium. Pro and PA reduce mitochondrial activity and ROS in oocytes. The reduction in ROS by Pro and PA may be due to one or more of a variety of mechanisms: (i) The reduction in mitochondrial activity per se, (ii) the direct scavenging of ROS by these molecules, and (iii) their metabolism leading to increased production of GSH. We suggest that these may be some of the mechanisms responsible for the beneficial effect of Pro and PA. The beneficial actions of Pro are prevented when other amino acids are present in the IVF medium due to competitive uptake by amino acid transporters. Very little attention has been given to the amino acid composition of media used during fertilisation; rather, research has focused on culture media used during embryo development. This study shows that specific amino acids present during the process of fertilisation have impacts on later development. Indeed, the composition of currently used commercial IVF media may prevent the beneficial effect of Pro by oversupplying amino acids which prevent Pro uptake. We suggest a simplified fertilisation medium that contains key elements required to drive development in vivo that is a favourable strategy for promoting oocyte competency and subsequent embryo viability in vitro.

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Conflicts of Interest: The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Abbreviations

IVF	In vitro fertilisation
ESC	Embryonic stem cells
GSH	Glutathione
P5C	Pyrroline-5-carboxylic acid
P6C	Piperidine-6-carboxylate
PA	Pipecolic acid
POX	Proline oxidase
ROS	Reactive oxygen species
SLC	Solute carrier transporter

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Chapter 3

Stage-Specific L-Proline Uptake by Amino
Acid Transporter Slc6a19/B⁰AT1 Is Required
for Optimal Preimplantation Embryo
Development in Mice

Article

Stage-Specific L-Proline Uptake by Amino Acid Transporter Slc6a19/B⁰AT1 Is Required for Optimal Preimplantation Embryo Development in Mice

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Abstract: L-proline (Pro) has previously been shown to support normal development of mouse embryos. Recently we have shown that Pro improves subsequent embryo development when added to fertilisation medium during in vitro fertilisation of mouse oocytes. The mechanisms by which Pro improves embryo development are still being elucidated but likely involve signalling pathways that have been observed in Pro-mediated differentiation of mouse embryonic stem cells. In this study, we show that B⁰AT1, a neutral amino acid transporter that accepts Pro, is expressed in mouse preimplantation embryos, along with the accessory protein ACE2. B⁰AT1 knockout (*Slc6a19*^{−/−}) mice have decreased fertility, in terms of litter size and preimplantation embryo development in vitro. In embryos from wild-type (WT) mice, excess unlabelled Pro inhibited radiolabelled Pro uptake in oocytes and 4–8-cell stage embryos. Radiolabelled Pro uptake was reduced in 4–8-cell stage embryos, but not in oocytes, from *Slc6a19*^{−/−} mice compared to those from WT mice. Other B⁰AT1 substrates, such as alanine and leucine, reduced uptake of Pro in WT but not in B⁰AT1 knockout embryos. Addition of Pro to culture medium improved embryo development. In WT embryos, Pro increased development to the cavitation stage (on day 4); whereas in B⁰AT1 knockout embryos Pro improved development to the 5–8-cell (day 3) and blastocyst stages (day 6) but not at cavitation (day 4), suggesting B⁰AT1 is the main contributor to Pro uptake on day 4 of development. Our results highlight transporter redundancy in the preimplantation embryo.

Keywords: proline; amino acid transporter; *Slc6a19*; B⁰AT1; preimplantation embryo; mouse



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1. Introduction

Amino acids (AAs) play important roles in mammalian preimplantation development [1–4]. Reproductive fluid in the oviduct and uterus contains a mixture of all essential (EAA) and non-essential AAs (NEAA) which support embryo development from fertilisation to implantation [5,6]. During in vitro culture of mouse embryos, addition of NEAAs to the medium promotes cleavage to the 8-cell stage [1,7] whereas addition of EAAs has no effect until after the 8-cell stage, when they promote an increased number of cells in the inner cell mass (ICM) [1].

Some AAs, such as alanine, glycine, glutamine and taurine, are present at millimolar concentrations in reproductive tract fluid [5] and can be accumulated to the same or higher

concentrations within the developing embryo to prevent cell volume changes caused by the high osmolality (300–310 mOsm/kg) of reproductive fluid [8]. In addition to their role as organic osmolytes, AAs are also important for protein synthesis [9], as an energy source for production of ATP [10], for buffering of intracellular pH, and regulation of reactive oxygen species (ROS) either by use in the production of glutathione or by direct ROS scavenging [11]. L-proline (Pro), for example, is able to directly scavenge ROS and when added to culture medium (in the absence of all other AAs), at a concentration similar to that found in reproductive fluid [6], improves mouse preimplantation embryo development [4]. Recently, we have shown that adding Pro and/or its analogue pipecolic acid to fertilisation medium during in vitro fertilisation (IVF) of oocytes increases subsequent embryo development, blastocyst formation and the number of cells in the ICM [12]. The improvement in development induced by Pro may be attributed to a reduction of mitochondrial activity and ROS levels in oocytes [12]. Collectively, these and other results show that Pro (and its metabolites) act in a growth factor-like manner by a variety of interconnected mechanisms including signal transduction activation, regulation of ROS levels and mitochondrial activity, and modulation of the epigenetic landscape as shown either in embryos themselves [4,12] or using embryonic stem (ES) cells as an in vitro model of embryo development [13–16].

For AAs to have a beneficial effect on preimplantation development they must be taken up into the embryo via AA transporters (AATs). During early embryo development, AATs are expressed in a stage-specific manner reflecting, in part, the changing nutritional needs of the developing embryo. For example, there are multiple transporters for Pro in the preimplantation embryo, which are expressed at various stages. In the cumulus oocyte complexes (COCs), Pro can be taken up by cumulus cells surrounding the oocyte [17] via y+LAT2 (*Slc7a6*) [18] and GlyT (*Slc6a5* and/or *Slc6a9*) [19] and Pro is then transferred to the oocyte via transzonal gap junctions. In oocytes themselves, Pro is taken up by one or more transporters, including GlyT1 (*Slc6a9*) [19,20], PROT (*Slc6a7*), and PAT1 and/or 2 (*Slc36a1*, *Slc36a2*) [12]. After fertilisation, Pro is transported via SIT1 (*Slc6a20*), which is active in the zygote and 2-cell stage [21], as well as by GlyT1, which is active in 8-cell and blastocyst stages [20]. System A transporters are the major transport system in the ICM [22]. In mouse ES cells, Pro uptake by SNAT2 (*Slc38a2*) promotes differentiation [13,23].

Improved development of zygotes to the blastocyst stage induced by Pro in culture medium is prevented by the addition of a molar excess of glycine (Gly), betaine (Bet) and leucine (Leu) [4]. This profile for AA transport is consistent with that for the transporter B⁰AT1 (*Slc6a19*). In somatic cells, B⁰AT1 is expressed in the kidney, intestine, colon and prostate, and is a major transporter of neutral AAs, including Pro, across the plasma membrane [24]. Autosomal recessive inheritance of *SLC6A19* mutations in humans leads to poor absorption of AAs from the gut, or poor retention of AAs by the kidneys, causing Hartnup disorder [24,25]. Mice in which *Slc6a19* has been knocked out (*Slc6a19*^{−/−}) [26] exhibit symptoms of neutral aminoaciduria characteristic of Hartnup disorder. However, the effect of the loss of B⁰AT1 expression on reproductive capacity and in vitro development of embryos lacking B⁰AT1 has not been reported.

In this study, we therefore investigated whether B⁰AT1 is expressed during mouse preimplantation embryo development, along with known accessory proteins, and examined the role of B⁰AT1 in fertility and embryo development by utilising B⁰AT1 knockout (*Slc6a19*^{−/−}) mice. Our findings show that B⁰AT1 is expressed throughout preimplantation development. The accessory protein ACE2 was also expressed, while its paralogue collectrin (TMEM27) was not present at any pre-implantation stage. *Slc6a19*^{−/−} mice had decreased litter size compared to wild-type (WT). In addition, the development of embryos obtained from *Slc6a19*^{−/−} mice to the blastocyst stage was decreased compared to embryos from WT mice, suggesting an important role for B⁰AT1 in normal embryo development.

2. Methods

2.1. Animals (*Mus musculus*)

Generation N8 *Slc6a19*^{−/−} mice [26] were fully backcrossed to N10 on C57Bl/6 background (Centenary Institute). Wild-type C57Bl/6 mice were obtained from Australian BioResources. Outbred Quackenbush Swiss (QS) mice (Animal Resource Centre, Perth, WA, Australia and Lab Animal Services, The University of Sydney, NSW, Australia) were housed under a 12 h light: 12 h dark cycle and experiments were performed under the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and in accordance with the University of Sydney Animal Ethics Committee, as required by the NSW Animal Research Act (5583 and 824) and by Sydney Local Health District Animal Ethics Committee (2016-031).

2.2. Isolation of Oocytes and Embryos

Female mice (4–8 weeks of age) were superovulated by intraperitoneal injection of 10 IU pregnant mare serum gonadotropin (PMSG; Intervet, Vic., Australia) followed by 10 IU human chorionic gonadotropin (hCG; Intervet) 48 ± 2 h later. Immediately after hCG injection, female mice were individually housed and paired with QS, C57BL6 (WT) or *Slc6a19*^{−/−} male mice (2–8 months of age). *Slc6a19*^{−/−} embryos were obtained by mating *Slc6a19*^{−/−} female with *Slc6a19*^{−/−} male mice. Female mice were sacrificed by cervical dislocation at either 13, 24, 46, 56, 68, 80 or 92 h post-hCG to obtain oocytes, zygotes, 2-, 4-, 5–8-cell or morula stage embryos or blastocysts, respectively. Unfertilised oocytes and embryos were dissected from the oviduct or flushed from the reproductive tract into HEPES-buffered modified human tubal fluid (HEPES-modHTF). Bovine serum albumin (BSA; 0.3 mg/mL) was added to all media at a reduced amount to minimise the possibility of BSA breakdown into free AAs in the media. The concentration of NaCl in media was adjusted to give an osmolality of 270 mOsm/kg, as described previously [4]. Cumulus cells were removed by gently pipetting oocytes with a fine glass pipette for 1 min in HEPES-modHTF + 1 mg/mL hyaluronidase, followed by washing oocytes through three drops of modHTF + 0.3 mg/mL BSA. Denuded oocytes and embryos were allocated for embryo culture, L-[³H]-Pro uptake or immunostaining experiments.

2.3. Culture of Zygotes from WT and *Slc6a19*^{−/−} Mice In Vitro

Freshly isolated zygotes (day 1) were washed through at least three drops of HEPES-modHTF in the absence of AAs and then cultured in 96-well plates (Corning, NY, USA) to the blastocyst stage (120 h, day 6) at low density (1 embryo/100 µL) in modHTF + 0.3 mg/mL BSA ± 0.4 mM Pro at 37 °C in humidified air with 5% CO₂ under mineral oil. Embryos were scored for developmental stage every 24 h. The concentration of Pro used was previously shown to improve in vitro embryo development [4] and is similar to its physiological concentration in fluid of the reproductive tract [6]. Culture experiments were repeated 4–6 times with 5–21 embryos per treatment group per experiment.

2.4. Measurement of L-[³H]-Pro Uptake in Oocytes and Embryos

To measure the uptake of L-[³H]-Pro from medium, groups of 1–4 freshly isolated oocytes or embryos were allocated to 20 µL drops containing 1 µM L-[³H]-Pro (L-[2,3,4,5-³H]-proline (1 mCi/mL; Perkin-Elmer, Vic., Australia, NET483001MC) in HEPES-modHTF ± molar excess unlabelled AAs (5 mM for all AAs except Pro, which was used at 0.4 mM) covered with mineral oil and incubated for 60 min at 37 °C in humidified air with 5% CO₂. The L-isomer was used for all unlabelled chiral AAs (Sigma Aldrich, St. Louis, MO, USA). A time-course experiment was previously performed to show L-[³H]-Pro uptake was linear for at least 60 min (data not shown; [12]). After incubation in each treatment, oocytes and embryos were immediately washed through cold HEPES-modHTF media (4 °C) and aspirated onto a filter mat (Perkin-Elmer, Vic., Australia, #1450-421) with 4 mL scintillation fluid (Perkin-Elmer, Vic., Australia, #6013371). Each sample group was analysed for 30 min in a MicroBeta TriLux Plate Counter (PerkinElmer, Vic., Australia). A standard curve was

created from a serial dilution of 1 μM L-[^3H]-Pro and fitted by linear regression to enable calculation of uptake in $\text{fmol min}^{-1} \text{oocyte}^{-1}$ (or embryo^{-1}) for each separate experiment.

2.5. Immunofluorescent Staining and Confocal Microscopy of COCs and Embryos

After isolation or following culture, oocytes and embryos were fixed in 4% (*w/v*) paraformaldehyde (PFA) for 30 min at room temperature then washed three times in PBS + 1 mg/mL polyvinyl acid (PBS + PVA; Sigma-Aldrich, St. Louis, MO, USA). Oocytes/embryos were permeabilised with PBS + PVA + 0.3% (*v/v*) Triton X-100 for 30 min and blocked by incubation in PBS + PVA + 0.1% (*v/v*) Tween-20 + 0.7% (*w/v*) BSA (PPTB) for 30 min at room temperature. Primary antibodies were diluted 1 in 200 in PPTB and included polyclonal chicken anti-human B⁰AT1 (custom antibody from Aves Laboratories; immunising peptide CZ-DPNYEEFPKSQK representing aa 564–574 of B⁰AT1 protein [27]), rabbit anti-human ACE2 (Abcam, Cambridge, UK, Cat. No. ab15348; RRID:AB_301861) and mouse anti-collectrin (Alexis Biochemicals, San Diego, CA, USA). Appropriate control sera for each staining included pre-immune IgY serum (chicken) and purified IgG (rabbit and mouse). Oocytes and embryos were incubated in primary antibody for 2 h at room temperature and washed three times in PPTB. Secondary antibodies used were Alexa Fluor 594-coupled goat anti-chicken IgG (diluted 1:500), Alexa Fluor 488-coupled goat anti-rabbit IgG (diluted 1:200) and anti-mouse IgG (diluted 1:200). For staining of embryos for only B⁰AT1, the secondary antibody was diluted in PPTB containing FITC-conjugated phalloidin (1:200, Invitrogen, Carlsbad, CA, USA, Cat. No. F432). Oocytes and embryos were incubated in the dark for 1 h at room temperature and washed three times in PPTB then mounted in 3 μL Vectashield containing 1.5 $\mu\text{g/mL}$ DAPI (Vector Laboratories, Newark, CA, USA). Images of oocytes and embryos were taken using confocal microscopy (LSM 510 Meta, Carl Zeiss, Oberkochen, Germany) using 405, 488 and 516 nm lasers and a 40 $\times$ objective. Images were prepared using Fiji by Image J and are representative of 6–9 embryos stained from three separate embryo isolations.

2.6. Statistical Analyses

Embryo culture experiments were performed 4–6 times with 5–21 embryos per treatment group per experiment. The number of embryos developing to a particular stage was summed together from each replicate and the percentage development calculated. The total number of embryos in each group is provided in each figure and differences between treatment groups were determined by chi-square analysis. AA uptake experiments were conducted at least three times with 1–4 oocytes or embryos per treatment group. The average uptake of L-[^3H]-Pro for each treatment was calculated and the difference between groups compared by Student's *t*-test or 1-way ANOVA with Dunnett's post hoc test, as indicated in the figure legends. Data analysis was performed using GraphPad Prism v7.

3. Results

3.1. B⁰AT1 Is Expressed in Preimplantation Embryos

The stage-specific expression of the B⁰AT1 transporter in the preimplantation embryo was investigated by immunostaining. Embryos were counterstained with phalloidin, which binds to F-actin including subcortical F-actin, thereby enabling the identification of the periphery of each blastomere. Expression of B⁰AT1 was detected with F-actin at or near the plasma-membrane of embryos at all developmental stages examined, but not at the surface of contact between blastomeres (Figure 1). In 1-cell through to 8-cell stages, B⁰AT1 staining was observed in the cytoplasm close to the plasma membrane. B⁰AT1 was also detected in the apical and basolateral membranes of trophoblast cells and at the plasma membrane of inner cell mass cells in blastocysts (Figure 1).

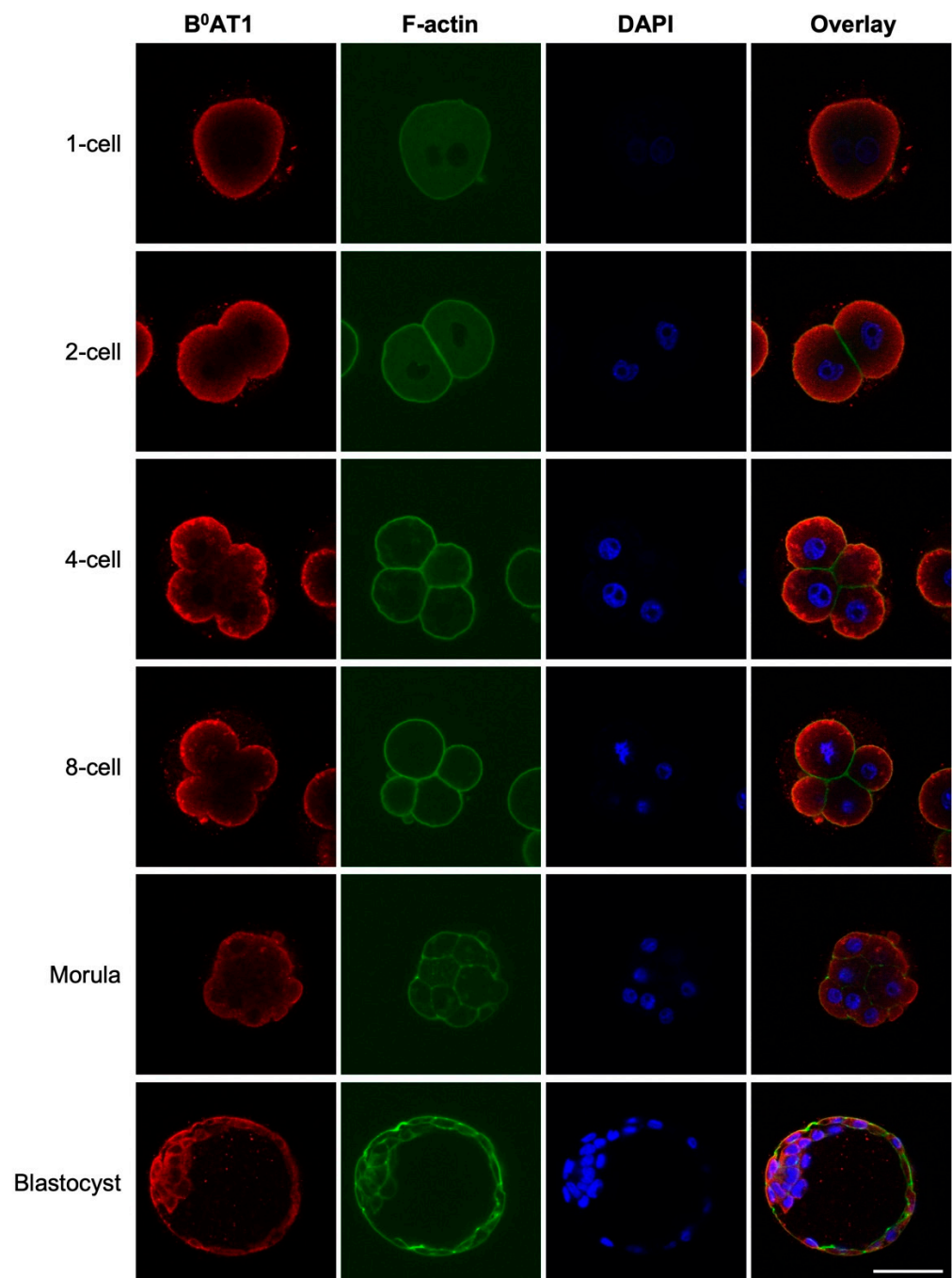


Figure 1. B⁰AT1 is expressed throughout preimplantation mouse embryo development. Embryos from QS mice were freshly isolated at 1-, 2-, 4-, and 8-cell, morula and blastocyst stages, fixed and immunostained for B⁰AT1 (red) and counterstained for F-actin with phalloidin (green) and nuclei with DAPI (blue). Fluorescent images were captured by confocal microscopy using a 40× objective. No staining was observed when a pre-immune IgY equivalent was used as a negative control for B⁰AT1 staining (not shown). Scale bar = 50 µm for all images.

3.2. The B⁰AT1 Accessory Protein ACE2 Is Expressed in the Preimplantation Embryo

Trafficking of B⁰AT1 to the plasma membrane is dependent on the co-expression of an accessory protein, either angiotensin converting enzyme 2 (ACE2) or collectrin [28–30]. Embryos were therefore immunostained with the B⁰AT1 antibody together with either ACE2 or collectrin antibodies to investigate whether one or both accessory proteins associated with B⁰AT1 in preimplantation embryos. B⁰AT1 colocalised with ACE2 in all preimplantation

stages up to and including the blastocyst (Figure 2A). No collectrin staining was observed at any embryonic stage (Figure 2B).

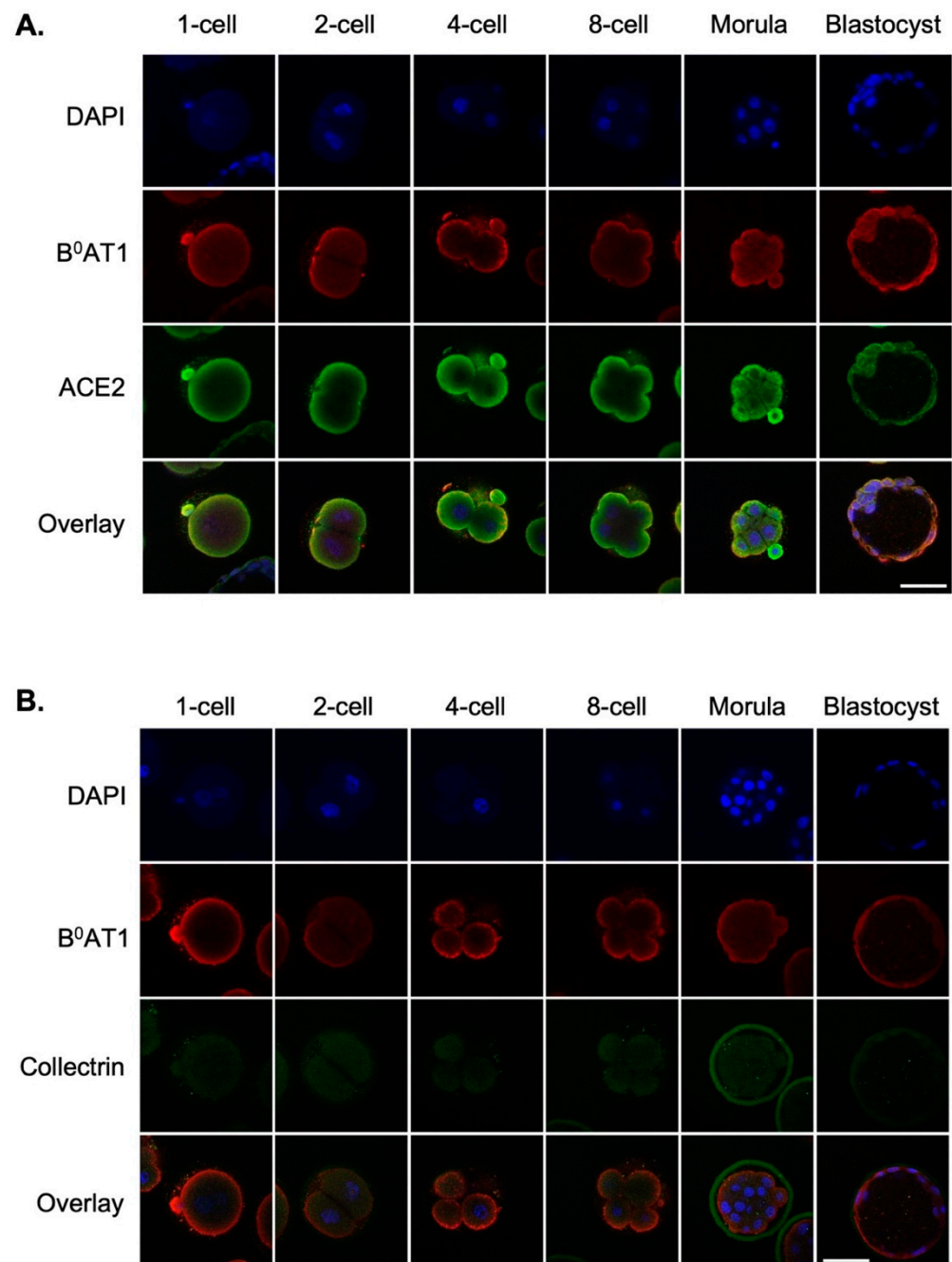


Figure 2. ACE2 colocalises with B⁰AT1 in the mouse preimplantation embryo. Embryos were freshly isolated from QS mice at 1-, 2-, 4-, and 8-cell, morula and blastocyst stages, fixed and immunostained for B⁰AT1 (red) and (A) ACE2 (green), or (B) collectrin (green). Nuclei were counterstained with DAPI (blue). Fluorescent images were captured by confocal microscopy using a 40× objective. No staining was observed when a pre-immune IgY or IgG equivalent was used as a negative control for the primary antibodies (not shown). Scale bar = 50 µm for all images.

3.3. Fertility of *Slc6a19*^{−/−} Mice Is Reduced

To confirm the role of B⁰AT1 in early embryonic development, we examined a *Slc6a19*-nullizygous mouse model previously reported by us [26]. Firstly, the absence of B⁰AT1 protein in COCs and blastocysts from *Slc6a19*^{−/−} mice was confirmed by immunostaining

(Figure 3A). In viable mice, litter size in *Slc6a19*^{-/-} mice (KO × KO; 3.8 ± 1.5 (mean ± SD)) was significantly reduced compared to that in both WT (WT × WT; 6.4 ± 1.3 (mean ± SD), $p < 0.001$) and heterozygous mice (het × het; 6.2 ± 2.2 (mean ± SD), $p < 0.0001$) (Figure 3B), indicating B⁰AT1 deficiency negatively impacts fertility and/or live birth rate.

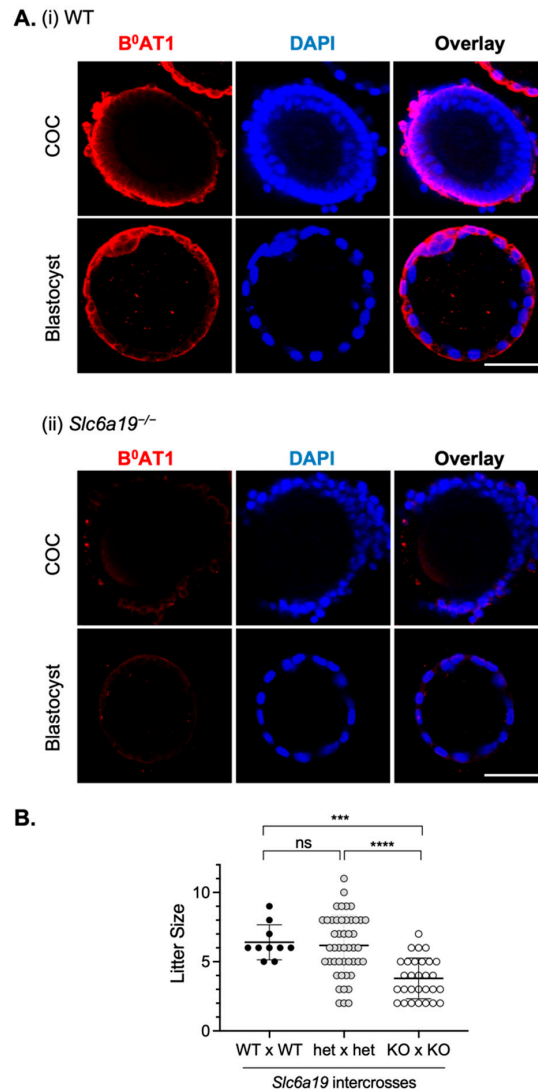


Figure 3. B⁰AT1 deficiency impairs mouse fertility. (A) COCs and blastocysts were freshly isolated from (i) WT and (ii) *Slc6a19*^{-/-} mice and immunostained for B⁰AT1 (red) and DAPI (blue) to confirm loss of B⁰AT1 protein in knockouts. Scale bar = 50 µm for all images. No staining was observed when a pre-immune IgY was used as a negative control for B⁰AT1 staining (not shown). (B) The number of pups born from wild-type (WT × WT) [31], heterozygous (het × het) and homozygous *Slc6a19*^{-/-} mice (KO × KO) intercrosses. Data show mean ± SD; *** $p < 0.001$, **** $p < 0.0001$, ns is not significant. Statistical analysis was performed using a one-way ANOVA with Tukey's post hoc test for multiple comparisons.

3.4. Pro Uptake by Embryos from WT and *Slc6a19*^{-/-} Mice Is Dependent on Developmental Stage

The rate of uptake of L-[³H]-Pro by oocytes and 4–8-cell stage embryos from WT and *Slc6a19*^{-/-} mice was examined in the absence (No AA) and presence of excess unlabelled Pro. In both of these developmental stages from WT mice, excess unlabelled Pro significantly decreased the rate of L-[³H]-Pro uptake (Figure 4), indicating the presence of one or more AAT(s) for Pro. Whereas in oocytes and 4–8 cell embryos from *Slc6a19*^{-/-} mice, unlabelled Pro failed to prevent L-[³H]-Pro uptake (Figure 4), suggesting B⁰AT1 is normally an active AAT at these stages in WT mice. Any uptake now in the *Slc6a19*^{-/-} mice is via a

compensatory mechanism, which is not inhibited by the presence of excess unlabelled Pro (see Discussion).

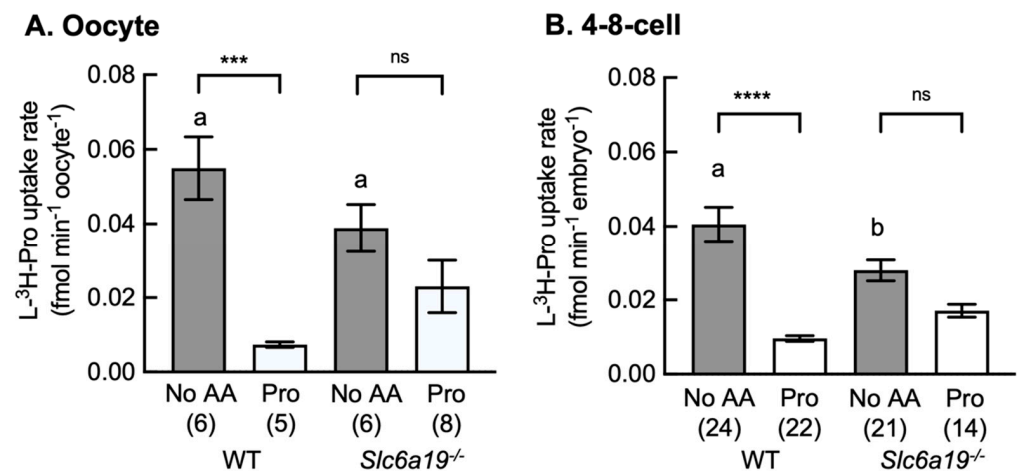


Figure 4. Excess unlabelled Pro reduces the rate of L-[³H]-Pro uptake in WT but not *Slc6a19*^{-/-} embryos. Rate of uptake of 1 μ M L-[³H]-Pro in (A) oocytes, and (B) 4–8-cell embryos from wild-type (WT) and *Slc6a19*^{-/-} mice without (No AA) or with molar excess of unlabelled Pro (0.4 mM). Each bar represents the mean $\pm$ SEM with the number of replicates given in parentheses. Each replicate was performed on 1–4 oocytes or embryos over at least 3 independent experiments. Data were analysed by Student's *t*-test. 'No AA' bars with different letters (a,b) are significantly different ($p < 0.05$). *** and **** indicate $p < 0.001$ and $p < 0.0001$ respectively; ns, not significant ($p > 0.05$) for No AA vs Pro.

3.5. Pro Is Taken up by B⁰AT1 and/or an Unknown Betaine-Sensitive Pro Transporter

B⁰AT1 is a sodium-dependent neutral AAT which accepts Pro, Leu and Ala but not Bet [32]. Previously, we have shown that PROT and PAT1/2 transporters are responsible for the uptake of Pro in oocytes [12]. Here, we also investigated the competitive effect of B⁰AT1 substrates on the rate of Pro uptake at the 4–8-cell stage. All 3 AAs significantly decreased the rate of L-[³H]-Pro uptake in embryos from WT mice (Figure 5A) but not in embryos from *Slc6a19*^{-/-} mice (Figure 5B).

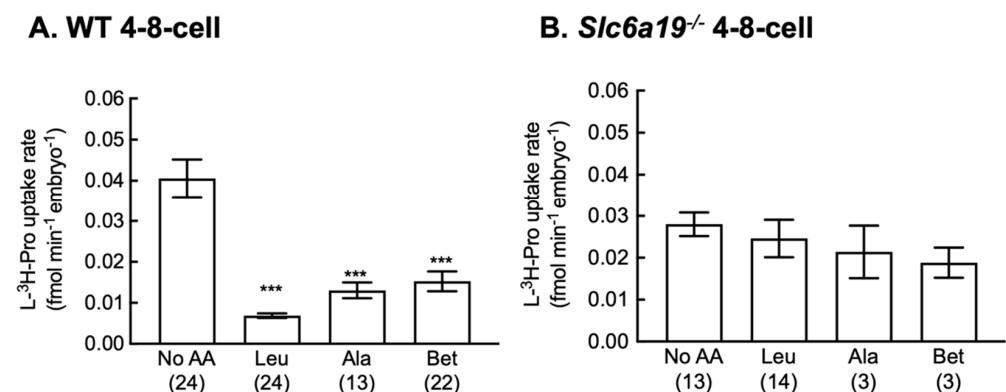


Figure 5. Characteristics of AA transport are different in embryos obtained from *Slc6a19*^{-/-} mice compared to embryos from WT mice. Rate of uptake of L-[³H]-Pro was measured in 4–8-cell stage embryos from (A) WT or (B) *Slc6a19*^{-/-} mice without (No AA) or with molar excess (5 mM) of unlabelled AAs. Each bar represents the mean $\pm$ SEM with the number of replicates given in parentheses. Each replicate was performed on 1–4 embryos over at least 3 independent experiments. Data were analysed using one-way ANOVA with Dunnett's post hoc test. *** $p < 0.001$ compared to No AA. Bet = betaine, Leu = leucine, Ala = alanine. Note that bars for No AA and Pro in this figure are the same data as in Figure 4.

3.6. B⁰AT1 Is Needed for Pro Uptake and Optimal Development at Day 4 of Development

The effect of loss of B⁰AT1 on embryo development was examined by culturing embryos from WT and *Slc6a19*^{−/−} mice from the zygote to blastocyst stage in medium $\pm$ 0.4 mM Pro (Figure 6). On day 2 (i.e., at the 2-cell stage), there was no significant difference in development between WT and *Slc6a19*^{−/−} embryos with no AAs. Fewer *Slc6a19*^{−/−} embryos developed in medium containing no AA to the $\geq$ 5-cell stage by the appropriate time (i.e., by day 3) and to the blastocyst stage (days 5 and 6) (Figure 6A). Addition of 0.4 mM Pro to the culture medium largely rescued the decreased development of embryos from *Slc6a19*^{−/−} mice, except at day 4, when fewer embryos had cavitated, suggesting slower development at this point (Figure 6B). Note, that in embryos from WT mice, Pro significantly increased development to the appropriate stage on day 4 ($p < 0.001$). Whereas in *Slc6a19*^{−/−} embryos, Pro increased development on days 3 and 6 ($p < 0.05$) compared to embryos cultured in medium containing no AA. These data suggest that in the absence of B⁰AT1, Pro can be taken up by other transporter(s) on days 3 and 6 but not on day 4 of preimplantation development.

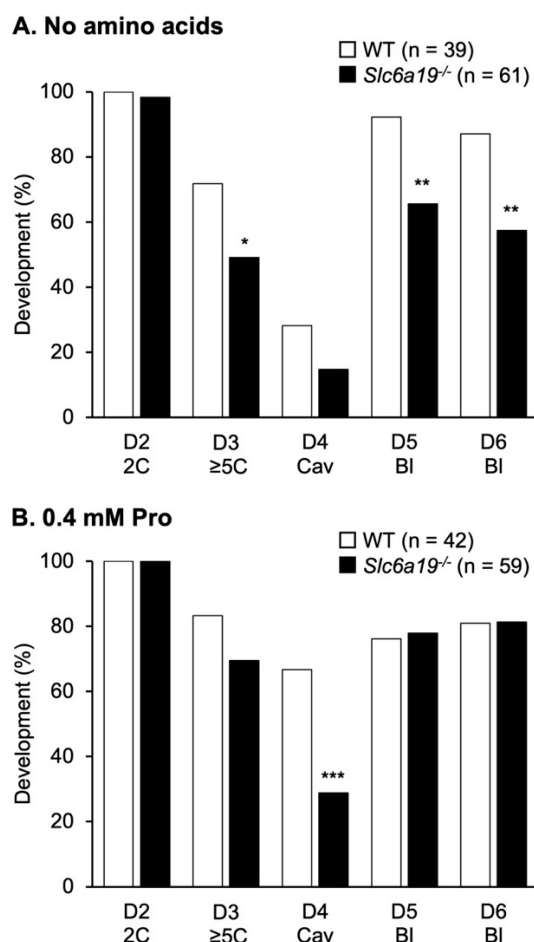


Figure 6. Pro improves development of *Slc6a19*^{−/−} embryos at $\geq$ 5–8-cell and blastocyst stages. Zygotes were cultured in the (A) absence of AAs, or (B) in the presence of 0.4 mM Pro and scored for development to the appropriate stage every 24 h (i.e., to the 2-cell (2C) on day 2 (D2), 5–8-cell ($\geq$ 5C) on D3, cavitation (Cav) on D4, and blastocyst stage (BI) on D5 and D6. Data on each day of development were compared by chi-square analysis of pooled data from 4–6 replicate experiments, with the total number of embryos in each group given in parentheses. Bars with an asterisk are significantly different to WT at each timepoint: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

4. Discussion

In this study, we investigated the effect of Pro on embryo development to determine: (1) if Pro is taken up by preimplantation embryos; and (2) if Pro transporters are expressed in embryos from WT and *Slc6a19*^{−/−} mice. In vitro, the developmental potential of an oocyte and/or embryo is improved by the exogenous addition of Pro to the culture medium in a time-dependent manner. For example, when Pro is added to fertilisation medium, cleavage rates are not affected but improvement in development occurs at compaction through to the blastocyst stage, and blastocysts produced have more cells in their inner cell mass [12]. If Pro is added to culture medium from the zygote stage onwards, improved embryo development occurs from the 5-cell stage and the proportion proceeding to blastocyst development and hatching is increased [4]. Stage-specific uptake of Pro by AATs is needed for these Pro-mediated improvements in development. Since Gly and Leu both inhibit Pro-mediated improvement in development in culture [4], and are common substrates of the neutral AAT system B⁰, we hypothesised that B⁰AT1 (*Slc6a19*) is expressed in preimplantation embryos where it is responsible for Pro uptake.

B⁰AT1 was detected in the plasma membrane of embryos at all stages from WT mice. B⁰AT1 was also present in the cumulus cells of COCs and suggests a role for B⁰AT1 in the increased Pro uptake into oocytes by coupled cumulus cells [19]. B⁰AT1 staining was co-localised with ACE2 at all embryo stages, suggesting a role for ACE2, rather than collectrin, in the trafficking of B⁰AT1 to the plasma membrane in embryos.

The functional role of B⁰AT1 in uptake of Pro into embryos was determined by measuring the rate of radiolabelled Pro uptake in oocytes and 4–8-cell stage embryos and competition for Pro uptake by other substrates of B⁰AT1, including Leu and Ala [32,33]. Due to the decreased fertility of *Slc6a19*^{−/−} mice, we were unable to elucidate the complete inhibition profile of Pro transport in embryos from *Slc6a19*^{−/−} mice. AAT-mediated uptake of Pro was present in oocytes from WT mice. However, despite the fact that the rate of Pro uptake into oocytes lacking B⁰AT1 is the same as for WT, it was not inhibited by excess Pro, indicating Pro uptake in the knockout is not transporter-mediated. Other studies have shown that Pro uptake in WT oocytes can occur via a number of AATs including PROT (*Slc6a7*), GlyT1 (*Slc6a9*), PAT1 (*Slc36a1*) and/or PAT2 (*Slc36a2*) [12,19], but none of these seem to be active in this knockout since excess unlabeled Pro did not reduce uptake of labelled Pro. Thus, in this study, Pro was taken up in B⁰AT1 knockout oocytes and embryos via a non-saturable route. This may be due to transport via channels that also conduct AAs, such as the volume-sensitive anion channel known to be present in oocytes [34–36].

At the 4–8-cell stage, Pro was taken up by embryos from both WT and *Slc6a19*^{−/−} mice with the uptake reduced in *Slc6a19*^{−/−} mice. The AAs Leu and Ala inhibited the uptake of Pro in WT but not in embryos from *Slc6a19*^{−/−} mice. Bet, which is not a substrate for B⁰AT1 [32], also inhibited Pro uptake in 4–8-cell WT embryos. PAT1 and PAT2 transport Pro, Bet and Ala [37] and are therefore good candidates for the Bet-sensitive portion of Pro uptake in 4–8 cell embryos. Recently, we have shown that PAT1 and 2 are present in the plasma membrane of mouse oocytes and may be responsible for Na⁺-independent uptake of Pro in oocytes [12]. System A and BGT1 (*Slc6a12*), which also transport Bet, are not present in preimplantation embryos [20,38]. Further studies are needed to examine the expression profile of PAT1/2 in later embryo stages. A proportion of Pro uptake at the 4–8-cell stage could be via GlyT1, which is expressed in 4- and 8-cell embryos [39,40]. Again, none of these AATs seem to be active in the knockout. It is not clear why the Bet-sensitive uptake was also lost when B⁰AT1 was knocked out. Further investigation is needed to determine whether the AAT profile in 4–8-cell embryos represents more than one transport system.

Development of embryos from *Slc6a19*^{−/−} mice was decreased at the ≥5-cell and blastocyst stages when the medium contained no AA. These data suggest that B⁰AT1 is required in vivo for uptake and storage of Pro in the oocyte and during fertilisation, which subsequently improves later embryo development [12]. Exogenous addition of Pro to culture improved development of embryos from *Slc6a19*^{−/−} mice at the ≥5-cell

and blastocyst stages but not on day 4 when cavitation occurs. In embryos from WT mice, Pro improved development at the cavitation stage. These findings indicate that B⁰AT1 is normally expressed on day 4 of in vitro embryo development, or at a cleavage stage just before cavitation, to accumulate intracellular Pro for activation of Pro-mediated intracellular mechanisms, such as regulation of reactive oxygen species and mitochondrial activity [12,41]. Furthermore, the expression of AATs during pre-implantation development is very dynamic. We speculate that during or soon after day 4 in *Slc6a19*^{−/−} mice, new Pro transporters are now expressed, and Pro is then taken up in sufficient quantity for cavitation and further development to proceed.

There was no decrease in litter size in Het × Het intercrosses and the appropriate Mendelian distribution of litter genotypes was observed [26], suggesting the maternal reproductive tract environment of *Slc6a19*^{+/-} mice enables survival of *Slc6a19*^{−/−} embryos, as seen with improved development in vitro of *Slc6a19*^{−/−} embryos in the presence of Pro. Note though, a maternal diet low in vitamin B3 in pregnant *Slc6a19*^{+/-} mice results in embryo loss and malformations [42]. We postulate that both intrinsic (i.e., *Slc6a19* KO embryo) and extrinsic factors (i.e., *Slc6a19* KO reproductive tract) are responsible for the decreased litter size in KO × KO intercrosses. This has implications for pregnancy in humans who carry loss-of-function mutant alleles in *SLC6A19*. These findings suggest that embryonic development and fertility could be improved in *Slc6a19*^{−/−} mice by supplementation of the maternal diet with Pro.

This is the first study to demonstrate expression of the neutral AAT B⁰AT1 in preimplantation embryos. Absence of this transporter adversely impacts fertility and embryo development, although the effect on embryo development can be overcome by the presence of Pro in embryo culture medium. This study demonstrates the importance of Pro for embryo development and that Pro can be taken up at each developmental stage by one or more transporters. The presence of multiple AATs for Pro and the ability to compensate for the loss of an individual AAT by upregulation of non-saturable transport mechanism indicates the critical importance of Pro for embryo development.

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Informed Consent Statement: Not applicable.

Data Availability Statement: All data are included in the published article. Data and materials will be made available upon request.

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Chapter 4

A Simplified Medium Supports Development of Parthenotes for Therapeutic Cloning

This chapter has been written as a pre-publication manuscript.

A Simplified Medium Supports Development of Parthenotes for Therapeutic Cloning

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Abstract: Parthenotes are a source of pluripotent stem cells that have therapeutic applications in regenerative medicine. In this study we investigate the suitability of IVF discarded immature oocytes for parthenogenesis. The *in vitro* maturation rate of 174 immature human oocytes was 78% with similar results between maturation medium (SAGE or Vitrolife). The successfully matured oocytes were parthenogenetically activated with DMAP; 72% forming diploid heterozygous embryos. 64% of these parthenotes developed to the 2-cell stage and 43% to 8-cells, with 10% reaching 32 cells; however, none formed blastocysts. There was no significant difference in the overall outcome regardless of which culture medium was used or whether the matured oocytes were fresh or frozen. However, frozen matured oocytes had reduced development in SAGE medium, not developing beyond the 8-cell stage. Recently we have shown that L-proline (Pro) is taken up by mouse oocytes and improves subsequent development when present in fertilisation medium. It is not known if Pro is taken up by parthenotes. In our next experiment we parthenogenetically activate mouse oocytes and culture parthenotes in the absence of amino acids or in the presence of Pro or a combination of amino acids. Mouse parthenotes had increased development to blastocyst stage when cultured with Pro compared to parthenotes cultured in medium with no amino acids or groups of amino acids. This study demonstrates a simplified medium can be beneficial to maintain development of parthenotes to blastocyst stage for the purpose of therapeutic cloning.

Keywords: Immature oocytes, parthenogenesis, parthenote, proline

1. Introduction

Therapeutic cloning, also called somatic cell nuclear transfer (SCNT), is a process which removes and replaces the oocytes DNA with a somatic cell nucleus and results in the formation of embryonic stem cells (ESCs) that are pluripotent and have the same genetic makeup as the donor. The advantage of using such cells for therapeutic purposes is that they can be used to create universal cell lines for cell clinical replacement therapy (e.g. for treating Parkinson's Disease (Barker et al., 2021) and will not be rejected by the immune system of the recipient (Schwartz et al., 2012). In mice ESCs have been stimulated to differentiate into cardiac cells (Doetschman et al., 1985), skeletal tissue (Braun et al., 1994), and neurons (Bain et al., 1995), and there are an increasing number of clinical trials using ESCs for the treatment of stroke and spinal cord injury (Bonn 1998). An important factor in undertaking therapeutic cloning is to ensure oocytes are of high quality to produce clinical grade cell lines. Donation of quality oocytes by women for financial gain is not permissible in Australia although it is in California, New York and Newcastle, England. However, immature oocytes in the form of Germinal Vesicles (GV) and Metaphase I (MI) oocytes are available in Australia. They constitute 15 - 20% of oocytes retrieved during human Assisted Reproductive Technology (ART) cycles (Chian et al., 2004) and are routinely discarded as unusable for IVF. In Australia, the *Research Involving Human Embryos Act 2002* (RIHE Act) prohibits the creation of a human embryo purely for research and there is a complete ban on therapeutic cloning for reproductive purposes. Therapeutic cloning of human embryos for purposes that are not reproductive (i.e. harvesting stem cells for regenerating tissue) is permitted in Australia if approved by the National Health and Medical Research (NHMRC) Licensing Committee. However the process of retrieving ESCs from viable human embryos leads to their destruction and raises moral and ethical concerns over their use (Caulfield 2003). Parthenotes are an alternative source of ESCs that are obtained from activated oocytes which have not been fertilised by sperm and are not

viable as they contain only maternal DNA (Kaufman et al., 1975). Parthenogenesis rarely occurs naturally in mammals (Winberg et al., 2010) as oocytes normally arrest after second meiosis at metaphase II (MII) and are maintained in this state by maternal hormones in the antral follicle (Mehlmann 2005). Human oocytes under certain conditions spontaneously activate and begin developing (Ye et al., 2020) but arrest at mid-gestation due to defective paternal imprints (Barton et al., 1984, Combelles et al., 2011, Ye et al., 2020) or cause ovarian teratoma formation (Linder et al., 1975). Spontaneous activation is thought to occur due to defects in cell-cycle regulators c-Mos/MAPK which control the pathways of meiotic arrest (Hirao et al., 1997, Madgwick et al., 2007). Simple mechanical and chemical manipulations *in vitro* can trigger oocyte activation by increasing intracellular Ca^{2+} influx, which is normally initiated at the site of sperm-egg fusion (Mitalipov et al., 2001, Tachibana et al., 2013).

GV and MI oocytes obtained from unstimulated ovaries can be matured via *in vitro* maturation (IVM) and when fertilised and implanted, can result in a pregnancy. This was first reported by Cha and associates (Cha et al., 1991). Not all such oocytes are suitable for fertilisation; for example, those with no cumulus have suboptimal nuclear and cytoplasmic maturation in IVM media (Sato et al., 2007, McElroy et al., 2008). Amino acids (AAs) are routinely added to medium to improve maturation of oocytes (Hong et al., 2007) and embryonic development to blastocyst stage (Brinster 1965, Summers et al., 2000, Gardner 2008, Kane 2019), however, it is not known whether the nutritional requirements are the same for parthenote development. The use of *in vitro* matured oocytes in ART clinics and providers is generally restricted to women with polycystic ovaries, but the practice is not wide spread. In contrast to immature oocytes collected from unstimulated ovaries, immature oocytes collected during ART cycles are thought to have limited developmental capacity, as evidenced by their failure to mature *in vivo* (Lacham-Kaplan et al., 2008). A screening test to examine the quality of such oocytes, once matured, is used to determine if they can be stimulated to develop like embryos in the

absence of sperm. Others have shown that it is possible to create blastocysts by parthenogenesis using matured oocytes obtained during ART, and tailor make human embryonic stem cells (hESC) derived from these (Revazova et al., 2007). The efficiency was high, with 50% of activated oocytes developing into blastocysts, and hESC lines being produced from 30% of these. The same rate has yet to be reported with *in vitro* matured human oocytes, although blastocysts have occasionally been produced from such oocytes, with a frequency of 1-3% (McElroy et al., 2010, Versieren et al., 2010, Yan et al., 2011). This has been achieved when the maturation medium was supplemented with growth factors and peptides, including follicle stimulating hormone (FSH) and human chorionic gonadotrophin (HCG) (McElroy et al., 2010); the oocytes were activated by electrical and chemical stimulation (Versieren et al., 2010); or when the culture medium was supplemented with FSH and luteinizing hormone (LH) (Yan et al., 2011).

In this study we investigated the suitability of immature human oocytes for parthenogenesis. GV and MI oocytes collected during ART were not suitable for therapeutic cloning as they did not develop to the blastocyst stage in commercial media. Next, we examined the effect of culturing mouse parthenotes in the absence of AAs or in the presence of Pro alone or in the presence of groups of AAs. Parthenotes had increased development to blastocyst stage when cultured in Pro and formed ESCs. This study shows that a simplified medium has a beneficial effect on parthenote development and may provide a useful approach for the production of quality hESCs for therapeutic purposes.

2. Materials and methods

2.1 Regulatory approval

Human: Approval was obtained from the Human Research Ethics Committees of Northern Sydney Central Coast Health (08/HAWKE/70/71) as well as Monash University (CF10/2006 - 2010001122). A Licence to conduct the study was obtained from the National Health and Medical Research Council Embryo Licensing Committee, Licence Number 309716. A condition of the Licence was to report every time an oocyte was obtained and what happened with it as regards maturation and activation outcome.

Animals: Quackenbush Swiss (QS) mice (Animal Resource Centre, Perth and Lab Animal Services, University of Sydney) were housed under 12 h light:12 h dark conditions. Experiments were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and approved by the University of Sydney Animal Ethics Committee as required by the NSW Animal Research Act (protocols 5583 and 824).

2.2 Oocyte collection and maturation

Human: Informed consent for the study was obtained from 143 women who attended the Fertility East clinic for ART. The ovaries of these women were hormonally primed, and oocytes subsequently collected by OPU. Mature oocytes were used for reproductive purposes; only the immature oocytes were used in the study. They were of two types, those with a germinal vesicle (GV) and in metaphase 1 (MI). Where it was not possible to identify if the oocyte was mature, the cumulus was denuded with hyaluronidase to allow for characterising the oocyte. Immature oocytes were cultured for 24 - 48 h in either SAGE (Gytech, Australia) or GIVM (Vitrolife, Australia) culture medium. Additionally, GIVM medium was supplemented with growth factors and peptides (Supplement B) as follows: 75 mg/mL FSH,

75 mg/mL LH, 0.025 µg/mL recombinant human chorionic gonadotropin (rHCG) (Merck Serono), 0.1 mL serum protein substitute (Gytech SAGE #ART3011), 3 ng/mL brain-derived neurotrophic factor (Bioscientific), 100 ng/mL Insulin like growth factor 1 (IGF1) (Bioscientific), 30 ng/mL glial cell derived neurotrophic factor (Bioscientific), 10 ng/mL leptin (Bioscientific), 0.5 ng/mL basic fibroblast growth factor (FGF2) (R&D Systems, Bioscientific) and 1µg/mL oestradiol (Sigma Aldrich). IVM was performed at 37°C in air and 5% CO₂. Pronuclear assessment and presence of a polar body determined matured oocytes which were cryopreserved by rapid freezing for future parthenogenesis.

Animals: Female mice aged 4–6 weeks were injected intraperitoneally with 10 IU pregnant mares' serum gonadotropin (PMS; Intervet, Vic, Australia). Immature oocytes were dissected from the ovaries, 48 h after PMS, into HEPES-buffered modified human tubal fluid (Hepes-modHTF; Table 1) and then cultured in Dulbeccos Modified Eagle Medium (DMEM; Thermofisher Scientific) supplemented with 10 % fetal bovine serum (FBS) and 2 mM L-Glutamine for 16 – 18 h at 37°C and 5% CO₂. The pH of mod-HTF and hepes-buffered mod-HTF was adjusted to 7.4 using 1 M HCL or 2 M NaOH respectively, and osmolarity adjusted to 270 mOsm/kg. A low concentration (0.3mg/mL) of bovine serum albumin (BSA) (Sigma-Aldrich, USA) was added to the medium and filter sterilised with 0.22 µm filter (Millipore, USA).

Table 4.1 Composition of mouse embryo collection and culture media

Component	Mod HTF (mM)	Hepes-buffered mod HTF (mM)
Lactic Acid (60%)	21.4	21.4
NaCl	85	85
KCl	4.6	4.6
KH ₂ PO ₄	0.4	0.4
MgSO ₄	0.2	0.2
NaHCO ₃	25	25
Na ₂ EDTA	0.11	-
Na Pyruvate	0.4	0.4
Glucose	2.8	2.8
Phenol Red	0.001%	0.001%
Penicillin	0.06 mg/ml	0.06 mg/ml
CaCl ₂ • 2H ₂ O	2.04	2.04
HEPES	-	21

2.3 Vitrification and Thawing

The Medicult Vitrification Cooling system (CooperSurgical, USA) was used to freeze mature human oocytes. Briefly, the matured oocytes were washed in G-MOPS Plus medium (Vitrolife), before being transferred to the equilibration for 5 min and then the vitrification medium for 45 sec. The frozen oocytes were loaded into CryoLogic Vitrification Method (CVM) Fibreplugs, one per device, and stored in liquid nitrogen. Thawing was carried out using the Medicult Vitrification Warming Kit protocol (CooperSurgical, USA). Briefly, the CVM Fibreplugs containing the frozen oocytes were placed in GMOPS Plus handling medium and a micropipette used to transfer the oocytes into the warming medium (37°C) for 3 min. Oocytes were transferred through to 2 drops of warming solution for a total of 6 min, then

through 2 drops of cleavage medium for a further 6 mins and then placed in 20 μ l drops SAGE or GIVM medium overlayed with 500 μ l sterile paraffin oil (Ovoil, Vitrolife) in a MINC Benchtop Incubator (Cook Medical), for 20 mins to recover.

2.4 Parthenogenetic Activation and Culture of Embryos

Human: Matured oocytes were parthenogenetically activated with 5 μ M ionomycin in 300 μ l SAGE or GIVM medium for 5 min, washed through 300 μ l drop cleavage medium and then incubated at 37°C and 5% CO₂ in 300 μ l cleavage medium containing 1 mM 6-DMAP for 4 h overlayed with 500 μ l sterile paraffin oil (Ovoil, Vitrolife). DMAP blocks the extrusion of the second polar body and the polar body genetic material is retained which results in a pseudo-diploid embryo. Parthenotes were then cultured in SAGE or GIVM cleavage medium, the latter being supplemented with growth factors and peptides (see section 2.2). Embryonic development was checked every 24 h for 7 days, with the number of cells in the embryo counted.

Animals: After IVM, oocytes which had extruded a polar body were transferred to a treatment HEPES-buffered modHTF containing 1 μ M ionomycin (Ca²⁺ salt; Sigma-Aldrich) and incubated for 5 mins at 37°C and 5% CO₂ to activate the oocytes. The oocytes were then washed through 3 drops of HEPES-buffered mod-HTF and transferred to a drop of mod-HTF containing 2 μ g/ml cytochalasin-D (CCD) which prevents actin polymerisation and blocks second polar body emission, and incubated for 3 h at 37°C and 5% CO₂. Parthenotes were washed thoroughly through at least 4 drops of HEPES-buffered mod-HTF and cultured for 96 h in mod-HTF medium containing no AAs or 0.4 mM Pro or in commercial culture medium (ORGIO Sequential Cleav), which contains all 20 common AAs.

2.5 Immunofluorescent Staining of Mouse Parthenotes

Following culture for 96 h mouse blastocysts were fixed in 4% paraformaldehyde (PFA) for 30 min at room temperature then washed three times in PBS + 1 mg/mL polyvinyl acid (PBS + PVA; Sigma-Aldrich, St. Louis, MO, USA). Parthenotes were permeabilised with PBS + PVA + 0.3% Triton X-100 for 30 min and blocked by incubation in PBS + PVA + 0.1% (Tween-20 + 0.7% BSA for 30 min at room temperature. Antibodies used were Nanog (Cell Signaling Technology, MA, USA #8822) Alexa Fluor 488-coupled goat anti-rabbit IgG (Invitrogen, Vic, Australia #A11034), diluted 1:500 and 1:200, in PBS + PVA + 0.1% Tween-20 + 0.7% BSA, respectively. Samples were incubated in primary antibody for 2 h at room temperature or overnight at 4 °C and then washed 3 times in PBS + PVA + 0.1% Tween-20 + 0.7% BSA. Samples were incubated in the dark in secondary antibody for 1 h at room temperature and then washed 3 times in PBS + PVA + 0.1% Tween-20 + 0.7% BSA before being mounted in 3 µL Vectashield containing 1.5 µg/mL DAPI (Vector Laboratories, CA, USA) to visualise nuclei. Images of parthenotes were taken using confocal microscopy (LSM 510 Meta, Carl Zeiss, Oberkochen, Germany) using 405, 488 and 516 nm lasers and a 40× objective. Images were prepared using FIJI by ImageJ.

2.6 Statistical analysis

Maturation rate and parthenote development were analysed using χ^2 test with $P < 0.05$ being accepted as the cut-off for significance. Development of mouse parthenotes in the different treatment groups was compared by calculating the percentage of embryos that developed to blastocyst stage. Differences between groups were determined using 1-way ANOVA and Tukey's or Dunnett's multiple comparisons test as indicated in the figure legends.

3. Results

3.1 Human parthenotes arrest at the 32-cell stage

Of the 174 immature eggs collected over a period of 2 years, 136 of these (78%) matured *in vitro* within 24 hrs to MII stage, developing a polar body (Table 2). There was no significant difference in maturation or activation rate regardless of the media used in this study. Of the 136 matured oocytes, 98 became activated by parthenogenesis, an efficiency of 72%. Human parthenotes were successfully developed to the cleavage stage in commercial media (see Fig. 4.1). Most of the activated parthenotes (64%) developed beyond the 1 cell stage, 21% reached 2-4 cells, 24% 4-8 cells, 9% 9-32 cells, and 10% developed into morulae, but none continued to the blastocyst stage (i.e. 128 or more cells). It is from blastocysts that pluripotent stem cells are most frequently derived for purposes of therapeutic cloning.

There was no significant difference between SAGE (n = 36) or supplemented GIVM Plus (n = 100) culture medium concerning the efficiency of maturation, activation by parthenogenesis, or stages at which developmental arrest occurred. There was no difference in outcomes regardless of whether matured oocytes were activated fresh or activated after being cryopreserved and thawed. However, there was an effect of GIVM Plus with supplement B (growth factors and hormones) compared to GIVM Plus with no supplement, which caused a significant increase in the number of parthenotes that developed ($p < 0.05$) (data not shown).

Table 4.2 Nuclear maturation of human oocytes *in vitro* from hormone stimulated cycle.

Maturation medium	No of oocytes	No of matured oocytes	% Maturation
SAGE	46	37	80
GIVM Plus + Supplement A	18	14	78
GIVM Plus + Supplement B	110	85	77
All	174	136	78

Table 4.3 Embryo development following parthenogenetic activation of matured human oocytes. Immature oocytes that were fresh or frozen were matured, activated by chemical means and cultured in SAGE, or GIVM Plus + supplement B, medium. Cleavage stage was recorded every 24 h. * indicates significant difference ($p < 0.05$).

Culture medium	Matured oocytes	Activated	2-4 cell	5-8 cell	9-31 cell	32 cell
SAGE						
Fresh	26	19 (73%)	13 (68%)	11 (58%)	4 (21%)	2 (11%)
Frozen	10	9 (90%)	3 (33%)	1 (11%)	0	0
GIVM Plus + supplement B						
Fresh	32	24 (75%)	18 (67%)	13 (54%)	7 (29%)	3 (13%)
Frozen	68	36 (53%)	29 (81%)	17 (47%)	8 (22%)	5 (14%)
	Total					
Fresh	58	43 (74%)	31 (72%)	24 (56%)	11 (26%)	5 (12%)
Frozen	78	55 (71%)	32 (58%)	18 (33%)	8 (15%)	5 (9%)

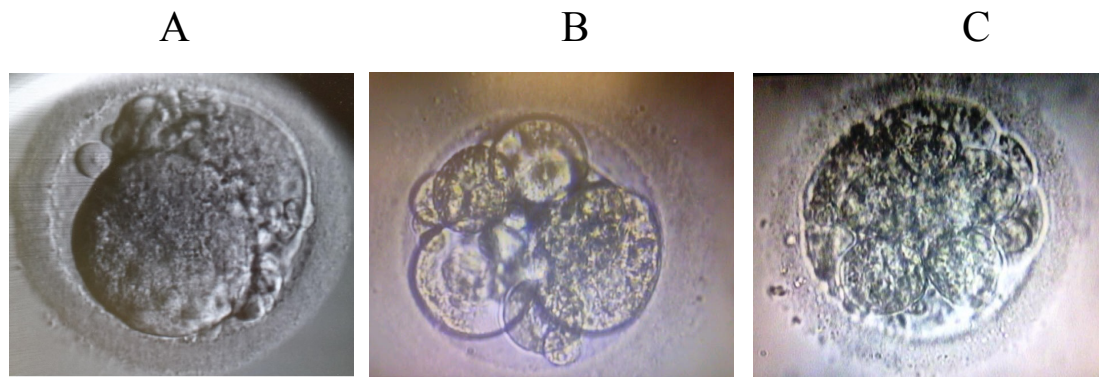


Figure 4.1 Human parthenotes at stages of development. (A) 2-cells; (B) 8-cells; and (C) 32 cells. Parthenotes were imaged using industry software on a light microscope every 24 h.

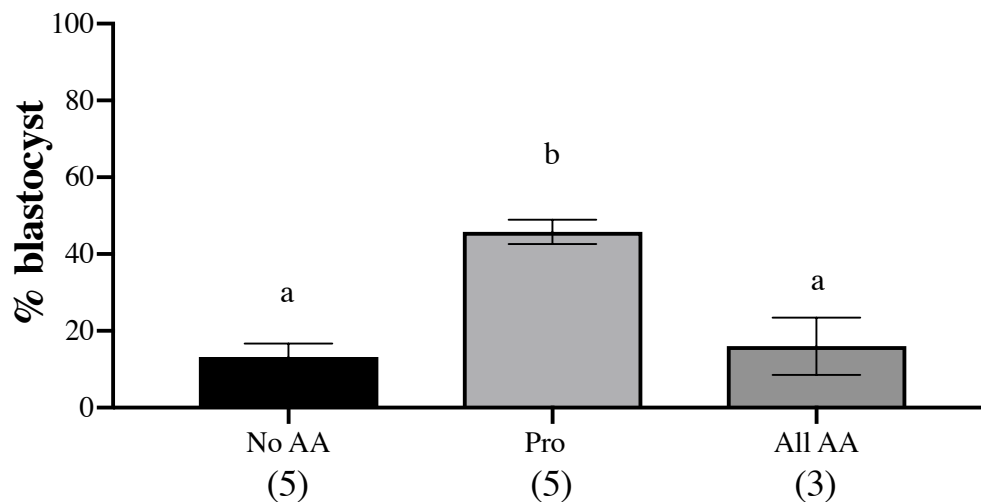


Figure 4.2 Blastocyst development in mice following parthenogenetic activation with or without amino acids. Parthenotes were activated and cultured for 96 h in mod-HTF in the absence of amino acids (No AA) or the presence of 0.4 mM L-proline (Pro) or in commercial medium (All AA). Each bar represents the mean $\pm$ SEM from at least 3 independent experiments, each with replicates performed of 10 parthenotes. Data were analysed using GraphPad Prism using 1-way ANOVA with Tukey's post hoc test. Bars with different letters are significantly different ($p < 0.05$).

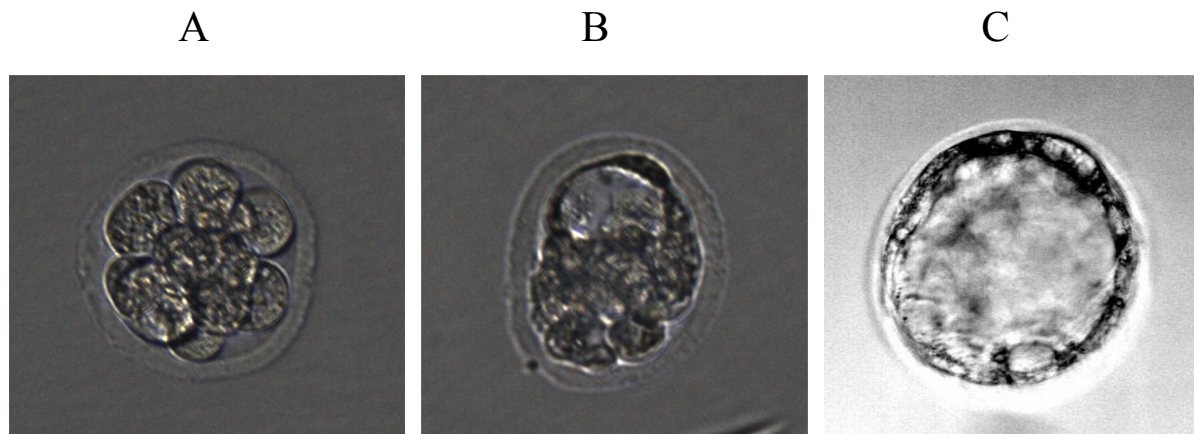


Figure 4.3 Preimplantation development of mouse parthenotes. *In vitro* matured oocytes were parthenogenetically activated and cultured with a physiological concentration of Pro (0.4 mM) in mod-HTF medium. Mouse parthenotes developed to (A) 12-cell stage (B) formed a blastocoele cavity between 72 h - 96 h (C) and blastocyst with inner cell mass after 96 h. Images are representative of 3 blastocysts taken from 3 independent experiments.

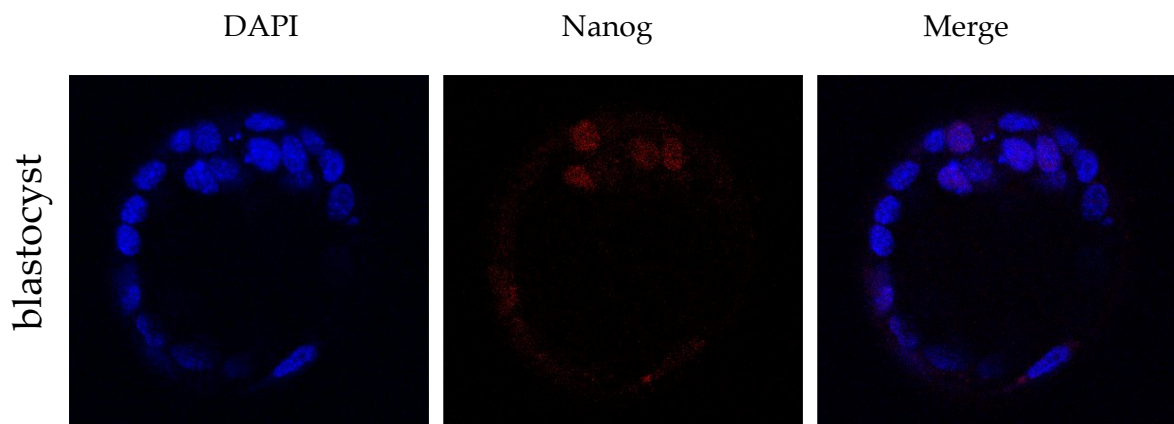


Figure 4.4 Nanog protein in parthenote-derived embryonic stem cells. Parthenogenetically activated blastocysts exposed to Pro (0.4 mM) were fixed, and immunostained for NANOG (red) and maternal chromosomes were counterstained with DAPI and analysed by Zeiss Meta confocal microscopy using a 40 $\times$ optical zoom. Images are representative of 3 blastocyst taken from 3 independent experiments. Scale bars represent 20 μ m.

3.2 Pro fosters development of parthenotes to the blastocyst stage

Mouse oocytes were assessed after IVM in DMEM + BSA + FSH (16 h – 18 h) and only MII oocytes were developed further in culture medium. The IVM rate of oocytes was 60% (data not shown), which was similar to rates reported by others (Miraki et al., 2017). Previous studies have identified that diploid parthenotes have increased developmental potential compared to haploid embryos (Liu et al., 2002). In the present study we treated oocytes with CCD to produce heterozygous diploid parthenotes, which extrude one polar body and retained chromosomes of the second polar body. Mouse oocytes cultured in Pro had significantly increased development to blastocyst stage compared to parthenotes cultured in the absence of AA or with groups of AA which are present in commercial media (Figure 2). The activated parthenotes developed a blastocoel cavity (Figure 3B) and formed an ICM at or before 96 h (Figure 3C).

3.3 Nanog is expressed in the inner cell mass of blastocysts

Expression of Nanog protein is associated with pluripotency in embryonic stem cells (Mitsui et al., 2003). Thus we immunostained with Nanog to ascertain the cell type of parthenogenetically-activated blastocysts cultured with a physiological concentration (0.4 mM) of Pro. Nanog protein was observed in cells of the ICM but not in the trophectoderm. Nanog expression is ubiquitous in ICM cells (Figure 4).

4. Discussion

In countries, such as the United Kingdom and parts of the USA, where it is permissible for mature oocytes to be harvested from women for research purposes, it is possible to create blastocysts and ESC lines by parthenogenesis (Revazova et al., 2007). In countries where it is not permissible, such as Australia, the best that can be done is to start with immature oocytes which are not normally used for the purpose of IVF. In the present study we were successful in maturing oocytes *in vitro* at a similar efficiency (78%) as that reported by others (68%-87%) (McElroy et al., 2010, Versieren et al., 2010, Yan et al., 2011). This was regardless whether we used SAGE medium or GIVM Plus containing supplements. The activation rate of mature oocytes to become parthenotes (72%) was also within the range reported by others (56% - 77%) (McElroy et al., 2010, Versieren et al., 2010, Yan et al., 2011). As might be expected, the activation rate was, however lower than the 94-96% achieved with in vivo matured oocytes (Revazova et al., 2007, Versieren et al., 2010). The rate of subsequent development of parthenotes was slightly greater than reported by others, at least until the 8 and 32 cell stage, 7 - 31% matured oocytes vs 5 - 27% (McElroy et al., 2010, Yan et al., 2011) and 3 - 5% (McElroy et al., 2010, Versieren et al., 2010). In the present study blastocysts did not form from IVM oocytes. Blastocyst formation has also not been successful from activated-aged human oocytes (Winston et al., 1991, Nakagawa et al., 2001), whereas others have reported a success rate of 1-3% (McElroy et al., 2010, Versieren et al., 2010, Yan et al., 2011), vastly less than the 50% achieved with in vivo matured oocytes (Revazova et al., 2007). Insufficient information is available to determine if age of oocytes contributed to the lack of blastocyst formation in the present study. The approximate age of our donors ranged between 35-45 years, and age of donors in previous studies were > 31 years (Revazova et al., 2007) and 26-35 years (Yan et al., 2011). Other studies do not report the age (McElroy et al., 2008, Versieren et al., 2010). The addition of gonadotrophins and growth factors to culture medium provided some benefits as

has been previously demonstrated (McElroy et al., 2008) but was insufficient to support parthenotes to develop to blastocyst stage. The maturation medium used in the present study may have played a role. The media we used was SAGE or GIVM Plus combined with supplements; others have used SAGE or an in-house medium combined with supplements (TCM) (McElroy et al., 2008, Versieren et al., 2010, Yan et al., 2011). Versieren and associates activated a small supply of in vivo matured eggs, with TCM achieving a better rate of activation 94% vs 76%, cleavage 94% vs 67%, and formation of morulae 13% vs 5%. However, blastocyst formation was not reported. There is growing evidence from mouse studies that the nutritional formulation of commercial culture media may not support embryo development potential (Ermisch et al., 2020, Morris et al., 2020). Suboptimal culture medium is likely to be a contributing factor to failed blastocyst development in the present study.

Our improved results with freshly matured as compared to cryopreserved oocytes subsequently matured in SAGE medium, indicates the negative effect of vitrification on the cellular material (Paynter et al., 1999), however we did not observe an effect on subsequent development from fresh or frozen oocytes when GIVM Plus with supplements was used. Moreover, fertility rates are not adversely affected when cryopreserved embryos are used in infertile women (Pereira et al., 2019). One explanation is that the stress of either freezing and/or thawing oocytes caused an increase in reactive oxygen species (ROS), which damages cellular membranes (Guerin et al., 2001, Krishnan et al., 2008), or there was protein denaturation (Rusciano et al., 2017). It might be that the nutrients or growth factors required to maintain oocyte competence are insufficient or absent in SAGE medium but not GIVM Plus with supplements. However, in this study no parthenotes from human oocytes developed to blastocyst stage in any treatment group, and this could be directly related to the low developmental competence of immature oocytes or chromosome abnormalities.

Previously we have reported that mouse oocytes fertilised in the presence of L-proline (Pro) have improved subsequent embryo development, and this is due to Pro's ability to reduce ROS (Treleaven et al., 2021). In the present study exogenous addition of Pro to culture medium supported mouse parthenote development to blastocyst stage. Blastocyst formation occurred at a higher rate when parthenotes were cultured with Pro compared to culture with no AAs or with a combination of AAs. Pro-mediated improvement in parthenote development is likely due to expression of Pro transporters. A previous study has reported that mouse parthenotes exhibit Pro transport activity between the 1- and 4-cell stage and this unknown transporter also accepts betaine (Anas et al., 2007). The AA transporters PROT (SLC6A7), PAT1 (SLC36A1), PAT2 (SLC36A2) and GLYT1 (SLC6A9) are expressed in the plasma membrane and can transport Pro in oocytes (Van Winkle et al., 1990, Steeves et al., 2003, Treleaven et al., 2021). It is likely that the Pro-mediated improvement in blastocyst development observed in the present study is due to the expression of one or more of these previously characterised Pro transporters.

Others have been successful in birthing mice from parthenogenesis (Kono et al., 2004, Chen et al., 2009), however, defective imprinting in parthenotes caused by transcription of only maternal genes makes the quality of these blastocysts and cell lines difficult to elucidate. In this study the benefit observed by exogenous addition of Pro to culture medium of mouse parthenotes has yet to be confirmed with human parthenotes, especially those obtained from oocytes matured *in vitro*. Future research of the effects of Pro on human parthenote development would be beneficial. The production of parthenotes for therapeutic purposes is a good strategy to address the ethical and legal constraints of using blastocysts from fertilised zygotes. Improvement of culture medium composition may improve the quality of these parthenote-derived stem cell lines.

Chapter 5

Australian Public Attitudes on Gene Editing of the Human Embryo

Australian Public Attitudes on Gene Editing of the Human Embryo

Tamara Treleaven and Bernard E Tuch*

Technology is now available which facilitates gene editing and has recently been applied internationally to embryos in the laboratory. A 2002 law in Australia prohibits making heritable changes in embryos, regardless of whether the treated embryo is discarded thereafter. We sought to begin to understand public opinion in Australia about this matter, using a questionnaire given to the audience attending a Q and A panel of experts. We found majority support for allowing heritable changes for health purposes. If this is confirmed in a larger survey of the population, we suggest the existing law should be reviewed.

Keywords: gene editing; embryo; ethics; health law

INTRODUCTION

Genome editing technology and laws governing its use in humans is a worldwide issue. The discovery of CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats -associated protein-9 nuclease), a technology used by scientists to alter DNA sequences and modify gene function,¹ has rapidly progressed germline² and somatic cell³ research, and several countries have already used CRISPR to investigate its therapeutic potential in the human embryo.⁴ Legislation governing the use of gene editing of embryos varies across the globe. In China, human embryo research is currently not restricted by legislation, while in Europe, the Convention on Human Rights and Biomedicine allows research on human embryos *in vitro*, but prohibits the creation of human embryos for research.⁵ In the United Kingdom, the Human Fertilization and Embryology Authority recently approved a project to use gene editing on donated embryos left over from Assisted Reproductive Technology.⁶ In the United States, while the use of federal funding for human embryo research is banned, privately funded research on donated embryos is allowed. In these cases, embryos are not permitted to develop beyond 14 days.

Australian law is more conservative. It bans all inheritable changes to be made to the human genome for research and therapeutic purposes, with the laws governing this being made in 2002,⁷ well and truly before CRISPR technology was known. Public opinion on whether scientists should be permitted to use gene editing technology for advancing research and medical applications appears to be inconsistent

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¹ C Mussolino, T Mlambo and T Cathomen, "Proven and Novel Strategies for Efficient Editing of the Human Genome" (2015) 24 *Current Opinion in Pharmacology* 105.

² P Liang et al, "CRISPR/Cas9-mediated Gene Editing in Human Trippronuclear Zygotes" (2015) 6 *Protein Cell* 363.

³ M Jinek et al, "RNA-programmed Genome Editing in Human Cells" (2013) 2 *eLife* e00471.

⁴ L Tang et al, "CRISPR/Cas9-mediated Gene Editing in Human Zygotes Using Cas9 Protein" (2017) 292 *Molecular Genetics and Genomics* 525; H Ma et al, "Correction of a Pathogenic Gene Mutation in Human Embryos" (2017) 548 *Nature* 413; NME Fogarty et al, "Genome Editing Reveals a Role for OCT4 in Human Embryogenesis" (2017) 550 *Nature* 67.

⁵ FW Dommel and D Alexander, "The Convention on Human Rights and Biomedicine of the Council of Europe" (1997) 7 *Kennedy Institute of Ethics Journal* 259.

⁶ Fogarty et al, n 4.

⁷ *Prohibition of Human Cloning for Reproduction Act 2002* (Cth); *Research Involving Human Embryos Act 2002* (Cth).



with current policy. A recent consensus report from the United States National Academies of Science, Engineering, and Medicine formally suggests that scientists should be permitted by law to modify human embryos for eliminating genetic disease.⁸ Public attitudes in the United States are largely in favour of this, provided the technology is used to prevent a child inheriting a genetic disease but not for purposes of enhancing appearance or intelligence.⁹

Little is known about public attitudes in Australia on gene editing of human embryos. There is one international study involving 12,562 respondents from 185 countries including Australia, which looked at this issue,¹⁰ with almost two-thirds of respondents supporting gene editing of human embryos for health purposes. However, country differences were not looked at and the majority of respondents were not Australian. We attempted an initial foray into this area, during Sydney Science Festival Week in August 2017, by running an evening Q and A on the topic with a panel of experts including an adult gene therapist, a paediatric geneticist, a lawyer with an interest in ethics, a clinician with an interest in stem cells and policy development, and a person with a disability. A science journalist, who was editor-at-large of the science journal, *Cosmos*, was the host. The public audience was self-selected, and members were asked to complete an anonymous questionnaire both before and after the panel discussion.

ATTITUDES OF THE AUSTRALIAN PUBLIC

Of the 57 attendees, 46 completed and handed in the pre-questionnaire (response rate 80%), slightly more than the 68% who completed the post questionnaire. Two-thirds of the responders were between 20 and 30 years of age, with most being professionals (48%) and students (26%). Almost a quarter (22%) of the respondents classified themselves as scientists.

Three quarters (72%) of the respondents were knowledgeable about the topic prior to the event, with those who classified themselves as scientists knowing significantly more than others (χ^2 10.6, P = 0.005). Most respondents were supportive of (1) allowing heritable changes to embryos for health purposes (86%), (2) gene editing for clinical trials in both adults and children (98%) and (3) research to be carried out in both human (89%) and animal embryos (96%) (see Figure 1). In contrast, the majority of respondents (69%) were not supportive of allowing heritable changes to embryos for cosmetic or non-health related purposes. Gender, age and occupation had no effect on the attitudes of the respondents, except for animal research, which was supported significantly more by scientists and students than others (χ^2 10.4, P = 0.03). Following the seminar, the discussion appeared to increase support slightly for allowing heritable changes in embryos for health purposes (Figure 2), but this did not reach statistical significance (χ^2 6.41, P = 0.17).

TIME FOR A CHANGE

This is the first study of its kind in Australia to collect public opinion of gene editing of the human embryo. It supplements previous public studies in this country which show that the majority of the public supports gene therapy in general, human embryonic stem cell research for health-related purposes,¹¹ and the use of human embryos for medical research.¹² The *Prohibition of Human Cloning for Reproduction Act 2002* (Cth)¹³ and the *Research Involving Human Embryos Act 2002* (Cth)¹⁴ state that heritable

⁸ National Academies of Sciences, Engineering, and Medicine, *Human Genome Editing: Science, Ethics, and Governance* (The National Academies Press, 2017).

⁹ DA Scheufele et al, "U.S. Attitudes on Human Genome Editing" (2017) 357 *Science* 553; RJ Blendon, MT Gorski, and JM Benson, "The Public and the Gene-Editing Revolution" (2016) 374 *New England Journal of Medicine* 1406.

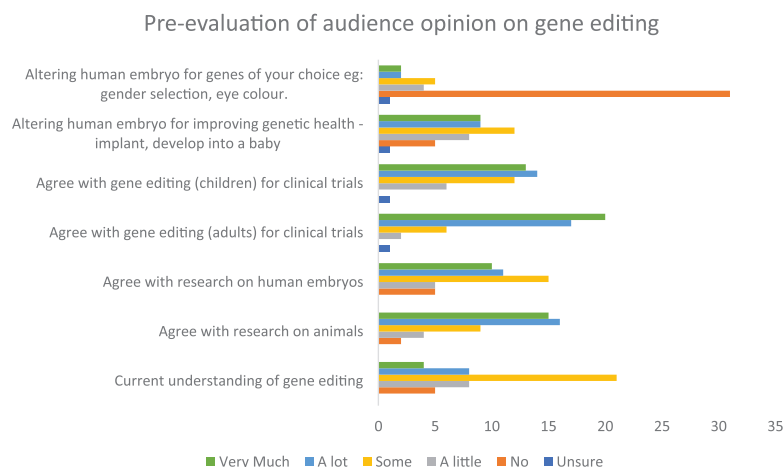
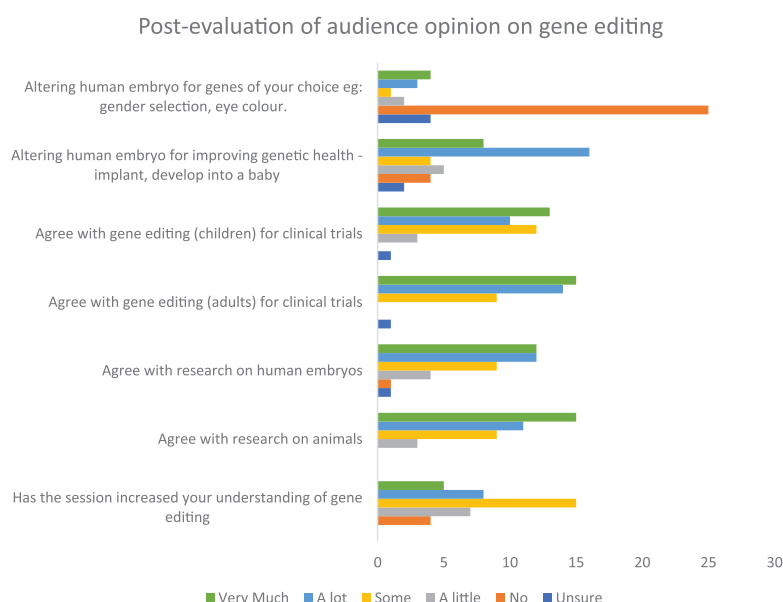
¹⁰ T McCaughey et al, "A Global Social Media Survey of Attitudes to Human Genome Editing" (2016) 18 *Cell Stem Cell* 569.

¹¹ Department of Industry, Innovation and Science and Research, Australian Government, *Public Attitudes towards Biotechnology in Australia: Results of a Survey of Public Attitudes 2009–10* <<https://archive.industry.gov.au/Pages/default.aspx>>.

¹² M Gilding and C Critchley, "Technology and Trust: Public Perceptions of Technological Change in Australia" (2003) 1 *Australian Journal of Emerging Technologies and Society* 1.

¹³ *Prohibition of Human Cloning for Reproduction Act 2002* (Cth).

¹⁴ *Research Involving Human Embryos Act 2002* (Cth).

FIGURE 1. Response to survey questions on gene editing before panel discussion.**FIGURE 2. Response to survey questions on gene editing after panel discussion.**

changes cannot be made to the human embryo, even in the laboratory, and that genetic material to create an embryo can come only from two people. Pre-implantation genetic testing does allow for selection of unaffected embryos in most would-be parents with an inherited genetic disorder, thereby obviating the need for gene editing of the embryo. However, potential parents with genetic conditions of a dominant nature, such as Huntington's disease, may have no unaffected embryos. In these cases, gene editing using CRISPR technology is a possible option. At present, this is not an available option in Australia, but it is in the United States, China, Sweden and England.

Of course, it is as yet unknown what effect gene editing of an embryo will have on a child born from this altered embryo. It may be that deleting the abnormal genetic message and replacing it with the normal genetic sequence may cause unexpected and unwanted other changes to the child. This is a good reason why one would not want to proceed too rapidly down this pathway. It may also be the reason that

even the countries permitting genetic modification of embryos in the laboratory have yet to permit the implantation of such embryos into a woman. At present, in Australia it is illegal even to explore *in vitro* the effect of CRISPR technology on an embryo, which would subsequently be discarded.

There are some who would argue that one is opening Pandora's box by developing the art of altering the genome of an embryo for health purposes. Some might then attempt to use this knowledge to produce tailor-made embryos for seemingly insignificant reasons such as selecting a particular eye colour. This is the slippery slope argument. The majority of the respondents in our questionnaire did not agree with using the technology for this purpose. We believe that guidelines can reasonably be developed to protect our society against this scenario. We suggest that a Committee, such as the NHMRC Embryo Licensing Committee, could consider each application for gene editing of embryos on its merits, and make a ruling based on (1) ability to advance scientific knowledge and (2) the likely health benefit to be achieved, both generally and when appropriate, for a specific couple.

The Q and A session was a robust one, with audience interaction. Panel members were by no means in agreement with one another – for example, on when it would be appropriate to implant a genetically modified embryo. This is similar to where the field of *in vitro* fertilization was in its infancy four decades ago. Despite this disparity of views, at least in some areas, respondents were slightly more supportive of gene editing of human embryos after the Q and A session. That this did not reach statistical significance may have been because the responses to the questionnaire were not linked at an individual level. Greater support after discussion would suggest that the public had increased awareness of the issues as a result of the debate. This has implications for public awareness, understanding and engagement processes of a larger public audience in the future.

The questionnaire did not have sufficient questions to help understand why some respondents were opposed to society changing its practices. A study in the United States found that people who have high religious affiliation, and those with low factual knowledge of the technology, were less likely to support human genome editing.¹⁵

There has been a call in both the United States and Europe to involve the public in the discussion of possible gene editing of embryos. We believe it is timely for Australia to take up this call and survey our fellow citizens about what they believe we should be allowed to practise. If there is a groundswell of support for change, we would then call to action policymakers to review legislation which will allow scientists more freedom to investigate the potential of this technology to improve human health. The current parliamentary inquiry of the Senate Standing Committees on Community Affairs into the *Science of Mitochondrial Donation and Related Matters* is an excellent start to pursuing this line of discussion. If the Committee recommends changes to the *Prohibition of Human Cloning for Reproduction Act 2002* (Cth), to allow people with mitochondrial defects to implant embryos with normal mitochondria, further changes to the Act, to allow genetic modification of embryos, might just be possible.

¹⁵ Blendon, Gorski and Benson, n 9.

Chapter 6

General Discussion

6.1 Overview

Technology used to aid in the treatment of infertility has been the focus of extensive research and development (Meseguer et al., 2012). However actual success rates for patients undergoing infertility treatment have shown little improvement in the last decade even with the introduction of novel technologies (Goodman et al., 2016, Li et al., 2018). By today's technological standards ART could be considered a suboptimal technology. Increasing the rates of pregnancies and live births is a key performance indicator for fertility clinics and the healthcare industry.

The neutral AA Pro exhibits a complexity of mechanisms that are critical for normal physiological functioning of cells. In Chapter 2, I demonstrate for the first time the beneficial effect on embryo development from the inclusion of Pro or the Pro analogue, L-pipecolic acid (PA), in fertilisation medium. In Chapter 2 and 3, I identify transporters of Pro novel to the oocyte and preimplantation embryo responsible for Pro uptake. Pro transporters have an important role in preimplantation development in AA accumulation (Dall'Asta et al., 1994, Franchi-Gazzola et al., 2004, Hundal et al., 2009), redox regulation (Phang et al., 2008, Hundal et al., 2009, Tan et al., 2011, Comes et al., 2013, Phang 2019) and as a receptor for growth-factor-like cell signalling (Tan et al., 2011, Morris et al., 2020). I discuss the significance of Pro transporter redundancy in maintaining critical development processes. In this final chapter I discuss why the results in Chapters 2 and 3 support the growing body of evidence that competitive inhibition of uptake of AAs in medium blocks the beneficial effect that select AAs have on development. In Chapter 4, I have described culture conditions that are more optimal for preimplantation embryo development, and I describe how this has clinical significance for sourcing quality pluripotent cell lines for gene therapy.

Certain research in Australia that involves human gametes or embryos is restricted by legislation. In Chapter 5, I have presented findings from our survey of a portion of the Australian public on their knowledge and attitudes towards human embryo gene editing. In this final chapter, I go on to discuss the wider implications of these results.

6.2 Effect of Pro transport in oocytes and cumulus cells

Previous studies have demonstrated that individual AAs can benefit embryo development (Gardner et al., 1994, Devreker et al., 1998, Morris et al., 2020) and are accumulated by oocytes (Summers et al., 2000, Summers et al., 2005). But it was not known how specific individual AAs impact later development when present in fertilisation medium or even whether these AAs are taken up by oocytes. Therefore the first aim of this thesis was to investigate the effect of selected AAs in fertilisation medium to determine whether oocytes take up Pro or Pro analogues. IVF of mouse oocytes in the presence of exogenous Pro or PA resulted in an increased percentage of embryos that compacted and developed to blastocysts, and an increased number of cells in the ICM. These data indicated that Pro and PA are able to be taken up by oocytes in sufficient quantities to exert an effect on subsequent development. Although it is well known that oocyte quality is critical for embryo development potential there is a lack of published articles on effects of the fertilisation medium on maintenance of oocyte competency. A previous study which added groups of EAA and NEAA, including Pro, to fertilisation medium reported some benefits to subsequent embryo development, such as increased hatching and number of cells in the ICM. But all formulations failed to increase the percentage of embryos that develop to the blastocyst stage (Summers et al., 2000, Summers et al., 2005). Addition of molar excess AAs, including Bet, Gly and His, to fertilisation medium inhibited the improvement effect mediated by Pro and PA indicating these AA prevented the uptake of Pro and PA into the oocyte. Collectively, these and other results present evidence

that current medium formulations, which contain groups of AAs, are overwhelming embryos with unnecessary nutrients, causing metabolic stress (Lane et al., 1996, Leese et al., 2008) or prevent optimal embryo development by blocking the uptake of beneficial AAs (Gardner et al., 1993, Sonoda et al., 2003, Krishnan et al., 2008, Richards et al., 2010, Casalino et al., 2011, Arnaiz et al., 2013, Ermisch et al., 2020).

Cumulus cells have previously been reported to take up Pro (Colonna et al., 1983, Corbett et al., 2014) but not oocytes (Anas et al., 2008). Pro is likely taken up into cumulus cells via B⁰AT1(SLC6A19) and/or y+LAT2 (SLC7A6) and transferred to oocytes via GJC in the COC (Colonna et al., 1983, Corbett et al., 2014). However oocytes must take up Pro via alternate mechanisms when cumulus cells are removed, i.e. when stripping oocytes in preparation for ICSI, or in vivo shortly after ovulation when the connection between COCs begins to break down (Pelland et al., 2009). In the present study, GJC was absent as cumulus cells were removed to confirm the maturation status of oocytes. Data presented in Chapters 2 and 3 indicated that Pro was taken up by oocytes via the expression of multiple transporters. These were the Na⁺ -dependant Pro transporter PROT (SLC6A7), Na⁺ -independent proton exchangers PAT1 (SLC36A1) and PAT2 (SLC36A2). The distribution of Pro transporters in the oocyte varies. High affinity Pro transporter, PROT, is expressed in the plasma membrane above the metaphase II spindle and is also localised to vesicle membranes; revealing a novel role for PROT in maintaining intracellular concentrations of Pro, and/or acting as an intracellular AA sensor for signalling pathways. PAT1 and PAT2 is expressed on the plasma membrane of oocytes and low affinity B⁰AT1 is expressed in cumulus cells (see Fig. 6.1).

The depletion of other AAs from the fertilisation medium may have caused the upregulation of AAT observed in the present study. In other cell types such as human fibroblasts, AA starvation causes an increase in SNAT2 mRNA and also System A transport activity (McGivan

et al., 1994, Gazzola et al., 2001) and in human cell lines, extracellular Arg depletion causes an increase in B⁰AT1 expression (Torrente et al., 2014). Although the data presented in this thesis cannot exclude that the addition of exogenous Pro to medium triggers the synthesis of transporter proteins that may not normally be expressed during in vivo development, the results conclusively demonstrate when Pro is added to fertilisation medium more Pro transporters are expressed on the plasma membrane.

Pro and its metabolites play a considerable role in redox homeostasis in oocytes (Hardy et al., 2021) although it was not known how the uptake of Pro effects oocyte competency. The data presented in Chapter 2 revealed a novel role for Pro transport in reducing ROS levels in oocytes. The antioxidant role of Pro in protecting oocytes from oxidative stress has also been demonstrated by others (Zhang et al., 2016). In that study spindle configuration of oocytes was maintained, and ROS levels were reduced, by the presence of 2 mol/L Pro in vitrification medium (Zhang et al., 2016). In the present study, the reduction of ROS in oocytes is likely due to uptake of Pro or PA (1) directly scavenging ROS, or (2) reducing electron transport chain (ETC) activity, which then reduces ROS production, or (3) their metabolism; which likely increases production of antioxidant glutathione (GSH) (see Fig. 6.1). Previous studies have shown that oocytes contain high levels of GSH and this supports fertilisation (Zuelke et al., 2003) (Calvin et al., 1986, Sutovsky et al., 1997). Culturing mouse cumulus cells with Pro increases GSH production (Liu et al., 2023), thus the Pro-mediated improvement in embryo development observed in the present study is likely due to transport of Pro which increases GSH production. Future studies measuring GSH concentration or GSH enzyme activity of oocytes fertilised in the presence of Pro or PA would be beneficial. The results presented in this thesis describe a functional role for transporters of Pro, as vehicles of AA transport, and initiators of important metabolic processes that reduce ROS levels in oocytes. The presence of

multiple transporters of Pro highlights the adaptive expression of Pro transport systems to maintain oocyte competency *in vitro*.

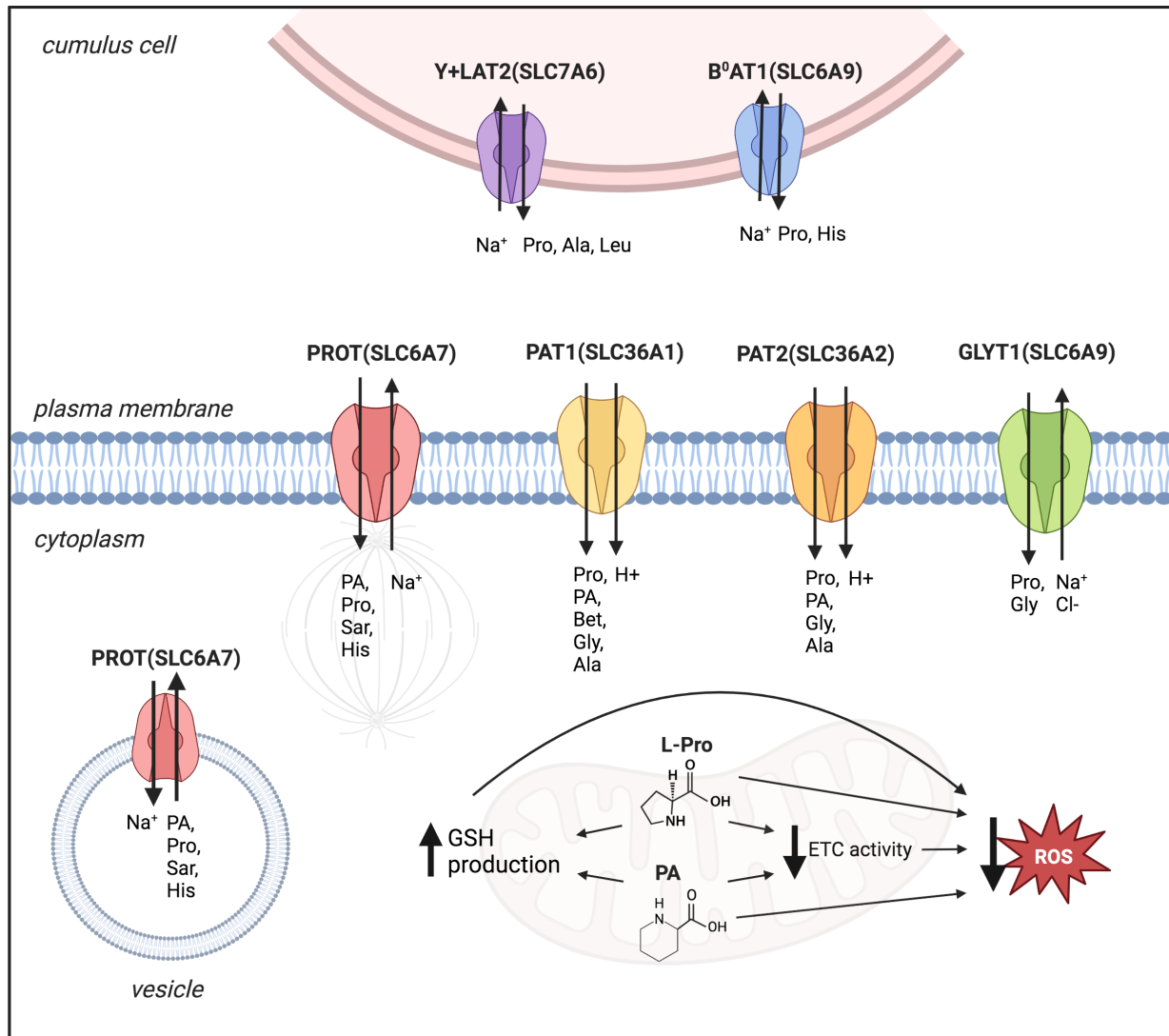


Figure 6.1. Role of Pro transporters in the cumulus oocyte complex. Pro is accumulated in oocytes via multiple transporters of Pro. B⁰AT1 and Y⁺LAT2 are expressed in cumulus cells and transport Pro to the oocyte via GJC. PAT1, PAT2 and GLYT1 are expressed on the plasma membrane and take up Pro from extracellular fluid into the cytoplasm of the oocyte. PROT is expressed over the meiotic spindle and on the membrane of intracellular vesicles, and may function to maintain intracellular levels of Pro and/or is involved in signalling processes. Uptake of Pro and PA from fertilisation medium improves embryo viability via metabolic mechanisms including decreasing electron transport chain (ETC) activity, ROS reduction, and likely increasing intracellular GSH synthesis.

6.3 Effect of Pro transport in preimplantation embryos

Pro transport is needed to support embryo development in a time-dependant manner. When Pro is added to medium of fertilised embryos, development is improved during cleavage stages, but the presence of Pro has no effect on blastocyst cell numbers (Morris et al., 2020). In this thesis I have demonstrated that when Pro is added at the time of IVF (and then subsequently removed) development is improved from compaction, and blastocyst viability is increased. Upregulation of plasma membrane AAT allows the embryo to actively move Pro, Na^+ and/or Cl^- from the extracellular fluid and between gap junctions of the cells to support the embryos changing nutritional requirements (Lane 2001, Houghton 2005). In the preimplantation embryo ASCT1 (SLC1A4) is expressed in the ICM of blastocysts (Jamshidi et al., 1995, Formisano et al., 2016), SIT1 (SLC6A20) during early cleavage stages (Anas et al., 2007), GLYT1 at the zygote, 8-cell and blastocyst stage (Van Winkle et al., 1988) and SNAT2 (SLC38A2) in mouse ESCs (Tan et al., 2011) (see Fig. 6.2). However it was not known which specific stage Pro is needed to improve development or if transporters of Pro are expressed at these stages; and therefore an aim of this thesis was to identify the specific stages when Pro is taken up by preimplantation embryos. Pro was taken up by embryos at all preimplantation stages. Exogenous addition of Pro to culture medium increased embryo development on day 4 in WT, and day 3 and day 6 in $\text{B}^0\text{AT1}$ knockout embryos, and improved the percentage of blastocysts that formed from parthenogenically-activated oocytes. This confirmed the hypothesis that Pro uptake in embryos was due to expression of $\text{B}^0\text{AT1}$. These data indicate that this occurs on day 4, or a cleavage stage just before cavitation (see Fig. 6.2.). However in $\text{B}^0\text{AT1}$ knockout embryos Pro uptake occurred at other stages, indicating that mouse embryos have compensatory mechanisms to take up Pro. This is a significant finding as it provides evidence of Pro transporter redundancy in the oocyte and embryo and highlights the extraordinary developmental plasticity of the preimplantation embryo.

Cavity formation in embryos is an important time-point that contributes to the developmental capacity of blastocysts. The B^{0,+} transport system is activated at compaction, just prior to this stage, and regulates AA accumulation so development can progress and cell differentiation can occur (Van Winkle et al., 1990, Van Winkle, 2001, Richards et al., 2010). B⁰AT1 has a low affinity for Pro transport (Broer et al., 2004, Bohmer et al., 2005). Low affinity transporters increase net substrate uptake by reducing efflux. The expression of B⁰AT1 at this stage of embryo development may be to increase the net uptake of Pro for (1) protein synthesis and/or (2) to stimulate metabolic mechanisms of ROS reduction and decreased metabolism and/or (3) activate mTOR signalling pathways (Bosdriesz et al., 2018, Morris et al., 2020), or a combination of these mechanisms. Others have shown that Pro causes differentiation of mouse ESCs and this is due to the transport of Pro for intracellular activation of mTORC1 signalling pathways (Washington et al., 2010, Tan et al., 2011, Comes et al., 2013). Pro also activates mTORC1 signalling in blastocysts (Morris et al., 2020) which increases trophectoderm development (Deming et al., 2013, Liu et al., 2021) so it is likely that expression of B⁰AT1 on day 4 is also to function as a receptor upstream of mTOR signalling pathways needed for cell growth and differentiation.

In the present study, the absence of B⁰AT1 adversely impacted fertility and blastocyst development, although the effect on embryo development could be overcome by the presence of Pro in embryo culture medium. This indicates that compensatory mechanisms are activated when important genes are knocked out, as in the case of SLC6A9 (Van Winkle, 2021). Due to the challenges of obtaining embryos from B⁰AT1 KO mice we were unable to determine if uptake of Pro in these embryos was completely via non-saturable mechanisms.

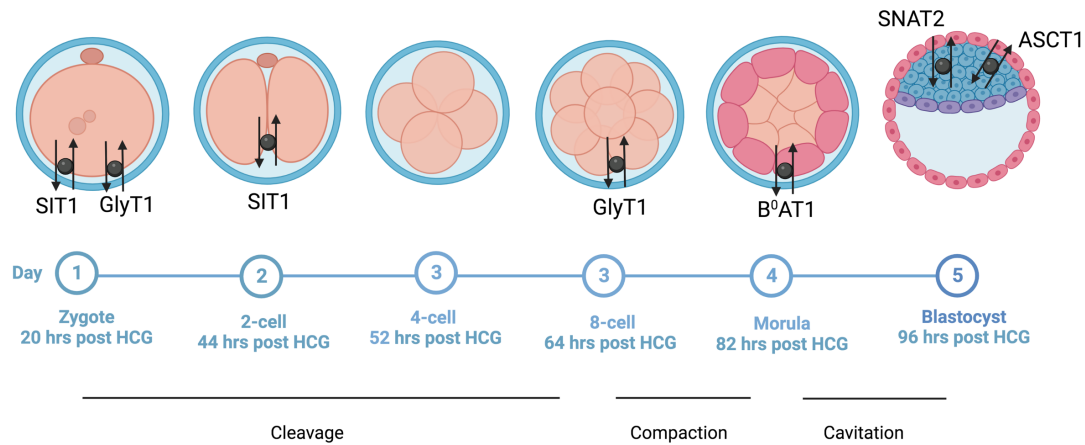


Figure 6.2 Pro transporters are expressed throughout preimplantation development in a stage-specific manner. Pro is taken up in by embryos via SIT1 after fertilisation until the 2-cell stage. GLYT1 is expressed in zygotes, 8-cell and blastocyst stage. B⁰AT1 is expressed on day 4 or at a cleavage stage just before cavitation. SNAT2 and ASCT1 are expressed in the ICM of blastocysts. References (Van Winkle et al., 1988, Jamshidi et al., 1995, Anas et al., 2007, Tan et al., 2011, Formisano et al., 2016).

Previous studies have identified that mouse ESCs can be stimulated to differentiate into a number of cell types including cardiac cells (Doetschman et al., 1985), skeletal tissue (Braun et al., 1994), and neurons (Bain et al., 1995, Shparberg et al., 2019), and there are an increasing number of early phase clinical trials using pluripotent cell lines for the treatment of stroke and spinal cord injury (Bonn, 1998), indicating that research into the procurement of quality human ESC (hESC) would be beneficial for clinical therapies. Current law in Australia bans the creation of human embryos for purposes other than achieving pregnancy, which limits the availability of hESC for research or therapeutic purposes. Parthenotes are an alternative source of embryonic stem cells (pESCs) that are pluripotent and have therapeutic applications in regenerative medicine. An aim of the preliminary experiments carried out prior to my thesis commencing was to determine if parthenogenically activated immature human oocytes which are unusable for IVF can be created for therapeutic cloning purposes. In the preliminary study

human oocytes that were matured *in vitro* and activated by chemical means arrested in commercial medium at or before blastocyst formation. Perhaps the most likely reason for the arrested development of parthenotes into blastocysts is inefficient genomic activation, or aberrant imprinting (McElroy et al., 2008) with retention of residual mRNA from immature oocytes, or perhaps because of the suboptimal quality of oocytes. An indication of low quality is that these oocytes did not develop *in vivo* despite stimulation with gonadotrophins. What would be required to enhance their quality is unknown, but it is likely that commercial culture medium is suboptimal. Thus the second aim of this study was to determine the effect of Pro supplementation in culture medium of parthenogenetically-activated mouse embryos. Exogenous addition of a physiological concentration of Pro improved development of mouse parthenotes to blastocyst stage which confirmed the hypothesis that a simplified medium with Pro is beneficial for development. Due to the lack of readily available human oocytes I was not able to investigate if Pro-mediated improvement of mouse parthenotes also benefits development of human parthenotes for the purpose of therapeutic cloning. Future studies should determine if human parthenotes take up Pro. Findings in the mouse embryo may not be fully transferrable to human embryos and this is a limitation of my study that needs further investigation. However studies on mouse embryos are an effective method to gather information on the safety and efficacy of the human ART culture medium.

6.4 Public opinion on human embryo research in Australia

There is a moral imperative for scientists and governments to regulate the responsible use of embryo modification technology for public safety, but also allow for the progression of research which has the potential to reduce severe inherited disease or to improve human health. Recent advances in embryo modification technology have increased knowledge of early developmental processes (Jinek et al., 2012, Fogarty et al., 2017) and therapies to treat inherited

diseases (Ma et al., 2017, Tang et al., 2017, Depil et al., 2020, Doudna, 2020, Wu et al., 2020). However intentionally applying technology to embryos that causes developmental and heritable changes raises significant scientific, moral and ethical considerations. A good strategy to guide legislation on the issue of embryo modification technology is to increase public understanding of the science, canvass public opinion and separate safety concerns from ethical and moral concerns (Jinek et al., 2012, Fogarty et al., 2017). It is not known if the Australian public have knowledge of genome editing technology; or if the Australian public agree with embryo modification for health or other purposes. An aim of this study was to determine the opinion of at least a segment of the Australian public on the topic of embryo modification for research purposes. In Australia, published literature pertaining to the attitudes of the public towards the use of germline editing of human embryos is limited. Current Australian legislation dealing with research that involves embryos was enacted in 2002 and it has yet to be reviewed despite significant medical advances, particularly with the discovery of CRISPR-Cas9 technology being made available in 2016. In Chapter 5, I have presented the results of a questionnaire given to participants of a Q and A event at Sydney Science Festival. The majority of the respondents agreed with allowing genome editing on human and animal embryos for research purposes or on human embryos for health purposes. These data are consistent with other studies that have reported the public would be supportive of scientists using gene editing in research to enhance human health (Critchley et al., 2018) or to prevent a child inheriting a genetic disease (Scheufele et al., 2017) or using human embryos to test the safety of gene editing technology (Scheufele et al., 2017, Kobayashi et al., 2022). In the present study respondents support for allowing heritable changes in embryos for health purposes did not increase after the event and this was due to respondents being mostly knowledgeable about the topic of gene editing prior to the event. Other studies have shown that acceptance of technology related to cell and gene therapies generally increases after the science, risks and

benefits are explained (Aked et al., 2017, Strong et al., 2017). Together these findings highlight the importance of quality public engagement activities on science and health issues.

It would seem there is a strong social objection to the use of gene editing technology on human and animal embryos when being used for enhancement purposes (Scheufele et al., 2017, Kobayashi et al., 2022). In the present study the moral objection to germline editing of embryos was shown to be strongest when its application is linked to cosmetic enhancement. There appears however to be a distinct difference between the impact on public attitude from moral concern and that from hereditary concern. Findings from a study of 1004 randomly selected Australians reported public attitude to be most strongly influenced by hereditary concern when related to human health or human research (Critchley et al., 2018), and moral concern over embryo modification, and this was strongest in respondents that were female or Australian born (Critchley et al., 2018). In the present study, gender, age or occupation had no effect on attitude towards embryo modification for research or other purposes. Future research of a larger cross-section of the Australian public would be helpful to elucidate if gender-related differences exist for moral concern over embryo modification.

It is important that the public are informed on issues that have a potentially significant impact to human health. Scientists are concerned that current regulations in Australia are holding back research that could reduce the incidence of human genetic disease (Nicol et al., 2017) and have called for a moratorium to permit fundamental research on genome editing technology (Baltimore et al., 2015). In the present study respondents were supportive for the use of human embryos in research, which is inconsistent with current policy that prohibits making heritable changes to human embryos for research, regardless of whether the treated embryo is discarded after 14 days. In Chapter 4 we describe a method to acquire high quality pESCs by artificially activating oocytes and developing embryos which have not been fertilised by sperm. Human

parthenotes are not viable and cannot be implanted and so are not considered to be a human embryo by Australian law. In 2020 the National Academies/Royal Society Report on Heritable Human Genome Editing published a report that provides a pathway for the limited approval of germline editing under certain circumstances and assuming various criteria are met (Angrist et al., 2020). Recently (2021) the Australian government passed an amendment to the *PHCR* and *RIHE* known as *The Mitochondrial Donation Law Reform (Maeve's Law)* which will allow a human embryo to contain the genetic material of more than two people and heritable changes to be made to the embryonic genome providing certain regulations are met by the licenced clinic or research facility. However this amendment does not permit human embryos to be used in genome editing research or for embryos donated to research to be developed beyond 14 days. In Australia committees such as the NHMRC Embryo Licensing Committee, could consider each application for genome editing of human embryos for research purposes on a case-by-case basis i.e. (1) ability for the research to advance scientific knowledge or (2) the likely benefit to human health to be achieved. However a case-by-case approach is likely to slow scientific advancement. Future research that increases the public's awareness of the risks and benefits of human embryo research, including a larger cross-section of Australian stakeholders would be beneficial. This may guide policymakers to review current legislation that governs human embryo research in Australia.

6.5 Summary and Conclusions

This study has identified multiple transporters responsible for Pro uptake in the oocyte and has demonstrated that stage-specific uptake of Pro by transporters is required for optimal preimplantation development. The data presented in this thesis provides evidence that supplementation of fertilisation and culture medium with a physiological concentration of Pro improves preimplantation embryo development. A simplified fertilisation medium that

contains key elements required to maintain development is a favourable strategy for promoting oocyte competency and subsequent embryo viability *in vitro*. It would be beneficial for future studies to investigate if the positive effects of Pro observed in this thesis are reflected in human oocytes and embryos. This may direct the development of a more optimised medium for human ART. Research will be ethically acceptable if the risks justify the potential benefits to human health. Results from this study suggest that public opinion on embryo modification for research is inconsistent with current policy and it is timely for Australian law on embryo research to be reviewed.

Chapter 7

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Appendix



Predicting Public Attitudes Toward Gene Editing of Germlines: The Impact of Moral and Hereditary Concern in Human and Animal Applications

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Background and Objective: New and more efficient methods of gene editing have intensified the ethical and legal issues associated with editing germlines. Yet no research has separated the impact of hereditary concern on public attitudes from moral concern. This research compares the impact these two concerns have on public attitudes across five applications including, the prevention of human disease, human and animal research, animals for the use of human food and the enhancement of human appearance.

Methods: A sample of 1004 Australians responded to either a telephone ($n = 501$; randomly selected) or online survey ($n = 503$; sourced by Qualtrics). Both samples were representative in terms of States and Territories as well as gender (51% female), though the online sample was younger ($M = 40.64$, $SD = 16.98$; Range = 18–87) than the telephone sample ($M = 54.79$, $SD = 18.13$; Range = 18–96). A 5 (application) by 3 (type of cell) within groups design was utilized, where all respondents reported their level of approval with scientists editing genes across the 15 different contexts. Multilevel modeling was used to examine the impact of moral (embryo vs. germ) and hereditary (germ vs. somatic) concern on attitudes across all applications.

Results: Australians were comfortable with editing human and animal embryos, but only for research purposes and to enhance human health. The effect of moral concern was stronger than hereditary concern, existing in all applications except for the use of animals for human purposes. Hereditary concern was only found to influence attitudes in two applications: improving human health and human research. Moral concern was found to be accentuated amongst, women, more religious individuals and those identifying as Australian, while hereditary concern was strongest amongst non-Australians, those with stronger trust in scientists, and more religious respondents.

Conclusion: Moral and hereditary concerns are distinct, and require different approaches to public education, engagement and possibly regulation. Further research needs to explore hereditary concern in relation to non-human applications, and the reasons underlying cultural and gender differences.

Keywords: gene editing, public opinion survey, cell type, morality, germline, CRISPR-Cas9, multilevel modeling, religiosity

INTRODUCTION

Since its first emergence in 2012, CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats) has become the most dominant of a suite of new generation of genetic editing methods. Extensive media and scholarly comment have declared that CRISPR-Cas9 will “supercharge” our understanding of biological life. A multitude of applications and “breakthroughs” are predicted, including the elimination of pests and diseases, advances in food production, improved human health, and even changes to the course of evolution (Funnell, 2016; Park, 2016; Doudna and Sternberg, 2017). These claims are not new, with the promise of cures for diseases and more nutritious food crops, for example, existing since the advent of genetic engineering in the 1970’s (Hogan, 2016). Relative to earlier methods however, the reduced cost, enhanced efficiency and ease of use mean that potentially any molecular biology laboratory can use CRISPR-Cas9 to insert, replace or delete sections of DNA in any living organism.

There are indeed signs that an explosion in CRISPR-Cas9 research has already commenced, with Science magazine’s 2015 “Breakthrough of the Year” already being employed to alter crops (Cai et al., 2015) and animals (Whitworth et al., 2014) for food production and the first clinical trials on humans beginning in 2016 (Cyranoski, 2016). The acceleration of CRISPR-Cas9 and other gene editing techniques have however outpaced legal and regulatory guidelines, especially in relation to heritable changes. Scientists are therefore concerned that outdated legislation and guidelines established prior to 2012 will impede progress, particularly the development of much needed human genetic therapies (Nicol et al., 2017). For example, modifications to the genome within human embryos and germ cells that can be theoretically inherited are considered especially promising in eradicating genetic disorders and understanding human development, yet this practice is currently restricted (in varying degrees) in many jurisdictions (Isasi and Knoppers, 2015; Isasi et al., 2016; Nicol et al., 2017).

In most jurisdictions, legislation attempting to guide the ethical use of genetic engineering and human embryonic research prior to 2012 was driven by political, economic, religious and community pressure to protect the moral status of the human embryo, and avoid potential dystopian outcomes such as human clones, designer babies, genetic inequity and eugenics (Nuffield Council on Bioethics., 2016). Legislation associated with the modification of animal and plant genes was also associated with pressure to prevent disruption to our ecosystem, environmental damage and, importantly, to ensure the safety of genetically

modified organisms for human consumption (Tsatsakis et al., 2017). With the availability of new gene editing methods, these ethical legal and social concerns have intensified, especially in relation to heritable edits of the human genome (Nuffield Council on Bioethics., 2016). In 2014 the scientific community was forced to confront these issues when Liang et al. (2015) modified the gene responsible for a potentially fatal blood disorder in 86 non-viable human embryos. The work was rejected by both the prestigious journals *Nature* and *Science* partly due to ethical issues, leading to a media frenzy and calls amongst the scientific community for a moratorium on editing human germlines (Cyranoski and Reardon, 2015). Acknowledging the ethical concerns as well as the potential benefits, the US National Academies of Sciences, Engineering, and Medicine convened the Committee on Human Gene Editing: Scientific, Medical, and Ethical Considerations to review the science, ethics and governance of human genome editing. A comprehensive report followed, culminating in guiding principles for governance, and recommendations for the oversight and use of human genome editing. While acknowledging that “basic laboratory research involving genome editing of human cells and tissues is critical to advancing biomedical science” (National Academies of Sciences, 2017, p4), the report recommended that both somatic and germline editing should only occur for the purpose of preventing disease or disability. Germline editing was considered only acceptable if there are compelling reasons and “a stringent oversight system able to limit uses to specified criteria” (p13).

The NASEM report also recommended an ongoing “reassessment of both health and societal benefits and risks, with broad ongoing participation and input by the public” (p178). Other scholars agree arguing, that “public sentiment has considerable influence over allocation of resources, political policy and participation rates in studies, all of which affect the course of research” (McCaughy et al., 2016, p. 571). More specific to gene editing, a survey of 301 attendees (74% basic scientists) at a plenary entitled “Scientific, Clinical and Ethical Implications of Genome Editing” at an American Heart Association conference in 2017 found that 72% indicated that they would not support “clinical applications of human germline genome editing if the public was not asked about their opinion on the issue” (Musunuru et al., 2017, p. 2). Lawyers and ethicists also advocate public engagement to prevent a potential backlash against media hype associated with germline editing obscuring or biasing the potential benefits gained from gene editing somatic cells within the clinic (Lanphier et al., 2015; Nicol et al., 2017). The aim of this research was therefore to conduct a national survey to extend our knowledge of attitudes toward gene

editing, inform policy and, importantly, provide a voice for the public.

A first step in considering public opinion is to determine the extent of support and its drivers. Apart from a handful of studies, little is currently known about lay perceptions of gene editing of germline cells and embryos in humans or animals. To date, only five peer reviewed surveys have been published post 2012 (McCaughey et al., 2016; Gaskell et al., 2017; Rose et al., 2017; Weisberg et al., 2017; Scheufele et al., 2018), reporting results that coincide with earlier findings that people tend to support genetic and genomic research generally (including genetic engineering/modification) (Lucht, 2015) if it is safe and the purpose is altruistic (e.g., to improve human health) (Henneman et al., 2006; Condit, 2010). The first peer reviewed study within the context of CRISPR also found more support for therapeutic relative to enhancement applications, but support for using embryos depended on the purpose (McCaughey et al., 2016). Amongst a young (Median age = 24) predominately male (62.1%) sample of 12,562 social media users across 185 countries (primarily the US, UK, and China), McCaughey et al. found that the majority supported editing cells in embryos to prevent a debilitating (63%) or life threatening disease (63%) (And where “all future generations would not have the disease”), and within children or adults to cure a debilitating (59%) or life threatening disease (59%) (And where “the disease could still be passed on to their children”). Support was low for editing genes within an embryo to alter any non-disease characteristics such as memory, eye color or height (27%)¹. They also found significantly greater support for all applications amongst males, younger people and those without a religious affiliation. Support for editing genes to treat diseases was also significantly higher for those who had finished a tertiary education and for those in wealthier countries². Self-reported wealth, however, was not associated with support, nor was a family history of a monogenic Mendelian disease.

Gaskell et al. (2017) also found significantly high support in 2017 for therapeutic, relative to enhancement applications. An online panel sample of 11,716 respondents across the US and UK, Austria, Denmark, Germany, Hungary, Iceland, Italy, the Netherlands, Portugal, and Spain was used to investigate the perceived morality of different applications of gene editing. They also found strong support for therapeutic applications and low support for the purposes of enhancement across all countries. Specifically altering “the gene sequence in some affected cells” to improve symptoms of dementia in an adult patient (Median = 8 from a possible 11 point scale) and in an unborn child genetically at risk for dementia (Median = 6) were viewed as more morally acceptable than enhancing memory and learning capacities in a healthy adult (Median = 2) or unborn child (Median = 0). Altering the genes of adults was also viewed as more morally acceptable than altering the genes of an unborn child, again in all

countries. The level of moral acceptability was not significantly associated with age or education, but females were generally less supportive than males of all applications.

A recent (late 2016 and early 2017) national survey of 1600 U.S. adults found similar results (Scheufele et al., 2018). Higher approval was reported for therapeutic applications (e.g., human gene editing to treat human medical conditions or restore health) than for human enhancement (e.g., human gene editing to enhance or improve human abilities). Support was similar and relatively high for both somatic and germline therapies. More religious respondents and women were less supportive of gene editing for both applications.

A fourth study utilized 1,249 online panel members (recruited by Amazon’s Mechanical Turk) to investigate the impact of metaphors or how genetic editing is framed (i.e., genetic editing, genetic engineering, genetic surgery, and genetic hacking) on beliefs in whether we should “be actively researching these technologies” (−3 = Absolutely not, 0 = Unsure, 3 = Absolutely yes) (Weisberg et al., 2017). They also explored whether exposure to the risks of gene editing³ influenced beliefs in whether the technology should be used. They found, amongst the relatively young (Median = 37.2 years), left wing and educated sample of predominantly males, strong general support for gene editing ($M = 1.65$, $SD = 1.32$). There were no differences in relation to framing, but the study found support decreased for respondents who received the vignette containing the risks ($M = 1.43$, $SD = 1.36$) relative to those who did not ($M = 1.87$, $SD = 1.24$). Men, younger people, non-African Americans and those with more than 4 years of college education and reporting a more left wing political ideology were significantly more supportive. Having a genetic disease or being related to someone with a genetic disease was not associated with support.

Finally, two studies reporting on participants of public scientific engagement events also reported strong support. The first was conducted with 183 members of the US public who participated in the 2015 Wisconsin Science Festival. A total of 34 participants attended a panel discussion including scientists, academics and ethicists on human gene editing titled “Designer Genes: Should We Be Able to Edit Our Genomes?” A survey administered to the participants before ($n = 34$) and after ($n = 26$) the festival found that participants’ generally believed that “genetic editing is morally acceptable” ($M = 3.38$, $SD = 1.00$; 1 = Strongly Disagree–5 = Strongly Agree), and that this belief increased significantly after the panel discussion ($M = 4.00$, $SD = 0.71$). Knowledge of gene editing also significantly increased after the event as well as perceptions that there are both benefits and risks to society.

Another evaluation of 57 public attendees to an Australian Q and A expert panel discussion on gene editing (as part of the Sydney Science Festival) also found support for editing the genes of adults and children for clinical trials, human embryos for research, and animals for research (Treleaven and Tuch, 2018). The majority also approved of the use of gene editing within human embryos to improve health and where the embryo is

¹ Although this study also assessed attitudes towards genetically modified food, the level of agreement for the question, “How much do you agree with the use of genetically modified food?” was not reported.

² Prior knowledge of gene editing was also shown to be significantly associated with support for the two adult and children disease conditions, but the direction of the effect could not be ascertained as the question or coding of responses for this question were not available.

³ The risks stated were: For individuals, it could have unintended consequences, or lead to unexpected mutations. For society, it could lead to eugenics.

allowed to develop into a baby, although support was lower for this than other applications. Respondents did not agree that altering human embryos for enhancement purposes (i.e., gender selection, eye color) was acceptable. Although support appeared to increase for altering the genes of embryos after the discussion, the differences were not significant.

Research examining public opinion relating to new forms of genomic editing is therefore in its infancy. Emerging evidence appears to suggest support varies according to the application and particularly in relation to editing within human embryos, but this also appears to vary alongside individual differences unique to particular samples (i.e., those derived from online panels or social media). There has also been no direct investigation into the relative influence of both the source of cell (human, animal, embryo, reproductive, somatic) and the purpose of editing (to prevent disease, research purposes, enhance attributes). In particular, there has been no attempt to separate the effects of editing germ cells from those that occur within embryos. Establishing greater public awareness of the distinction between germline and somatic cell editing has been repeatedly emphasized (Lanphier et al., 2015; Cartier-Lacave et al., 2016; Olson, 2016), yet we know little about whether public support is influenced by concerns about inherited edits being passed on to offspring. Previous studies have only compared support for gene editing somatic to embryonic cells (McCaughy et al., 2016; Gaskell et al., 2017) which may confound concern with inherited edits with moral or religious values attached to human embryos (Allum et al., 2017).

We therefore directly investigated for the first time whether public support for gene editing differs across somatic, germ cells and embryos suggesting distinct underlying concerns. Differences between germ and embryonic cells were defined as concern for the moral status of the human embryo (referred throughout as moral concern), while variation in support for somatic compared to germ cells was considered concern for passing edits on (referred to throughout as hereditary concern). Using both a probabilistic population survey (randomly digit dialed telephone interview) as well as an identical online panel survey (sourced by Qualtrics), we also examined the effect of cell type on attitudes in combination with different applications: for research purposes only; to improve health or prevent disease; in animals for human use; and for human enhancement (e.g., to change appearance). Finally we also explored the role of demographic and individual difference variables (i.e., trust in scientists, political orientation, knowledge) in explaining moral or hereditary concerns and general support for gene editing. Whilst current research suggests support will be lower for enhancement than for improving human health, we had no prior expectations of which factors and what combinations would be most important in predicting support. Nevertheless, the ability to directly compare multiple factors within the same study was expected to shed light on which issues cause most concern, and thereby provide valuable direction for policy makers keen to address public confidence and trust in regulatory procedures governing gene editing, and on where to direct educational strategies.

MATERIALS AND METHODS

Participants and Procedure

A total of 1004 respondents were surveyed with an identical measurement instrument, 501 via a computer assisted telephone interview (CATI) and 503 via an online panel sample (OLP) sourced through Qualtrics⁴. Both samples were stratified by state and territory, with the OLP being also stratified by gender. The CATI survey consisted of randomly generated mobile (43.5%) and landline (56.5%) numbers sourced by Sampleworx⁵. The response rates for the CATI survey according to the American Association for Public Opinion Research (2009) definitions and calculations (i.e., RR1–RR4) ranged from 6.0 to 9.0% with a cooperation rate of between 13.8 and 15.4%.

CATI and OLP respondents were not significantly (at $p < 0.05$) different in terms of state and territory, gender (CATI = 51.3% female; OLP = 51.1%), and political orientation (CATI: $M = 5.00$, $SD = 2.17$; OLP: $M = 4.97$, $SD = 2.12$; Range: 1 = Left wing–10 = Right wing). The CATI sample ($M = 54.79$, $SD = 18.13$; Range = 18–96) was however significantly ($p < 0.001$) older than the OLP sample ($M = 40.64$, $SD = 16.98$; Range = 18–87), more likely to have a university degree (CATI: 47.9%; OLP: 36.0%), and less likely to state that their ethnicity was Australian (CATI = 76%; OLP = 83.3%). The OLP sample was more likely to be working full (38.2%; CATI = 28.9%) and part time (20.9%; CATI = 15.7%), engaged in home duties (9.9%; CATI = 3.4%) and unemployed (10.9%; CATI = 6.8%), while the CATI sample were more likely to be retired (36.5%; OLP = 12.5%). The CATI sample also reported being significantly ($p < 0.001$) more spiritual (CATI: $M = 2.67$, $SD = 1.17$; OLP: $M = 2.33$, $SD = 1.06$; Range = 1 = Spirituality is not at all important–4 = Very important), though there were no differences in church attendance (apart from weddings, baptisms or funerals) ($p = 0.877$) or trust in the churches ($p = 0.10$). There were no significant differences between samples in terms of their self-reported knowledge of gene editing or trust in scientists. Both groups demonstrated relatively low knowledge on a 10-point scale where 0 = I know nothing about gene editing and 10 = I know a great deal about gene editing (CATI: $M = 2.80$, $SD = 2.57$; OLP: $M = 2.91$, $SD = 2.71$). Trust in scientists was relatively high for both groups (CATI: $M = 3.83$, $SD = 0.99$; OLP: $M = 3.70$, $SD = 1.05$) in response to being asked “how much do you trust scientists” (0 = Don’t trust at all–5 = Trust a very great deal) (see **Supplementary Materials** for actual wording of all questions).

Materials

The survey questions were included in the 2017 Swinburne National Technology and Society Monitor, and were identical for the CATI and online sample. Before introducing the topic of gene editing, all respondents were asked about their knowledge of gene editing and were then read the following information:

“Genome editing is when scientists deliberately alter the genes in a living cell to change how a gene functions. So far most uses

⁴Qualtrics online panels. Available online at: <https://www.qualtrics.com/au/online-sample/>

⁵Sampleworx.

of gene editing have been for research purposes only, however there are many possible applications. For example, it could be used to alter the genes of plants and animals to increase resistance to disease. It could also be used to develop new drugs, prevent the inheritance of diseases, and determine the attributes of babies.”

Questions were designed to vary across the type of application (improving health, research only, changing appearance and animals for food) and cell source (embryo, germ cell, somatic cell). Respondents were first asked about cells or embryos where the edit could be inherited. Specifically they were asked, “The first lot of scenarios relate to editing genes that could potentially lead to the change being inherited (passed onto another human or animal).” They were then presented with 10 different options (described below) that varied by source and aim, and asked, “For each of the following do you agree with editing the genes of?” The presentation of each option was random across respondents, and the response options were: Strongly Disagree = 1, Disagree = 2, Agree = 3, Strongly Agree = 4, and Unsure.

In this section, two items assessed the application of improving health or preventing disease: “Human embryos [reproductive cells (i.e., egg or sperm)] to improve health or prevent disease.” For research purposes four items were used to assess attitudes across embryos and reproductive cells and animal and humans. That is, “Human [Animal] embryos for research purposes only” and “Human [Animal] reproductive cells (egg or sperm) for research purposes only.” Enhancing attributes was assessed by two items: “Human embryos [reproductive cells (i.e., egg or sperm)] for reasons of producing a baby with certain genes e.g., for hair color, gender selection.” Finally, two items measured differences in source across the aim animals for human purposes, “Animal embryos [reproductive cells (i.e., egg or sperm)] for human purposes (e.g., improving the quality of beef).

Participants were then asked about somatic cells. Specifically, interviewers read out the following:

“The next lot of scenarios relate to editing the genes within a somatic/body cell that cannot pass on the change to another human or animal. For each of the following again, let us know the extent to which you agree or disagree with editing the genes of.”

Six scenarios were randomly presented that assessed attitudes across the five aims. That is, “A human body cell (e.g., eye or heart cell) to improve health or prevent disease (e.g., blindness)?”, “A human [animal] body cell for research purposes only,” “A human body cell to change one’s appearance,” and “An animal body cell to alter its characteristics for human purposes (e.g., leaner beef in cows).”

RESULTS

Preliminary Analyses

To check for significant differences across the CATI and OLP samples, a series of one way analyses of covariance (ANCOVA) were computed for all 15 scenarios using SPSS Version 25. The demographic differences between the two samples determined the covariates since differences in support may be due to differences in age, education or ethnicity for example rather than because of the different survey administration. A series of binary logistic regression equations suggested that the differences in

employment status across CATI and OLP respondents was due to older people being more likely to be retired and less likely to be working full or part time and unemployed. Differences across the groups in education, ethnicity, spiritual beliefs, and home duties were independent of differences in age. Thus, all (except for occupational status) were used as covariates along with age in the ANCOVA analyses. After controlling for the demographic factors, the results revealed no significant differences in mean agreement across the two samples in all but one of the 15 scenarios (see **Table 1** for the adjusted means across samples). The exception was for human embryos for research purposes, where support was significantly, though only slightly higher ($p = 0.028$) for the CATI ($M = 2.63$, $SE = 0.04$) than OLP sample ($M = 2.48$, $SE = 0.05$). Given the lack of differences it was decided to combine the two samples for all subsequent analyses.

Table 1 suggests that, for both samples, support dropped markedly for applications involving altering human cells to change appearance and altering animals for human purposes. All means for both samples were below the midpoint of 2.5 for these applications, and above for all other applications. Support appears particularly high for applications aimed at improving human health across all three cell types. The OLP was significantly more likely to select unsure to nearly all scenarios apart from those involving editing of germ and somatic cells for human research purposes and changing appearance, and editing embryos of animals for human purposes. This was after controlling for the effects of all covariates in a binary logistic regression, comparing the probability of being unsure relative to providing an answer across the OLP and CATI samples. This is likely due to a modality difference between the online and phone surveys where “unsure” was presented as a response option for the OLP but not for CATI (although it was accepted if a respondent stated to an interviewer that they were unsure).

Predicting Attitudes

To assess change in attitude across cell type within each application, a multilevel model approach (Bryk and Raudenbush, 1992; Goldstein, 1995; Longford, 1995 using MLWIN Version 1.10; Rasbash et al., 2001) was employed. Two analyses were undertaken, the first to assess whether support for gene editing differed across cell type and application separately (i.e., main effects), and whether variation across cell type depends upon the aim (i.e., interaction effects). The second set of analyses examined the ability of the demographic and individual differences factors to predict variation across cell type. Both analyses utilized a three level model where variation in the outcome variable (gene editing support) across respondents was level 3, variation across applications was level 2, and variation across cell type was level 1. Unsure responses in all models were treated as missing.

Moral and Hereditary Concern Across Gene Editing Applications

Table 2 contains the results of the 3-level model that examined the main effects of cell type and application as well as their interaction on attitude. The reference category for cell type was germ cell, meaning the parameter estimates for the predictor embryo compared support for embryos to germ cells (indicating

TABLE 1 | Adjusted mean support and percent unsure for all scenarios across CATI and OLP samples.

Application	Cell type	CATI			OLP				
		Mean	SE	n	Unsure %	Mean	SE	n	Unsure %
Improve Health or prevent disease	Embryo	3.07	0.04	450	7.2	2.97	0.04	451	10.3*
	Germ	3.15	0.04	465	4.0	3.06	0.04	465	7.6**
	Somatic	3.21	0.03	475	1.8	3.15	0.03	479	4.8**
Research purposes only (Human Cells)	Embryo	2.63	0.04	453	6.4	2.48°	0.05	428	14.9***
	Germ	2.82	0.04	444	8.6	2.80	0.04	445	11.5
	Somatic	2.85	0.04	459	5.2	2.90	0.04	462	8.2
Research purposes only (Animal Cells)	Embryo	2.80	0.04	454	6.2	2.75	0.04	436	13.3**
	Germ	2.85	0.04	466	4.0	2.81	0.04	450	10.5**
	Somatic	2.83	0.04	459	5.2	2.85	0.04	457	9.1*
Change Appearance (Human cells)	Embryo	1.74	0.04	474	2.6	1.84	0.04	468	7.0*
	Germ	2.01	0.04	446	8.0	2.12	0.04	449	10.7
	Somatic	1.97	0.04	466	4.2	1.90	0.04	465	7.4
Animals for human purposes (food)	Embryo	2.41	0.04	435	10.2	2.38	0.04	440	12.5
	Germ	2.41	0.04	442	8.6	2.40	0.04	442	12.1*
	Somatic	2.28	0.04	451	6.8	2.29	0.04	446	11.3**

Means are adjusted for age, education, ethnicity, spiritual beliefs and home duties. ° = ANCOVA results revealed mean was significant from CATI sample at $p < 0.05$; SE, Standard Error; Range for attitude, Strongly Disagree = 1, Disagree = 2, Agree = 3, Strongly Agree = 4. *Results of binary logistic regression predicting unsure compared to provided an answer across OLP and CATI samples (controlling for age, education, ethnicity, spiritual beliefs and home duties) significant at $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

moral concern), and those for somatic cell compared support for germ cells with somatic cells (indicating hereditary concern). Application predictors were constructed to reflect deviation contrasts or change in support across a specific aim (e.g., Improving health) compared to all other aims. Cell type by application interactions were used to assess whether any change in support across cell type depended on the gene editing application. Separate models were computed for each application (using deviation contrasts) and the results are shown in **Table 2**.

The random effects in **Table 2** for the Null model reveal that there was significant variation in support for gene editing across applications and cell type and, as would be expected, across respondents. The relative size of the effects reveals that overall what gene editing is used for (i.e., the application) has a greater influence on support compared to the type of cell (0.36 vs. 0.26). In relation to the main effects for application, models 2–6 reveal that in comparison to all other applications (and averaged over cell type), support was significantly higher for improving human health, and for human and animal research (see **Figure 1** for predicted means resulting from the estimates in **Table 2**). Support was significantly lower for enhancement and the use of animals for food. The fixed, main effect for cell type in Model 1 reveal that, averaged over all applications, there was significantly higher support for gene editing when scientists use germ cells compared to embryos, suggesting that the level of support for gene editing is dependent on moral concerns for the embryo. Model 1 also suggests that there was no significant difference between germ and somatic cells, thereby providing no evidence

that hereditary concerns influence support for gene editing in general.

The pattern of results for the interaction effects, however, reveal that in some contexts, hereditary concerns may matter. When scientists use gene editing to improve human health or prevent disease, the somatic by health interaction (in Model 2) was significant. This suggests that support increases when somatic cells are used relative to germ cells when gene editing is used to improve human health, but when it is used for other purposes, the tendency for support to be higher for somatic relative to germ cells is not apparent (see **Figure 1**). In other words, hereditary concerns are important when gene editing is used to improve human health and for human research but not for animal research, enhancement, or using animals for food. There were no significant somatic vs. germ cell differences for animal research, but unexpectedly for human enhancement and using animals for food, the significant somatic cell interactions revealed that support was higher for germ than somatic cells (see **Figure 1**).

The interactions relating to embryos (relative to germ cells) were also significant across all applications. Gene editing in embryos generated significantly lower support than germ cells across all applications except animals for food, but the extent of this effect varied across applications (see **Figure 1**). The effect was largest for human research and human enhancement (represented by the negative interaction effect), and smallest for human health and animal research. There was no difference in support for embryos relative to germ cells for the use of gene editing in animals for food.

TABLE 2 | Parameter estimates for predicting support for gene editing across application and cell type.

Effect	Null model	Model 1 (cell type)	Model 2 (health)	Model 3 (human research)	Model 4 (animal research)	Model 5 (appearance)	Model 6 (animals for food)
Intercept	2.58(0.02)	2.63(0.02)	2.52(0.02)	2.59(0.02)	2.59(0.02)	2.78(0.02)	2.69(0.02)
FIXED EFFECTS							
Embryo		−0.13(0.01)	−0.14(0.01)	−0.11(0.01)	−0.15(0.01)	−0.09(0.01)	−0.16(0.01)
Somatic		−0.02(0.01)	−0.04(0.01)	−0.03(0.01)*	−0.02(0.01)	0.01(0.01)	0.01(0.01)
Health			0.57(0.03)				
Health × Embryo			0.06(0.02) *				
Health × Somatic			0.12(0.03)				
Human Res.				0.20(0.03)			
Human Res. × Embryo				−0.13(0.03)			
Human Res. × Somatic				0.09(0.03)			
Animal Res.					0.23(0.03)		
Animal Res. × Embryo					0.10(0.03)		
Animal Res. × Somatic					0.03(0.03)		
Enhancement						−0.71(0.03)	
Enhancement × Embryo						−0.17(0.03)	
Enhancement × Somatic						−0.13(0.03)	
Animals for food							−0.29(0.03)
Animals for Food × Embryo							0.16(0.03)
Animals for Food × Somatic							−0.11(0.03)
RANDOM EFFECTS							
Level 3 Respondent	0.21(0.01)	0.21(0.01)	0.22(0.01)	0.21(0.01)	0.21(0.01)	0.23(0.01)	0.21(0.01)
Level 2 Application	0.36(0.01)	0.36(0.01)	0.28(0.01)	0.35(0.01)	0.34(0.01)	0.22(0.01)	0.34(0.01)
Level 1 Source	0.26(0.00)	0.26(0.00)	0.26(0.00)	0.26(0.00)	0.26(0.00)	0.26(0.00)	0.25(0.00)
−2*loglikelihood	29570.5	29397.13	28600.65	29268.9	29255.88	27969.17	29167.65

The fixed effects for Embryo, Somatic, Health, Food, Human Research, Animal research, appearance, and Animals for food represent main effects. Effects with “×” represent interactions. Standard errors are contained within parentheses. All parameters were significant at $p < 0.001$ unless otherwise stated. * $p < 0.01$. Bolded parameters were not significant at $p < 0.05$. The −2*loglikelihood value represents overall model fit, with lower values representing better fit. The Null model is a model that contains no predictor variables. N was 923 for all models.

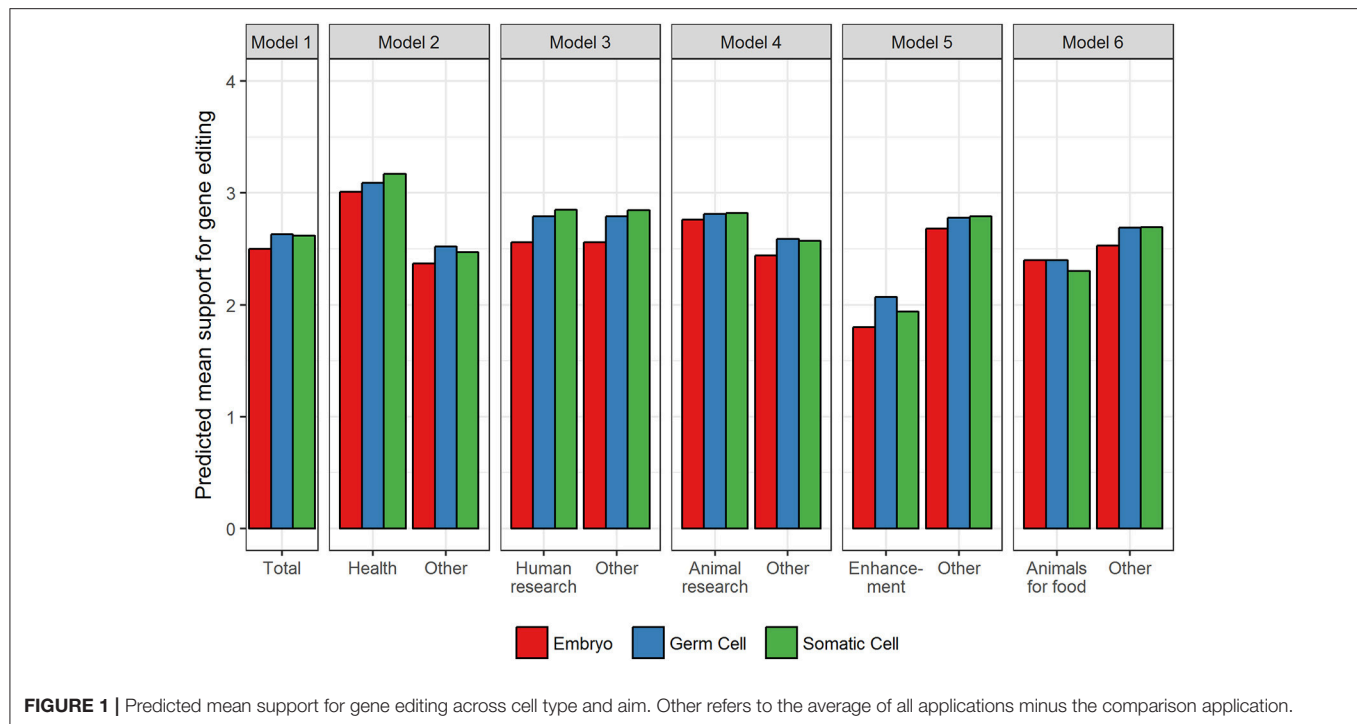
Individual Differences in Moral and Hereditary Concern

The second set of analyses examined individual difference predictors of variation in support for gene editing across cell type. The first model contained the following fixed effect predictors entered simultaneously: CATI vs. OLP sample; Embryo vs. Germ Cell; Somatic vs. Germ Cell; Age, Gender, Education, Religion, Ethnicity, Gene Editing Knowledge, Political Orientation and Trust in Scientists (see **Table 3** for parameter estimates). A second model added interactions between the individual difference factors and the two cell type predictors, embryo and somatic, to investigate whether moral or hereditary concern varied across different respondents. Given that hereditary concerns were only found to occur in gene editing to improve human health and human research, a third model was tested using only these two applications in the analysis. Interactions were entered one at a time in the order presented in **Table 3**. If the interaction was significant at $p < 0.05$ it was retained in the model. **Table 3** shows the results for the final models.

The fixed main effects for the individual difference variables in **Table 3** for Model 1 suggest that attitudes toward gene editing significantly vary according to age, gender, religion, knowledge

and trust in scientists. Averaged over cell type and application, higher support was evidenced amongst younger people, males, those with lower religiosity, and those with more self-reported knowledge and trust in scientists. Education, ethnicity and political orientation were not associated with general support for gene editing. The interaction effects for Model 2 averaged over all applications in **Table 3** suggest that only gender and ethnicity were significantly associated with moral concern. Specifically, the predicted means (derived from the regression equation) show that males ($Y' = 2.38$) and females ($Y' = 2.23$) demonstrated similar support for gene editing using germ cells, but support was lower amongst females ($Y' = 2.11$) than males ($Y' = 2.32$) for the use of embryos. This suggests that the moral status of the embryo was more of a concern for females ($Y'_{\text{embryo}} - Y'_{\text{germ}} = -0.12$) than males ($Y'_{\text{embryo}} - Y'_{\text{germ}} = -0.06$). The predicted means for ethnicity also show that moral concern was stronger for Australians ($Y'_{\text{embryo}} [2.05] - Y'_{\text{germ}} [2.26] = -0.21$) compared to non-Australians ($Y'_{\text{embryo}} [2.11] - Y'_{\text{germ}} [2.31] = -0.12$).

Interestingly, when only the applications of improving human health and human research were analyzed, the interaction effects in Model 3 show that moral concern still differed across males and females, but also across different levels of religiosity. An



effect for ethnicity was no longer significant, suggesting that Australians demonstrate greater moral concern overall, but not when the purpose of the editing is to improve human health or human research. The predicted means for gender show that moral concern was stronger for women ($Y'_{\text{embryo}} [2.29] - Y'_{\text{germ}} [2.54] = -0.25$) compared to men ($Y'_{\text{embryo}} [2.45] - Y'_{\text{germ}} [2.61] = -0.16$) when the purpose of gene editing is for human health and research. The predicted means for religion showed that as religiosity increased the gap between support for using embryos compared to germ cells increased (see **Figure 2**). Thus, greater moral concern was associated with stronger religiosity when gene editing is used to improve human health and human research.

In relation to hereditary concern, the interaction effects in Model 2 in **Table 3** reveal that variation in support across somatic vs. germs cells was significantly associated with ethnicity, trust, and religion. Interestingly, this was only the case when all gene editing applications were included in the analysis. When only human health and research were examined, there was no evidence for hereditary concerns either across the entire sample or amongst specific individual difference groups. The predicted means for ethnicity (using the coefficients from Model 2) showed a reversed hereditary concern effect, in that averaged over all aims, support for germ cells was higher than somatic cells. This was the case for both Australians and non-Australians, but the difference was greater for Australians (0.13) than non-Australians (0.05). The predicted means for the effect of religiosity revealed that hereditary concern increased alongside stronger religiosity. For example, for those who scored zero on religiosity, support for germ cells was greater than somatic cells (0.08). This difference however decreased with stronger religiosity, disappearing for those who scored on the extreme end

of the scale (i.e., religiosity = 5) (-0.03). The effect for trust in scientists showed higher predicted support for germ relative to somatic cells for those demonstrating low levels of trust (i.e., trust = 0) (0.07), whilst slightly higher support for somatic relative to germ cells was estimated for those reporting high levels of trust (i.e., trust = 5) (-0.04).

DISCUSSION

In general, the results of this Australian survey correspond with US and European findings, suggesting public support for gene editing depends on the application (McCaughy et al., 2016; Gaskell et al., 2017). That is, support is relatively high when the purpose is to enhance human health, and low when the purpose is to enhance attributes or appearance in otherwise healthy individuals. Extending on existing knowledge, we also found the sample supported editing genes within the context of animal as well as human research, and that both generated significantly more support than enhancing human appearance and animal attributes for the purpose of improving human food. These results coincide with general research suggesting that Australians demonstrate consistently high levels of trust in scientific research (Bruce and Critchley, 2003–2017), general support for the use of animal models in medical research (under certain conditions) (Critchley et al., 2013) and a distaste of designer babies and genetically modified animals for food (Marques et al., 2015). They also validate the findings generated from recent online panel studies, given the similarities we found between our OLP and CATI samples (after statistically controlling for demographic differences).

TABLE 3 | Parameter estimates for predicting variation in support across cell type from individual differences.

Effect	Model 1	Model 2	Model 3 (human health and human research only)
Intercept	2.20(0.09)	2.24(0.10)	2.43(0.13)
FIXED EFFECTS			
MAIN EFFECTS			
OLP	−0.01(0.03)	−0.01(0.03)	−0.03(0.04)
Embryo	−0.12(0.01)***	−0.12(0.05)*	−0.12(0.08)
Somatic	−0.02(0.01)	−0.139(0.05)**	−0.06(0.08)
Age	−0.002(0.001)*	−0.002(0.001)*	−0.002(0.001)*
Female	−0.18(0.03)***	−0.15(0.03)***	−0.07(0.04)
University educated	0.03(0.03)	0.03(0.03)	0.02(0.04)
Religion [†]	−0.06(0.01)***	−0.06(0.01)***	−0.08(0.02)***
Australian	−0.02(0.04)	−0.03(0.04)	0.05(0.05)
Knowledge	0.02(0.006)***	0.02(0.006)***	0.015(0.008)
Political orientation	0.01(0.01)	0.01(0.01)	0.01(0.01)
Trust in Scientists	0.18(0.02)***	0.17(0.02)***	0.19(0.02)***
INTERACTIONS			
Embryo × Female		−0.06(0.02)**	−0.09(0.04)*
Somatic × Female		−0.03(0.02)	−0.01(0.04)
Embryo × Religion		−0.003(0.01)	−0.037(0.015)*
Somatic × Religion		0.02(0.01)*	0.02(0.015)
Embryo × Australian		0.06(0.03)*	
Somatic × Australian		0.08(0.03)**	
Embryo × Trust		0.004(0.01)	
Somatic × Trust		0.02(0.01)*	
RANDOM EFFECTS			
Person	0.20(0.01)	0.20(0.01)	0.20(0.02)
Type	0.31(0.01)	0.31(0.01)	0.15(0.01)
Cell	0.25(0.00)	0.25(0.00)	0.26(0.007)
−2loglikelihood	25727.0	25696.6	9860.59

Standard errors are contained within parentheses. [†]Religion consisted of the mean score of church attendance, spirituality and trust in the churches ($\alpha = 0.73$). The Null model was identical to that presented in **Table 2**, so was not reported here. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. n was 821 for all models.

Moral Concern Associated With Editing Embryos

In line with other recent studies (Critchley et al., 2013; Treleaven and Tuch, 2018) our results suggest that Australians are relatively comfortable with the idea of editing human and animal embryos, but only for research purposes or to enhance human health. Editing human and animal embryos for the purpose of enhancing human or animal attributes, respectively, was not supported. Thus, this study aligns with earlier research suggesting that Australians are generally supportive of embryonic research if it will lead to cures for serious human disease (Critchley and Turney, 2004; Critchley, 2008). Moral concern was however found to occur in all applications assessed by this research except for editing animal embryos for the purpose of improving human food. The low support for this application combined with the absence of a moral concern effect suggests the influence of other factors.

Strong public opposition to genetically modified animals for food has been consistently demonstrated within Australia (Bruce and Critchley, 2003–2017; Marques et al., 2015) and elsewhere (Schuppli et al., 2015), and has often been attributed to unease associated with safety, animal welfare and low trust in regulators (Kronberger et al., 2014; Shriver and McConnachie, 2018). It is possible, therefore, that these factors may override the influence of moral concern for animal embryos when the purpose is for human food. Moral concern did however reduce support in the context of using animals for research. Perhaps moral concern in a research context reflects concern with animal welfare rather than safety or lower trust which are more likely to occur within the context of genetically modified animals for food.

In the two research contexts, the impact of moral concern on support for gene editing was stronger for human compared to animal embryos. While this may reflect a value orientation emphasizing the importance of human life over all other forms (at least for those opposed to embryonic research more generally) (Kass, 1997), it does not support the findings of a national survey of 2700 Australians that stem cell research using animal embryos generated statistically equal levels of comfort (or discomfort) as human embryos (Critchley et al., 2013). The fact that moral concern was also less important for the application of enhancing human health than the human research context may provide a clue to this inconsistency. The human research condition was articulated to respondents as simply, “do you agree with editing the genes of human embryos for research purposes only,” while the enhancing human health condition was, “human embryos to improve health or prevent disease.” The question relating to human research could have been interpreted as including multiple purposes (including some that are potentially undesirable such as cloning) resulting in higher concern for the embryo than when health benefits are directly promised, and where ethical concerns are more likely to be compromised (Caulfield et al., 2006).

Similarly, an underlying willingness to compromise ethical or moral principles if research is likely to cure disease may have also explained the lower moral concern for animal embryos in the research context. In the Australian study where similar support was found for research using animal (i.e., mice) and human embryos (Critchley et al., 2013), respondents were clearly informed in both contexts that the aim of the stem cell research was to find cures for untreatable diseases in humans. The only other animal scenario included in this research was the unpopular application of editing animal cells for the purposes of human food. With the negative food application statistically controlled for, the research purposes for animals could have been perceived to include research that was beneficial for animals as well as humans. Because no question assessing specific health benefits using animals was included, this was not controlled for as it was for human research. Thus, it was possible that animal research was perceived to include more beneficial purposes on average than human research (as preventing disease via human editing was accounted for). Clearly future research is needed to confirm this finding by also including a scenario where the use of gene editing within animals is described as being conducted

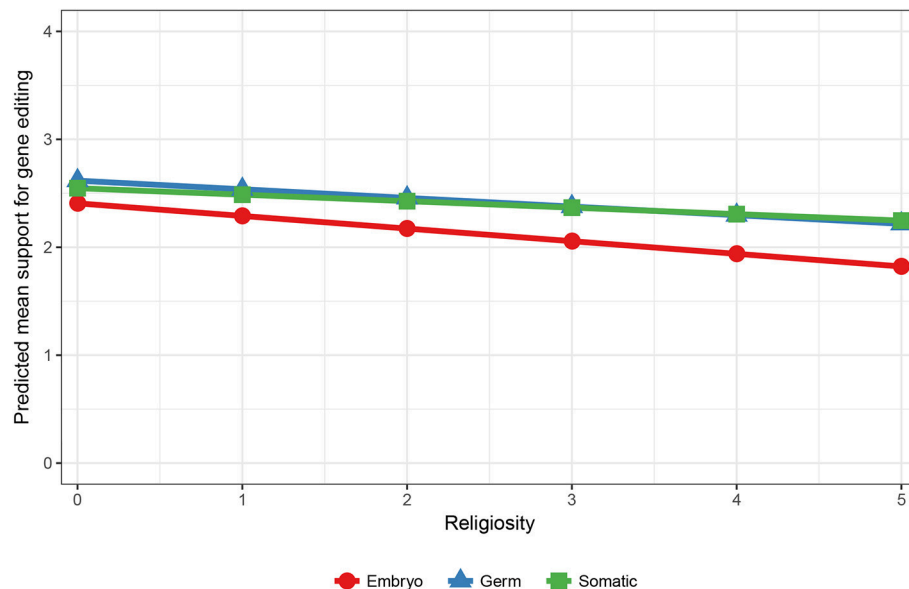


FIGURE 2 | Predicted mean support for gene editing across cell type and religiosity. 5 = Higher religiosity.

specifically to improve human health or animal health and welfare.

Hereditary Concern

Previous studies have confounded hereditary and moral concerns by only using embryos (or unborn children) to compare with somatic or adult cells (McCaughey et al., 2016; Gaskell et al., 2017). The design of this study allowed for the separating of these two effects to confirm for the first time, that public concern about the moral status of the embryo is independent from any hereditary concern. Although moral concern was found to be more pervasive than hereditary concern, the latter was found to be present for applications involving humans. Averaged over all applications there was no significant difference in support between cells that were specifically described as being able to transmit edits to offspring (i.e., germ cells) and those that could not (i.e., somatic cells). However, when human genes are edited for the purpose of improving human health or for human research, support for the use of germ cells was significantly less than somatic cells. Gene editing animals for research or human purposes did not generate concern for edits being passed on to future generations. Although future research is needed to examine the reasons for these findings, a possible explanation for the lack of hereditary concern amongst animal applications is that the breeding and behavior of domesticated research and farm animals is tightly regulated within Australia, arguably posing little threat that their modified genes will be transferred to the wider ecosystem.

This is inconsistent however, with earlier research concluding people are not comfortable with the possibility of genetically engineered animals passing on harmful defects to offspring (Schultz-Bergin, 2018). This would suggest hereditary concern due to fear for the offspring's welfare rather than detrimental

safety or environmental effects. If moral concern was associated with welfare concern, then any worry about edited animals passing on harmful effects to offspring may have been removed when the effects of moral concern on gene editing support were accounted for (at least in the animal research application). Future research should therefore assess support for gene editing within animals in additional contexts that directly specify the potential for harmful consequences to the animal's offspring (e.g., the type of gene edited and resulting phenotype) as well as the environment (e.g., gene editing in animals that are not in captivity). Comparing reactions to the editing of non-domesticated animals would also be helpful, particularly those whose breeding could permeate the wider ecosystem.

Individual Differences in Support for Gene Editing

Averaged over cell type and application, the current research found significantly higher support amongst males, younger people, and those reporting lower religiosity, more knowledge and stronger trust in scientists. This supports the work of McCaughey et al. (2016) as well as Weisberg who also found amongst a sample of social media users and online panel members, respectively, higher support for gene editing amongst males and younger respondents. Gaskell et al. (2017) also found higher support amongst males, but age was not associated with attitudes. In line with the findings of McCaughey et al., we also found that those with lower religiosity were more supportive. In contrast to previous studies however (McCaughey et al., 2016; Weisberg et al., 2017), and similar to the results reported by Gaskell et al., higher support was not found to be associated with higher education. Finally in contrast to Weisberg, a more left wing political ideology was not associated with more support in this study.

Possible explanations for the demographic differences in attitudes to gene editing in general are likely to be complex involving psychological, socio-political and cultural factors (Hartman et al., 2017). Indeed there is still debate within the general attitudes toward science literature about why certain demographic differences influence attitudes. The complexity is also compounded by the different measures, analyses techniques and combination of demographic variables used across studies (Sturgis and Allum, 2001). For example Weisberg et al. found that support for gene editing rose for those with a more left wing political ideology but did not measure or control for religiosity. We controlled for both of these correlated variables (Rutjens et al., 2017) and found religiosity rather than political orientation was important. Thus, understanding why certain demographic characteristics may or may not influence attitudes to gene editing also requires much more research including direct comparisons of cultural or country differences in the importance of predictors (especially religion and political orientation), as well as comparisons across different types of samples. Indeed our research demonstrated extensive differences between the demographic background of the OLP and CATI surveys, which could further alter the impact of certain demographic variables on support. Despite this, our results add to previous findings that males, younger respondents and those with more education are more supportive of gene editing. Given that higher support for science and emerging biotechnologies (including GM foods) in general have also often been found amongst these groups (Rutjens et al., 2017), future research should also investigate whether gene editing is viewed differently from other biotechnology applications amongst different groups or whether this is simply a reflection of support for science in general (Hartman et al., 2017).

In relation to moral concern for embryos, it was found that women and those who were Australian born displayed significantly more unease when all applications were considered. However, when the application involved only human embryos (for human health enhancement and human research) the effect remained for gender but disappeared for ethnicity, and became significant for those with stronger religiosity. This suggests that women are more concerned than men about embryonic editing regardless of its purpose, and that Australian born respondents are only more concerned in applications involving animals and enhancing human attributes. In support of previous research indicating religious beliefs are associated with lower support for embryonic research (Nisbet, 2005; Ho et al., 2008; Critchley and Nicol, 2009; McCaughey et al., 2016) increasing discomfort with editing embryos was displayed as religiosity increased. The fact that religion did not significantly predict increased moral concern for embryos when the application involved animals or enhancing human attributes provides support for the idea that even when the editing is being conducted to cure or prevent disease, human embryos are considered sacred and morally distinct from animal embryos for religious individuals.

The findings associated with gender and moral concern coincide with previous findings that women are generally less accepting of biotechnology and science in general than men. The reasons for more general gender differences are not clear

and have been attributed to women's lower interest, knowledge and educational opportunities (Hayes and Tariq, 2000), decreased trust in science and increased religiosity compared to men. Some scholars have also suggested that gender differences are due to men attributing more importance to science due to stereotypical beliefs associating science with male rather than female attributes (Rutjens et al., 2017). While we cannot assess differences due to stereotypical beliefs, gender effects in this study were still found to significantly reduce support for editing embryos (in all applications) after removing the effects of knowledge, trust, education and religion, thereby suggesting alternative explanations.

Interestingly, Simon (2012) argues that women are less supportive of biotechnology because they are more likely than men to be directly affected by any negative consequences. Potentially harmful outcomes of new and untested biotechnological developments have more direct implications for women's bodies, their reproductive processes, offspring and the selection and preparation of food. Feminist scholars also point out that women's bodies are often at the center of biotechnological controversies, especially reproductive technologies which are at particular risk of increasing commodification of the human body (Kirejczyk, 2008). This may further generate an emotive "yuck factor" type response amongst women providing another source of discontent to explain their decreased support for research involving embryos. These explanations remain speculative however, as the complex reasons for gender differences in relation to concerns for human and animal embryos need to be further investigated. There are also other possible reasons for gender differences, such as women being more sensitive to genetic inequality given their historical oppression by men. Research needs to directly capture the underlying values, emotions, motivations and world views of both men and women, and how they may interact with other contextual factors to drive gender differences in support for gene editing.

There were no gender differences in hereditary concern which is interesting given women's consistent reduced general support relative to men for a range of biotechnologies. Perhaps the description of germ cells could have been attributed to either gender effectively removing Simon's (2012) gender salience effect. General hereditary concern was however significantly associated with ethnicity, trust and religion. Possibly reflecting a general distaste toward scientists playing god, higher religiosity was associated with greater hereditary concern. The result for ethnicity was intriguing as both Australians and non-Australians demonstrated a reverse hereditary effect in that editing germ cells were viewed as more positive than somatic cells, but the effect was even more pronounced for those reporting an Australian identity.

After controlling for all demographic and individual differences as well as moral concern and any associated welfare concerns, the reverse hereditary effect may indicate that editing germ cells is perceived to be more beneficial than containing an edit within a somatic cell. Improving human attributes, removing diseases or improving animals for food, if safe and not morally objectionable (and conducted by trusted scientists),

would have more impact if unwanted characteristics are no longer passed onto future generations. The phrasing of the information provided to respondents before they answered any questions emphasized the ability of gene editing to prevent the inheritance of disease and increase resistance. This may have primed some respondents to view the passing on of desirable edits as positive rather than negative, supporting Shriver and McConnachie's (2018) notion that new forms of gene editing (especially within animals) may be viewed as more acceptable due to not mixing species and a reduction in off-target edits resulting in harmful consequences. They further argue that increasing an understanding amongst the public of these issues may therefore increase support for editing germlines in some contexts if it were used in the interests of animal and human welfare as well as the environment.

Clearly more research is needed to unravel why hereditary concern may or may not translate into support for germline editing. After statistically removing the effects of moral concern, trust and religion, Australians were curiously even more supportive of germline editing than other ethnic identities. An explanation for this interesting result may be that Australian ethnic identity could have acted as a proxy for other factors associated with a preference for germ cells, such as increased exposure to positive media portrayal of the benefits of gene editing. But this is purely speculative and difficult to determine given the vast array of ethnic identities contained in the non-Australian category and the absence of measuring media exposure in this study. It is also curious that increased knowledge was associated with support overall, but was not associated with reversed hereditary concern. If increased knowledge included an awareness of the ability of more recent gene editing tools to reduce ethical and safety concerns it should be associated with more support for editing germ cells. Clearly what self-reported knowledge (as measured in the current study) is measuring needs to be examined, and more objective measures of knowledge across a number of areas of gene editing should be constructed, as well as instruments to capture the extent and type of media exposure.

Another explanation for the reversed hereditary finding is that it may be an artifact of a methodological effect. Support for gene editing measures were presented to respondents in two sections. The first described edits that can be passed onto offspring and contained the embryo and germ cell questions together. The second section contained only the scenarios with somatic cells. By placing the embryo and germ cells together a contrast bias may have occurred resulting in inflated support scores for germ cells. The more emotionally charged embryo examples may have been used as the perceptual anchor in which to evaluate or compare the germ cell applications. If an individual views embryo editing as undesirable, they may perceive the less negative comparative option (i.e., germ cells) to be more positive than they normally would (Skowronski and Carlston, 1989). In other words to emphasize their negative orientation toward embryos higher support is attributed to the apparent opposition which was germ cells. Because negative behaviors or attributes are more likely to be used as the perceptual anchor (Skowronski and Carlston, 1989), and perceptions of them are less resistant to change (Baumeister et al., 2001), it is more likely that an increased

positivity bias would be in effect for germ cells (Wänke, 1996) rather than an inflation of disapproval for embryos. This could therefore explain why support was higher for germ rather than somatic cells in some applications.

Even higher support for germ relative to somatic cells was found for those lower in trust. This is counter intuitive given that it would be expected that those who do not trust scientists would be more concerned about edits being passed on (if harmful). In line with previous research in the wider biotechnology literature, higher trust was strongly associated with greater support for gene editing across all applications and cell types. After controlling for all demographic and individual difference variables and the significant interactions between the somatic/germ comparison and ethnicity, religion and gender, the effect of trust on hereditary concern may have inadvertently captured a susceptibility to a contrast bias. After accounting for religion and gender which were also associated with hereditary concern, lower trust in scientists may have represented a range of factors responsible for an increase in bias such as a general tendency for skeptical individuals to polarize comparisons (Gunther, 1988), decreased cognitive engagement with the survey questions (Bukovskaya and Shmukler, 2016) or susceptibility to cognitive biases in general (Spithoven et al., 2017). It would therefore be beneficial for future research to reduce the potential for contrast effects by using a between groups design where different individuals are presented with each cell type and application.

CONCLUSIONS

This study has revealed that the nature of support for gene editing is complex and determined by the application, the type of cell and also individual differences. Importantly, it has revealed for the first time, that concerns about edits being passed onto human offspring are present alongside those associated with the moral status of the embryo. It also suggests, albeit tentatively that germline editing may not be controversial amongst some members of the public if moral (and possibly other) concerns are dealt with. One of the most important ethical issues arising from the gene editing community is the risk of passing on unintended or detrimental genetic modifications to future generations. This is distinct from the use of embryos in research more broadly, and as such requires a different approach to public education and engagement. It also arguably supports the implied recommendations of the International Summit on Human Gene Editing that called for distinct regulatory responses to ensure the protection of future living organisms and the environment, as well as the protection of the embryo at one point in time. Current bans in some countries on all germline editing may be considered by a large majority of the public to be too restrictive. Our result that hereditary and moral concern are independent therefore suggests that policy makers and regulators keen to accommodate public opinion should consider carefully the separate issues involved with editing embryonic compared to other types of germ cells.

The editing of somatic cells was found to be supported in all applications assessed by this research apart from editing the genes of animals for human use. This suggests that the likelihood of public opposition to editing is low within the clinic where

therapeutic developments by altering the genetic information responsible for Mendelian diseases is currently most promising. Though lower in some applications, support for editing cells within gametes was also generally supported, though again not within animals for human purposes. Although more research is needed to validate the findings, this study implies that the public do support edits that can be passed on to future offspring and that this support may increase alongside an awareness of new methods such as CRISPR-Cas9. That is, specific knowledge relating to CRISPR-Cas9's potential to reduce harmful off-target edits and not needing to introduce the genetic material from one species into another. Education and engagement strategies targeting religious individuals and non-Australians may also increase this support further.

DATA AVAILABILITY STATEMENT

Datasets are available on request—the raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the National Statement on Ethical Conduct in Human Research, National Health and Medical Research Council (Australia) with informed consent from all subjects. The protocol was approved by the Swinburne University Human Research Ethics Committee.

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AUTHOR CONTRIBUTIONS

CC and DN conceived the study. CC, DN, GB, and JW contributed to the design of the survey instruments. GB and JW collected the data. CC drafted the manuscript and analyzed the data. All authors made a substantial contribution toward the development of the final manuscript and approved publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fgene.2018.00704/full#supplementary-material>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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