ACCUMULATION OF BETAINE IN THE DEVELOPING MOUSE OOCYTE REQUIRES CHOLINE DEHYDROGENASE

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Abstract

In the developing mouse oocyte, as well as in the preimplantation embryo, betaine (N,N,N-trimethylglycine) plays an important role first as a mechanism for cell volume regulation and second as a major methyl donor. Thus, the presence of betaine has implications both during development, and throughout the lifespan. It has previously been observed that betaine accumulates in the mouse oocyte as it matures, however its origin in the egg is unknown.

Here I explore the enzyme choline dehydrogenase (CHDH; EC 1.199.1) as a method by which the mouse oocyte synthesizes the betaine that we observe prior to initiation transport activity in the preimplantation embryo. I carefully monitored betaine transport throughout meiotic maturation to confirm that no other previously unobserved membrane transport existed in the maturing oocyte. However, no betaine transport into oocytes was detected during meiotic maturation suggesting *de novo* synthesis. Previous data suggests that the enzyme is expressed (at the transcript level) in the developing oocyte, and becomes active during meiotic maturation. I demonstrated the presence of CHDH protein in the oocyte and preimplantation embryo.
I then examined whether the mouse oocyte synthesizes betaine autonomously and addressed whether CHDH is a requirement for this process. *Chdh* knockout oocytes did not accumulate betaine *in vivo,* while normal betaine levels were observed in *Chdh* wildtype oocytes. CHDH-mediated synthesis of betaine was directly confirmed by detection of increased betaine in oocytes matured *in vitro* in the presence of choline. *Chdh*$^{-/-}$ oocytes failed to produce betaine when similarly cultured in choline. This establishes the production of betaine as an autonomous process in maturing oocytes. Overall, I have built upon previous data to demonstrate that betaine accumulation is a feature of meiotic maturation that occurs by *de novo* synthesis of the molecule, a process that requires transient activation of the enzyme choline dehydrogenase.

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List of Abbreviations

AC = adenylyl cyclase

ANOVA = analysis of variance

APC = anaphase promoting complex

ART = Assisted Reproductive Technologies

ATP = adenosine triphosphate

BGT1 = betaine transporter

BHMT = betaine homocysteine methyltransferase

BMP15 = bone morphogenetic protein 15

BMP6 = bone morphogenetic protein 6

bp = base pairs

BSA = bovine serum albumin

cAMP = cyclic adenosine monophosphate

cGMP = cyclic guanocine monophosphate

CDC25 = cell division cycle 25 phosphatase

CDK1 = Cyclin dependent kinase 1

cDNA = complementary deoxyribonucleic acid

CEEF = cumulus enabling expansion factor

CHDH = choline dehydrogenase

CNS = central nervous system

COC = cumulus oocyte complex

CPM = counts per minute

CSF = cytostatic factor

Cx = connexin

EGF = epidermal growth factor

dbcAMP = dibutyryl cyclic adenosine monophosphate

Fig. = figure

Fmole = femtmole

FSH = follicle stimulating hormone

GABA = γ-aminobutiric acid

GC = granulosa cells

GDF9 = growth differentiation factor 9

GLYT1 = glycine transporter 1

GTP = guanylyl triphosphate

GV = Germinal Vesicle

GVBD = Germinal Vesicle Breakdown

Has2 = Hyaluronan synthase 2

hCG = human chorionic gonadotropin

HEPES = 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid

HPLC = high performance liquid chromatography

h = hour

i. p. = intraperitoneally

ICM = inner cell mass

IU = international units

IVF = *In Vitro* Fertilization

IVM = *in vitro* maturation

KSOM = K^+ supplemented optimized medium

LC-MS/MS = liquid chromatography with dual mass spectroscopy

LH = luteinizing hormone

MAPK = Mitogen-activated protein kinase

MEM = minimum essential medium Eagle

MGCs = mural granulosa cells

MI = meiosis I

MII = meiosis II

mKSOM = modified K+ supplemented optimized medium

MPF = Maturation Promoting Factor

NPPC = natriuretic peptide precursor C

P1-P21 = Post-natal day 1-21

PB = polar body

PBS = phosphate buffered saline

PCR = polymerase chain reaction

PDE = phosphodiesterase

PGCs = primordial germ cells

PKA = protein kinase A

PKC = protein kinase C

PLC = phospholipase C

PMSG = pregnant mare's serum gonadotropin

PN = pronuclei

PVA = polyvinyl alchohol

PVS = perivitelline space

RIPA buffer = radioimmunoprecipitation assay buffer

RNA = ribonucleic acid

RT-PCR = reverse-transcribed polymerase chain reaction

SAM = S-adenosylmethionine

SDS = Sodium Dodecyl Sulfate

SDS-PAGE = Sodium dodecyl sulfate polyacrilamide gel electrophoresis

SEM = standard error of the mean

SMIT = Na^+/myo-inositol transporter

SOM = simplex optimized media

TAUT = taurine/β-amino acid transporter

TGFβ = Transforming growth factor β

TonE = tonicity responsive enhancer

TonEBP = tonicity enhancer binding protein

UV = Ultraviolet

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1. Introduction

1.1 Folliculogenesis,

1.1.1 Mammalian follicular and oocyte development

Oogenesis in mammalian females begins during fetal development when the diploid primordial germ cells (PGCs) migrate from their extra-embryonic origin to the gonadal ridges in the primitive gonads (Falconer and Avery, 1978; Hogan et al., 1994). The PGCs rapidly undergo multiple mitotic divisions until they reach the primitive undifferentiated gonad to produce a large population of germ cells. In females the presence of the PGCs in the gonad stimulates sexual differentiation of the ovary and stimulates oogenesis and folliculogenesis. As oogonia within the ovary cease to proliferate, the cells become differentiated into primary oocytes. These clusters of germ cells become enclosed by early granulosa cells (GCs) and a rigid layer of glycoproteins known as the zona pellucida and are known as primordial follicles. The primary oocytes within primordial follicles enter meiosis, but are arrested at the diplotene stage of the first meiotic prophase. Oocytes arrested at prophase I contain a large nucleus covered by a nuclear envelope also known as the germinal vesicle (GV) (Hogan et al., 1994).

More than half of the primordial follicles that develop in the ovary degenerate at birth during a large wave of oocyte death (Faddy et al., 1983). However, during every reproductive cycle once sexual maturity is reached, follicle stimulating hormone (FSH) released by the pituitary initiates groups of remaining primordial follicles to develop (Figure 1). These recruited oocytes begin a period of extensive growth. The early GCs that surrounded the primordial follicle differentiate into cuboidal GCs. This involves the entire follicle becoming a multi-

laminar structure as the GCs proliferate to encircle the oocyte with multiple layers of cells. This forms the preantral or secondary follicle. Late secondary follicles develop a clear layer of theca cells separated from granulosa cells by a basement membrane. As fluid accumulates between the GCs the follicle begins to swell and a fluid filled cavity forms known as the follicular antrum. Fully mature or Graafian follicles have several layers of GCs surrounding the oocyte and a large fluid-filled antrum. This antrum divides the GCs into two populations: cumulus cells directly surrounding the oocyte which promote its growth and development, and the mural GCs that line the follicular basement membrane and are primarily active in steroidogenesis.

As the oocyte gradually increases to 80% of its mature size, it acquires the ability to progress beyond arrest at prophase I of meiosis, known as meiotic competence (Wassarman et al., 1979). Mature, fully-grown and meiotically competent oocytes in the antral follicle will resume meiosis in response to correct hormonal stimulus or will resume meiosis spontaneously if removed from the follicle and cultured in supportive media (Handel and Eppig, 1997). In humans, a single dominant follicle containing a fully grown and competent oocyte will be ovulated in response to luteinizing hormone (LH) and released into the oviduct to be fertilized (Wassarman et al., 1979; Demestere et al., 2012).

1.1.2 Cell communication in the ovarian follicle

From the beginning of follicular growth in the ovary, gap junctions connect both the oocyte to GCs, as well as the GCs to each other (Kidder and Vanderhyden, 2010). Gap junctions act as intercellular channels to allow the passage of small molecules between cells. This couples the growing follicle into a functional unit in which all cells within

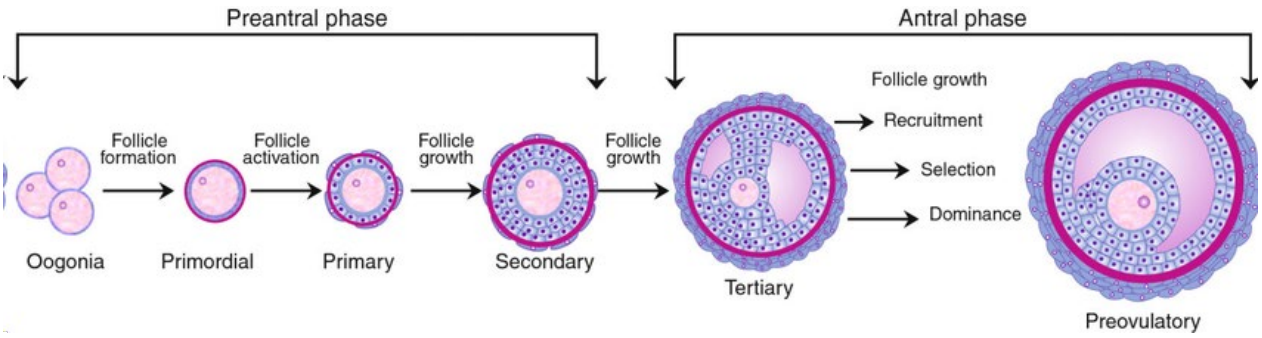

Figure 1: Folliculogensis

Schematic sequence of complete follicular development. Light pink = oocyte, dark pink = zona pellucida, light purple = granulosa cells, dark purple = theca cells. Preantral phase: Formation and beginning of growth and activation of primordial follicles and growth of primary and secondary follicles. Antral phase: formation of tertiary follicle (antral-filled follicular fluid cavity). Follicle growth continues through the phases of recruitment, selection, dominance, and preovulatory stage of follicular waves. Oogonia develop from a primordial germ cell and differentiate into an oocyte in the ovary. Primordial follicles have one layer of granulosa cells. Primary follicle has a single layer of cuboidal granulosa cells. Secondary follicle has two or more layers of cuboidal granulosa cells and a small number of theca cells. All the preantral follicles have a primary oocyte. Tertiary follicle has several granulosa cell layers, theca cells, and primary oocyte and is characterized by an antral cavity which contains follicular fluid. Preovulatory or also called as Graafian follicle is the last stage of follicle development; these follicles are larger, have more antral fluid and contain a secondary oocyte. This image was obtained with permission from Araújo et al. (2014).

the basal lamina are able to share metabolites and signaling molecules. Proper follicular and oocyte development requires cooperativity between different cell populations within the follicle by both paracrine signaling and gap-junctional communication. This allows the follicle to respond to stimuli as a functional unit. The cells that make up the follicle must act in synergy to produce a fertilizable female gamete, and mediate successful ovulation of a mature oocyte.

To this end the components of gap junctions connexin43 (CX43) and connexin37 (CX37) play critical roles in oogenesis. CX43 forms gap junctional connections between the granulosa cells which will remain metabolically coupled with each other until the LH surge causes their closure, and CX37 forms the link between the oocyte to its surrounding granulosa cells (Norris et al., 2008; Simon et al., 1997). The connections the oocyte forms with its surrounding follicle provide nutritional support, by allowing the movement of amino acids, glucose and ribonucleosides (Eppig, 1991). Additionally, the follicle connections provide ionic support for oocyte pH regulation (FitzHarris and Baltz, 2006), regulatory signals which mediate meiotic maturation (discussed below) and influence the differentiation and luteinization of the GCs (Edry et al., 2006). Gap junctional communication is required for the follicle to respond optimally to the paracrine factors that constitute another main form of cell to cell communication within the follicle.

Paracrine signals allow bidirectional communication within the follicle. Oocyte-derived factors like growth differentiation factor – 9 (GDF-9), bone morphogenic protein – 15 (BMP-15) or bone morphogenic protein – 6 (BMP-6) are required for proper granulosa cell proliferation and differentiation throughout folliculogenesis (Dong et al., 1996; Galloway et al., 2000; Otsuka et al., 2001). This suggests that the oocyte exerts paracrine control over granulosa cell

development. Conversely, the somatic GCs also support the oocyte's growth and development via the effects of KIT/KITL signaling. (Brower and Schultz, 1982; Matzuk et al., 2002) and modulate the endocrine signals needed in order for the oocyte to acquire meiotic competence, become mature and ovulate successfully (Chesnel et al., 1994; Matzuk et al., 2002).

1.1.3 Prophase I arrest

Prior to antral follicle formation, oocytes are maintained in a state of arrest at prophase I of meiosis. These oocytes are meiotically incompetent. They develop competence progressively during oocyte growth in direct relation to follicle and oocyte size as they accumulate sufficient levels of maturation-promoting factor (MPF). This molecule is comprised of two parts - cyclin-dependent kinase (CDK1) and cyclin B1 (Eppig et al., 2004). The oocytes continued arrest at prophase I, despite its physiological ability to mature further, is attributed to MPF inactivation via synthesis and maintenance of high levels of cyclic adenosine monophosphate (cAMP). Adenylyl cyclase is activated by its G-protein coupled receptor to produce cAMP. cAMP is maintained at high level by inhibition of PDE3A, which would otherwise degrade the molecule. This is achieved via production of natriuretic peptide precursor C (NPPC) by the mural GCs which stimulates the production of cGMP by its guanylyl cyclase receptor present in the cumulus GCs. cGMP enters the oocyte through the gap junctions and prevents PDE3A from hydrolyzing cAMP. The high levels of cAMP activate protein kinase A (PKA) which in turn activates Wee1B kinase and inhibits the Cdc25B phosphatase. This leads to the inactivation of CDK1. The constant degradation of cyclin B1 prevents MPF activation and therefore oocyte progression through the cell cycle (Adhikari and Liu, 2014; Wigglesworth et al., 2013).

1.1.4 Meiotic maturation

Prophase I-arrested oocytes are named after the prominent nucleus with a single nucleolus, also known as a Germinal Vesicle (GV) that is clearly visible at their center (Hogan et al., 1994). The stimulatory LH surge that triggers ovulation (discussed below) also initiates a series of distinct nuclear and cytoplasmic changes as the oocyte undergoes meiotic maturation from GV oocyte to mature egg that can be successfully ovulated and fertilized (Figure 2). Nuclear maturation occurs prior to the release of the oocyte from the ovarian follicle. The oocyte is stimulated by LH to resume meiosis and progress from prophase I of meiosis to the subsequent developmental steps. This occurs as LH binds to its receptors on the mural GCs, which is thought to induce release of epidermal growth factor (EGF)-like ligands from mural granulosa cells which bind cumulus granulosa cell EGF receptors leading to increased intracellular MAPK signaling in cumulus cells. This allows meiotic progression of the oocyte. LH acts to stimulate a decrease in cAMP concentrations within the oocyte leading to the inactivation of PKA. CdC25 phosphatase is activated and catalyzes the de-phosphorylation of CDK-1, producing activated MPF to allow progression (van den Hurk and Zhao, 2005). The activation of MPF allows germinal vesicle breakdown (GVBD) and chromosome condensation and the formation of the meiotic spindle. At this point the oocyte is a metaphase I (MI) oocyte.

Active MPF is required for progression to MI, but its levels decline during the transition from first and second meiosis (Dekel, 2005). The chromosomes move toward the outer edges of the cell and the first meiotic division takes place. The first polar body, containing one set of homologous chromosomes, is extruded while the oocyte retains the majority of the cytoplasm and organelles. High MPF levels are then reestablished and prevent entry into interphase, forcing the oocyte to transition immediately into second meiosis (Dekel, 2005). MPF remains active and

signals an arrest in the second metaphase (MII), as a mature MII oocyte or egg. The MII oocyte will only resume and ultimately complete meiosis after fertilization occurs.

MII arrest is maintained by cytostatic factor (CSF) activity via the MOS-MEK-MAPK pathway (Phillips et al., 2002). The high levels of MPF trigger the translation of *Mos* mRNA and therefore the expression of MOS, a MEK kinase found in germ cells. MOS protein synthesis results in MEK activation, followed by mitogen-activated protein kinase (MAPK) activation. MAPK induces MII arrest by initiating a phosphorylation cascade which results in the inhibition of anaphase promoting complex (APC). APC is a ubiquitin ligase which targets cyclin B for degradation at the transition from metaphase to anaphase and allows progression (Maller et al., 2002). It is in this MII-arrested state that the oocyte will be released from the follicle.

Cytoplasmic maturation must also occur in the oocyte prior to ovulation. It has been observed that fully mature oocytes will fertilize and develop at low rates if collected from improperly matured follicles (Pavlok et al., 1992). Oocytes must acquire factors which allow successful fertilization, progression through early embryogenesis, implantation and ultimately development of the embryo. One major factor is the ability to produce normal Ca^{2+} oscillations immediately after fertilization which will allow completion of meiosis II, initiate oocyte cortical granule exocytosis (to prevent polyspermy), and recruitment of maternal mRNA during embryonic genome activation (Ducibella *et al.*, 2006). This requires cytoplasmic changes including the reorganization of the endoplasmic reticulum which houses the oocyte's calcium reserve (Mehlmann, 1995) and an increase in the number, location and sensitivity of 1,4,5-inositol triphosphate (IP3) receptors (Mehlmann, 1996) to allow an effective response to fertilization.

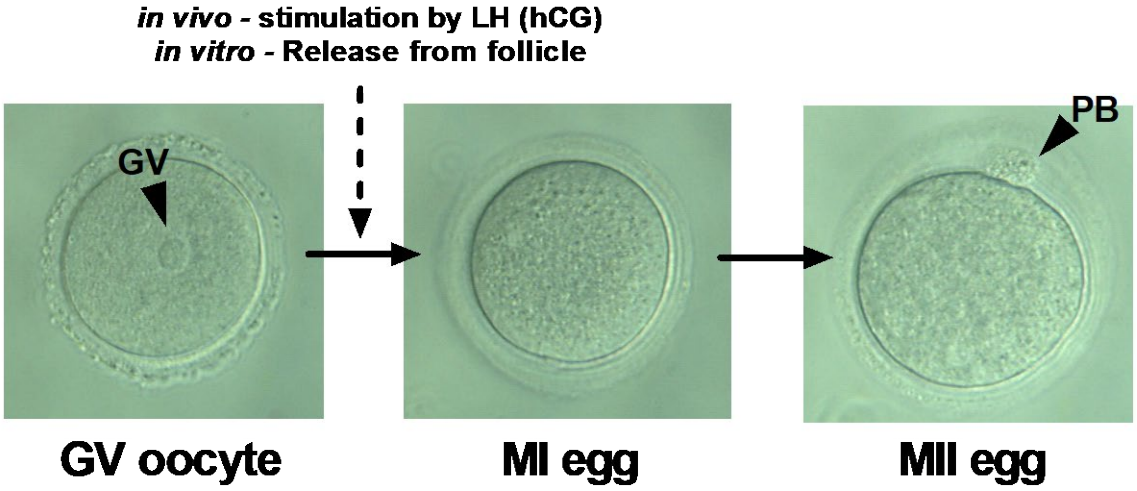

Figure 2: Oocyte meiotic maturation

Photos of mouse oocytes at various stages of meiotic maturation. The germinal vesicle (GV) stage oocyte is recognizable by its prominent nucleus with single nucleolus that is clearly visible at its center. LH stimulates meiotic maturation *in vivo,* while *in vitro* meiotic maturation is triggered by the oocyte's release from the follicle. Meiotic maturation is marked by germinal vesicle breakdown (GVBD), followed by progression through meiosis I (MI oocyte). First meiosis is completed when the oocyte extrudes the first polar body and forms the MII spindle producing a mature MII oocyte. GV = germinal vesicle, PB= polar body

On a more complex level cytoplasmic maturation comprises all the changes occurring within the oocyte allowing the accumulation of mRNA, proteins and nutrients that are required to achieve oocyte developmental competence, many of which remain unknown. However, it has been shown that the competence of the oocyte directly fosters embryonic developmental competence (Gandolfi and Gandolfi, 2001; Krisher, 2004; Sirard et al., 2006).

1.1.5 Ovulation

Ovulation is the rupture of the antral follicle and the subsequent release of the enclosed oocyte into the oviduct. In rodents, much like in humans, this process occurs mid-cycle, several hours after the stimulatory LH (and FSH) surge from the pituitary. This activates a variety of signaling pathways and affects all cell types of the ovarian follicle. LH stimulates the antral follicle to undergo a number of changes including inducing meiotic maturation of the enclosed oocyte (discussed above). The oocyte has previously been arrested at prophase of the first meiosis. The LH surge induces its progression through the first meiotic metaphase to re-arrest in the second meiosis (MII) as a mature egg (Wassarman et al., 1979; Eppig, 1996; Eppig et al., 2004).

LH signaling cannot act directly on the oocyte or on the surrounding cumulus-granulosa cells, as neither population expresses the required LH receptors (Amsterdam et al., 1975; Lawrence, 1980; Peng et al., 1991). Therefore, signaling must be transferred through the LH-responsive mural GCs to induce an ovulatory response. When LH binds to its G-protein coupled receptor on mural GCs multiple intracellular signaling pathways are activated (Salustri et al., 2004; Norris et al., 2008; Park et al., 2004). These are EGF-like peptides which lead to changes

in transcription factor expression and therefore induce gene transcription of products that will allow successful ovulation.

Upon binding of LH the oocyte excretes cumulus expansion enabling factors (CEEFs), which will activate the cumulus-cell dependent pathways leading to cumulus expansion (mucification) (Eppig, 2001; Ochsner et al., 2003). GDF9 is an oocyte-secreted CEEFs (Salustri et al., 2004). Activation of cumulus expansion pathways by CEEFs lead to transcription of cumulus-specific transcripts including Hyaluronan synthase 2 (*Has2*) (Richards, 2005), which is essential for the production of hyaluronic acid (HA). HA secretion by the GCs cells closest to the antrum will lead to the breakdown and mucification of the cumulus mass. This is required for successful ovulation and later fertilization of the cumulus oocyte complex (COC), allowing the oocyte to be captured and moved into the oviduct by the ovidutcal fimbriae. The gap junctions between the oocyte and granulosa cells will begin to close as the oocyte becomes independent, and able to sustain itself without the follicle in which it is enclosed (Gilula et al., 1978; Norris et al., 2008).

Stimulation of LH receptors on the mural granulosa and theca cells also trigger both inflammatory and pro-inflammatory gene expression cascades. This process results in a local inflammatory reaction which is associated with proteolysis and degradation of the apical follicular tissue and follicle rupture. This causes a small hemorrhage on the ovarian surface which enables expulsion of the mucified cumulus mass containing the matured oocyte into the oviduct (Espey et al., 2004). The remaining granulosa and theca cells form the corpus luteum, which will secrete progesterone and play an important role in maintaining early pregnancy (Kidder and Vanderhyden, 2010).

1.1.6 Preimplantation embryo development

As the ovulated, expanded COC is trapped by the oviductal fimbriae, the cilated cells of the oviductal epithelium transport it towards the expanded ampulla portion of the oviduct for fertilization. At this stage the oocyte remains arrested in metaphase II (MII egg) and becomes reactivated by sperm binding. Penetration of the egg plasma membrane results in release of the second polar body and the production of a fertilized diploid zygote. Following fertilization, both sets of chromosomes are surrounded by nuclear membranes. The 1-cell embryo is recognizable by the two distinct pronuclei (PN) containing maternal and paternal genetic material (Figure 3). These PN migrate to the center of the embryo and will undergo syngamy. First cleavage occurs yielding a 2-cell embryo in which each cell contains the entire new diploid genome (Cooper, 2000).

Critical to embryogenesis is the activation of the embryonic genome which allows the transition from maternal to embryonic developmental control. In mice, embryonic genome activation occurs during the 2-cell stage (Shultz, 1993). The early embryo travels down the oviduct towards the uterus while undergoing consecutive cycles of mitosis. Reductive cleavages occur and very little change in total volume is observable as the embryo becomes a 2-, 4- and 8-cell unit. During these stages the embryo cells are totipotent as each cell, referred to as a blastomere, if separated from its sister blastomeres has the potential to give rise to a complete new organism. As cleavage progresses to the 16-cell stage, the blastomeres begin to flatten out to form a morula in which the cells begin to express surface adhesion molecules (E-cadherin) and form tight junctions and gap junctions between blastomeres. This allows maximal surface

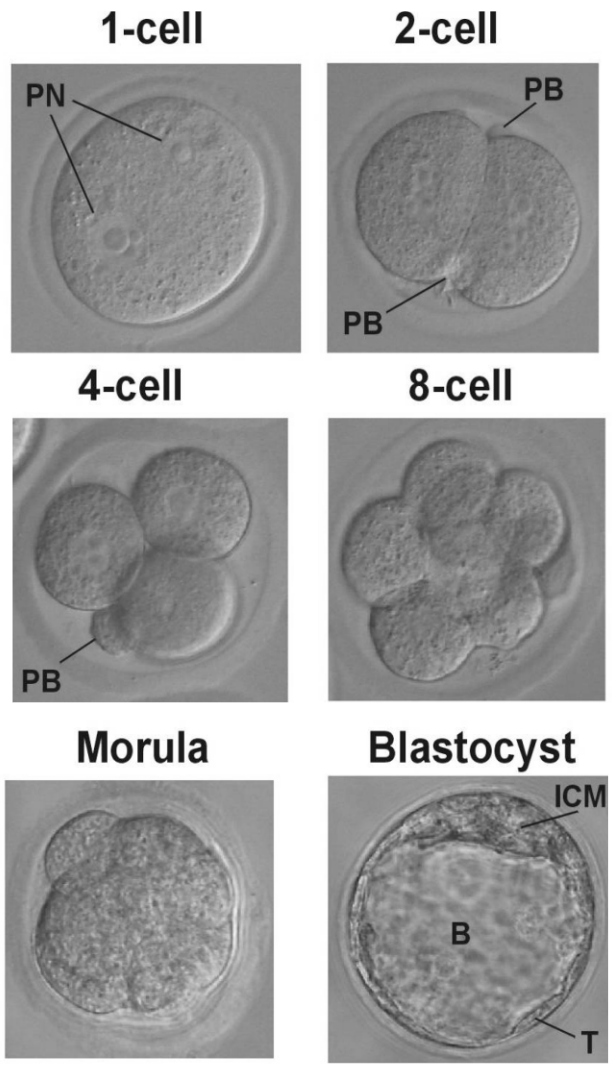

Figure 3: Preimplantation embryos

Photos of mouse preimplantation embryos. Embryos were flushed from oviducts at the 1-cell stage and cultured *invitro* for up to 4 days. Shown: 1-cell, 2-cell, 4-cell, 8-cell, morula and blastocyst stages. PN = pronuclei, PB = polar body, ICM = inner cell mass, T = trophectoderm, B = blastocoele.

contact between neighboring cells and allows intercellular communication (Hogan et al., 1994). Late morulae undergo the process of cavitation in which a fluid-filled cavity (blastocoel) forms. At this stage the once totipotent embryonic cells experience a restricted developmental potential and the cells become differentiated into distinct lineages. By the blastocyst stage the outer cells surrounding the blastocoel cavity form the trophectoderm which will ultimately form a large portion of the placenta, while the inner embryonic cells form the inner cell mass (ICM), which will eventually develop into the distinct structures of the fetus. In mice, on the fifth day following fertilization, the blastocyst hatches from the rigid zona surrounding it and will implant into the uterine wall.

1.2 In Vitro culture of growing, maturing oocytes and embryos

1.2.1 Development of embryo culture media

The first embryo transfer of rabbit embryos which resulted in live offspring, as well as the first successful culture of rabbit oocytes (Heape, 1891; Brachet, 1912) initiated a long series of historical developments to develop techniques, media and protocols which would allow the *in vitro* production of viable mammalian oocytes and preimplantation embryos. These early techniques were developed, modified and applied to a variety of species (reviewed in Betteridge, 1981), until Steptoe and Edwards reported the first successful human embryo *in vitro* fertilization (IVF), culture and transfer resulting in a live birth almost a century later (Steptoe and Edwards, 1978). Early attempts to culture embryos *in vitro* were performed in undefined complex biological fluid such as blood plasma and serum (Alexandre, 2001). These fluids were both largely unsuccessful culture mediums, as well as difficult to manipulate in a scientific manner due to their undefined and highly variable nature. A more complete, orderly assessment of

culture techniques and media characteristics such as osmolarity, pH, amino acid composition and energy sources continued through the mid-20th century (reviewed in Hammer, 1998; Brinster, 1965).

Mouse oocytes could be cultured in a variety of media (Pincus and Enzmann, 1934; Pincus and Werthessen, 1938), and later it was determined that simple salt solutions such as Krebs-Ringer, Tyrode's and Gey's (Adams, 1956; Austin, 1961; Brinster, 1963) would maintain development through various embryonic stages. The addition of bovine serum albumin and glucose to Krebs-Ringer solution constituted a major breakthrough in the development of a defined complete *in vitro* culture medium, as this allowed the successful progression of mouse embryos from the 8-cell stage to blastocyst (Whitten, 1956). The addition of lactate as a metabolic substrate extended the range of preimplantation embryo stages that could be supported *in vitro* from 2-cell to blastocyst (Whitten, 1957). The addition of pyruvate supported the earliest stages of mouse embryo development from cleavage to the 2-cell stage (Biggers et al., 1967). As such, classic mouse embryo culture media such as M16 (Whittingham, 1971) were developed and used for many years, with corresponding media for human embryo culture.

1.2.2 The "2-cell block"

A fundamental flaw in the media described above existed. Though they permitted development of the fertilized 1-cell mouse embryo from cleavage to the 2-cell stage, as well as 2-cell embryos (collected *in vivo*) to blastocysts, culture media would not support development of embryos from 1-cell stage through to the blastocyst stage. Mouse embryos would become arrested at the late 2-cell stage (Goddard and Pratt, 1983), at what was known as the '2-cell block' in mice. Similar developmental blocks existed in other species with embryos becoming

arrested at specific points throughout *in vitro* preimplantation development e.g., 4-8 cell block in humans (Baltz and Tartia, 2010).

The origin of the 2-cell block in mice, and the similar developmental blocks in other species remains somewhat mysterious. This developmental arrest was not due to the lack of an absolutely required substance provided by the oviduct for development through the 2-cell stage, since embryos produced from oocytes of some specific F_1 hybrid female mice did not block in identical media conditions. Instead, it was thought that blocking is the result of imbalances or deficiencies in media causing stress to the embryo (Biggers, 1998). The 2-cell

block coincides with the transition of G2/M phase which suggests a role for cell-cycle control mechanisms. The 2-cell block also coincides with the onset of transcription of the new embryonic genome. Thus, it appears that the developmental block which occurs at the 2-cell stage in mice is indicative of the embryo's very specific metabolic requirements which had not previously been satisfied by early *in vitro* culture media (Goddard and Pratt, 1983).

1.2.3 Overcoming the "2-cell block"

Inability to culture the zygote through complete preimplantation development prevented embryo culture to progress for uses in the treatment for human infertility, in the development of animal models and in technology for animal husbandry. When it appeared the research into the bourgeoning field had stalled in the 1980s the U.S. National Institute of Child Health and Human Development launched a National Cooperative Program on Non-Human In Vitro Fertilization and Development, which became more colloquially known as the "Culture Club." The focus of this group was to specifically to overcome the blocks to *in vitro* preimplantation embryo development (Baltz, 2013).

Remarkably, within just a few years this group successfully designed a defined media capable of supporting development of mouse eggs through to blastocyst stage (Baltz, 2013). The successful media were CZB (Chatot et al., 1989) and simplex optimized medium (SOM) (Lawitts and Biggers, 1991a; Lawitts and Biggers, 1991b). SOM was further optimized with additional potassium to (K^+ simplex optimized medium) KSOM, and is currently the most widely used mouse embryo culture media (Lawitts and Biggers, 1993). Both have essentially the same basic components as M16 with the simple addition of glutamine and the divalent metal chelator EDTA. However, both new media had significantly lower osmolarities than previous media. M16, one of the original media, had an osmolarity of 290 mOsM, while KSOM was approximately 250 mOsM, and CZB had an osmolarity of 275 mOsM (Baltz, 2013). The success of lower osmolarity media to eliminate *in vitro* blocks to development suggests that preimplantation embryogenesis is sensitive to increased osmolarity. The sensitivity is so pronounced that increased osmolarity could produce the stress necessary to cause complete developmental blocks.

1.3 Osmolarity and cell volume regulation in embryos

1.3.1 Osmolarity, osmolality and tonicity

Osmosis describes the movement of water across a semi-permeable membrane. This process is driven by the concentration gradient of water present on either side of the membrane. Water flows from an area of high water concentration into an area of lower water concentration. This flow will continue through the membrane until the two concentrations are equalized (Strange, 2004). Osmolarity is defined as the measure of osmotic pressure exerted by a solution across a semi-permeable membrane and is dependent on the concentration of particles in solution. One

osmole per liter (OsM) is the equivalent of osmotic pressure exerted by one mole of a completely dissolved solute in one liter of water. The cell membrane is semi-permeable as it allows water to pass freely through aquaporin channels, but prevents movement of most other molecules, and therefore is affected by osmotic pressure which leads to changes in cell volume (Baltz, 2001). Osmolality (osmoles per kilogram) is conceptually similar to osmolarity (osmoles per litre) (Baltz, 2001). These are conceptually different from tonicity, which is more context specific as it describes the pressure of one solution relative to another across a semipermeable membrane (Baltz, 2001). In mammalian cells, blood plasma is known to be ~300 mOsM.

1.3.2 Oviductal osmolarity

The success of *in vitro* embryo culture without developmental block in solutions of lower osmolarity indicated that embryos may perceived solutions with higher osmolarities to be hypertonic. It was hypothesized that the embryo's *in vivo* environment of the oviduct had a lower osmolarity than other biological fluids. However, this does not appear to be the case as measurements of the major ionic concentration in oviductal fluid were not lower than in other biological fluids (Borland et al., 1977). Direct measurement of oviductal fluid osmolarity by Fiorenza et al., (2004) suggested an oviductal fluid osmolarity of 302 ±4 mOsM. Historically this measurement was difficult to perform due to issues obtaining reliable measurements in small volumes such as the mouse oviduct. However, several different methods of measuring the osmolarity of oviductal fluid have indicated mouse oviductal fluid has an osmolarity of approximately 300 mOsM (Collins and Baltz, 1999). This is very similar to the documented osmolarity of mouse blood plasma. Thus, the developmental blocks in culture media such as

M16 (~290 mOsM) do not occur due to the media being hypertonic to the normal *in vivo* environment of embryos.

1.3.3 Osmolarity and Cell Volume

Homeostasis of intracellular osmolarity is generally well controlled; however, cell functions such as nutrient uptake, membrane transport and metabolic activities can create changes in the osmolarity of the microenvironment of the cell (Lang et al., 1998). Small differences in osmolarity between the intracellular and extracellular solute concentrations can create an osmotic pressure differential across the mammalian cell membrane (Baltz, 2001). Mammalian cells lack a rigid cell wall, and are highly permeable to water. This allows them to respond to osmotic membrane differentials by immediately changing their volume to equalize the osmotic pressure (Baltz, 2001; Lang et al., 1998). A cell exposed to high tonicity will shrink due to water efflux, and the opposite, cell swelling, will occur under low tonicity. These alterations in volume change not only cell size, but also may disrupt optimal metabolic and enzymatic concentrations for cell functioning (Mongin and Orlov, 2001).

1.3.4 Preimplantation embryo sensitivity to osmolarity

It was suggested that early culture media lacked at least one substance which was critical to embryo survival at high, *in* vivo osmolarities, but that was not required at lower osmolarity. Thus, embryos must be able to sense osmotic changes and respond to the osmolarity of their environment. In somatic cells two main types of MAP kinase pathways initiate cellular responses to hypertonicity. The p38 family is activated by increased osmolarity as well as cell shrinkage (Sheikh-Hamad and Gustin, 2004), and the SAPK/JNK pathway is activated by hypertonic conditions (Hoffmann et al., 2009). These somatic pathways are both also present in the mouse

embryo. P38 MAPKs are present in the preimplantation mouse embryo from mature unfertilized egg to blastocyst (Natale et al., 2004), as are components of the p38 signalling pathway (Fong et al., 2007). SAPK/JNK is also present in the blastocyst and is upregulated by stress (Xie et al., 2006), including hypertonicity (Xie et al., 2007). Thus, the preimplantation embryo contains functional pathways which respond specifically to osmotic stress.

1.3.5 Osmolarity and cell volume regulation

To survive perturbations in osmolarity and the corresponding changes in cell volume, cells have developed mechanisms which allow fast cell volume recovery, as well as mechanisms which allow long-term volume preservation when tonicity alteration persists.

1.3.5.1 Acute responses to hypertonic conditions

Mammalian somatic cells can respond acutely to changes in volume by accumulating or releasing inorganic ions (Hoffman et al., 2009). When volumes become too large the swelling is eliminated via ionic release using K^+ channels, Cl^- (anion) channels or K^+, Cl^- cotransporters (Lang et al., 1998; Mongin and Orlov, 2001). When cell volume decreases ions are accumulated along with osmotically driven water influx to reduce shrinkage. This occurs primarily via accumulation of Na^+ and Cl^- by the coupled activation of Na^+/H^+ antiporters and HCO_3^-/Cl^- exchangers (Strange et al., 1996; Lang *et al.*, 1998; Baltz, 2001). The embryo develops in a hypertonic environment in the oviduct, therefore the major issue in oocytes and preimplantation embryos is cell volume shrinkage. Briefly, decreased cell volume activates the NHE1 isoform of the Na^+/H^+ exchanger which imports Na^+, but also slightly raises intracellular pH (Hoffman et al., 2009). The AE2 isoform of the HCO_3^-/Cl^- exchanger is subsequently activated by the increase in intracellular pH and imports Cl^- while neutralizing the pH with bicarbonate (Humphries et al., 1995; Baltz, 2013).

This coupling of NHE1 and AE2 results in the pH and net charge within the cell remaining unchanged, while Na^+ and Cl^- are accumulated. This allows the cell to recover from volume decrease within a matter of seconds to minutes.

1.3.5.2 Mechanisms ensuring long-term adaptation to hypertonic conditions

Although accumulation of inorganic ions is an effective and fast-acting rescue mechanism for cell volume recovery, ion accumulation can be deleterious over time for overall cell homeostasis. The increased ionic content of the cell can effect protein structure, protein function, membrane potential and metabolism (Lang et al., 1998). Therefore cells (such as the oocyte and developing embryo) that exist in chronic hypertonic conditions preferentially accumulate intracellular organic osmolytes to replace intracellular inorganic ions (Figure 4) (Baltz and Tartia, 2010).

Organic osmolytes are small, neutral organic compounds which are capable of providing intracellular osmotic support over a wide range of concentrations without altering cellular functions or compromising cell biochemistry (Yancey *et al*., 1982; Yancey et al., 1994; Garcia-Perez and Burg, 1991). In mammalian cells these are a diverse group of molecules which includes polyalcohols such as sorbitol and inositol, glycerophosphorylcholine, and amino acids or their derivatives such as glycine, glutamine, taurine, proline or betaine (Lang et al., 1998; Baltz, 2001). Organic osmolytes have also been shown to have the ability to stabilize native protein structure (Street et al., 2006; Burg and Ferraris, 2008). By utilizing organic osmolytes in the place of inorganic ions, intracellular ionic strength and physiological function can be maintained, while controlling intracellular osmolarity and cell volume.

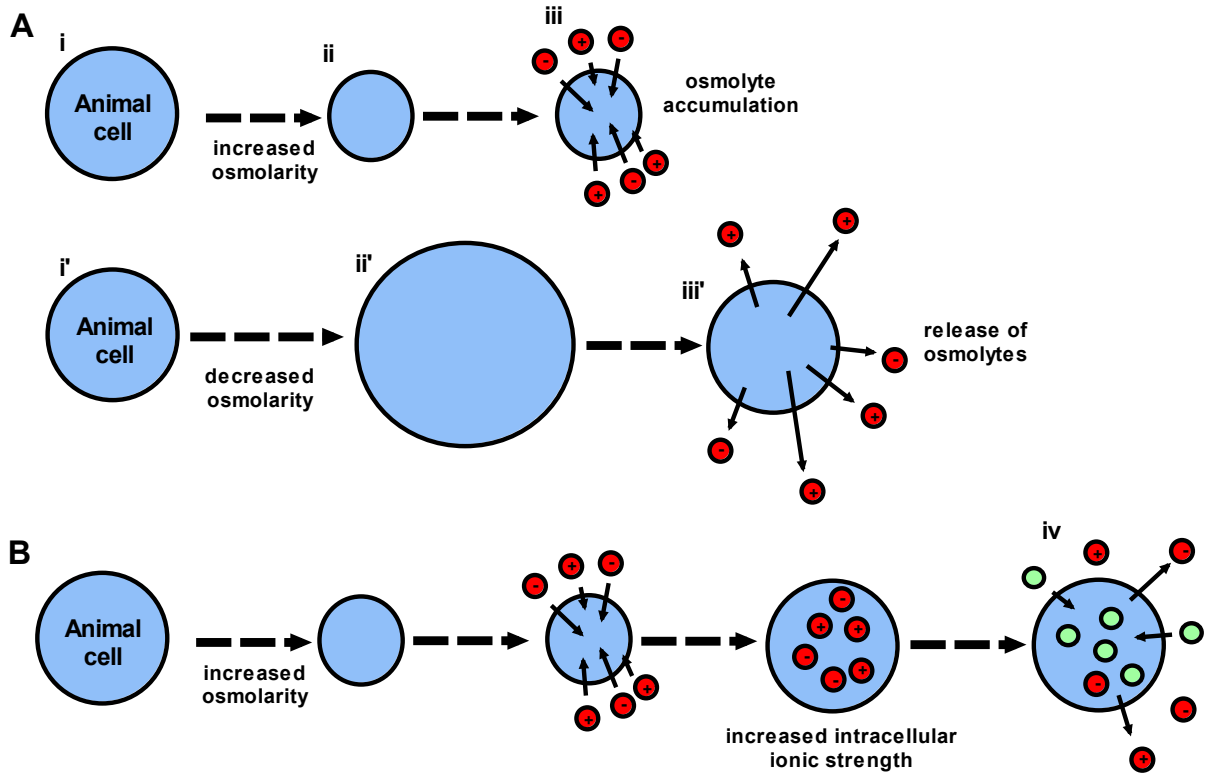

Figure 4: Schematic representation of volume regulation in animal cells

A) Mammalian cells that are maintaining a certain volume (i, i') respond to an increase or decrease in osmolarity of their environment by altering their volume by shrinking (ii) or swelling (ii') respectively. The typical response is to activate ion transporters such as Na+, K+ and Cl- to import or export ions (represented by red circles) allowing the cell to recover from the deviation in volume (iii, iii').

B) In some cells such as the mammalian oocyte, the increased ionic strength of the cytoplasm would be detrimental in the long term. These cells use organic osmolytes (represented by green circles), to replace a portion of the intracellular inorganic osmolytes (iv) without disrupting the ionic balance of the cytoplasm.

This image adapted with permission from Baltz and Tartia (2010).

The accumulation of organic osmolytes can occur via *de novo* cellular synthesis, but most occurs by transport into the cell from the external environment (Yancey et al., 1982, Baltz, 2013). The mammalian kidney medulla has previously been the focus of organic osmolyte research as this tissue is exposed to hypertonic conditions as a result of the concentrating mechanism which creates an osmotic gradient in the extracellular fluid of the medulla. Thus, cells in these tissues must adapt to their environment by accumulating organic osmolytes to counteract the osmotic stresses. Several organic osmolytes have been identified at high concentration in mammalian kidney tissues of different species, including myo-inositol, sorbitol, glyercophosphorylcholine and betaine (Balaban and Burg, 1987). These osmolytes are transported into the kidney via the Na^+/myo-inositol transporter (SMIT), betaine transporter (BGT1), taurine/β-amino acid transporter (TAUT) that transports β-amino acids such as taurine and β-alanine, and the System A amino acid transporter (ATA2) that transports small, neutral amino acids (Garcia-Perez and Burg, 1991; Kwon and Handler, 1995; Pastor-Anglada *et al*., 1996). Somatic tissues outside of the kidney also use subsets of these four transporters as means to acquire organic osmolytes.

In mammalian somatic cells only one transcription factor is known to be sensitive to osmolarity. Accumulation and synthesis of organic osmolytes is controlled by tonicity enhancer binding protein (TonEBP) (Miyakawa et al., 1998; Miyakawa, 1999; Woo et al., 2002). The cell's exposure to hypertonic conditions induces synthesis of TonEBP, which will be phosphorylated and translocated to the nucleus (Woo et al., 2000). TonEBP in the nucleus binds to its enhancer elements on several genes. This stimulates transcription of genes encoding

organic osmolyte transporters and key enzymes for organic osmolyte synthesis (Woo et al., 2002; Burg and Ferraris, 2008). This ensures the accumulation of organic osmolytes and ultimately the maintenance of cell volume. Conversely, cells can also release organic osmolytes in situations of hypotonic swelling. Both inorganic ions and organic osmolytes can be removed quickly through swelling activated channels or more slowly as organic osmolyte synthesis and uptake is down-regulated (Strange, 2004).

1.3.6 Cell volume and developmental blocks

A key feature of the optimized media which permitted development past the 2-cell block was their low osmolarity. As described above, cells exposed to high osmolarity accumulate deleterious levels of Na^+ and Cl^- to maintain cell volume. It has been shown that this method of acute cell volume regulation using the NHE1 Na^+/H^+ exchange and AE2 $HCO3^-/Cl^-$ exchange is present in mouse embryos (Zhou and Baltz, 2012). This was thought to be a major cause of the 2-cell block resulting from *in vitro* culture in previous, high osmolarity media. Thus, lowering the osmolarity eliminated the block as fewer intracellular inorganic ions were accumulated by the cell to maintain a normal embryo volume, and therefore these ions did not disrupt the cellular processes necessary for developmental progress.

It was also logically speculated that organic osmolytes may have been able to enhance or rescue embryo development past the 2-cell block in higher osmolarity solutions (Lawitts and Biggers, 1991). Lawitts and Biggers (1991) confirmed the interaction of glutamine with NaCl levels, showing that higher glutamine levels protected against the damaging effects of increased NaCl. Other osmolyte candidates were tested using KSOM media (with glutamine removed) and the osmolarity increased to such a degree that nearly all embryos once-again

became blocked at the two-cell stage. By systematically adding organic osmolytes to this media their efficacy at rescuing embryos could be determined. By this method glutamine, glycine, betaine, proline, β-alanine and hypotaurine were identified as effective organic osmolytes (Dawson and Baltz, 1997; Van Winkle et al., 1990; Lawitts and Biggers, 1992; Biggers et al., 1993). It was also shown that several of these organic osmolytes including glycine (Dawson, Collins and Baltz, 1998), betaine (Hammer and Baltz, 2002; Anas et al., 2007) and β-alanine (Hammer and Baltz, 2003) were present and functioning in mouse embryos. This confirmed the critical role of organic osmolytes, osmolarity and cell volume in successful embryo development.

1.4 Betaine as an organic osmolyte

1.4.1 Betaine

Glycine betaine is the trimethyl derivative of glycine (*N, N, N*-trimethylglycine). It is commonly be referred to simply as "betaine.". Betaine was first isolated from and named after the sugar beet (*Beta vulgaris)*. It is a significant component of many foods including wheat, shellfish and spinach and is thus available through the diet (Zeisel et al., 2003). It is also synthesized from choline in mammals (see below). Betaine carries a neutral charge at physiological pH despite having both positive and negative charges and is therefore a zwitterion.

Strictly speaking, the chemical term betaine applies to an entire family of zwitterionic compounds containing a positively charged functional group that is separated from a negatively charged functional group. Mainly, these are analogues of glycine betaine formed from other amino acids. Only glycine betaine is generically called betaine. In mammals such as humans and mice, the only other betaine known to be present in significant amounts is proline betaine (*N,N*-

dimethylproline or stachydrine). However, proline betaine does not participate in the physiological processes described below for glycine betaine and does not appear to be metabolized in mammals (Lever et al., 1994). Throughout, all references to betaine are to glycine betaine.

1.4.2 Betaine as an organic osmolyte in somatic cells

Betaine is known to be a major organic osmolyte in somatic tissues, especially in the kidney medulla where it assists in managing the osmotic gradient of NaCl and urea. Additionally, human blood plasma has been recorded to have a betaine concentration within the range of 20-144 μM (Lever, et al., 2004). However, the intracellular betaine levels have been shown to be much higher which suggests a concentrative uptake (Wehner et al., 2003).

The betaine transporter in renal tissues, as well as other tissues such liver and brain is BGT1, which also has high affinity for neurotransmitter γ-aminobutyric acid (GABA) (Yamauchi et al., 1992). Most of the betaine present in the kidney is obtained via this mechanism from the extracellular medium (Miller et al., 1996). BTG1 belongs to the SLC6 family, a large family of Na^+ and Cl^- coupled transport systems which allow the passage of neurotransmitters, amino acids and osmolytes (Chen et al., 2004). BGT1 transport activity is low in renal cells maintained in isotonic medium but is strongly activated in response to hypertonic stress. Transcriptional activation plays an important role in upregulation of BTG1 transport activity, as the *BTG1* gene expression increases in cells exposed to hypertonic medium. This occurs via activation of the TonE enhancer sequence of the *BTG1* gene which is bound by the transcriptional activator TonEBP under hypertonic conditions (as described above).

1.4.3 The role of betaine as an organic osmolyte in the oocyte and embryo

Organic osmolytes are present in the female reproductive tract and act to protect the developing oocyte and embryo from hypertonic stresses. Organic osmolytes that have been shown to have osmoprotective properties in the preimplantation mouse embryo include glycine, glutamine, proline, taurine, hypotaurine, β-alanine, and betaine (Van Winkle et al., 1990; Lawitts and Biggers, 1992, Biggers et al., 1993; Dawson and Baltz, 1997; Baltz, 2001). Betaine specifically, as the focus of this investigation, was found to be among the most effective osmoprotectants for mouse embryos (Biggers et al., 1993). Development of 1-cell mouse embryos past the 2-cell block was virtually impossible in medium with an osmolarity above 300 mOsM. The addition of betaine to this media of increased osmolarity restored blastocyst development (Biggers at al., 1993). Further study demonstrated that betaine could rescue the development of the 1-cell mouse embryo in media with artificially increased osmolarity via NaCl, but also if osmolarity was increased with raffinose, a trisaccharide not transported or metabolized by mammalian cells. This shows the osmoprotective effect is not specific to increased NaCl concentration (Dawson and Baltz, 1997). Additionally, the effect of betaine on the embryo development from 1-cell to blastocyst was shown to be dose-dependent, and the stimulatory effect of betaine on blastocyst development was demonstrated to occur only in instances of increased osmolarity (Hammer and Baltz, 2002; Dawson et al., 1998). Thus, betaine is an effective organic osmolyte *in vitro* for oocyte survival and embryo development.

For betaine to function *in vivo* as an osmolyte, it must be present in the *in vivo* environment. In two separate experiments the betaine content of murine oviducts was measured and found to contain measurable amounts of betaine calculated to an average concentration of 300-500 μM

(Anas et al., 2007). This suggests that betaine is present and available to play a physiological role as an organic osmolyte *in vivo* in addition to its demonstrated role *in vitro* though this has not been definitively shown.

1.4.4 Endogenous betaine in the mouse oocyte and preimplantation embryo

Our laboratory has previously identified the presence of betaine in the oocyte. Endogenous betaine was observed in MII eggs at a level of 1.48 pmol/egg. These measurements were performed as part of a series of measurements that included 1-cell embryos. At this time it was noted that the levels present in MII eggs, was not significantly different from that of 1-cell embryos at 1.38 pmol/embryo (Corbett et al., 2014).

Our lab then examined endogenous betaine content of developing mouse oocytes and embryos and observed lower levels of betaine in preantral follicles containing growing oocytes (from postnatal day 11, i.e., P11, ovaries) and in fully-grown GV oocytes. COCs isolated from antral follicles did not contain more betaine than GV oocytes indicating that the cumulus cells do not act as a significant reservoir of betaine. Betaine levels were low in MI oocytes, and a small increase was observed in MI oocytes at 6 h post-*in vivo* stimulation by human chorionic gonadotropin (hCG) injection. However, significant levels of betaine were observed in MII oocytes and 1- cell embryos (Figure 5). Work previously done by others in our laboratory also demonstrated that betaine levels decrease throughout preimplantation embryo development to the blastocyst stage (McClatchie et al.,2017), The increase in betaine during oocyte development would suggest that oocytes are themselves able to transport betaine or are capable of synthesizing betaine *de novo*.

1.4.5 Transport and regulation of intracellular betaine concentration in early embryos

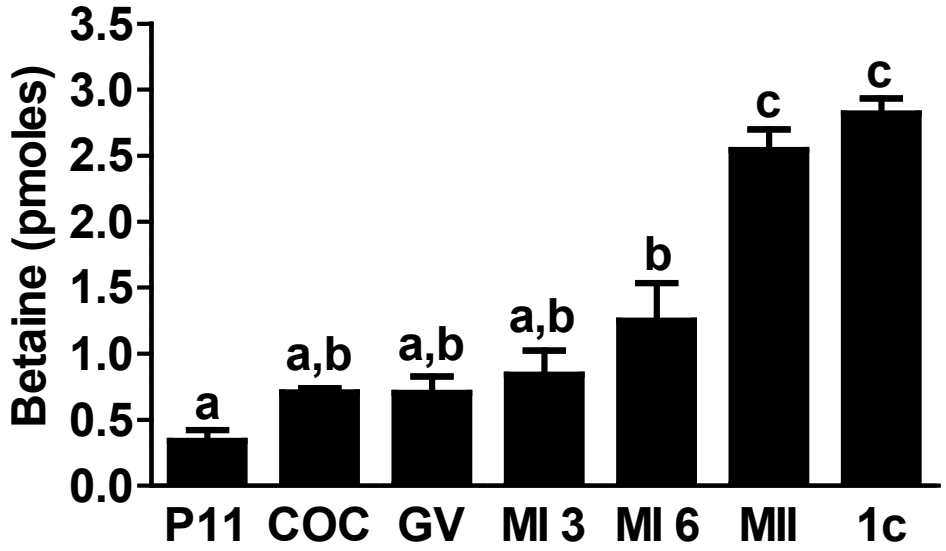

Figure 5: Endogenous betaine in follicles, oocytes and embryos

Betaine was measured by liquid chromatography tandem mass spectrometry (described below) in groups of 250 intact preantral follicles with enclosed oocytes (P11) and groups of 50 cumulus-oocyte complexes with enclosed GV oocytes (COC), GV oocytes (GV), first meiotic metaphase (MI) oocytes undergoing meiotic maturation at 3 (MI 3) and 6 (MI 6) h post-hCG injection, in mature eggs (MII) and in 1-cell embryos (1c). The amount of betaine is expressed as pmoles per follicle, COC, oocyte or embryo. The background levels of betaine measured in the final wash were subtracted from each paired measurement. Betaine was essentially undetectable in all paired background samples (<0.05 pmol per oocyte or embryo) except in the paired background for one MII egg sample (0.35 pmol per oocyte). Bars represent the mean ± S.E.M. of three independent repeats. Bars that do not share the same letter are significantly different ($P<0.001$ for a or b vs. c and $P<0.05$ for a vs. b) by ANOVA with Tukey's test). These images were adapted and used with permission from McClatchie et al. (2017).

The mechanism of transport of betaine into the developing mouse embryo was examined initially at the 1-cell stage, as it is at this stage that embryos are most osmosensitive (Davidson et al., 1988). Initial testing ruled out the transport of betaine into the embryo by BTG1, the transporter used in somatic cell types, since GABA did not inhibit the transport of radiolabelled betaine and betaine did not compete for transport of GABA in 1-cell embryos (Hammer and Baltz, 2002). Betaine is a glycine derivative, and osmosensitive accumulation of glycine occurs at the 1-cell stage, however transport of betaine by the GLYT1 transporter was shown to be non-existent (Hammer and Baltz, 2002). Betaine can be transported by the hypertonically activated amino acid transporter System A which is widely expressed in a variety of cells and tissues (Christensen et al., 1965; Petronini et al., 1994). However preimplantation embryos lack any detectable System A activity until the late blastocyst stage (Van Winkle et al., 1988). Hammer and Baltz (2002) attempted activation of normally inactive System A transport in cleavage stages by extended culture at high osmolarity, but were unsuccessful. This indicates that osmoprotection of embryos via this mechanism does not occur (Hammer and Baltz, 2002). Thus, of the four known organic osmolyte transport systems present in mammalian somatic cells, only system β, the β-amino acid transport mechanism (which does not transport betaine) is present and active in PI mouse embryos (Baltz, 2000). Our laboratory has previously identified a betaine/proline transporter with kinetic and inhibition profiles unlike any transporter previously shown to be present in mouse embryos as the putative mechanism for betaine accumulation in the developing mouse embryo (Baltz, 2001; Van Winkle, 2001; Hammer and Baltz, 2002). The dependence of this novel transporter on Na^+ and Cl^- led to the identification of possible candidates (Anas et al., 2007), and then eventual confirmation of the SIT1 (*Slc6a20a*) intestinal proline transporter as the transporter of betaine in the embryo. SIT1 is quiescent in the

unfertilized mouse egg, only becoming activated several hours after fertilization (Anas et al., 2008).

1.4.6 Transport and regulation of betaine concentration in developing oocytes

As outlined above the SIT1 transporter has been identified as the mechanism for betaine accumulation after fertilization, however the mechanism for betaine accumulation in the MII egg remains unknown. We have demonstrated that there is no saturable betaine transport into mouse oocytes prior to fertilization of MII eggs when SIT1 is activated (Anas et al., 2008).

To determine whether cumulus cells in the COC had a role in supplying betaine to the enclosed oocyte, kinetic and inhibitory profiles of the COC were examined and the y^+LAT2 (*Slc7a6*) transporter was identified. Metabolic coupling between the cumulus cells and the enclosed oocyte (i.e., transfer of specific compounds from the cumulus cells to the oocyte) is well documented (Haghighat and Van Winkle, 1990). Our lab demonstrated that the presence of cumulus cells significantly enhances the amount of betaine accumulated in the GV oocyte, and conversely demonstrated that inhibition of gap junctions caused a significant reduction. Thus, Corbett et al. (2014) demonstrated that transport into the cumulus cells and transfer to the enclosed oocyte via gap junctions could account for a portion of the endogenous betaine present in MII eggs. However, the cumulus mass becomes uncoupled from the enclosed oocyte, closing gap junctions and stopping any transfer of metabolites (including betaine) during meiotic maturation, before betaine levels have reached maximal endogenous levels in the egg. Thus, accumulation of betaine via the cumulus cells cannot account for most of the betaine in MII eggs.

1.5 Methylation and the role of betaine in the cellular methyl pool

Methylation is simply the addition of a methyl group either by the addition of a single group to a substrate, or the substitution of a methyl containing group to a substrate. Specific enzymes undertake this role with respect to cellular functioning. Methylation occurs in cells as a method of modifying gene expression, RNA or DNA processing or to change protein function. Methylation is a key process regulating epigenetics. The universal methyl donor for these reactions is S-adenosylmethionine (SAM) which forms the cellular methyl pool that is used by most methyltransferases. The methyl pool is produced through one of two known pathways of 1-carbon metabolism in mammals. Most cells use the folate cycle to supply 1-carbon methyl units, though certain cell types also use a pathway that uses betaine as a methyl donor.

1.5.1 Metabolic pathway of betaine

The transmethylation reactions that are involved in creating SAM involve the transfer of a methyl group to homocysteine to produce methionine, which is further processed by the addition of adenosine to yield SAM (Ikeda et al., 2012). In the betaine pathway, betaine is catabolized to dimethylglycine when it donates a methyl group to homocysteine which is mediated by the enzyme betaine homocysteine methyl transferase (BHMT). BHMT is a zinc metalloenzyme that until recently was thought to exist only in the liver and kidney cortex in rodents (Delgado-Reyes et al., 2001).

1.5.2 DNA methylation

DNA methylation refers specifically to the addition of methyl groups to the DNA strand. Methylation of the cytosine residues that make up DNA is a key epigenetic process in mammals. This process, like other forms of methylation, is also mediated by the methyltransferases using

SAM as a methyl donor. The addition of methyl groups can change the activity of a segment of DNA. Methylation of gene promoter regions often represses transcription of the gene. This mechanism has been implicated in a number of biological processes in mammals including normal development, genetic imprinting, X-chromosome inactivation, repression of transposable elements and carcinogenesis.

1.5.3 DNA methylation patterns in the developing embryo

Generally, tissues have a characteristic pattern of DNA methylation that is maintained throughout cell division across the lifespan. However, DNA methylation patterns are largely erased and re-established between generations in mammals. The first erasure occurs early in embryogenesis. At this time, genome-wide epigenetic reprogramming occurs and gamete-specific patterns of methylation from the maternal and paternal DNA are removed. These are replaced with the embryo-specific epigenome (Lucifero et al., 2004; Hiura et al., 2006). The second erasure occurs shortly after fertilization, before implantation. This is evidenced by low methylation that is detectable in the morula and early blastocyst in mouse embryos (Zhang et al., 2015; Reik et al., 2001; Smith et al., 2012). Global *de novo* re-methylation can be detected in the epiblast after implantation. This ensures fetal viability and offspring health through maintenance of gamete-specific methylation of imprinted genes and normal establishment of methylation patterns of non-imprinted genes (Lucifero et al., 2004). Thus, both the oocyte growth and peri-implantation periods likely have an increased requirement for methyl group availability. Disruptions in methylation processes are thought to be a major cause of adverse consequences in the development of the fetus, placenta and ultimately the offspring (Fleming et al., 2004; Sinclair and Singh, 2007; Sinclair et al., 2007). Perturbations in methylation at very early stages of

development can have permanent adverse consequences, such as fetal growth restriction and fetal overgrowth, as well as adult behavioural abnormalities, developmental delays and increased adult morbidity (Fernandez-Gonzalez et al., 2004; Fleming et al., 2004; Watkins et al., 2007; Ecker et al., 2004).

Genomic imprinting is closely tied to DNA methylation patterns of the genomic DNA which create a heritable epigenetic mark. Small subsets of mammalian genes are imprinted, resulting in maternal and paternal alleles which are functionally non-equivalent. The allele from only one parent is normally expressed in imprinted genes. These parent-specific imprints are created during gametogenesis, so that the fertilized egg carries a set of imprinted genes from each parent. These imprints must be maintained during embryo development. Similarly, DNA methylation modulates tissue-specific gene expression in non-imprinted genes. As a result, methylation patterns must be created and deleted in such a way as to maintain appropriate development of the embryonic and extra-embryonic tissues early in preimplantation development.

1.5.4 Betaine and BHMT in preimplantation embryos

The source of the methyl groups that allow methylation of the preimplantation embryo was largely unknown. It was known that betaine plays a role as an organic osmolyte (Anas et al., 2007; Hammer and Baltz, 2002) and that the preimplantation mouse embryo could transport considerable amounts of betaine via the SIT1 transporter (above) during the 1- and 2-cell stage (Anas et al., 2007; Anas et al., 2008). Thus, our lab hypothesized that betaine accumulated by the embryo could also serve as a substrate for BHMT, thus contributing to the methyl pool for embryonic methylation.

We previously demonstrated that *Bhmt* mRNA is present in the morula and that BHMT protein was detectable and enzymatically active in mouse blastocysts. This implies that the endogenously accumulated betaine could be used as a methyl source at the blastocyst stage during which remethylation of the genome is occurring (Lee et al., 2012). Metabolism by BHMT is consistent with the decrease in endogenous betaine observed at the blastocyst stage. These findings were supported by experiments demonstrating a detrimental effect on blastocyst development and fetal viability when BHMT was perturbed in vitro (Lee et al., 2012). The finding that BHMT is active in the embryo is surprising as its function was previously thought to be restricted to the liver.

We have also demonstrated cooperativity between BHMT and folate-dependent generation of methyl groups, demonstrating that total SAM levels in mouse blastocysts decreased significantly only when both pathways were inhibited (Zhang et al., 2015). DNA methylation was similarly minimally affected by knock-down of either pathway alone, but decreased substantially when both were inhibited simultaneously (Zhang et al., 2015). Thus, unexpectedly both the folate cycle and betaine/BHMT appear to contribute to a methyl pool required for normal development and establishing initial embryonic DNA methylation, unlike most other cells outside of liver where only the folate cycle contributes.

1.6 Betaine synthetic enzyme Choline Dehydrogenase (CHDH)

1.6.1 *Chdh* gene, CHDH protein structure and function

Betaine can be made *de novo* via a two–step reaction in which metabolic choline is oxidized by the enzyme choline dehydrogenase (CHDH) to betaine aldehyde in the specific and rate limiting step of the reaction. Betaine aldehyde is then further converted to betaine by an NAD^+

dependent aldehyde dehydrogenase identified as ALDH9A1, which accepts a broad range of aldehyde substrates. The conversion of choline to betaine takes place on the matrix side of the inner mitochondrial matrix where CHDH is located, following choline transport across the mitochondrial membranes (Huang and Lin, 2003; Chi-Shui and Ru-Dan, 1986). The betaine formed by the reaction diffuses out of the mitochondria (Porter et al., 1993).

CHDH was previously thought to exist primarily in the mammalian liver and kidney, with small amounts present in the brain, but negligible activity was detected in other organs (Grossman and Herbert, 1989). However, we have demonstrated that CHDH is also present and active in the developing oocyte (McClatchie et al., 2017), as will be discussed in the Results section.

1.6.2 CHDH in the mouse oocyte and embryo

The focus of this investigation, described below, is the ability of CHDH to function in the mouse oocyte during meiotic maturation. Previous experiments in our lab performed by Hannah Corbett and published in McClatchie et al., (2017) demonstrated the presence of *Chdh* mRNA at each oocyte stage tested, including growing oocytes, GV oocytes and fertilized eggs (Figure 6A). The identity of the band was confirmed by sequencing, and the highest numbers of Q-RT-PCR Chdh transcripts were in oocytes isolated from neonatal ovaries obtained on P11 and P21. Fully-grown GV oocytes also had high numbers of transcripts that persisted in the fertilized eggs, but not into further preimplantation stages (Figure 6B). COCs contained a roughly equal number of transcripts to GV oocytes indicating the protein is isolated to the oocyte itself and not the cumulus cells of the COC.

Our lab also previously examined CHDH activity and determined that CHDH is transiently active in oocytes during meiotic maturation. An existing assay (Herbert and Grossman, 1989) was adapted for small samples to demonstrate that CHDH activity increased in oocytes as meiotic maturation processed *in vivo* with maximal activity present in MI and MII oocytes from 3-16 h after meiotic maturation was induced with hCG (Figure 7A). CHDH activity decreased after fertilization with low levels of activity remaining in 1-cell embryos and all further pre-implantation stages. Similar results were obtained using *in vitro* matured oocytes (Figure 7B) (experiments performed by Hannah Corbett, published in McClatchie et al., 2017).

1.7 Betaine and CHDH in human health and fertility

The relevance of betaine to oocyte and embryo culture has been demonstrated at length using the mouse (*Mus musculus*) as a model organism. The extension of results obtained in the mouse to human oocyte growth, maturation and ovulation, as well as preimplantation embryo development has been supported by many reports demonstrating the conserved nature of the related processes between oocyte maturation and preimplantation embryo development in both species. Data supporting the relevance of research performed in mice as a model for human fertility is historically very prevalent. Development of culture media, culture conditions, and eventually exploration of osmolyte function *in vivo* as well as *in vitro* has all been established using mouse oocytes and preimplantation embryos (Baltz, 2013; Baltz and Tartia, 2010). Performing these types of experiments using human gametes and embryos poses ethical and practical dilemmas, and thus the mouse provides an appropriate, inexpensive, readily available and modifiable alternative.

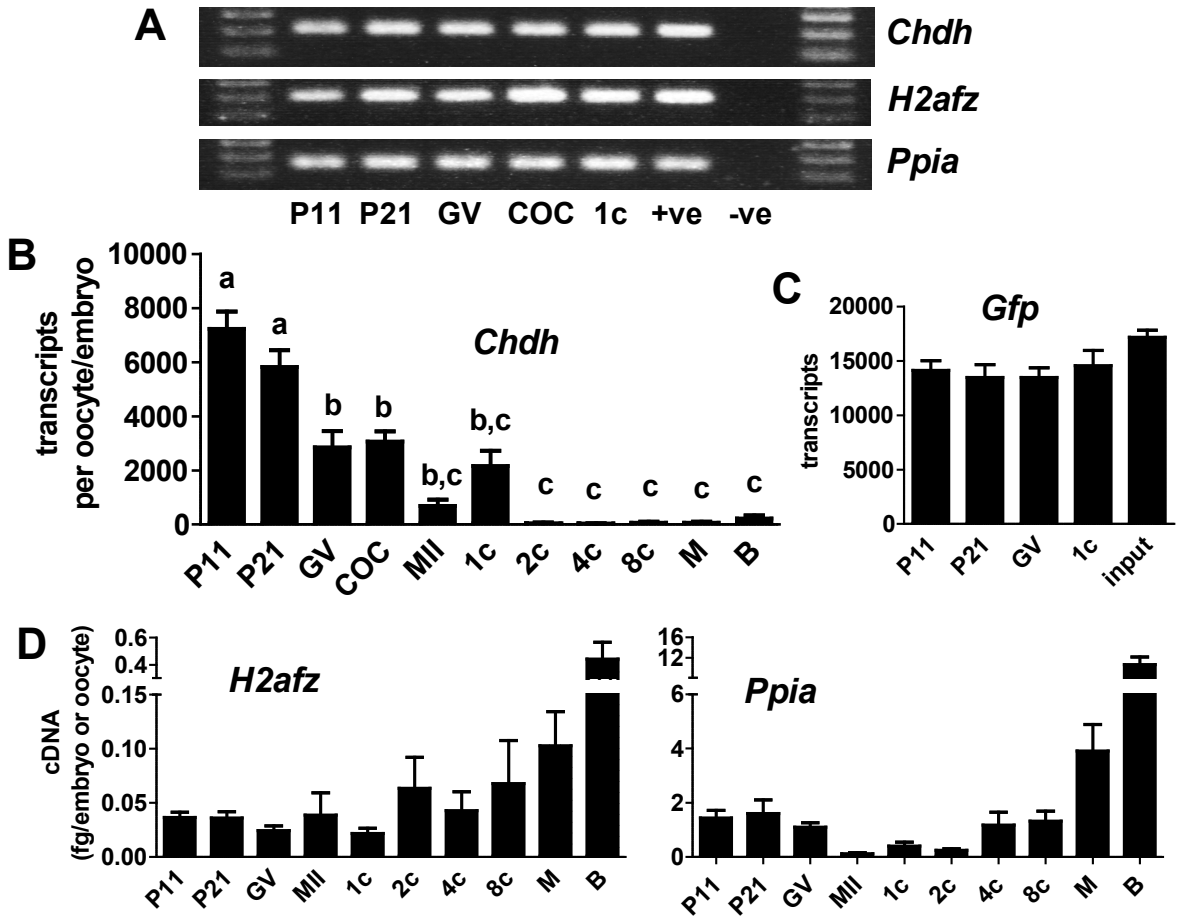

Figure 6: Expression of choline dehydrogenase mRNA transcripts

A) *Chdh* transcripts were detectable by conventional RT-PCR (40 cycles) in growing oocytes from postnatal day 11 ovaries (P11), fully grown oocytes from postnatal day 21 (P21), GV oocytes from adult females, cumulus-oocyte complexes containing GV oocytes (COC), and 1-cell embryos. Kidney served as a positive control (+ve), while water added to reaction mix was a negative (-ve). Transcripts for *H2afz* and *Ppia* controls were detected as expected. The gels shown represent one example of three similar repeats.

B) Quantitative RT-PCR confirmed the presence of *Chdh* in growing (P11) oocytes and fully grown (P21) oocytes from neonatal ovaries, GV oocytes, and 1-cell embryos, but not in 2-cell (2c), 4-cell (4c), 8-cell (8c), morula (M) or blastocyst (B) stage embryos. Eggs (MII) also showed a somewhat lower amount of *Chdh* transcript, although yield of transcripts from eggs is typically lower for technical reasons. Bars represent the mean ± S.E.M. of five independent repeats, except 10 repeats for GV oocytes. Bars that do not share the same letter are significantly different ($P<0.001$ for a or b vs. c and $P<0.05$ for a vs. b) by ANOVA with Tukey's test).

C) A fixed amount (1 pg) of *Gfp* cRNA was added to four sets of selected samples, and Q-RT-PCR performed to assess the efficiency of recovery. Mean recovery ranged from 79-84%. Thus, the transcript levels in (A) likely represent approximately 80-85% of the actual levels at each stage.

D) Q-RT-PCR was performed to detect *H2afz* and *Ppia* as controls. The relative expression levels are essentially as previously reported (Mamo et al., 2007). Transcript numbers were not calculated for these, and the results are expressed in fg of amplicon.

These images were adapted and used with permission from McClatchie et al. (2017).

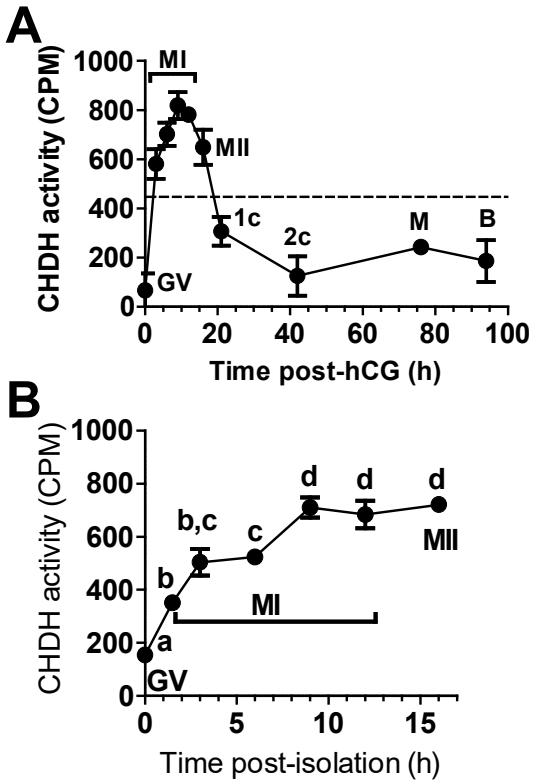

Figure 7: Choline dehydrogenase activity

A) Choline dehydrogenase activity measured in *in vivo*-developed oocytes and preimplantation embryos as a function of time post-hCG. Activity in GV oocytes was not significantly different from 0 (P=0.43, one-sample t-test). Activity peaked during meiotic maturation in maturing oocytes (MI) and eggs (MII). Significantly lower activity was present after fertilization in 1-cell (1C), 2-cell (2C), morula (M) and blastocyst (B) stage embryos. Each point represents the mean ± S.E.M. of three independent repeats. Points lying above the dashed line are significantly different from those below (P<0.05 by ANOVA with Tukey's test).

B) Choline dehydrogenase activity measured in oocytes undergoing spontaneous meiotic maturation *in vitro* after removal from antral follicles. Each point represents the mean ± S.E.M. of three independent repeats. Points that do not share the same letter are significantly different ($P<0.05$ by ANOVA with Tukey's test). Stages are indicated as in (A).

These images were adapted and used with permission from McClatchie et al. (2017).

Though the use of mice as a model of human fertility is well established, the relevance of betaine and CHDH to human biology has much less historical support. It is known that betaine is present in human serum at a concentration of 20-60uM (Lever et al., 1994). It is known to function as an osmolyte to assist in renal function and has been shown to improve various health outcomes and a variety of chronic diseases (reviewed in Craig et al., 2004). However, very little is known with regard to its impact on fertility in humans.

Perhaps less is known still about the relevance of CHDH to these processes. CHDH is highly unstable once removed from the mitochondrial membrane and has therefore not been successfully characterized in vitro. Thus, there is a general lag in understanding of the enzyme's precise biochemical structure and function in any species. The CHDH enzyme is highly conserved between human and mouse (not shown) and the functional interactions of the enzyme also appear conserved. The *CHDH* transcript has been detected at high levels in human MII oocytes (deposited data from Kocobas et al., 2006). Additionally, CHDH dysfunction (and modifications such as SNPs of the gene) have been associated with human disease states, including male infertility (Johnson et al., 2012). Likewise, betaine deficiencies and surpluses of its precursor choline have been implicated in human cardiovascular disease (Ueland, 2011).

Though the link between betaine, CHDH and fertility is not yet firmly established in humans due to the difficulty of performing the relevant research on human oocytes and embryos, the potential for betaine to improve culture and potentially long-term health is well supported in mice. Thus far, evidence gathered in this model organism suggests that further studies in humans should be pursued.

1.8 Summary

Betaine is a key molecule in the developing oocyte acting as a methyl donor and as an organic osmolyte in embryos after fertilization, with implications for human health and fertility. The presence of betaine in MII eggs prior to SIT1 activation was unexplained. Transfer into the oocyte through the cumulus mass likely accounts for some, but not all of the betaine present. It is possible that betaine may be synthesized in the growing oocyte by the enzyme CHDH as the mRNA is present and enzyme activity has been detected. It remains to be explored whether CHDH is essential to the unexplained appearance of betaine prior to embryo fertilization and subsequent SIT1 activation.

2. Objectives and Specific Aims

2.1 Significance

The increasing prevalence of Assisted Reproductive Technologies (ART) in recent decades has required an increased effort towards developing a more complete understanding of the developing oocyte and pre-implantation embryo. ART encompasses all the treatments that involve the manipulation of eggs or embryos outside of the body. The ability to use these eggs and embryos to respond to the increasing rates of human infertility, as well as the ability to manipulate animal models for domestic, commercial and research purposes requires an ever-improving knowledge of their physiology. To successfully mature, manipulate and maintain oocytes and embryos *in vitro* we must have a clear picture of the physiological processes surrounding gamete physiology and early embryo development *in vivo.*

Early embryos are sensitive to perturbations in their cell size (Baltz, 2001), suggesting that proper progression in culture occurs only under appropriate osmotic conditions. It has also been demonstrated that relatively mild stresses occurring during pre-implantation development can result in substantial disruption of imprinted genes that are normally differentially methylated, resulting in detrimental hypomethylation of normally methylated alleles (Zhang et al., 2015). Thus, it is crucial to eliminate osmotic stresses from the oocyte and embryo culture techniques used in ART, as these may impact oocyte and embryo physiology directly, as well as result in major health effects on the offspring throughout the lifespan.

The molecule betaine has been shown not only to function as an organic osmolyte in embryos (Biggers et al., 1997), but also as a major source of methyl groups used in the mouse blastocyst for epigenetic marking by methylation (Lee et al., 2012). This suggests betaine plays a

role in the link between embryo culture and physiology. Accumulation of betaine via SIT1 at the 1-cell stage contributes to embryonic control of cell volume at oviductal osmolarities, as well as development and maintenance of the intracellular methyl pool.

The ability to create and maintain competent, fertilizable eggs would streamline current treatment protocols for women undergoing infertility or seeking to preserve their oocytes. Outside of the protective *in vivo* environment the immature GV must successfully progress through meiotic maturation and cytoplasmic maturation in conditions that are currently, most-likely less than ideal. Understanding the key processes that keep an oocyte healthy and allow it to develop successfully will both directly and indirectly advance oocyte culture. Although betaine has been previously demonstrated to be present in oocytes as they develop, neither method of transport nor method of *de novo* synthesis has been proven to account for its presence prior to SIT1 activation.

Therefore, **the overall objective of my thesis was to determine the mechanism of betaine accumulation in the oocyte prior to fertilization.** By using the mouse as an animal model and a combination of functional studies and molecular biology techniques, including the *Chdh* gene knockout mouse, I have investigated whether the enzyme CHDH is required for betaine accumulation during meiotic maturation. Information provided by my research provided insight into the physiological processes allowing the oocyte to store betaine prior to fertilization. A more complete understanding of betaine and its physiological processes serves to clarify the requirements of maturing and maintaining healthy oocytes *in vitro*, a necessary step in the treatment of infertility and the preservation of oocytes for women.

2.2 Specific Aims and Hypotheses

Specific Aim 1: Determine if betaine is transported into the oocyte from the external environment during the period of meiotic maturation.

Betaine is transported into 1-cell and 2-cell stage embryos via the SIT1 transporter (Anas et al., 2008). Little is known about betaine transport and its role before fertilization. Data obtained at a few points during oocyte maturation indicated little betaine transport prior to SIT1 activation (Anas et al., 2008), but it was unknown whether there was a transient appearance of transport during maturation.

Therefore, I hypothesized that betaine is not transported into the oocyte throughout meiotic maturation.

Specific Aim 2: Determine if CHDH protein is present in oocytes during meiotic maturation when betaine increases.

If, as hypothesized above, there is no transport of betaine directly into the oocyte detected throughout meiotic maturation, another mechanism would be required for its accumulation. The SLC7A6 transporter in the cumulus cells (Corbett et al., 2014) can account for only the relatively low level of betaine already present in GV oocytes due to uncoupling of the gap junctions linking the oocyte and the cumulus granulosa cells. Thus, we focused on the enzyme CHDH, an enzyme specific to the catalysis of choline to betaine aldehyde in a two-step reaction that creates betaine *de novo* from choline. Work done in our laboratory has previously demonstrated *Chdh* mRNA, as well as CHDH enzyme activity in oocytes prior to fertilization.

Therefore, I hypothesized that CHDH protein would be detected in oocytes and PI embryos, specifically in GV oocytes and throughout meiotic maturation.

Specific Aim 3: Confirm that betaine is synthesized from choline by CHDH in the developing oocyte.

CHDH synthesizes the first of two steps converting choline to betaine. It is the specific step and therefore the focus of molecular biology techniques to demonstrate the physiological reaction in question. Having demonstrated the presence of CHDH at the molecular level, as well as previously demonstrating its activity I sought to link CHDH to the observed betaine accumulation in the oocyte.

I hypothesized that betaine accumulation would be confirmed to follow CHDH activity during meiotic maturation, and therefore I also hypothesized that a lack of CHDH would suppress betaine accumulation in the maturing oocyte.

Choline is taken up by mouse oocytes and is therefore available as a substrate for this reaction during meiotic maturation (Eppig, 1982; Barlow and Marchbanks, 1985).

Thus, I hypothesized that choline is converted to betaine during meiotic maturation thus implicating CHDH, and further that betaine would not accumulate in the absence of CHDH.

3. Materials and Methods

3.1 Chemicals and solutions

3.1.1 Chemicals and pharmaceutical agents

All chemicals and solutions were embryo tested or cell culture grade. All chemicals and compounds were obtained from the sources listed below in Table 1. Pharmaceutical agents were obtained from the sources listed in Table 2.

3.1.2 Oocyte and embryo culture and handling media

Oocyte and embryo culture media were based on KSOM embryo culture medium (Lawitts and Biggers, 1993), in which glutamine was omitted and polyvinyl alcohol (PVA, 1mg/ml) substituted for bovine serum albumin. KSOM medium contained: NaCl (95 mM), KCl (2.5 mM), KH_2PO_4 (0.35 mM), $MgSO_4.7H_2O$ (0.2 mM), Na lactate (10 mM), Glucose (0.2 mM), Na pyruvate (0.2 mM), NaHCO3 (25 mM), $CaCl_2.2H_2O$ (1.7 mM), EDTA (tetra Na) (0.01 mM), K penicillin G (0.16 mM), Streptomycin SO_4 (0.03 mM). Where specified, oocytes were cultured for up to 16 h in MEMα culture medium (Life Technologies/Gibco, Burlington, ON; catalog #12561) to which PVA (1 mg/ml) was added.

4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-KSOM was used for oocyte, follicle and embryo collection and handling. This additionally contained 21 mM Hepes and 4 mM $NaHCO_3$ (pH adjusted with NaOH to 7.4) The osmolarities of KSOM and Hepes-KSOM were 250 and 240 mOsM (± 5 mOsM), respectively. Osmolarities were measured and confirmed by osmometer (Vapro 5520, Wescor, Logan, UT).

3.2 Animals, oocytes and embryo manipulations

3.2.1 Animals

Animal protocols were approved by the Animal Care Committee at the University of Ottawa Faculty of Medicine. CF1 strain females (Charles River Canada, St-Constant, QC) aged 6-8 weeks were used to obtain growing and fully-grown oocytes, as well as preimplantation embryos. This strain is known produce oocytes and embryos that are sensitive to *in vitro* conditions, including osmolarity (Biggers, 1998; Hadi et al., 2005) and thus have been used as a model system in which to investigate culture effects. To obtain fertilized embryos, females were mated with BDF1 (B6D2F1) males overnight. Animals were maintained on a 12 h light: 12 h dark cycle. All animals were sacrificed by cervical dislocation.

The *Chdh* null mouse line was obtained from Dr. Steven H. Zeisel (University of North Carolina). These animals have a C57Bl/6 background, as previously described (Johnson et al., 2010). The null mice have no detectable CHDH protein, and drastically reduced CHDH activity compared to wild-type littermates, and males lacking *Chdh* are infertile (Johnson et al., 2010). *Chdh* $^{+/+}$ offspring were obtained from *Chdh*$^{+/+}$ ♀ *x Chdh*$^{+/+}$♂ matings and *Chdh*$^{+/-}$ and *Chdh* $^{-/-}$ offspring were obtained from *Chdh*$^{-/-}$ ♀ x *Chdh*$^{+/-}$ ♂ matings. These animals were maintained under identical conditions to CF1 mice and sacrificed by cervical dislocation at 4-5 weeks of age.

All adult animals were maintained on Teklad Global 18% Protein Rodent Diet 2018 (Envigo, Indianapolis, IN). Lactating females were maintained on Teklad Global 19% Protein Extruded Rodent Diet 2019 (Envigo, Somerset, NJ). Both include choline supplementation (1200 mg/kg), but do not include betaine. Neonates requiring nutritional support for weight gain immediately

after weaning were provided with DietGel Recovery Purified Dietary supplement (ClearH_2O, Portland, ME). All animals had free access to water.

3.2.2 Superovulation

To obtain a sufficient number of oocytes or preimplantation embryos a standard superovulation protocol was observed (Hogan et al., 1994; Dawson and Baltz, 1997). Stimulation of ovarian folliculogenesis was accomplished by priming adult females with pregnant mare serum gonadotropin (PMSG), an FSH-like hormone, while ovulation was triggered with human chorionic gonadotropin (hCG), an LH-like hormone. Female mice, between 4 and 8 weeks old, received an intra-peritoneal (i.p.) injection with 5IU PMSG. GV oocytes were collected directly from mice after receiving only PMSG injections. For embryos, the female mice were mated with BDF1 males overnight immediately after hCG injection.

3.2.3 Oocyte collection

Fully grown GV oocytes and unexpanded COCs were isolated approximately 44 h after PMSG injection. Ovaries from stimulated mice were surgically removed and separated from the oviduct, cleaned of fat and mechanically minced with a razor blade in mHEPES-KSOM. COCs were retrieved from the minced ovarian tissue. GV oocytes were isolated from the surrounding cumulus cells by gentle repeated pipetting with a narrow-bore flame-pulled Pasteur pipette.

In vitro- matured MI and MII oocytes were obtained by culturing GV oocytes 1.5 to 4 hours, or overnight 22 h respectively, after isolation from the cumulus cells in minimum essential medium eagle - **Alpha** (MEMα) (or mKSOM where specified) microdrop cultures (Wassarman et al., 1979).

Table 1: Summary of chemicals and compounds used

Name of chemical or compound	**Source**
4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES)	Sigma (St. Louis, MO, USA)
[3H]-betaine; ~1mCi/ml	American Radiolabelled Chemicals (St. Louis, MO, USA)
B-mercaptoethanol	Sigma (St. Louis, MO, USA)
Agar	Sigma (St. Louis, MO, USA)
Agarose	Sigma (St. Louis, MO, USA)
Aprotinin	Sigma (St. Louis, MO, USA)
Ascorbic acid	Sigma (St. Louis, MO, USA)
Bovine serum albumin (BSA)	Sigma (St. Louis, MO, USA)
BSA Standard (1.38 mg/ml)	Bio-Rad Laboratories (Mississauga, ON, Canada)
$CaCl_2$	Sigma (St. Louis, MO, USA)
Choline chloride	Sigma (St. Louis, MO, USA)
EDTA (tetra Na)	Sigma (St. Louis, MO, USA)

Ethanol	Sigma (St. Louis, MO, USA)
Glucose	Sigma (St. Louis, MO, USA)
Glutaraldehyde	Sigma (St. Louis, MO, USA)
Glycine	Sigma (St. Louis, MO, USA)
Hyaluronidase	Sigma (St. Louis, MO, USA)
K penicillin G	Sigma (St. Louis, MO, USA)
KCl	Sigma (St. Louis, MO, USA)
KH_2PO_4	Sigma (St. Louis, MO, USA)
Laemelli buffer (2x)	Bio-Rad Laboratories (Mississauga, ON, Canada)
Leupeptin	Sigma (St. Louis, MO, USA)
Methanol	Sigma (St. Louis, MO, USA)
$MgSO_4$	Sigma (St. Louis, MO, USA)
Mineral oil	Sigma (St. Louis, MO, USA)
Na lactate	Sigma (St. Louis, MO, USA)
Na pyruvate	Sigma (St. Louis, MO, USA)

NaCl	Sigma (St. Louis, MO, USA)
$NaHCO_3$	Sigma (St. Louis, MO, USA)
Phenylmethylsulphonyl fluoride (PMSF)	Sigma (St. Louis, MO, USA)
Potassium Hydroxide (KOH)	Sigma (St. Louis, MO, USA)
Polyvinyl alcohol	Sigma (St. Louis, MO, USA)
Scintillation fluid	Scintiverse BD, Fisher Scientific (Pittsburg, PA, USA)
Sodium deoxycholate	Sigma (St. Louis, MO, USA)
Sodium Dodecyl Sulfate (SDS)	Sigma (St. Louis, MO, USA)
Sodium Hydroxide (NaOH)	Sigma (St. Louis, MO, USA)
Streptomycin SO_4	Sigma (St. Louis, MO, USA)
Tris	Sigma (St. Louis, MO, USA)
Tris-buffered saline (TBS, pH 7.4)	Bio-Rad Laboratories (Mississauga, ON, Canada)
Tris-HCl (pH 7.4)	Sigma (St. Louis, MO, USA)
Triton X -100	Sigma (St. Louis, MO, USA)
Tween20	Sigma (St. Louis, MO, USA)

Table 2: Summary of pharmaceutical agents used

Pharmaceutical Agent	**Source**	**Action**
hCG (Chorulon, human chorionic gonadotropin)	Merck, supplied by: Intervet Canada Corp, Kirkland, QC, Canada	Stimulation of ovulation *in vivo* in mice (hormone)
PMSG (Folligon, pregnant mare serum gonadotropin)	Merck, supplied by: Intervet Canada Corp, Kirkland, QC, Canada	Stimulation of ovarian follicle growth *in vivo* (hormone).

To obtain *in vivo*-matured MI oocytes, expanded COCs and mature MII eggs female mice were injected with hCG 47 h post-eCG injection. MI oocytes and expanded COCs were obtained 2 to 6 h after hCG injection using an identical method to fully-grown oocytes. If required (beginning 4 to 6 h post-hCG when cumulus mass has expanded), KSOM containing 300 ug/ml hyaluronidase and mechanical pipetting were used to remove expanded cumulus cells surrounding the MI and MII oocytes.

In vivo matured MII oocytes were collected 15 h after ovulation was trigged by hCG, by flushing mouse oviducts with 300 μg/ml hyaluronidase in KSOM to remove the cumulus cells (Hogan et al., 1994).

3.2.4 Preimplantation embryo collection

1-cell embryos were collected from females caged overnight with BDF1 males approximately 24 h after ovulation was triggered with hCG. Embryos were flushed from excised oviducts using a blunt-tipped syringe containing KSOM. The cumulus mass was transferred to a drop containing KSOM with hyaluronidase (300 μg/ml) to facilitate dispersion of the cumulus cells. Isolated embryos were transferred through 5 successive wash drops of KSOM to remove any residual hyaluronidase. Fertilization was confirmed by observation of two pronuclei (Hogan et al., 1994).

Two-cell embryos were obtained directly by flushing surgically removed oviducts with HEPES-KSOM, 42-44 h following hCG injection (Hogan et al., 1994).

Blastocysts were retrieved 93-94 h after hCG injection via flushing of surgically removed uterine horns with HEPES-KSOM (Hogan et al., 1994).

3.2.5 Embryo and oocyte culture

Oocytes and embryos were cultured using standard protocols used routinely in our laboratory (Dawson and Baltz, 1997; Lawitts and Biggers, 1993). Unless otherwise specified each replicate used pooled oocytes or embryos from several female mice. Three 50 uL drops of culture media were placed in 35mm culture dishes (Falcon, Corning NY) and overlaid with KSOM-washed mineral oil. These microdrop dishes were preequilibrated in an incubator with 5% CO_2 in air at 37°C and 100% humidity (Lawitts and Biggers 1993), for a minimum 2 h prior to use. Oocytes and embryos were washed through the three drops to eliminate any carryover from collection or previous treatments to the culture media and left in the final drop for culture. Oocytes and embryos were returned to the incubator for culture according to experimental requirements. Oocytes and embryos were manipulated using mouth-operated Pasteur pipettes, pulled in a flame to a narrow opening.

3.2.6 Male reproductive tract dissection (for protein extraction)

Epididymis and testis were isolated from male *Chdh* $^{-/-}$ and *Chdh* $^{+/+}$ mice for use as Western blot control tissues. Tissues were extracted at ~4 weeks of age, and fat was completely removed. Tissues were stored in lysis buffer until protein was extracted for Western blot (described below).

3.3 Rate of transport measurements in oocytes and embryos using [^{3}H]-labelled betaine

All measurements performed using standard laboratory protocols previously described (Baltz, 2013). The rate of transport of betaine was measured by incubating oocytes or embryos in medium containing [methyl-^{3}H]-betaine (American Radiolabelled chemicals, St. Louis, MO; #ART 1903). Oocytes, MII eggs and 1-cell embryos were collected exactly as described above.

Groups of 6-11 oocytes or embryos were incubated in KSOM at 37ºC for 30 minutes with 1 μM [methyl-^{3}H]-betaine before being removed and quickly washed through 5 successive drops of ice-cold HEPES-KSOM. Subsequently, the washed oocytes or embryos were transferred to scintillation vials together with 4 ml of scintillation fluid. Non-saturable transport was determined using 5 mM unlabeled betaine present during incubation in addition to 1μM [^{3}H]-betaine. After incubation, the ^{3}H content of oocytes or embryos was quantified using a scintillation counter (LS 6500, Beckman Coulter, Brea, CA) with a 5 minute counting period for each sample (Dawson *et al.*, 1998).

In addition to the oocyte or embryo samples, background was measured by taking medium from the last wash drop and residual counts were determined. These counts were subtracted from the counts obtained from the corresponding oocyte or embryo samples. To allow conversion from counts per minute to concentration, standard curves were constructed weekly using known serial dilutions of the ^{3}H-labeled compound. The rate of transport was calculated as femtomoles of substrate per oocyte per minute of incubation.

3.4 Endogenous betaine measurements

Endogenous betaine was measured in blinded samples by liquid chromatography with tandem mass spectrometry (LC-MS/MS). For *in vivo*-matured GV oocytes and MII eggs, groups of 50 oocytes or eggs were isolated as described in HEPES-mKSOM, and dried before being stored at -20ºC in 1.5 mL Eppendorf tubes. For measurement of *in vitro* matured oocytes, sets of 30 freshly isolated GV oocytes were processed immediately for betaine or oocytes were cultured for 18 h in mKSOM media in the presence or absence of 500 μM choline before being dried and

processed for betaine. Any measurements using genotyped *Chdh* mice were performed after confirming genotype by PCR. Genotypes were performed in duplicate.

All samples were processed blind by liquid chromatography followed by tandem mass spectrometry by Jeremy Zhang of the Pharmacokinetics Laboratory of the Clinical Investigations Unit of the Ottawa Hospital. He provided the following description for publication in McClatchie et al. (2017):

"Measurements were obtained using a Thermo Accela HPLC system (0.4 mL/min flow rate) with Thermo TSQ Quantum Access Max Triple Quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, MA). Separation was by Thermo Accucore HILIC column (100 mm x 2.1 mm, 2.6 um). The mobile phase was Line A: 0.1% Formic Acid and Line B: Acetonitril+0.1% Formic Acid with a gradient of 10% to 90% B in 2 min and back to 10% B in 5 min, then holding at 10% B for another 2 min, for a total run time of 4.5 min.

Samples were re-suspended in 1 mL of 10% methanol, 90% acetonitrile with deuterated D9-betaine (10 μmol/L) internal standard. The injection volume was 10 μL and the oven temperature was 40^{o}C. Betaine was detected in positive ion mode by electrospray ion source with a mass transition 118 →59 and 127 →68 for D9-betaine. The betaine content of an equal volume of each final wash drop was also determined to ensure that there was no contamination."

Limit of detection was determined by the Clinical Investigations Unit prior to analysis of oocyte samples to ensure that a small magnitude (pmole levels) of betaine could be accurately measured. Samples with known betaine content were measured exactly as described above. The measured value was compared to nominal value and plotted, with duplicate measurements taken for every sample (Figure 8). We accepted a standard error rate of < 10%, which allowed us to

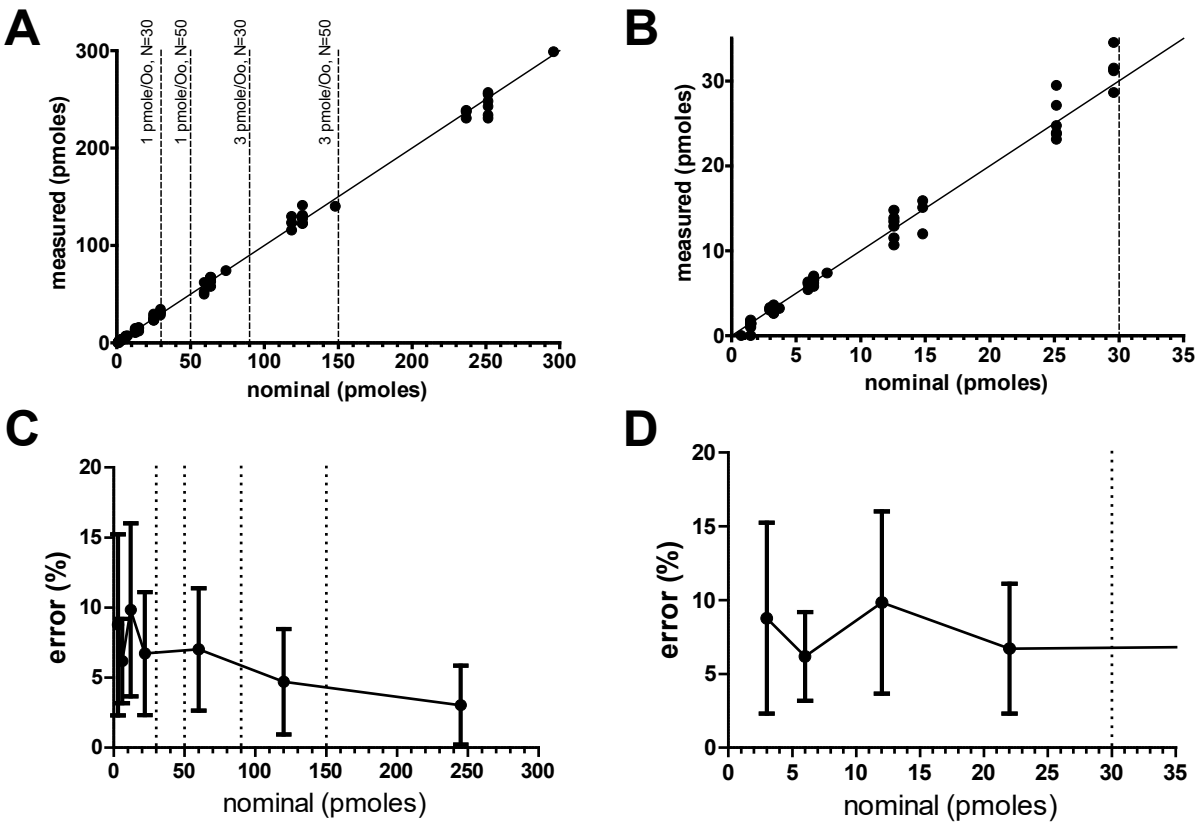

Figure 8: Limit of detection

A) Measured betaine levels of nominal samples from 1 to 300 pmoles, measured by LC-MS/MS. Samples sizes of 30 oocytes and 50 oocytes, with 1 pmole/oocyte and 3 pmole/oocyte values are superimposed. Actual endogenous betaine per oocyte has been hypothesized to be between 1 and 3 pmole/oocyte or embryo (Anas et al., 2008; Corbett et al., 2014; McClatchie et al., 2017), therefore these nominal values are highlighted for the equivalent betaine level we would expect in sample sizes of 30 and 50 oocytes or embryos.

B) Nominal betaine values below 30 pmole are shown to be within the linear range indicating reasonable accuracy below sample sizes of 30 oocytes or embryos, if the predicted betaine content is less than 1 pmole/oocyte or embryo.

C) Percent error is shown for nominal values from 0 to 200 pmole, calculated from the residuals of the data in A.

D) Percent error is shown for nominal values from 3 to 30 pmole. Error ranges from ~10-15%. Errors were not plotted below 3 pmole.

The above measurements and subsequent calculation of % error were performed by Jeremy Zhang at the Pharmacokinetics Laboratory of the Clinical Investigations Unit of the Ottawa Hospital.

measure samples containing as little as 2 pmole of betaine per sample, which is well within the range of endogenous betaine expected for each sample of 30 oocytes.

3.5 Molecular Biology Techniques

3.5.1 Western blot

3.5.1.1 Protein extraction

Liver lysates were prepared by adding 1mL of lysis buffer containing 20mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% Sodium deoxycholate, 0.1% SDS, 1mM PMSF, 5 μg/ml aprotinin, 5 μg/ml leupeptin to sample and incubated for 10-15 min. Samples underwent several freeze-thaw cycles using liquid nitrogen/warm water before sample was homogenized by hand. Homogenate was centrifuged at 11000 rpm for 10 min at room temperature. Supernatant was collected and protein content was analyzed by Lowry Assay (BioRad) and read by spectrophotometer at 750nm (Ultrospec 3000, Pharmacia Biotech, Cambridge, England). Values were compared to a standard curve constructed from Bovine serum albumin (BSA) commercial standard of 1.38 ng/ml (BioRad, Hercules, CA; #500-0007).

3.5.1.2 General Western Blot

The following solutions were used for Western Blot analysis: Lysis buffer containing 20mM Tris-HCl pH 7.4, 150 mM NaCl, 1mM EDTA, 1% Triton X-100, 1% Sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 5ug/ml aprotinin, 5 μg/ml leupeptin; 2X Laemmli Buffer (BioRad; #161-0737); 10X SDS PAGE Buffer (AMRESCO Inc, Solon, OH, USA); Transfer Buffer

containing Tris 25 mM, and 192 mM glycine in distilled water; TBS buffer containing 20 mM Tris and 500 mM NaCl in distilled water (pH was adjusted to 7.5 with HCl); Tween TBS (TTBS) solution containing 0.05% Tween20 in TBS buffer; Blocking solution of 5% milk; Prestained Protein Molecular Weight Marker (Precision Plus Protein Standards – Kaleidoscope, #161-0375).

10 µL samples mixed with equal amounts of Laemlli buffer and 1 µL β-mercaptoethanol were boiled for 5 min at 100ºC, separated by SDS-PAGE on 12% acrylamide gel (1 h at 70V), and subsequently transferred to Protran nitrocellulose (0.45 µm) (Whatman, Florham Park, NJ; #10600002) (for 1 h 10 minutes at 100V at 4ºC, Bio-Rad transfer apparatus). Visualization of bands was performed using 1X Ponceau. After blocking nonspecific protein binding with 5% milk (1 h at room temperature), each blot was incubated with Proteintech (Rosemont, IL; 17356-1-AP) primary antibody against CHDH (1:1000) in 5% milk (overnight, 4ºC). After 3 washes in TBST, membrane was incubated with goat anti-rabbit HRP secondary antibody in 5% milk blocking solution (1 h, Room temperature). Visualization was with the ECL Prime Detection Kit (GE healthcare, Mississauga, ON; RPN 2232), on autoradiography film (Hyblot CL, Denville Scientific, Hollison, MA; #E3018). In all cases GAPDH was a loading control (MW ~36 kDa) done on the lower portion of the membrane using GAPDH FL-355 antibody (Santa Cruz Biotechnology, Dallas, TX; sc-25778,) (1:200) in 5% milk (overnight, 4ºC). After 3 washes in TBST, membrane portion was incubated with goat anti-rabbit HRP secondary antibody in 5% milk blocking solution (1 h, room temperature). Visualization was performed using the ThermoScientific Pierce ECL Kit (Waltham, MA; #32106).

Other antibodies (Table 3) were tested under similar conditions, though the preliminary testing suggested none would be suitable for our visualization of CHDH protein, as outlined below.

Table 3: CHDH antibodies tested

Antibody Supplier	**Type**	**Result**
Proteintech (PT; AT-17356-1-AP)	Rabbit, polyclonal, IgG	Single, specific band at ~65 kDa
MyBioSource (MBS; MBS6006905)	Rabbit, polyclonal, IgG, complete antibody	Multiple bands, single band at ~65 kDa, specificity not confirmed
Sigma (Sigma; SAB1401751)	Rabbit, polyclonal, IgG, complete antibody	Multiple bands, single band at ~65 kDa, specificity not confirmed

Intensity of Western Blot bands was analyzed using ImageJ with background intensity subtracted.

3.5.2 PCR

3.5.2.1 DNA extraction

Genomic DNA was isolated from ear-notch samples taken from mice at weaning (~3 weeks of age). 50 mM NaOH was added to the sample before incubation using Eppendorf, Mastercycler thermocycler (Hamburg, Germany) at 95ºC for 30 min. 17 µL 1mM Tris was added to samples before vortexing. Total DNA from ear notch sample was used at 1.5 µL per PCR reaction.

3.5.2.2 General genotyping PCR

All animals were genotyped for *Chdh* by multiplexing polymerase chain reaction (PCR). The following solutions were used for PCR mastermix: RNAse free water, 10x buffer-$MgCl_2$., 50mM $MgCl_2$, 10 µM dNTPs, Taq Polymerase (Invirtrogen, Carlsbad, CA; #103422-053). Initial protocol used primers previously described (Johnson et al., 2010). The forward (sense) wildtype primer was 5'-AGGGCCACAAGTGTGGGCTGGCTGAAACTG-3', for null was 5'-ACGCGTCACCTTAATATGC-3', with a common primer of 5'-GCTAGCTTGAACCCTTTGAAGGGTCTTCTCAGACTC-3'. These yielded amplicons of 2.4 kb for wildtype and 1.6 kb for null. PCR conditions were as follows: 90ºC for 3 min., 94ºC for

30 s, 56ºC for 30 s, 72ºC for 3 min (repeated 35 times), and 72ºC for 10 min. This protocol was later adapted as described below and the primary genotyping protocol used the following primers. The forward (sense) primer for wildtype was: 5'-GTGTGAGGCTGTCACGAGAA-3', for null was: 5'-ACGCGTCACCTTAATATGC-3'. A common reverse (antisense) primer was used for both: 5'-AGCCAAGAGTCGGGGTATCT-3'. These yielded amplicons of 525 bp for wildtype and 478 bp for nulls using the following PCR conditions: 95ºC for 5 min., 94ºC for 30 s, 58ºC for 30s, 72ºC for 2 min. (repeated 10 times), followed by 94ºC for 30s., 56ºC for 30s., 72ºC for 2 min (repeated 25 times), and 72ºC for 10 min. Both protocols were run as a 20 µL PCR reaction + 4 µL loading dye on 1.3% and 3% agarose gel respectively for 1 h 120V.

3.6 Statistics

Statistical analysis and graphing were done with Prism 7 (GraphPad Software, La Jolla, CA). Quantitative data were expressed and plotted as the mean ± the standard error of the mean (SEM). Comparisons between means were made by ANOVA followed by the Tukey-Kramer post-test when more than 2 groups were compared, or by Student's two-tailed *t*-test when 2 groups were compared.

4. Results

4.1 Betaine is not transported during meiotic maturation

Previous data indicated that betaine is transported by a single saturable transporter in the 1-cell mouse embryo that also accepts proline as a substrate (Anas et al., 2007). However, the origin of the increased betaine we have noted to be present in MII eggs was unclear as no betaine transport activity was detected in oocytes at the time-points tested throughout meiotic maturation as noted by Anas et al. (2008). Therefore, I sought to determine whether activation of any transport mechanism was present prior to SIT1 activation by measuring the transport of [^{3}H]-betaine throughout meiotic maturation (Figure 9).

To confirm that betaine transport was absent throughout the period of meiotic maturation *in vivo*, oocytes were retrieved from mice that had been stimulated to ovulate by hCG injection. Oocytes collected at time points 3, 6, 9, 12 and 16 h post-hCG were immediately utilized for measuring the rate of betaine transport, thus providing a time-course for betaine transport as a function of time after ovulation was triggered *in vivo*.

Oocytes retrieved immediately after stimulation of ovulation were all GV stage, as expected. These oocytes exhibited low betaine transport activity. Starting 3 h after hCG injection, oocytes that had undergone GVBD were found indicating meiotic progression to MI-phase. These MI oocytes continued to exhibit low betaine transport. Oocytes began to achieve

MII-status from 9 h post-hCG onwards, and by 12 h post-hCG injection most oocytes were mature eggs. At each assessed time-point, very low saturable betaine transport, less than 0.001 fmol/oocyte/min, was recorded. A positive control of 1-cell embryos was included in which

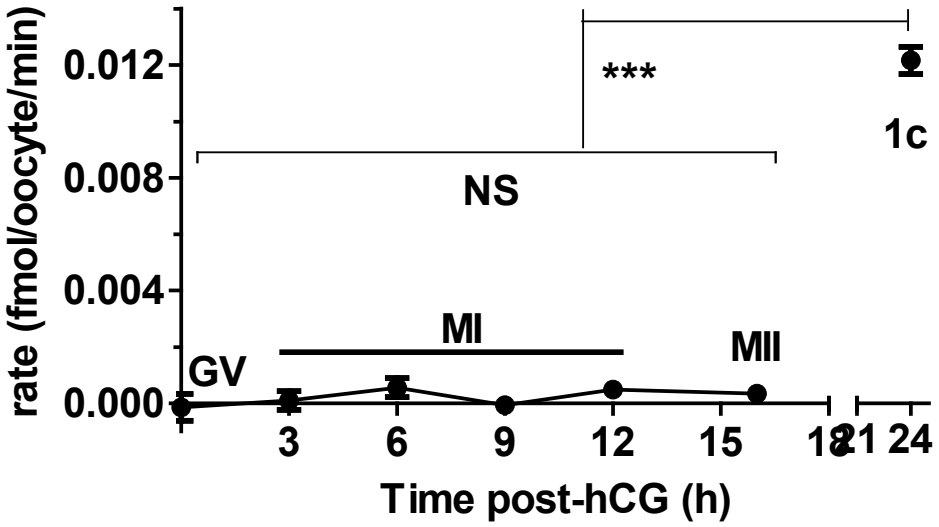

Figure 9: Transport of betaine throughout meiotic maturation

The transport of radiolabelled [^{3}H]-betaine was measured in GV oocytes, oocytes undergoing meiotic maturation through the first meiotic metaphase (MI, 3-12 h post-hCG), in mature MII eggs (MII, 16 h post-hCG) and in 1-cell embryos (1C). These were matured *in vivo* and collected from CF1 female mice at the indicated times after ovulation had been induced by injection of hCG. Oocytes, eggs and embryos were incubated with 1 uM [^{3}H]-betaine for 30 min, and the rate of transport is expressed as betaine (fmoles) per oocyte, egg or embryo per minute. The non-saturable betaine transport is subtracted in all cases. As expected no transport was detectable in oocytes or eggs, but substantial betaine transport was found in 1-cell embryos where the SIT1

transporter is known to be active. Each symbol represents the average rate of transport for three independent repeats ±SEM. The difference between mean rates of transport for oocytes and eggs is not significant, while the mean rate of transport for 1-cell embryos is significantly different from oocytes and eggs (*** $P<0.001$ by ANOVA with Tukey's test). This figure was obtained and adapted with permission from McClatchie et al. (2017).

betaine transport is known to be high due to SIT1 activity (Anas et al., 2008). As expected, substantial betaine transport was present in 1-cell embryos. The lack of saturable betaine transport detected at any point during meiotic maturation suggests that betaine transport cannot account for the high levels of betaine in MII-eggs.

4.2 Optimization of *Chdh* genotyping

Dr. Stephen Zeisel's lab provided the breeding pairs for our CHDH null mouse line. Dr. Zeisel provided the initial protocol used to genotype animals born into our developing colony. This was adapted directly from their 2010 publication (Johnson et al., 2010). This protocol generated very large amplicons of 2.4 kb for wildtype and 1.6 kb for null. Due to the large primer product size, we found that the smaller of the two products (the 1.6 kb null band) was often preferentially amplified in heterozygous genotyping, causing the wildtype band to be faint or undetectable (Figure 10). This resulted in confusion between heterozygotes and knock-out animals. Due to the breeding scheme described below, differentiation between heterozygotes and mutant animals was paramount to correct genotyping of the animals. Our colony was maintained via $Chdh^{+/+}$ *x* $Chdh^{+/+}$ matings to *generate* $Chdh^{+/+}$ offspring, *and* $Chdh^{+/-}$ x $Chdh^{-/-}$ (male x female) matings to generate $Chdh^{-/-}$ and $Chdh^{+/-}$ offspring (50:50). The latter pairing was necessary as $Chdh^{-/-}$ males are infertile due impaired sperm motility arising from mitochondrial

defects (Johnson et al., 2010). Thus, wildtype animals were the product of wildtype parents and genotyping was not required except for confirmation. In contrast, it was necessary to accurately differentiate between *Chdh*$^{+/-}$ and *Chdh* $^{-/-}$ offspring from the mixed genetic background parents. Thus, uncertain genotyping was not acceptable. We therefore attempted to improve the results using the original published primers, but in two separate reactions to allow better visualization of

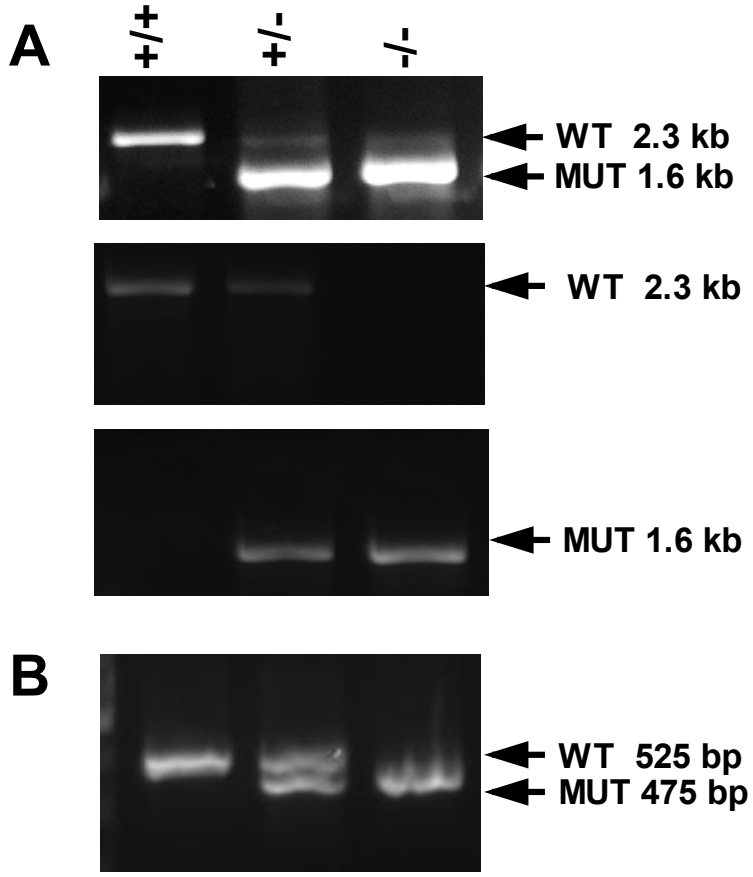

Figure 10: Comparison of choline dehydrogenase genotyping PCR reactions.

A) Control sample genotyping of *Chdh*$^{+/+}$, *Chdh*$^{+/-}$ and *Chdh*$^{-/-}$ animals by PCR using previously published PCR primers. Upper panel shows published genotyping using complete primer set. Faint wild type band visible in heterozygote samples. Middle panel

shows the same samples genotyped using wildtype and common primers only (neo primers removed) therefore producing only wildtype band. Lower panel shows identical samples genotyped using only neo and common primers (wildtype primers removed) therefore producing only mutant band. Wild type band at 2.4 kb and null band at 1.6 kb.

B) Control sample genotyping of *Chdh*$^{+/+}$, *Chdh*$^{+/-}$ and *Chdh*$^{-/-}$ animals by PCR using revised primers. Wild type band at 525 bp and knockout band at 478 bp. Demonstrating clear bands visible in all genotypes, allowing definitive genotyping of animals.

These images were obtained and adapted with permission from McClatchie et al. (2017).

the putative wildtype band. The first was according to the standard PCR protocol with all 3 primers, while the second used only the wildtype and common primers, but removed the null primer. This created a reaction in which only a wildtype band was present – allowing a positive or negative differentiation between heterozygote and knockout animals with reasonable accuracy. However, this was both time consuming and often still did not provide sufficient certainty. Therefore new primers were designed to have smaller amplicon sizes. These were designed by previous lab member, Chenxi Zhou. However, I confirmed primer binding locations within the mouse chromosome 14 using Ensemble. Predicted amplicon sizes were confirmed using NCBI Blast.

Thus, new primers (described above) were designed to yield 525 bp products in wildtype and 498 bp products in null animals. The smaller sized amplicons led to consistent, visible band patterns and accurate genotyping (Figure 10). These were used in all experiments where specified below. Primers, sequences and locations are outlined below (Table 4).

4.3 CHDH protein is present in oocytes and preimplantation embryos

Betaine synthesis from choline via CHDH is implicated by the lack of betaine transport during meiotic maturation. Previous data by Hannah Corbett demonstrated the presence of *Chdh* mRNA transcripts in developing oocytes, GV oocytes and fertilized eggs, but not in subsequent preimplantation embryo stages (McClatchie et al., 2017). Previously, no suitable antibody for

western blot use had been identified. I therefore sought to examine the presence of CHDH protein present in developing oocytes using previously untested antibodies.

Western blots were performed using various antibodies. Of the antibodies tested the most promising was a commercial polyclonal IgG directed against CHDH (17356-1-AP, Proteintech,

Table 4: Primer sequences and locations used for genotyping of *Chdh* mutant animals, specifically Mus musculus strain C57BL/6J chromosome 14.

	Name	**5' → 3' sequence**	**Location (chromosome 14)**
Original	Fwd WT	AGGGCCACAAGTGTGGGCTGGCTGAAA CTG	Exon 7
	Fwd MUT	ACGCGTCACCTTAATATGC	Neo cassette
	Com Rev	GCTAGCTTGAACCCTTTGAAGGGTCTTC TCAGACTC	3' UTR
Revised	Fwd WT	GTGTGAGGCTGTCACGAGAA	Exon 7
	Fwd MUT	ACGCGTCACCTTAATATG C	Neo cassette
	Com Rev	AGCCAAGAGTCGGGGTATCT	Exon 8

Chicago, IL; Proteintech). This antibody gave a single band at the predicted molecular size of ~66 kDa. Initial screening for specificity was performed using Human HEK293T transfected with human *CHDH* cDNA (LY413062, Origene, Rockville, ML) and therefore overexpressing CHDH protein, and an empty vector transfected control cell lysate (LY413062, Origene) as positive and negative antibody controls respectively. The Proteintech antibody was shown to be likely useful for CHDH western blotting by giving a band in the overexpression lysate, but not in the empty vector (Figure 11A).

To further confirm the band at 66 kDa was specific to CHDH, membranes were cut and half incubated with CHDH primary antibody and 1:100 of recombinant human CHDH protein (ag10614, Proteintech, Chicago, IL). The recombinant protein was expected to bind to the primary antibody, thus eliminating the CHDH band, while the band would remain present on the membrane incubated with primary antibody alone without recombinant protein. However, the presence of recombinant protein yielded generally dark unusable results due to the protein sticking to the membrane despite attempted correction with varying concentrations of the recombinant protein (Figure 11B). The membrane became lighter and no bands were visible at 1:1000, however CHDH band becomes visible at 1:750 suggesting that this concentration is the minimum recombinant protein required to maintain inhibition of the primary antibody. Increased number of TBST washes of the membrane prior to incubation with secondary antibody yielded a lighter result. No visible bands on the lightened membrane was taken to be a positive sign of

antibody inhibition by the recombinant protein, however the lack of clear result prevented confirmation of antibody validity by this method.

Therefore, I next used tissues obtained from *Chdh*$^{+/+}$ and *Chdh*$^{-/-}$ mice, which became available to us after the initial antibody testing. Testing of antibody specificity was performed using tissues isolated from the first litter of *Chdh* mutant mice successfully bred by our lab in the University of Ottawa animal care facility. CHDH protein was previously shown to be highly expressed in the male reproductive system and in kidney (Johnson et al., 2010). Kidney, testis and epididymal tissues were therefore isolated from male wildtype and null mice for antibody testing. Initial testing of wildtype samples showed kidney samples gave only one band, while testis and epididymal tissues showed several bands. Western blots were further optimized using kidney samples from *Chdh*$^{+/+}$ animals (Figure 11C). Following this, the Proteintech antibody banding was confirmed to be specific to CHDH using 50ng of kidney protein lysate per lane from each of *Chdh*$^{+/+}$ and *Chdh*$^{-/-}$ mice. A single clear band at ~ 66 kDa was visible in *Chdh*$^{+/+}$ mice, and absent at the same position in *Chdh*$^{-/-}$ kidney lysate (not shown).

I then confirmed the identity of the band detected in oocytes as CHDH using GV oocytes obtained from *Chdh*$^{+/+}$, *Chdh*$^{+/-}$ and *Chdh*$^{-/-}$ ovaries. Oocytes were cultured in MEMα for 6h after isolation to allow maximal CHDH enzyme activity to have developed (McClatchie et al, 2017). For this experiment 50 oocytes were loaded per lane. Samples were identified only by mouse number. Thus, gels were prepared blind and genotypes as determined by PCR were matched to oocyte samples only after the Western was completed. In all replicates a single band at 66 kDa was visible in mice identified by PCR to be *Chdh*$^{+/+}$, a fainter band present in *Chdh*$^{+/-}$ and an absence of any band in *Chdh*$^{-/-}$ samples. GAPDH was used as a loading control (Figure 11D).

The position of the band was indistinguishable from the band detected in kidney samples. As a further control, kidney tissue samples were collected from all mice and an additional western blot was performed using

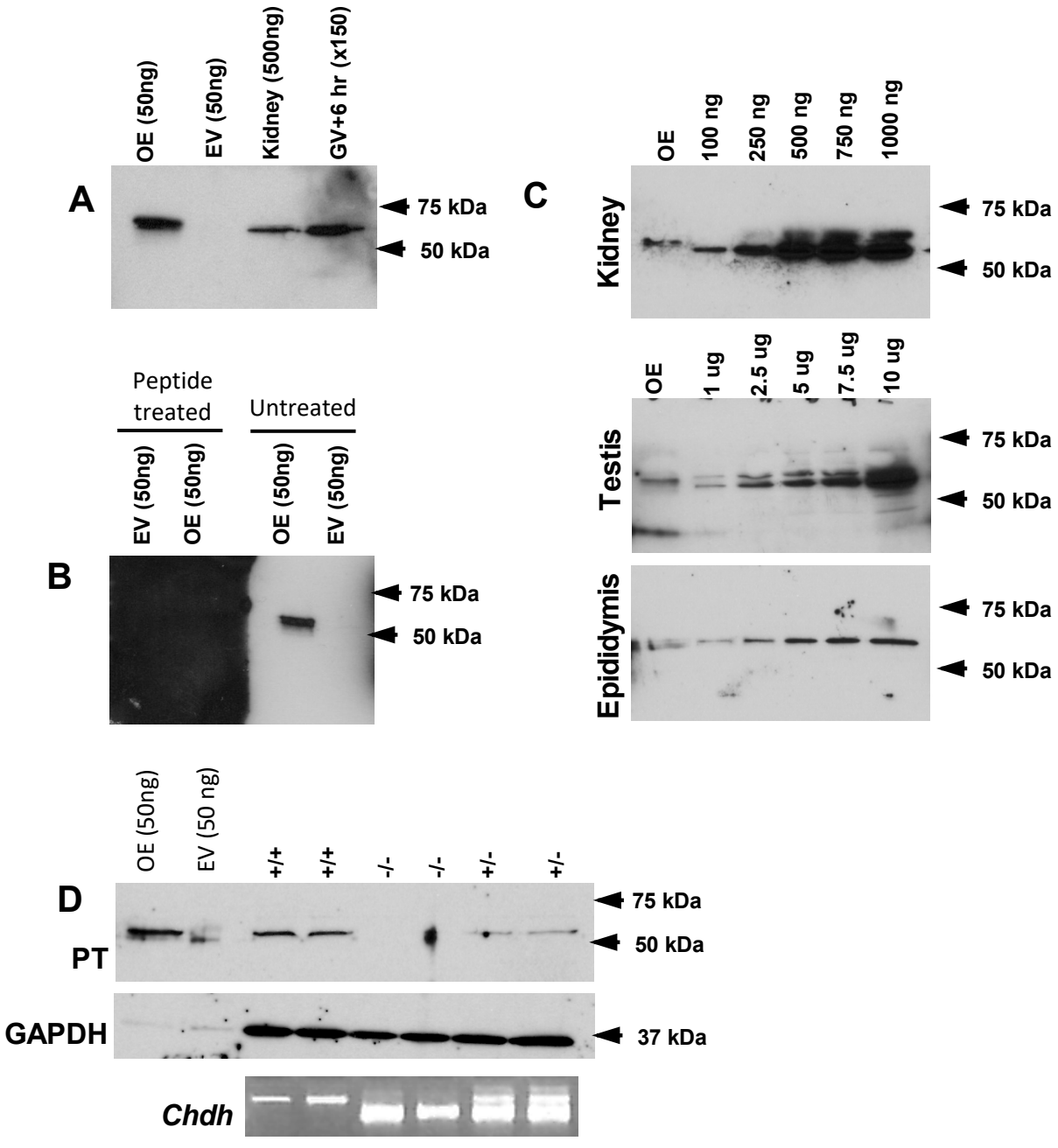

Figure 11: Expression of choline dehydrogenase, western blot optimization using Proteintech polyclonal antibody

A) Representative sample of polyconal IgG CHDH antibody (Proteintech; PT) banding pattern. Producing a single band at the predicted molecular weight of ~66 kDA in CHDH transfected HEK293T overexpression lysate and an empty vector transfected control cell lysate. Clear band is present in the overexpression (OE) lysate but not the empty vector (EV) lysate suggesting antibody specificity. Band is also present in control mouse kidney lysate (500ng) and in sample of 200 GV oocytes matured for 6 h in vitro. Band in mouse is slightly below band in human overexpression lysate.

B) Representative image of attempts to use recombinant human CHDH protein to confirm antibody specificity. Ideally, we sought to show the recombinant CHDH protein binding with Proteintech (PT) antibody in solution, thereby preventing membrane binding and consequently eliminating the banding pattern. However, due to excessive membrane sticking only inconclusive results could be obtained as shown. Various optimization techniques not shown.

C) Single replicate of each $Chdh^{+/+}$ kidney, epididymis and testis tissues at varying concentrations using PT antibody to determine ideal control tissue and loading dilutions. Single clear band was visible in kidney at 100ng and epidiymis tissue, while testis tissue gave multiple bands. Thus, kidney was selected as the control sample.

D) Western blot of GV oocytes (60 oocytes per lane) isolated from *Chdh*$^{+/+}$, *Chdh*$^{+/-}$, and *Chdh*$^{-/-}$ p21 neonatal ovaries. The oocytes were cultured for 6 h post-isolation in MEMα at which point maximal CHDH activity would have developed. CHDH (top) and GAPDH

loading controls (middle) are shown, with genotyping by PCR (bottom). One of three similar independent repeats is shown.

These images were adapted and used with permission from McClatchie et al. (2017).

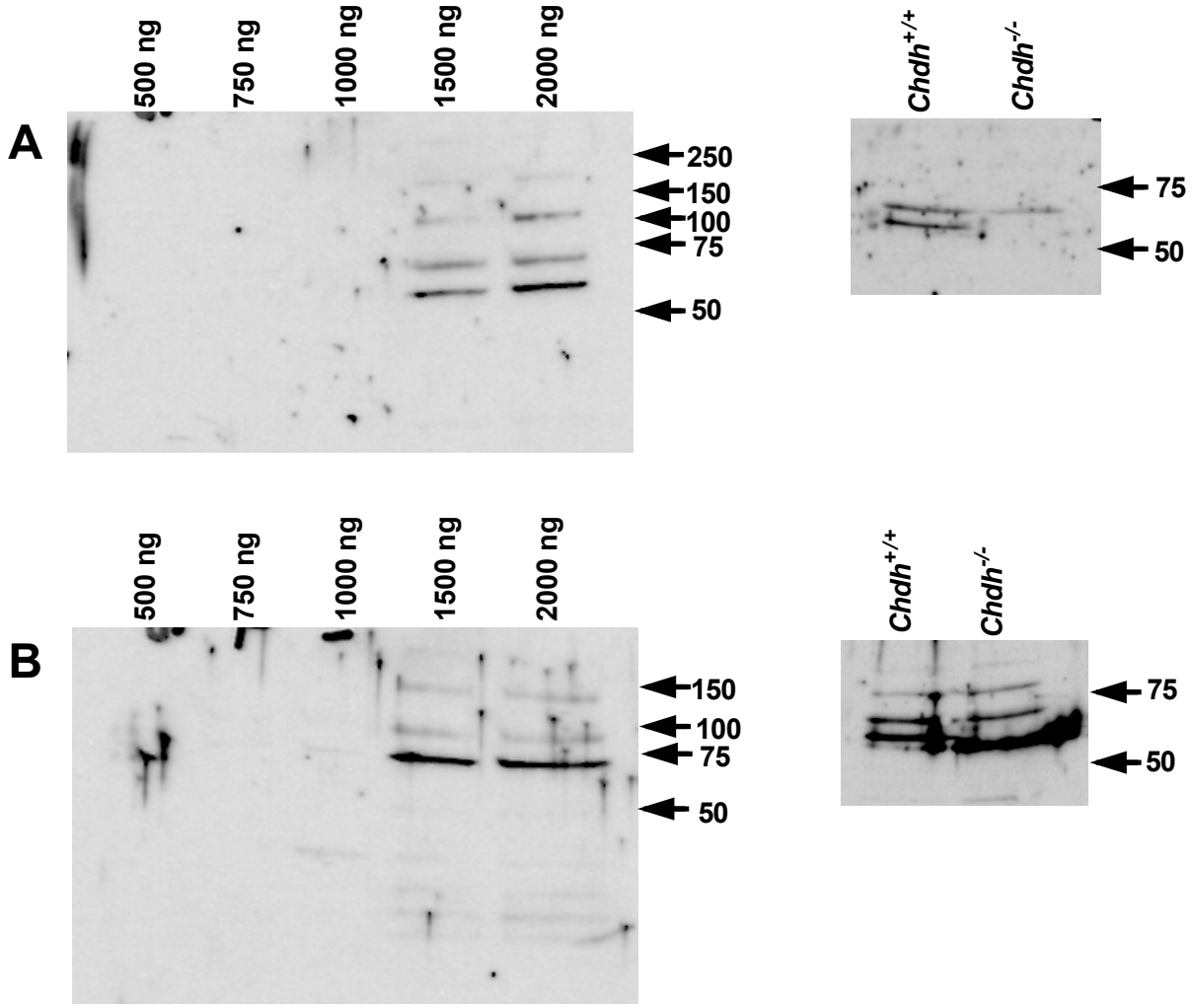

Figure 12: Expression of choline dehydrogenase, western blot validation using Sigma-Aldrich' (Sigma) and MyBioSource (MBS) complete CHDH polyclonal antibodies

A) Representative banding pattern produced by MyBioSource(MBS) complete CHDH polyclonal antibody in *Chdh*$^{+/+}$ kidney lysate. At ~1500 ng of lysate per lane, a clear banding pattern with a single band at the predicted molecular weight of 66 kDa is visible. This band is absent in *Chdh*$^{-/-}$ kidney lysate.

B) Representative banding pattern produced by Sigma Aldrich' complete CHDH polyclonal antibody Sigma) in *Chdh*$^{+/+}$ kidney lysate. At ~1500 ng of lysate a clear banding pattern with a single band at the predicted molecular weight of ~66 kDa is visible. This band appears to be absent in *Chdh*$^{-/-}$ kidney lysate

kidney lysate, to confirm the expected presence or absence of CHDH according to the genotype as determined (not shown).

Additionally, two other antibodies were tested with hopes of obtaining a second specific antibody. Loading controls using *Chdh*$^{+/+}$ kidney lysate for both MyBioSource (Figure 12A) (MBS6006905, MyBioSource, San Diego, CA) and Sigma (Figure 12B) (SAB1401751-100UG, Sigma-Aldrich, St. Louis, MO) polyclonal IgG full-length CHDH antibodies were initially tested, however both antibodies showed that significantly more protein (1500 ng) was required per lane to observe bands than the 250 ng required for the Proteintech antibody. Though both showed promising banding patterns in *Chdh*$^{+/+}$ and *Chdh*$^{-/-}$ lysates, the amount of protein required suggested that a prohibitive number of oocytes and embryos would be required to perform any testing with the samples of interest and further testing was not pursued.

4.4 CHDH is expressed throughout oocyte and embryo development

We have previously assessed CHDH enzyme activity in oocyte and embryo lysates. Our lab has previously adapted an assay from Grossman and Herbert (1989) to show that CHDH activity begins to increase in oocytes as meiotic maturation progresses *in vivo* (Figure 7). Maximal activity was present in MI and MII oocytes from 3-16 h after meiotic maturation was induced. CHDH activity decreased after fertilization with only low levels of activity remaining in the 1-cell embryo, or in any further preimplantation stages (Figure 7A) (McClatchie et al., 2017).

Having successfully demonstrated that CHDH protein can be detected in both kidney tissues as well as oocyte samples, I sought to demonstrate the presence of protein during the critical period for CHDH activity from the MI to MII stage. I also hoped to ascertain if and when the CHDH protein disappears in preimplantation embryos after CHDH activity diminishes at the 1- to 2-cell stage.

I performed western blots using the validated Proteintech antibody. Each lane contained 50 oocytes and embryos. *Chdh* activity is highest in MI and MII oocytes from 3-16 h post-hCG injection, becoming inactive by the 2-cell stage (McClatchie et al., 2017). *Chdh* mRNA is present throughout oocyte development, maturation, but likewise is absent by the 2-cell stage. Thus, I chose to test GV oocytes (negligible CHDH activity), MII eggs (high CHDH activity), 1-cell embryos (diminished activity), 2-cell embryos (negligible CHDH activity) and blastocysts (negligible activity) to observe any changes in protein levels at different stages of CHDH activity. Kidney lysate (250 ng) from confirmed $Chdh^{+/+}$ and $Chdh^{-/-}$ mice was used as a control. GAPDH was used as a loading control. CHDH protein was observed in GV oocytes and MII eggs, as well as in all stages of preimplantation embryo tested as a single band at ~66 kDa, at the same position as the band in kidney lysate (Figure 13).

4.5 CHDH is uniformly expressed throughout meiotic maturation

The above time-course contained GV oocytes and MII eggs. It appeared in these blots that the CHDH protein level did not increase from GV oocyte (no measurable activity) to MII egg (high activity). Preliminary testing performed by M. Meredith (not shown) indicated that the amount of CHDH protein did not appear to increase from GV to MI oocytes at 6 h post-hCG relative to GV oocytes despite CHDH enzyme activity increasing from negligible to maximal

(Figure 14A). To have better visualization of differences between GVs and MII I performed a dilution series of each. I hoped to visualize a difference in the limit of detection

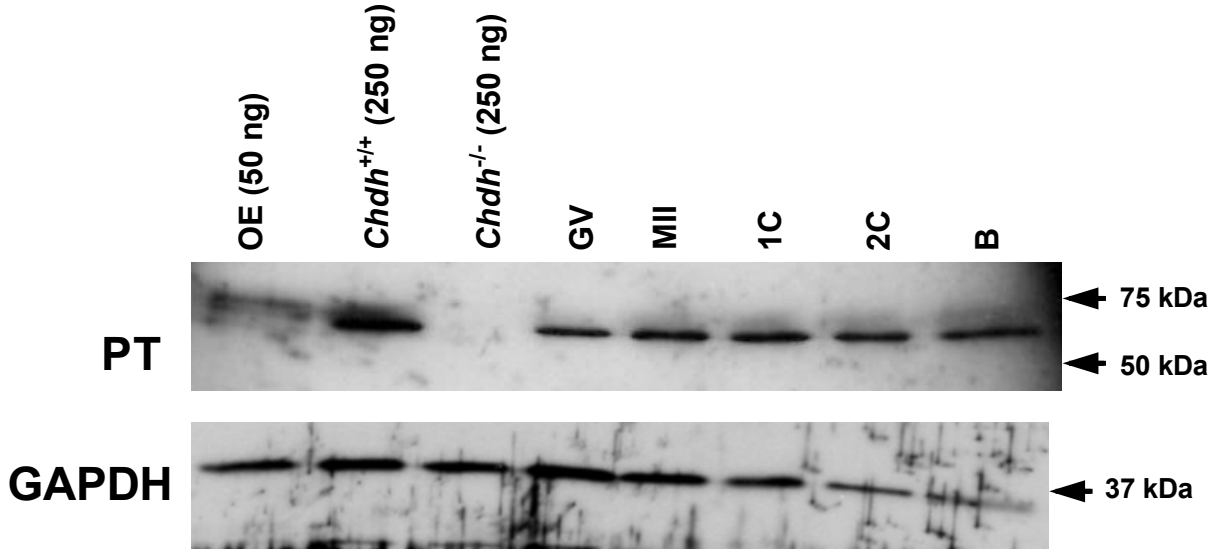

Figure 13: Expression of choline dehydrogenase protein throughout oocyte and embryo development

Western blot of GV oocytes, MII eggs, and 1-cell (1C), 2-cell (2C), and blastocyst (B) stage embryos showing CHDH (upper) using Proteintech polyclonal antibody (PT) and GAPDH as a loading control (lower). Fifty oocytes or embryos were loaded in each lane. Over-expression lysate (OE; 50ng protein), Kidney lysate (250 ng total protein) from *Chdh*$^{+/+}$ and *Chdh*$^{-/-}$ neonates demonstrates antibody specificity. Positions of MW markers are indicated and labeled at right. The example shown is representative of three independent repeats. This figure was obtained and adapted with permission from McClatchie et al. (2017).

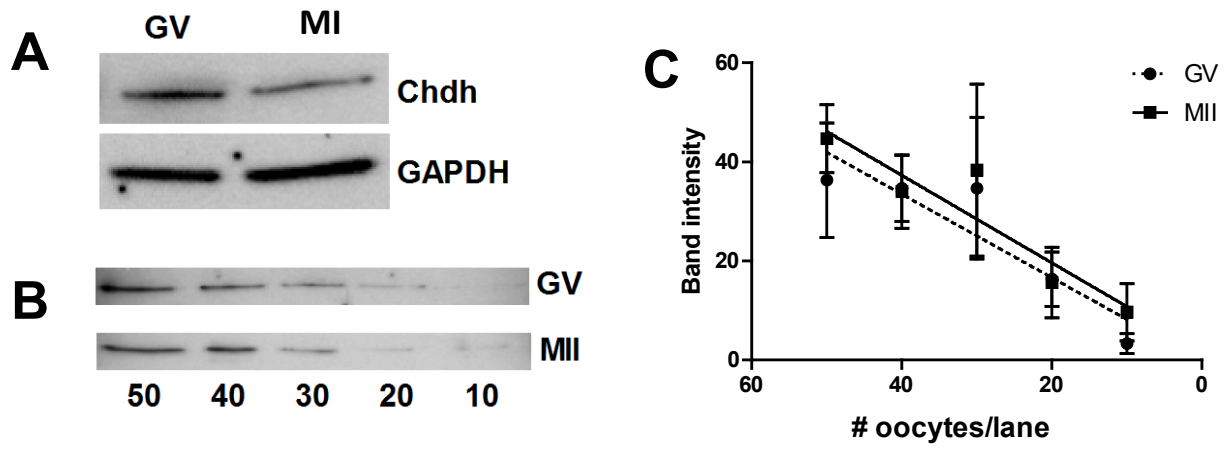

Figure 14: Expression of choline dehydrogenase protein in GV and MII oocytes

A) Representative image of western blot directly comparing GV oocytes and MI oocytes matured in vitro for 6 h. Each lane contained 50 oocytes. The example shown is representative of three independent repeats. Not shown is band intensity analysis which determined bands were not significantly different (P=0.109 by t-test).

B) Western blot of decreasing number of MII eggs and GV oocytes blotted simultaneously for direct comparison. Each lane contained the lysate of the number of GV oocytes or MII eggs indicated. The example shown is representative of three independent repeats.

C) Quantification of band intensity using ImageJ software. Intensity of western blot bands of decreasing numbers of GV and MII oocytes plotted to determine if differences in limit of detection (and therefore band intensity) were observable. There is no significant difference between GV and MII band intensity with any number of oocyte. Linear regression shown, as band intensity should decrease proportionally with number of oocytes. In each case background intensity is subtracted from band intensity.

This image was obtained and adapted with permission from McClatchie et al. (2017).

between the two sample types, I hoped this would be apparent through differences in band intensity. To this end, samples of 50, 40, 30, 20 and 10 of each GV oocytes and MII eggs were collected *in vivo* from adult CF1 mice. Western blot analysis was performed using an identical protocol for each sample, probing with Proteintech antibody (1:1000) to identify CHDH protein

(Figure 14B). For each replicate, samples of GVs and MIIs were run on the same gel to ensure consistency and allow comparison between bands. No distinct differences were immediately observable between GV and MII samples. Comparison of blot band intensity showed no significant difference in band intensity at any dilution of oocyte sample (Figure 14C).

4.6 Endogenous betaine measurements are consistent and follow CHDH activity

As previously described, the prior activity assay coincides with the period during which endogenous betaine is accumulated. Betaine levels significantly increased from those in GV oocytes by 6h post-hCG, and maximal levels were observed in MII eggs at 16h post-hCG injection. Conversely, activity peaked during meiotic maturation in maturing oocytes (MI) and in mature eggs. Activity was low in GV oocytes as well as post-fertilization in the 1-cell embryo and all subsequent embryonic stages (Figure 5). The following experiments seek to align the observed increase in betaine, with the known presence and activity of CHDH.

Groups of 50 GV oocytes or MII eggs were collected from CF1 female mice and immediately processed for endogenous betaine measurement by LC-MS/MS. Betaine levels were assessed blinded, along with background samples of the last wash drop from collection to ensure no contamination. This experiment did not yield novel data, as the endogenous betaine levels in GVs and MII-stage eggs had already previously been measured in New Zealand. Thus,

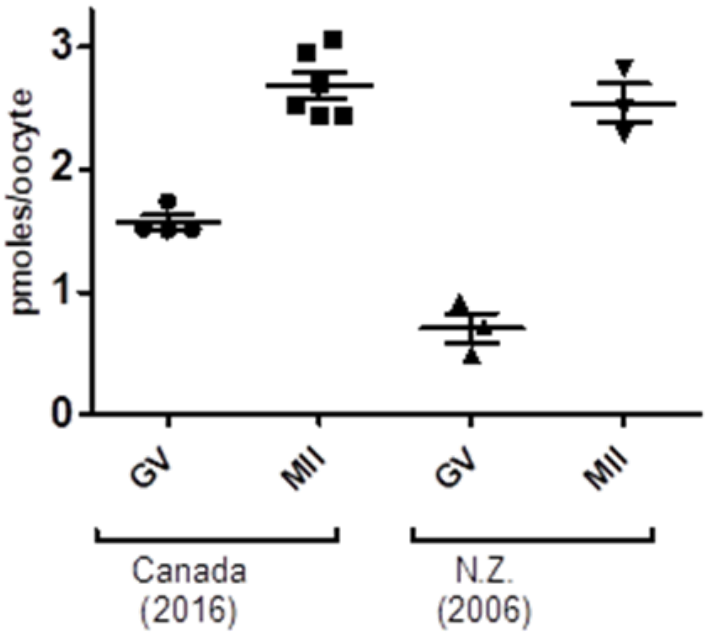

Figure 15: Endogenous betaine present in GV oocytes and MII eggs

Comparison of groups of 50 oocytes and MII eggs collected from CF1 female mice and processed for endogenous betaine both by LC-MS/MS at the Clinical Investigations Unit, Ottawa Hospital (2016) and previously by HPLC at Canterbury Heath Laboratories, New Zealand (2006) and published by Corbett et al. (2014). Both sets of data demonstrate a similar pattern with increased betaine present in MII eggs and significantly higher levels in mature MII eggs than in GV oocytes (P=0.0001 by t-test). Values were similar and not significantly different between the measurement method and location.

these preliminary measurements served to confirm that measurements yielded similar results in the two labs (Figure 15A). Consistent with previous results (Corbett et al., 2014), substantial betaine was present in MII eggs and levels were significantly higher in mature MII eggs than in GV oocytes. My results obtained at the Ottawa Hospital demonstrated a trend that was consistent with the results processed at Canterbury Health Laboratories. Baseline betaine levels in GV oocytes were slightly lower, while data produced at the Ottawa Hospital were slightly higher (Figure 15B). Values were similar and not significantly different. Therefore, these data support previous findings that high levels of betaine are accumulated during the period of maximal CHDH activity.

Samples of 50 GV oocytes and MII eggs were measured at the University of Ottawa and compared to those previously reported by Canterbury Health Laboratories. Data collected at different facilities appears similar, however for internal consistency each independent experiment was completed using measurements from only one location and method. Thus, the pattern of increasing CHDH activity coinciding with increasing endogenous betaine levels was confirmed.

4.7 Isolated oocytes are capable of producing betaine from choline

Previously reported results including the activity of CHDH (Figure 7) and its correlation with the appearance of betaine during oocyte maturation *in vivo* (Figure 5) implied that the increase in betaine could be attributed to choline conversion to betaine by CHDH. However, the possibility that betaine was instead accumulated by the maturing oocyte via another, unidentified mechanism remained. Thus, I sought to confirm that oocytes themselves were capable of producing betaine from choline during meiotic maturation.

Choline is present in blood (Wang and Haubrich, 1975) and has been shown to be present in other biological fluids including follicular fluid (Wallace et al., 2012; Marianna et al., 2017). Eppig et al. (1982) have also shown that choline can be taken up by the mammalian oocyte. This suggests that choline is both available to the oocyte by its presence in follicular fluid and able to be transported into the oocyte to be used as a substrate for betaine production during meiotic maturation. Choline uptake occurs primarily through the cumulus cells and is transferred to the enclosed oocyte until the cumulus and oocyte become uncoupled at ~6 h post-hCG. Choline transport by the oocyte itself becomes the sole route of choline uptake to the oocyte by 9 h post-hCG (Eppig, 1982).

To confirm that oocytes are capable of producing betaine from choline during meiotic maturation as would be predicted by the presence of CHDH mRNA, protein and enzyme activity in correlation with the appearance of endogenous betaine, I tested whether the amount of betaine would increase in isolated oocytes if they were matured *in vitro* in the presence of choline, with no betaine present in the medium.

I used mKSOM media as it contains no choline or betaine. We would usually culture developing oocytes through meiotic maturation in MEM-alpha medium, however, this commercial medium contains 1 mg/L choline. I only required the oocytes to be cultured overnight (18-24 h), and therefore specific oocyte medium was not necessarily a requirement. To confirm that oocytes could be successfully matured in mKSOM (which contains no choline), and the concentration of added choline that would be tolerated, I cultured groups of 10 CF1 oocytes, denuded of cumulus cells overnight in mKSOM in varying concentrations of choline between 0.25 mM and 1 mM, which is sufficient concentration to saturate choline transport in the oocytes

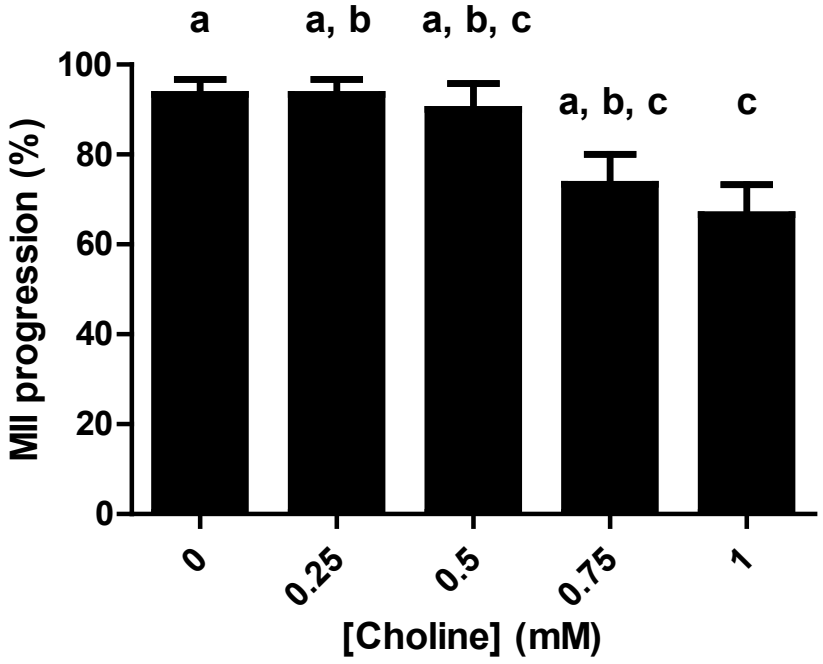

Figure 16: Maturation of GV oocytes to MII eggs in media containing choline

GV oocytes were isolated from CF1 females and cultured overnight in KSOM media. Percentage of GV oocytes progressing to MII eggs after overnight culture in increasing concentration of choline in KSOM was recorded. Bars represent the mean ± SEM of 3 independent repeats. Bars that do not share the same letter are significantly different ($P<0.05$ by t-test).

(Van Winkle et al., 1993). Oocytes survived and progressed from GV to MII stage in 0 mM, 0.25 mM and 0.5 mM with 90-100% progressing in each case (Figure 16). However, survival and progression diminished to ≤ 80% in 0.75 mM and 1 mM in KSOM (Figure 16). Thus, I selected 0.5 mM choline in mKSOM as the experimental conditions for further testing as this concentration of choline allowed good oocyte survival and would saturate choline transport into the maturingoocyte. This experiment was not repeated, but only used to determine the choline concentration used in the following experiments.

GV oocytes were isolated from CF1 female mice and immediately processed for endogenous betaine measurement or cultured for 18 – 24 h in the mKSOM medium in the presence or absence of choline (0.5 μM) before being processed. For cultured oocytes, only MII eggs that had successfully matured *in vivo* and had observable polar bodies were collected. The amount of betaine in the resulting MII eggs was determined by LC-MS/MS. MII eggs that had matured in the presence of choline had significantly more betaine than GV oocytes. In contrast, oocytes matured without choline showed only a slight increase in betaine from GV oocytes, and the difference was not significant (Figure 17). This suggests that betaine was synthesized during meiotic maturation from choline, as betaine was not accumulated in the absence of choline.

4.8 Isolated Cumulus-oocyte complexes produce highly variable amounts of betaine from choline

Cumulus cells are capable of transporting choline in the first hours post-hCG (Eppig, 1982). I hypothesized that increased choline transport via the cumulus mass might equate to increased betaine formation by CHDH in the enclosed oocytes. To test this, I isolated complete COCs from female CF1 mice and incubated them for 18-24 h in mKSOM medium in the presence or absence

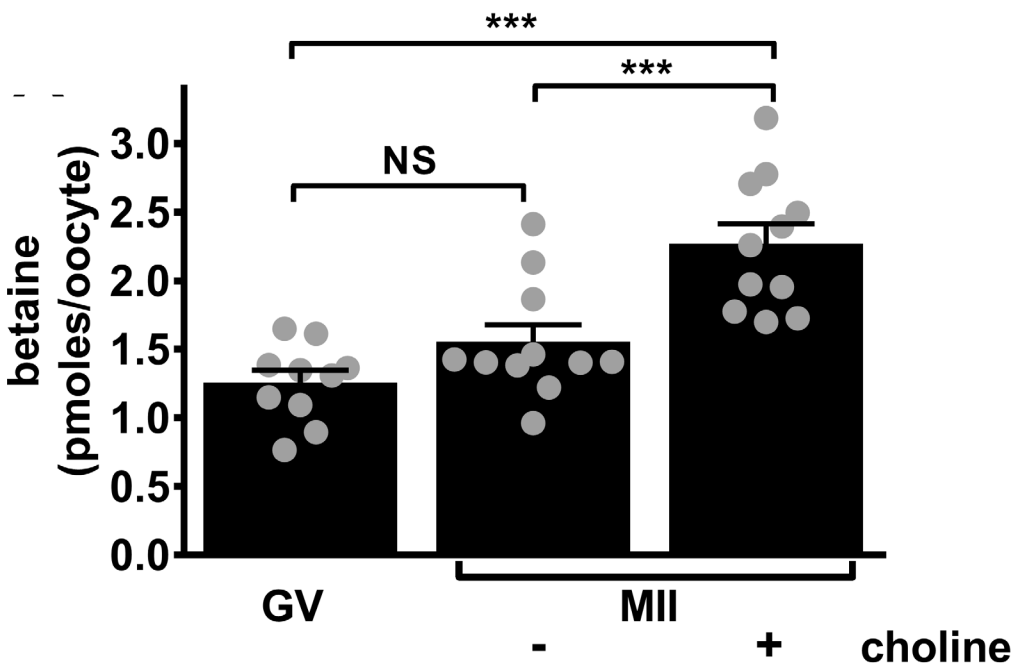

Figure 17: Endogenous betaine in GV oocytes cultured in the presence of choline

GV (GV) oocytes were isolated and immediately processed for analysis or isolated and placed directly in culture overnight to mature to MII eggs in the presence (+) or absence (-) of choline (0.5 mM) in mKSOM medium and then processed for endogenous betaine measurement by LC-MS/MS. Each point represents the measured betaine of a group of 25-30 oocytes or eggs, expressed as pmoles/oocyte or egg. Bars indicate means (±s.e.m.; N=10 for GV, N=11 for MII); grey circles indicate individual measurements. Means that do not share the same letter are significantly different ($P<0.01$ by ANOVA with Tukey's test). Eggs cultured in the presence of choline contained significantly higher betaine than GV oocytes, while eggs cultured in the absence of choline do not differ significantly from GV oocytes. This figure was obtained and adapted with permission from McClatchie et al. (2017).

of choline (0.5 mM). Before processing for analysis for LC-MS/MS any remaining cumulus granulosa cells were removed and only the enclosed MII egg was used for measurement. Oocytes from COCs cultured without choline had consistently comparable betaine levels to those previously measured in *in vivo* matured GV oocytes, suggesting that COCs do not act as a reservoir for betaine. However, oocytes from COCs cultured in the presence of choline had betaine levels that varied greatly between experiments (Figure 18). The large variability may perhaps have due to culture conditions causing a detrimental effect in COCs but not in GVs, where conditions were tested (Figure 16). Due to the inconsistent results we chose not to pursue this type of experiment further.

4.9 *Chdh* is required to produce betaine

The activity of CHDH enzyme has already been demonstrated under experimental conditions and correlated to the accumulation of betaine in oocytes *in vivo* and *in vitro.* To confirm that CHDH protein itself was required for the increase in betaine in oocytes, we utilized oocytes lacking CHDH due to the lack of a functional *Chdh* gene. Thus, I first sought to demonstrate the effect of a lack of functional CHDH protein *in vivo* on the endogenous betaine levels using the established line of *Chdh* knockout mice. To determine whether CHDH is absolutely required for betaine accumulation *in vivo* in the oocyte I isolated GV oocytes and MII eggs from $Chdh^{+/+}$, and $Chdh^{-/-}$ females and betaine levels were measured in the blinded samples by LC-MS/MS. Due to the time consuming and costly process of collecting oocytes from each genotype, $Chdh^{+/-}$ oocytes were not collected for this series of experiments. I predicted that *Chdh* null GV oocytes would have similar betaine levels to wildtype mice, and that these levels would not increase throughout meiotic maturation as a result of the knockout of the *Chdh* gene. Unexpectedly, both GV oocytes and MII eggs from $Chdh^{-/-}$ females had negligible endogenous betaine levels. The

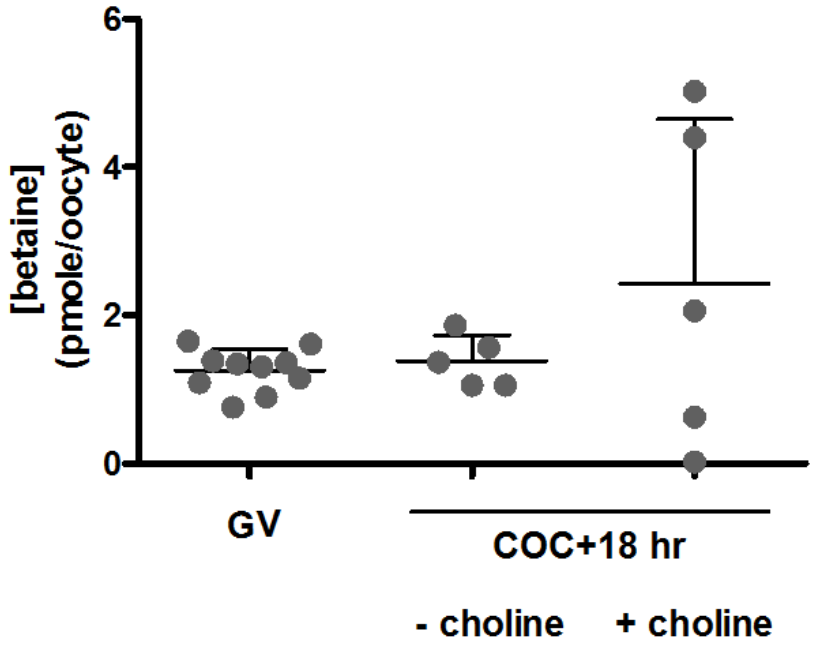

Figure 18: Total betaine present in COCs cultured in the presence of choline

Intact COCs were isolated immediately placed in culture overnight to mature to MII eggs in the presence (+) or absence (-) of choline (0.5 mM) in mKSOM medium and processed for endogenous betaine measurement by LC-MS/MS. Endogenous betaine of *in vivo* collected GV oocytes included for comparison. Each point represents the measured betaine of a group of 25-30 oocytes or cultured MII eggs, expressed as pmoles/oocyte. Bars indicate means (±s.e.m.); symbols indicate individual measurements. There was no significant difference between groups ($P<0.01$ by ANOVA with Tukey's test). However, we could not make any useful conclusions from these measurements since the variability in the group where cumulus cells were present during incubation with choline was extremely high, for unknown reasons. Therefore, we did not pursue this type of measurement further.

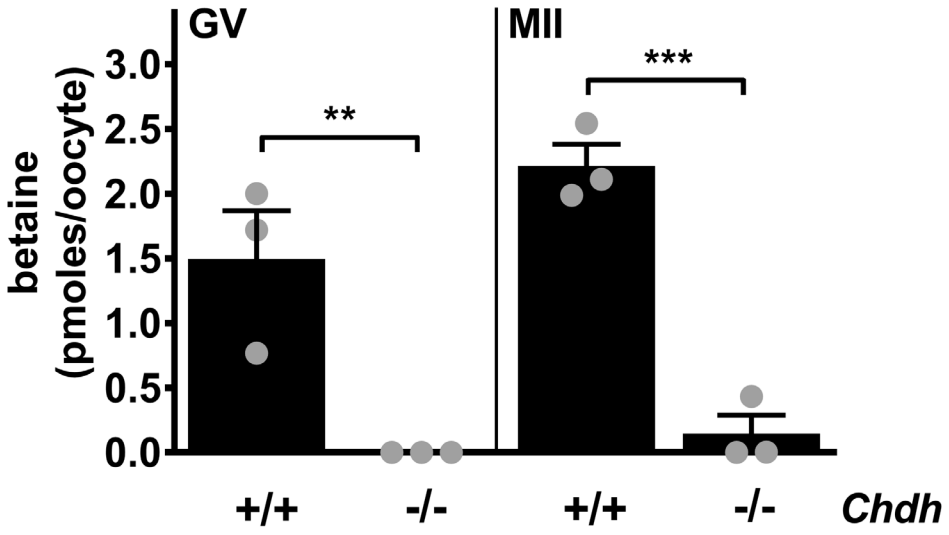

Figure 19: Endogenous betaine accumulation in oocytes with and without *Chdh*

In vivo matured GV oocytes and MII eggs were isolated from *Chdh*$^{+/+}$ and *Chdh*$^{-/-}$ females and processed immediately for endogenous betaine measurement by LC-MS/MS. GV oocytes were immediately processed for endogenous betaine measurements by LC-MS/MS. Both GV oocytes and MII eggs from null females contained little or no detectable betaine, while those from wild type females contained significantly higher levels of betaine (**P<0.01, ***P<0.001 by ANOVA). Limited data set (N=3 pools of 30 oocytes for each genotype) presented and *Chdh*$^{+/-}$ oocyte are omitted from analysis due to difficulty obtaining sufficient *in vivo* developed MII eggs from each genotype. This figure was obtained and adapted with permission from McClatchie et al. (2017).

lack of detectable betaine in GV oocytes and MII eggs from *Chdh*$^{-/-}$ females that had been matured *in vitro* implied that GV oocytes from null females lacked even the low level of betaine present in wild-type GV oocytes (Figure 19). Despite the GV oocytes being lower in endogenous betaine than predicted, MII eggs had the same low level and did not accumulated any betaine, thus implicating CHDH in the normal accumulation of betaine.

4.10 *Chdh* is required to convert choline to betaine

Finally, to directly confirm the role of CHDH in the conversion of choline to betaine during meiotic maturation I used oocytes from *Chdh*$^{+/+}$, *Chdh*$^{+/-}$ and *Chdh* $^{-/-}$ females cultured from GV oocyte to MII egg in media without betaine in the presence or absence of choline. Groups of 30 GV oocytes were isolated from each genotype and cultured for 18 – 24 in mKSOM in the presence or absence of choline (0.5mM) exactly as described above for oocytes from CF1 animals. The amount of betaine was then determined in the resulting MII eggs by LC-MS/MS. Betaine levels again were higher in oocytes from *Chdh*$^{+/+}$ females matured to MII eggs in the presence of choline. Levels were indistinguishable from wild-type in heterozygote oocytes. In contrast, no detectable betaine was present in MII eggs from *Chdh*$^{-/-}$oocytes matured to MII eggs either in the presence or absence of choline (Figure 20). This suggests that the ability to utilize choline to create betaine is directly tied to the presence of both copies of the CHDH gene *in vitro.*

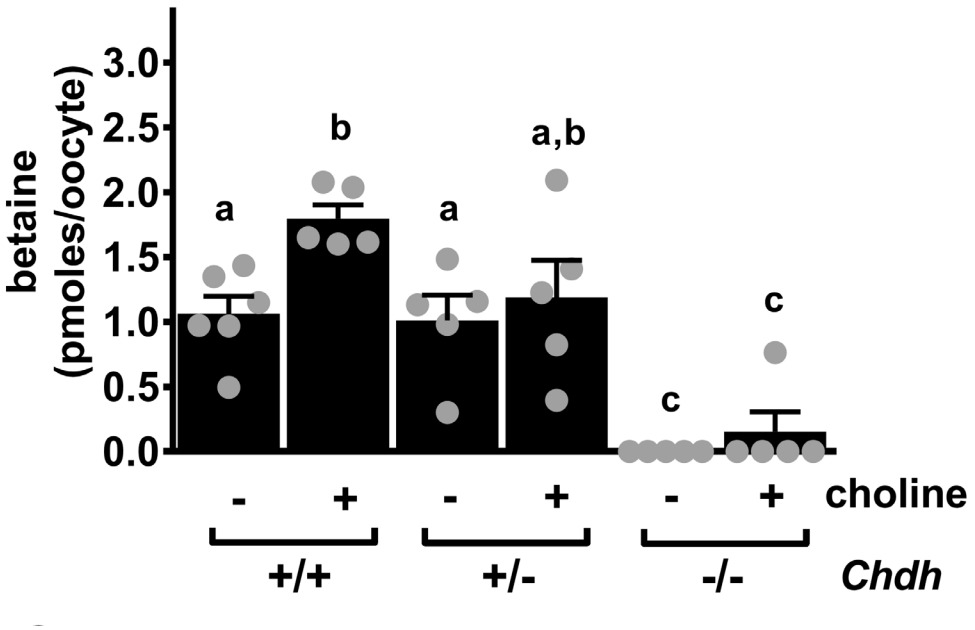

Figure 20: CHDH is required to convert choline to betaine

GV oocytes from *Chdh*$^{+/+}$, *Chdh*$^{+/-}$, and *Chdh*$^{-/-}$ females were *in vitro* matured to MII eggs in the presence (+) or absence (-) of choline, and then were processed for endogenous betaine as described previously. Indistinguishable levels of betaine were present in wild type (+/+) and heterozygous (+/-) oocytes, while betaine was not detected in null (-/-) oocytes. Only wild type oocytes accumulated betaine during meiotic maturation, while there was no significant increase in betaine in null or heterozygous oocytes. Means that do not share the same letter are significantly different ($P<0.05$ by ANOVA with Tukey's test). This figure was obtained and adapted with permission from McClatchie et al. (2017).

5. Discussion

The uses of betaine in the developing oocyte and early embryo firstly as a novel, embryo-specific mechanism of cell volume regulation during the 1-cell and 2-cell stages (Anas et al., 2007; Hammer and Baltz, 2002; Biggers et al., 1993; Anbari and Schultz, 1993) and secondly as a methyl donor at the blastocyst stage have recently been established (Lee et al., 2012; Zhang et al., 2015). Our research has investigated the mechanism by which the developing MII oocyte produces the high levels of betaine that are known to be present at this stage. We have conducted experiments to test the hypothesis that the enzyme CHDH is both present and active and is required for betaine synthesis and accumulation in oocytes during meiotic maturation. My results indicate that the substantial amount of betaine that is accumulated in the oocyte during meiotic maturation and then used by the preimplantation embryo is in fact synthesized autonomously from choline by the enzyme CHDH within the oocyte.

Lack of betaine transport supports hypothesis of CHDH-mediated betaine synthesis.

The amount of betaine present in the MII eggs is on the order of 10-12 mM, assuming the oocyte has a cytoplasmic volume of 180 (Collins et al., 1999) to 210 pL (Tartia et al., 2009) and an intracellular concentration of ~2.5 pmole/oocyte (McClatchie et al., 2017). This is comparable to that of the 1-cell embryo (Lee et al., 2012). Kidney and liver tissue were used as experimental controls throughout the described protocols, as mammalian liver and kidney were shown to have the highest concentration of betaine in measured tissues by Slow and Lever (2009).

The hypothesis that the enzyme CHDH is responsible for the betaine present in MII eggs is only logical if the cells are unable to utilize the betaine present in the extracellular medium of

the oviductal fluid, which Anas et al. (2007) estimated to have a concentration of 300 – 500 μM. We have shown that the enzyme CHDH functions to accumulate betaine *in addition* to the activity of the SIT1 transporter, SLC7A6/y+LAT2 and any other possible betaine transport mechanism by demonstrating a lack of betaine transport in the timeframe of interest. SIT1 transports betaine with high affinity, but only after fertilization, while SLC7A6/y^{+}LAT2 transport is only effective prior to the uncoupling of the follicular cells from the enclosed oocyte. We can observe the major accumulation of betaine occurs in the period between these, throughout meiotic maturation; however, we were unable to observe any saturable transport of betaine during this period. Only non-saturable betaine transport was recorded into denuded GV oocytes. This is likely the result of passive diffusion through the membrane and is often seen when measuring transport of substrates. Transport measurements only serve to measure the unidirectional flux of labeled substrate into the cell. Transporters that can move a substrate against a gradient are the only part of measured transport that can cause the accumulation above what is found in the extracellular environment. Diffusion or simple transmembrane exchangers can only equalize transmembrane concentrations. Thus, they cannot contribute to the accumulation of 10mM betaine in eggs when the external concentration is on the order of 100 uM (i.e. that of blood plasma). We have previously shown that GV oocytes only have this non-specific, non-saturable component, and in these investigations, we have further confirmed this finding using more finely spaced time points during meiotic maturation. This allowed us to confidently focus on CHDH as a mechanism of betaine accumulation via *de novo* synthesis, rather than via utilization of any available extracellular resources for transport (Anas et al., 2007; Anas et al., 2008).

The absence of any transport of betaine into the oocyte specifically during meiotic

maturation was not entirely unexpected. My initial series of experiments involving radiolabelled betaine transport measurements served to confirm that previous findings which indicated that no betaine was transported in the oocyte during meiotic maturation (Anas et al., 2007). These past findings had tested whether betaine transport was present only at one time point during meiotic maturation. My measurements extended this to narrower timepoints throughout the course of oocyte maturation and ruled out a transient appearance of betaine transport that had previously gone undetected. We were unable to identify a transporter whose action was restricted to the period of meiotic maturation and that would also be inactivated shortly after fertilization. Thus, we safely confirmed that betaine is not directly transported into the oocyte by a previously unknown transport mechanism. This validates that autonomous betaine synthesis was an appropriate area of investigation for this thesis.

Coupled with previous research, in this series of experiments I have shown that the substantial betaine accumulation is a feature of meiotic maturation that is mediated by the transient activation of betaine synthesis via CHDH. Based on my results, results of my co-workers and previous published results we have shown that endogenous betaine accumulation *in vivo* over the course of meiotic maturation coincides with the developmental window of CHDH activity, the presence of CHDH protein and by the oocyte's pattern of choline metabolism thus implicating CHDH-mediated betaine synthesis as the mechanism.

The overlap of the developmental window of CHDH activity and the accumulation of endogenous betaine over the course of oocyte maturation *in vivo* and *in vitro*, implicates CHDH-mediated betaine synthesis as the mechanism. This had previously been supported by the observation of CHDH activity evidenced by the conversion of radiolabelled choline to betaine in

lysates of developing oocytes, as well as by the presence of CHDH mRNA in the growing GV oocyte and maturing egg (Figure 6B) (performed by H. Corbett, published in McClatchie et al., 2017). Additionally, I provided evidence of the presence of CHDH protein itself in both GV oocytes and MII eggs, supporting the presence of CHDH throughout the period of interest. This was ascertained by Western Blot using a CHDH antibody whose specificity was confirmed using oocyte samples from CHDH WT and KO females. This confirmation of protein in conjunction with the activity of CHDH enzyme provided concrete evidence of CHDH's role in betaine accumulation in the oocyte.

CHDH catalyzes the reaction of choline to betaine. Mouse oocytes are known to actively take up choline (Eppig, 1982; Haghighat et al., 1990). The primary uptake in GV oocytes is into the cumulus granulosa cells, followed by gap junction-mediated transfer into the enclosed oocyte. This transport continues to occur until ~6 h post-hCG when the cumulus and oocyte become uncoupled. A more minor route of choline uptake directly into the oocytes persists through the end of maturation (Haghighat et al., 1990).). We propose that the early, robust transport of choline into the COC, and continued uptake of choline by the oocyte after uncoupling provides the choline substrate used by CHDH for betaine synthesis.

The implied ability of CHDH to produce betaine from choline in maturing oocytes was directly confirmed by detecting an increase in betaine levels in GV oocytes matured *in vitro* to MII eggs in the presence of choline. These oocytes were cultured in media free of other cells (that may have stored betaine from outside sources or could have been capable of synthesizing betaine from choline) and devoid of betaine. Therefore, the increased betaine levels must be the result of a mechanism within the oocyte itself – assumed to be CHDH conversion of choline to

betaine aldehyde, in conjunction with betaine aldehyde dehydrogenase completing the conversion of betaine aldehyde to choline. The lack of increased betaine observed in oocytes cultured in choline-free medium, establishes the production of betaine as an autonomous function of the maturing oocyte specifically in the presence of choline. The inability of *Chdh*$^{-/-}$ oocytes to produce betaine when cultured similarly in choline-containing medium indicates an essential role for CHDH in the accumulation of betaine in the oocytes.

Metabolic coupling provides choline as a substrate for CHDH-mediated betaine synthesis.

Follicular cells are known to enhance the uptake of various substances. Metabolic coupling, whereby substances are accumulated by cumulus cells and can be subsequently transferred to the oocyte via gap junctions, has been well documented (Haghighat et al., 1990; Pelland et al., 2009; Eppig et al., 2005). This transfer is selective, as some substrates are rapidly transferred to the enclosed oocyte, whereas others are not enhanced, or are complete excluded from the oocyte. It has previously been demonstrated that cumulus cells significantly enhance the total amount of betaine accumulated by the enclosed oocyte prior to meiotic maturation directly via metabolic coupling of the betaine transport via y^+LAT2 betaine transport into the COC and then moving betaine through gap junctions into the oocyte (Corbett et al., 2014).

However, as a consequence of ovulatory stimulation, the oocyte not only undergoes the process of meiotic maturation but also becomes independent from its supporting follicular environment. Previous research has indicated that during cumulus expansion, the connections between cumulus granulosa and oocyte are gradually lost, with the cumulus becoming substantially uncoupled by roughly 6 h following stimulation of ovulation (Eppig, 1982; Salustri and Siracusa, 1983; Norris et al., 2008). Since many signals are thought to be transferred from

different follicular compartments to the oocyte via gap junctions, these findings imply a functional isolation of the oocyte from the follicular environment in the initial hours of ovulation. This precludes the ability of y^+LAT2 that is present in cumulus cells but not oocytes to further mediate betaine accumulation in the oocyte, above the relatively low levels already present in GV oocytes at the time of ovulation. Endogenous betaine remains low in oocytes at 6 h post-hCG, after which the cumulus granulosa cells are no longer coupled to the oocyte through gap junctions. However, this coupling supports the hypothesis that CHDH is able to use choline as a substrate. Choline is actively moved into the oocyte primarily via uptake into the cumulus cells, and gap junctional coupled transfer into the oocyte itself (Eppig, 1982; Haghighat and Van Winkle, 1990). This continues until the cumulus cells become uncoupled. The more minor direct transfer into the oocyte (Eppig, 1982) then becomes the sole route of uptake for the remainder of maturation (Van Winkle et al., 1993). Thus, we proposed that though the metabolic coupling of the oocyte and its surrounding follicular cells are unable to account directly for the betaine present in the mature egg, it does provide the substrate for the formation of betaine by CHDH in the form of choline. We are suggesting that the robust transport of choline into the COCs prior to uncoupling and the continued uptake of choline by oocytes after the uncoupling serves to supply the maturing oocytes with the choline that is used for betaine synthesis. Thus, the cumulus cells not only enhance the accumulation of betaine early in development via direct betaine transport through metabolic coupling using SLC7A6/y^+LAT2, but also through metabolic coupling allowing choline to move from the extracellular environment into the oocyte to act as a substrate for CHDH.

We attempted to demonstrate that the prolonged presence of cumulus cells, which have high capacity for choline transport would provide increased choline as a substrate for CHDH,

and in turn increase the overall betaine production if complete COCs were incubated in choline rich media, compared to denuded GVs incubated in the same media. This protocol yielded widely inconsistent results. Some COCs incubated in the presence of choline had little or no betaine, while others had the highest levels of betaine recorded (ranging from 0.02 pmole/oocyte to 5 pmole/oocyte). This suggests that the COCs incubated with choline are capable of enhanced betaine synthesis in some instances. However, the inconsistent results were unexplained and we did not pursue this series further.

CHDH activity is initiated through an unknown mechanism during meiotic maturation.

We have demonstrated the ability of CHDH to mediate betaine synthesis throughout meiotic maturation, however the mechanism by which it becomes activated remains unknown. Though not the subject of this investigation, the mechanism of activation is directly tied to our focus in demonstrating the role of CHDH in betaine accumulation as well as the interplay between the different mechanisms of betaine accumulation. The results suggest that meiotic progression is required as CHDH activity has not arisen in GV oocytes maintained in GV arrest. This suggests a possible role for M-phase promoting factor which is required for meiotic progression or of MAPK which is activated during meiotic maturation. MAPK has many functions including maintaining the arrest of the mature egg in MII phase, but also regulates meiotic spindle integrity, oocyte cytoskeletal dynamics and transporter activity. Thus, it may also induce CHDH activity, though this is not supported by any data to date.

Chdh mRNA was present at each oocyte stage tested and was restricted to the oocyte. This indicates that the activity of the enzyme itself is not dependent on transcription immediately prior to maturation. A transcription-mediated activation of CHDH would also be inconsistent

with the well-known transcriptional silence of oocytes during maturation. Transcription does not become active until after fertilization, with most of the transcriptional reactivation occurring at the 2-cell stage (BouniolBaly et al., 1999). Therefore, enzyme activity cannot be directly initiated by mRNA transcription and subsequent translation of the protein during the period in which CHDH activity appears. However, many proteins are translated from previously transcribed and stored mRNA in oocytes (Chen et al., 2011). My western blot data demonstrating no difference in CHDH protein levels between GV oocytes (no activity) and MII eggs (maximal CHDH activity), in addition to preliminary data demonstrating a lack of effect of protein synthesis inhibition on CHDH protein levels, suggest that CHDH protein is not translated during meiotic maturation. However, the effect observed on CHDH activity with general protein synthesis inhibition may be the result of the effect on the synthesis of a regulatory protein, though no current candidates exist for this role. Little is known about the regulation of CHDH likely at least partially because active mammalian CHDH has not been successfully purified or produced (Salvi & Gadda, 2013). Alternatively, inhibition of protein synthesis could be interfering via secondary effect through inhibition of meiotic progression (which as mentioned above, is required for CHDH activity). Inhibition of protein synthesis blocks meiosis between chromosome condensation and the MII stage, for reasons that are still unknown, but that could contribute to inactivity of CHDH (Wassarman et al., 1976). Alternatively, CHDH activity could be regulated by the availability of choline as a substrate. Choline transporters are specific and could therefore regulate the production of betaine by the mitochondrial CHDH enzyme through restricted or enhanced choline availability. Further investigation, will be required to elucidate the regulation of CHDH during meiosis as its kinetic properties and expression patterns have not

been extensively studied. Its modulation by physiological status, diet or other factors is still unknown.

CHDH plays roles in other biological systems that are becoming more evident.

CHDH enzyme was historically known to be present and highly active in mammalian liver and kidney tissues where it is used to generate SAM and maintain osmotic homeostasis respectively. Dr. Stephen Zeisel's lab generated a *Chdh* null mouse line and Johnson et al. (2010) investigate their physiology with a somewhat novel focus on fertility. In our experiments these animals proved very useful in methods validation and allowed comparison of the CHDH and non-CHDH groups. However, the published observations from Johnson et al (2010), as well as our own breeding manipulations have generated questions regarding the long-term impact of the CHDH enzyme on male *and* potentially also, on female fertility. Most notably, it was observed that sperm function, though not sperm viability was severely compromised in $Chdh^{-/-}$ males. These homozygotes were infertile largely due to poor sperm motility. Mitochondria are localized to the sperm mid-piece, while glycolytic enzymes are found in the sperm principle piece. When CHDH enzyme was absent in the inner mitochondrial membrane, the mitochondria in the sperm mid-piece appeared grossly abnormal when examined by electron microscopy. In addition, these cells showed a decrease in mitochondrial dehydrogenase activity and adenosine triphosphate (ATP) content. A constant supply of ATP, both from oxidative phosphorylation in the mitochondria and from glycolysis, is required for sperm motility and for the sperm to successfully fertilize the oocyte (Johnson et al., 2010). As noted, mitochondrial abnormalities were not limited to sperm in $Chdh^{-/-}$ mice and were noted in other tissues including the liver, kidney and heart. Emerging data suggests that fertility in $Chdh^{-/-}$ females may also be impacted

by the lack of CHDH, perhaps also due to mitochondrial dysfunction. We have observed disrupted mitochondrial arrangement during meiotic maturation, decreased polar body emission and a possible earlier decrease in fertility with age in *Chdh*$^{-/-}$ compared to *Chdh*$^{+/+}$ females (Megan Meredith, Baltz lab, unpublished data) though further experiments are required to confirm these findings. It is important to note that in addition to the role CHDH plays in betaine accumulation in the oocyte and fertility, it is possible that more extensive studies of mitochondrial function will reveal more subtle abnormalities not yet detected by current assays with regards to fertility and other organ systems.

CHDH activity is supported by redundant biological pathways.

The biological significance of betaine has been made evident, and lack of CHDH protein has dramatic influence on male fertility. However, *Chdh* null females are fertile (Johnson et al., 2010), which indicates that activation of CHDH is not an absolute requirement for producing a fertilizable egg or for subsequent embryo development. We are suggesting that this is a consequence of parallel mechanisms for both cell volume homeostasis and methyl pool generation and maintenance. Redundant mechanisms suggest that if the deleterious effects of a hypertonic oviductal environment on the oocyte cannot be negated by the presence of betaine, other systems provide the organic osmolytes necessary to allow cell survival. In this case, the most likely candidate is glycine which acts as a major organic osmolyte in eggs and early preimplantation embryos (Steeves et al., 2003; Tartia et al., 2009). The accumulation of glycine occurs in the same period as CHDH activity in the oocyte, allowing it to successfully compensate for the lack of betaine to maintain cell volume control and prevent such consequences as developmental blockages.

The contribution of betaine to the development of the methyl pool can be compensated by the contribution of the folate cycle to the methyl pool in embryos (Zhang et al., 2015). Consistent with this suggestion, severe phenotypes are observed only when betaine-dependent mechanisms are impaired in conjunction with the parallel mechanisms of folate metabolism. (Zhang et al., 2015; Hammer at al., 2002). The folate cycle is a key component of one-carbon metabolism in mammalian cells, shuttling single carbon units onto a variety of substrates (Tibbetts and Appling, 2010). Like betaine the folate cycle works to generate the intracellular methyl pool by supplying carbon units to the methionine cycle that produces SAM. The interplay of betaine and the folate cycle has been tested *in vitro* but the availability of MTHFR (Methylene tetrahydrofolate reductase) knock-out mice will allow the cooperativity of the two methyl pool contributors to be tested further. MTHFR is the enzyme controlling the rate-limiting step of the methyl cycle. It catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which is a co-substrate for the methylation of homocysteine to methionine. To reiterate, this is the same reaction of homocysteine to methionine that can be undertaken by the folate-independent enzyme BHMT using betaine. The interplay of these three genes has yet to be fully explored.

Relative contribution of three betaine accumulation pathways in oocyte/embryo development

All the available data suggest the role of betaine in the developmental process of the oocyte and early embryo under *in vivo* and *in vitro* conditions. The obviously complex developmental regulation of betaine indicates the importance of the molecule both as an organic osmolyte and as a methyl source. Though the finer details are unknown, the obviously carefully regulated accumulation of betaine in the oocyte across various stages of development reflects the importance of the system to appropriate physiological functioning. We have found that betaine

was present in early GV oocytes even before CHDH activity was detectable, albeit at a lower level than in MII eggs. We hypothesized that this early betaine is transported from elsewhere in the body and delivered to the follicle by circulation. Since GV oocytes from *Chdh*$^{-/-}$ females lacked any detectable betaine, the betaine present in wildtype GV oocytes must have been synthesized from choline by the CHDH outside of the oocyte and delivered to the follicle, rather than a result of dietary betaine intake. We suggest that the circulating betaine is derived from CHDH-mediated synthesis in other tissues – mainly the liver which is known to be the main source of circulating betaine other than that which is taken up in the diet. As demonstrated, the oocyte itself possesses no channels by which to transport betaine prior to fertilization. Thus, we propose that the betaine present in oocytes prior to meiotic maturation is primarily synthesized elsewhere by CHDH before being taken up by cumulus cells via the SLC7A6/y^+LAT2 transporter and transferred to the cumulus-enclosed oocyte via gap junctions (Corbett et al., 2014). This transfer is active until the oocyte and the cumulus granulosa become uncoupled during meiotic maturation. Subsequently, and as outlined above, the major accumulation of intracellular betaine in MII eggs is a result of its synthesis from choline and is accumulated via transient activation of CHDH during meiotic maturation and inactivation of CHDH after fertilization. Third, the post-fertilization and pre-implantation embryo volume regulation is likely additionally regulated by the SIT1 transporter in 1-cell and 2-cell embryos (Figure 21) (Anas et al., 2008). Betaine maintained at high intracellular levels until it is metabolized and its methyl groups by BHMT in blastocysts. It is at this point that betaine levels return to low. However, further research is required to determine the relative contributions of each mechanism to the overall betaine pool. Additionally, it remains unknown what circumstances – whether tonicity,

osmotic pressure, methyl requirements or other cellular needs demands lead to the activation of each pathway.

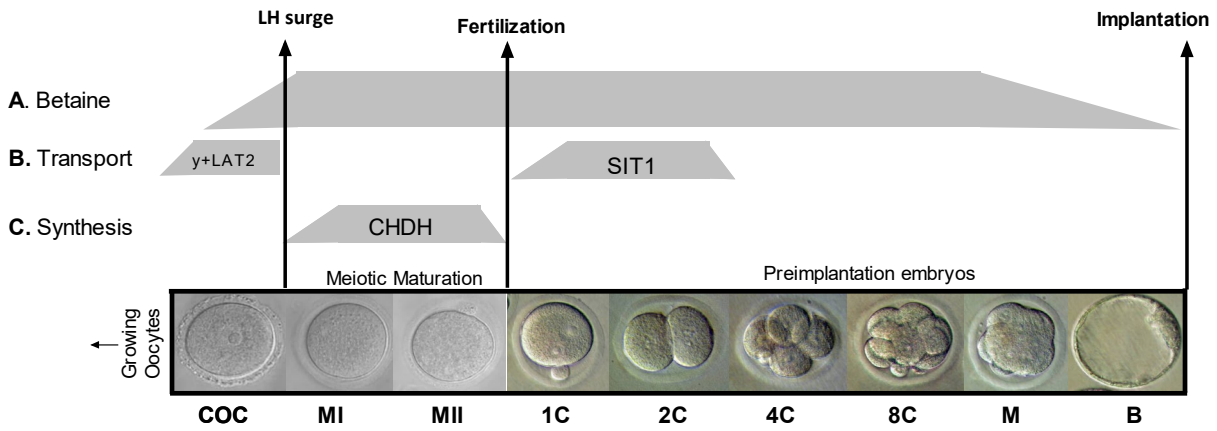

Figure 21: Schematic representation of methods of betaine accumulation

A) We have previously shown that endogenous betaine levels are high from 1-cell to morula and then decrease when BHMT consumes betaine. Our most recent published data indicate that endogenous betaine is also detectable in GV oocytes, and substantial betaine accumulation takes place during meiotic maturation.

B) We have found two periods during which betaine is transported. A modest amount of betaine is taken up by the cumulus cells via the y^+LAT2 transporter and transferred into the enclosed oocyte, likely accounting for the low levels of betaine detectable in GV oocytes. The second is uptake by the SIT1 transporter in 1-cell and 2-cell embryos.

C) We have shown that CHDH is present and active during meiotic maturation. I have shown above that CHDH is necessary to convert choline from the extracellular environment to betaine in the developing oocyte.

6. Conclusions

The results presented in this thesis indicate that *de novo* betaine synthesis occurs in the oocyte as a result of activity of the enzyme CHDH. My work has determined that CHDH is present and active in oocytes during meiotic maturation, that betaine is synthesized from choline by oocytes and that CHDH is required for betaine accumulation in oocytes.

In the immature GV oocyte within the ovarian follicle betaine is obtained though the SLC7A6/y^+LAT2 transporter located on the cumulus granulosa cells and is then transferred into the enclosed oocyte through gap junctions. As the oocyte matures, this physical interaction is gradually lost, and accumulation of betaine is no longer the result of transport, but rather autonomous synthesis via CHDH. The embryo is able to counteract increased osmolarity using betaine transport directly into the oocyte via the SIT1 identified transporter, activated only after fertilization. Thus, subsequent to ovulatory stimulation and the onset of meiotic maturation the oocyte switches from metabolically coupled betaine transport to an autonomous, *de novo* synthesis mechanism, before reverting to a direct transport route into the fertilized egg, all of which are carefully modulated to allow optimal oocyte and embryo development.

7. Significance

My research has revealed new information for the field of reproductive physiology and cell volume regulation, uncovering physiologically important aspects of oocyte behavior over the period of meiotic maturation in which the female gamete attains independence from its protective follicular environment. As the oocyte progresses to a mature, fertilizable egg, the acquisition of the organic osmolyte and methyl donor betaine occurs in a way not previously described for the mammalian oocyte. This transition seems to occur only between the immature oocyte stage and fertilization.

Besides answering basic physiological questions and filling in gaps in basic scientific knowledge, my findings could also be used to improve conditions for culturing and maturing oocytes in a clinical or experimental setting. The benefit to betaine inclusion in culture media has already been suggested. The presence of various methods of betaine regulation in the mouse embryo suggests similar interplay in human oocytes as well. Therefore, data collected in this investigation could find application in developing culture media and outlining optimal culture conditions for human ART.

The two roles of betaine in the oocyte and developing embryo indicate roles in pre-implantation volume regulation and post-fertilization development and even health outcomes in the offspring. However, it appears that betaine is not an absolute requirement for either volume regulation or DNA methylation, thus the implications of insufficient betaine in clinical applications are more subtle. Though more investigation is required to determine the potential clinical implications of betaine supplementation, overall the findings of my research have furthered the understanding of the basic biology of the molecule, which could improve the ability to successfully

culture, mature and preserve female gametes. A more complete understanding of all these aspects will undoubtedly be applicable for ART centers with the hopes of more reliably treating human infertility and preserving human fertility.

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