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Cell Volume Regulation Is Initiated in Oocytes at Ovulation

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CELL VOLUME REGULATION IS INITIATED IN OOCYTES AT OVULATION

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ABSTRACT

It was previously shown that the early preimplantation mouse embryo regulates cell volume by a novel mechanism involving the accumulation of glycine as an organic osmolyte via the glycine transporter GLYT1. GLYT1-mediated cell volume control is present in fertilized eggs and persists only until the 4- to 8-cell stage of embryo development. However, it was unknown when during development this mechanism first becomes active. Therefore, I have investigated the mechanisms of cell volume regulation in oocytes prior to fertilization.

To determine when glycine transport is initiated in oocytes and the mechanisms controlling its activation, I used the mouse as a model and various techniques including embryo and oocyte culture, ^{3}H-glycine transport measurements, amino acid microanalysis, RT-PCR, Western blot and immunocytochemistry.

GLYT1 was quiescent in GV oocytes freshly removed from follicles. However, transport developed within several hours of ovulatory stimulation. Endogenous free cytosolic glycine, the fraction that contributes intracellular osmotic support, was measured at high levels only after ovulation, in ovulated MII oocytes and 1- and 2-cell embryos, where GLYT1 was active. Once active, GLYT1 activity was necessary for protecting against volume decreases in oocytes. Thus, GLYT1-mediated cell volume-regulatory glycine transport was initiated in oocytes at ovulation. This activation was independent of meiotic maturation and did not involve *de novo* synthesis. The inhibitory action of unidentified follicular factor(s) over a constitutively active GLYT1 is most likely responsible for the quiescence of GLYT1 before ovulation.

Prior to GLYT1 activation, the oocyte was unable to independently control its

volume. Instead, its size was determined by a firm adherence between the oocyte's membrane and its extracellular matrix, the zona pellucida (ZP). Electron microscopy studies indicated that oocyte-ZP adherence occurs at the tip of the oocyte microvilli. When freed from the follicle or following ovulation, the oocyte released this adhesion, decreased in volume, and a perivitelline space appeared. This release from adherence and change in preferred volume coincided with GLYT1 activation.

Thus, the onset of cell volume regulation occurs at ovulation when the mammalian oocyte switches from a fixed cell volume determined by the ZP to active GLYT1 and glycine-dependent volume control.

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LIST OF ABBREVIATIONS

AEBSF = 4-(2-Aminoethyl)benzenesulfonylfluoride hydrochloride

Alpha1-PDX = alpha1 antitripsin Portland

ANOVA = analysis of variance

ART = Assisted Reproductive Technologies

BAPTA-AM = N, N'[1,2-ethanediyl bis(oxy-2,1-phenylene)]bis[N-[2-](acetyloxy)-methoxy]2-oxoethyl]]-,bis[(acetyloxy)methyl]ester

BGT1 = betaine transporter

BMP15 = bone morphogenetic protein 15

BMP6 = bone morphogenetic protein 6

bp = base pairs

BSA = bovine serum albumin

cAMP = cyclic adenosine monophosphate

CDC25 = cell division cycle 25 phosphatase

CDK1 = Cyclin dependent kinase 1

cDNA = complementary deoxyribonucleic acid

CEEF = cumulus enabling expansion factor

CFCS = consensus furin cleavage site

CNS = central nervous system

CSF = cytostatic factor

dbcAMP = dibutyryl cyclic adenosine monophosphate

DMSO = dimethyl sulfoxide

E11 = embryonic day 11

EHP = external hydrophobic patch

EM = electron microscopy

Fig. = figure

FSH = follicle stimulating hormone

GABA = γ-aminobutiric acid

GDF9 = growth differentiation factor 9

GLYT1 = glycine transporter 1

GLYT2 = glycine transporter 2

GPC = glycerophosphorylcholine

GV = Germinal Vesicle

GVBD = Germinal Vesicle Breakdown

Has2 = Hyaluronan synthase 2

hCG = human chorionic gonadotropin

hr = hour

i. p. = intraperitoneally

IBMX = Isobutylmethylxanthine

ICM = inner cell mass

IHP = internal hydrophobic patch

IU = international units

IVF = *In Vitro* Fertilization

IVM = *in vitro* maturation

KSOM = K^+ supplemented optimized medium

LH = luteinizing hormone

MEM = minimum essential medium Eagle

MI = meiosis I

MII = meiosis II

MPF = Maturation Promoting Factor

NMDA receptor = N-methyl-D-aspartate receptor

NTT = Neurotransmitter Transporter Family

PB = polar body

PBS = phosphate buffered saline

PDE = phosphodiesterase

PGCs = primordial germ cells

PKA = protein kinase A

PKC = protein kinase C

PLC = phospholipase C

PMA = phorbol 12-myristate 13-acetate

PMSF = Phenylmethylsulphonyl fluoride

PMSG = pregnant mare's serum gonadotropin

PVS = perivitelline space

RNA = ribonucleic acid

RT-PCR = reverse-transcribed polymerase chain reaction

RVKR = Decanoyl -Arg-Val-Lys-Arg-chloromethylketone

SDS = Sodium Dodecyl Sulfate

SEM = standard error of the mean

SMIT = Na^+/myo-inositol transporter

SNAP23 = synaptosomal-associated protein 23

SNARE = soluble N-ethylmaleimid-sensitive factor attachment protein receptors

Stx = Syntaxin

TAUT = taurine/β-amino acid transporter

TEM = Transmission electron microscopy

TMD = transmembrane domain

TonE = tonicity responsive enhancer

TonEBP = tonicity enhancer binding protein

VAMP = vesicle-associated membrane protein

VSOAC = volume sensitive organic osmolyte and anion channel

ZP = zona pellucida

ZP1, ZP2 and ZP3 = zona pellucida glycoproteins 1, 2, and 3

ZPD = ZP domain

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INTRODUCTION

1. Folliculogenesis, oogenesis and preimplantation embryo development

1. 1. Mammalian follicular and oocyte development

The mature female germ cells, the oocytes, are descendants of epiblast that gives rise to primordial germ cells (PGCs) (Falconer and Avery, 1978; Hogan *et al.*, 1994). In mouse fetuses, around day 11 post coitus (E11), PGCs start to migrate and colonize the genital ridges, located in the primitive gonads (Byskov and Hoyer, 1988; Hogan *et al.*, 1994). During their migration, PGCs in female fetuses undergo multiple rounds of mitotic division (Eppig *et al.*, 2004) resulting in the production of millions of oogonia, a process that in mice and humans terminates before birth (Dissen *et al.*, 2004; Gougeon, 2004). Late in the fetal period, female germ cells enter the first meiotic division where they arrest in the prophase and are now known as non-growing oocytes. At this stage they become surrounded by flat epithelial cells (precursors of follicular cells) originating from the surface epithelium covering the ovary (Byskov and Hoyer, 1988; Hirshfield, 1991).

A non-growing oocyte together with its pre-granulosa cells enclosed by a basement membrane is recognized as a primordial follicle (Sadler, 1990; Hirshfield, 1991). Shortly after birth, mouse ovaries contain only primordial follicles (Eppig *et al.*, 2002; Dissen *et al.*, 2004) with the oocytes arrested in the first meiotic prophase. The primordial follicle can stay in this arrested state for an extended period of time, up to 50 years in women (Gougeon, 2004). Once sexual maturity is reached, continuing throughout the reproductive life of the female, cohorts of primordial follicles are constantly recruited into the growth phase by unknown signals. When recruited, the

primordial follicle develops into a primary follicle, the oocyte within, known as a growing oocyte, begins an extensive growth phase, and the surrounding follicular cells, now called granulosa cells, become cuboidal and proliferative (Eppig, 2001) (Fig .1).

The preantral or secondary follicle stage is reached when the oocyte is in mid-growth stage and surrounded by multiple layers of granulosa cells. In the fully matured antral or Graafian follicle, granulosa cells have proliferated to form a multilayered epithelium around the oocyte and develop a fluid-filled cavity, called the follicular antrum, which divides the population of granulosa cells into two main groups: cumulus cells directly surrounding the oocyte and mural granulosa cells lining the follicular wall (Salustri *et al.*, 2004). Two layers of theca cells that have differentiated from the surrounding ovarian stroma (Erickson, 1983; Skinner, 2005), coat the external follicular wall (Fig. 1).

The oocytes complete their growth phase near the transition from preantral to antral follicle. Around this time they also acquire meiotic competence, defined as the ability of the oocyte to progress beyond prophase of first meiosis (Wassarman *et al.*, 1979). In the antral follicle, the oocyte is fully grown, meiotically competent and will resume meiosis spontaneously if removed from the follicle and cultured in supportive medium (Eppig, 2001). The mature antral follicle is ready for ovulation and awaits the stimulus of the preovulatory surge of luteinizing hormone (LH) from the pituitary. Throughout folliculogenesis, the somatic cells surrounding the oocyte have numerous projections forming specialized junctions with the oocyte. These projections pass through the zona pellucida, the extracellular matrix coating the oocyte, and form gap junctions with the oocyte. Similar junctions form between follicular

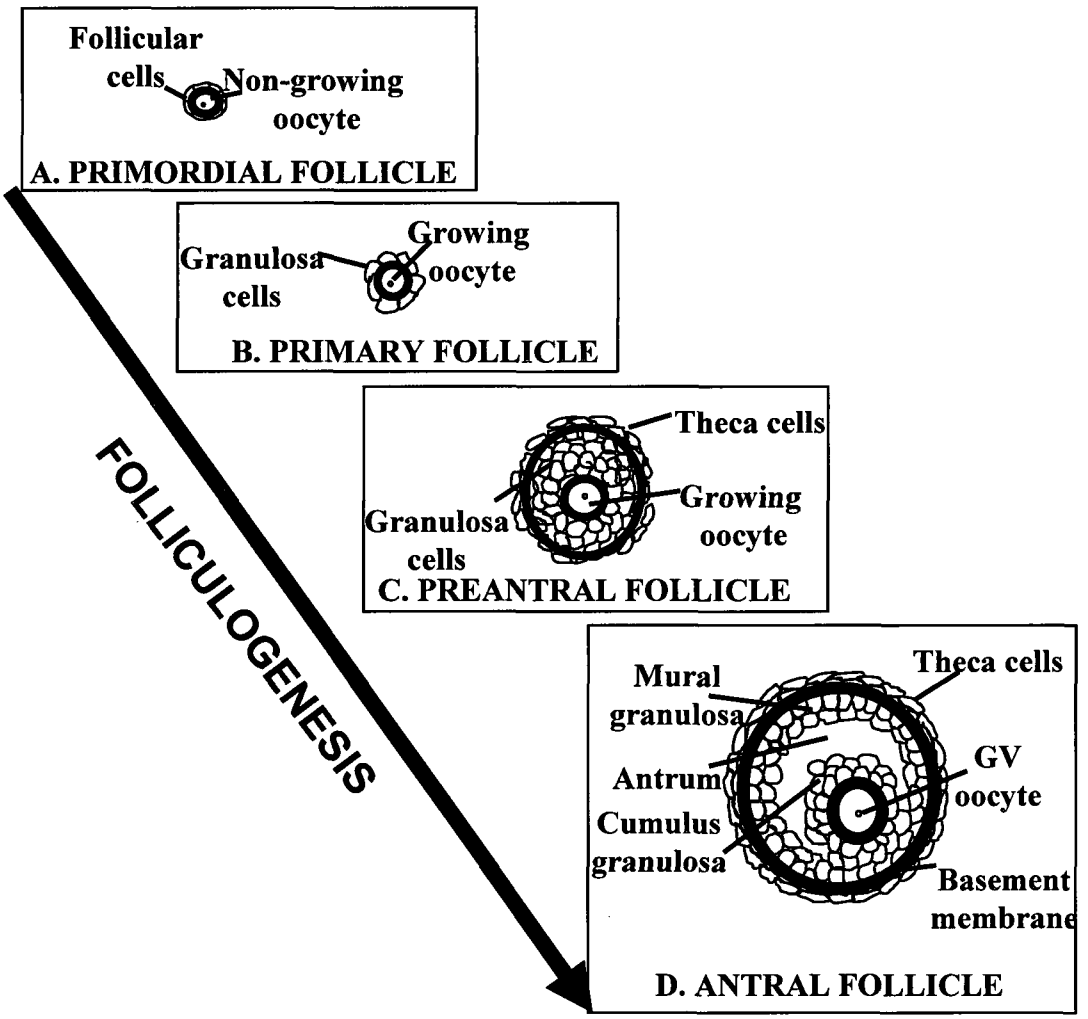

Follicular cells
Non-growing oocyte
A. PRIMORDIAL FOLLICLE
Granulosa cells
Growing oocyte
B. PRIMARY FOLLICLE
Theca cells
Granulosa cells
Growing oocyte
C. PREANTRAL FOLLICLE
Mural granulosa
Theca cells
Antrum
GV oocyte
Cumulus granulosa
Basement membrane
D. ANTRAL FOLLICLE
FOLLICULOGENESIS

Figure 1. Folliculogenesis. Follicle and oocyte growth is shown schematically, proceeding from the primordial (A) to the antral follicle (D). Primordial follicles containing a non-growing oocyte surrounded by flattened follicular cells are recruited every reproductive cycle into an extensive growth phase, becoming primary follicles (B). Primary follicles (B) containing growing oocytes increase in size as granulosa cells proliferate and become multilaminar, developing into preantral follicles (C). As the oocyte nears full size, an antrum (cavity) forms in the follicle. The antrum divides the granulosa population into cumulus granulosa associated with the oocyte and mural granulosa cells lining the follicular wall. The antral follicle (D) has an acellular basement membrane, outside of which lie theca cells. The antral follicle contains a fully-grown oocyte still arrested in first meiotic prophase, evidenced by the prominent prophase nucleus, or germinal vesicle (GV). The oocyte and surrounding cumulus granulosa cells form the cumulus-oocyte complex. Mural granulosa cells coat the interior follicular wall.

cells as well (Anderson and Albertini, 1976). The oocyte and granulosa cells remain connected through gap junctions until the time of ovulation (Gilula *et al.*, 1978). The pituitary FSH and LH represent the main endocrine factors controlling folliculogenesis and ovulation, respectively. It has been proposed that early folliculogenesis is gonadotropin-independent since follicles are able to develop to preantral stages in the absence of FSH (Dierich *et al.*, 1998) whereas the proliferation and differentiation of granulosa cells at later stages requires FSH (Gougeon, 2004).

1. 2. Cell communication in the ovarian follicle

Throughout follicular development, different cell populations contained by the follicle synergistically undergo changes in response to endocrine or local stimuli allowing the ovarian follicle to act as a functional unit. The main function of the follicular unit is to produce a mature, fertilizable female gamete and to mediate its ovulation. Two major means of communication allow rapid interaction between the oocyte and its surrounding somatic cells: paracrine factors and gap junctions. A series of studies, trying to elucidate the nature and importance of oocyte-somatic cell communication, indicated that this interaction is controlled mainly by the oocyte (Matzuk *et al.*, 2002). For instance, oocyte-derived secreted factors like GDF-9, BMP-15 or BMP-6 are required for proper granulosa cell proliferation and differentiation throughout folliculogenesis (Dong *et al.*, 1996; Galloways *et al.*, 2000; Otsuka *et al.*, 2001), providing evidence of paracrine control by the oocyte of granulosa development. In turn, the somatic cells support the oocyte growth and development (Brower and Schultz, 1982; Matzuk *et al.*, 2002) and

modulate endocrine signals needed for oocyte acquisition of meiotic competence, meiotic maturation and ovulation (Chesnel *et al.*, 1994; Matzuk *et al.*, 2002).

The steroids estrogen and progesterone are also important ovarian regulators supporting a synergistic behavior between different compartments throughout folliculogenesis. Based on the two cell/two gonadotropin model, ovarian estrogen production in mammals begins with androgen synthesis within theca cells under LH control, followed by transfer into granulosa cells and conversion to estrogen by FSH-inducible aromatase (Adashi, 1994). Estrogens have been shown to promote theca and granulosa cell proliferation (Adashi, 1994), and differentiation by mediating intercellular gap junction formation, estrogen receptor (Richards, 1980), LH and FSH receptor expression (Louvet and Vaitukaitis, 1976) as well as formation and enhancement of aromatase activity (Adashi and Hsueh, 1982). Although both theca and granulosa cells express the enzymes necessary for progesterone production, low amounts of progesterone are synthesized during the preovulatory period (Jamnongjit and Hammes, 2006). Subsequent to the ovulatory LH surge, granulosa cells start to secrete large amounts of progesterone (Wood and Strauss, 2002). Progesterone has been shown to act as an autocrine factor during ovulation, inducing expression of proteolytic enzymes responsible for follicle rupture (Richards *et al.*, 2002). Interestingly, it has been proposed that oocyte-derived factors control the steroidogenetic activity of cumulus cells throughout folliculogenesis (Vanderhyden and Macdonald, 1998).

Gap junctional communication is of critical importance throughout folliculogenesis and ovulation. Mice lacking connexin 37 (the major protein forming oocyte-granulosa gap junctions) or connexin 43 (forming granulosa-granulosa gap

junctions) are infertile, revealing an impaired folliculogenesis, aberrant follicle architecture and morphologically abnormal, meiotically incompetent oocytes (Simon *et al.*, 1997; Ackert *et al.*, 2001). These gap junctions are an important route for transfer of nutrients and metabolic precursors (like amino acids or pyruvate) from granulosa cells to support the growing and maturing oocyte (Biggers *et al.*, 1967; Donahue and Stern, 1968; Heller *et al.*, 1981; Senbon *et al.*, 2003) until it develops the capacity to sustain these activities on its own. The gap junction-mediated communication between oocyte and cumulus cells has been reported to be lost by 2 hours after stimulation of ovulation (Gilula *et al.*, 1978).

1. 3. Ovulation

Ovulation is a complex physiological phenomenon occurring every reproductive cycle. In rodents, this process ends several hours after the stimulatory LH surge with the antral follicle rupture and the expulsion into the oviduct of a mucified cumulus mass containing a few matured oocytes. Following stimulation of ovulation, the antral follicles undergo important changes. The oocyte, which had been arrested in prophase of first meiosis, proceeds through nuclear maturation, resuming first meiosis, progressing through first meiotic metaphase and rearresting in second meiosis metaphase as a mature MII oocyte (Wassarman *et al.*, 1979; Eppig, 1996; Eppig *et al.*, 2004), a process termed meiotic maturation (Fig. 2). At the cumulus granulosa cell level, the LH surge triggers a cascade of events that will lead to hyaluronic acid secretion and hydration, a process known as cumulus expansion or mucification (Eppig, 2001).

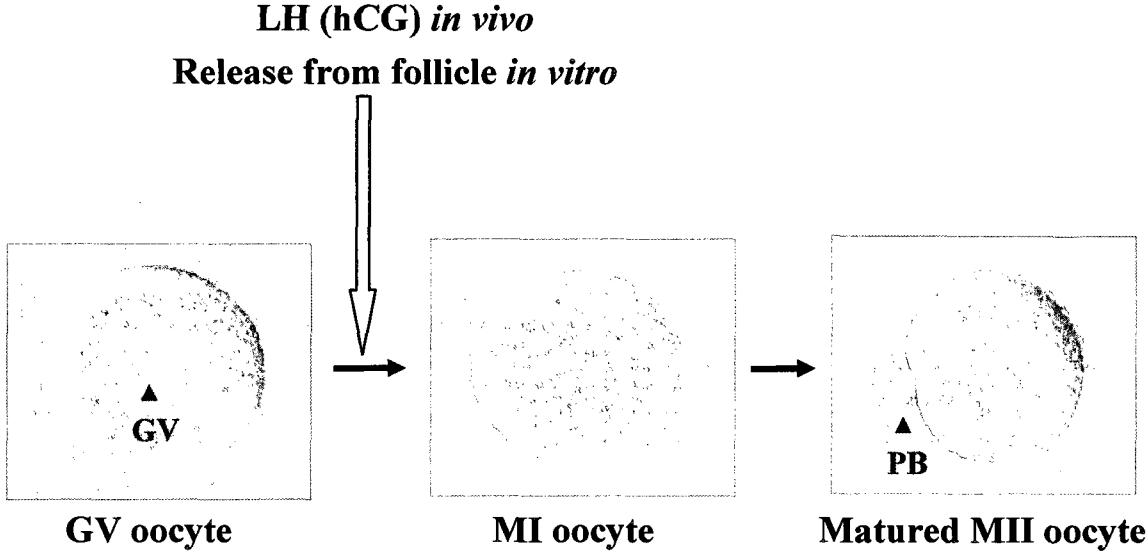


Figure 2. Oocyte meiotic maturation. Photomicrographs of mouse oocytes at several stages of meiotic maturation. The Germinal Vesicle (GV) stage oocyte is named after the prominent nucleus with single nucleolus (GV) that is clearly visible at their centre. The oocyte initiates meiotic maturation in response to an LH surge *in vivo*, or upon release from the follicle *in vitro.* Meiotic maturation is marked by germinal vesicle breakdown, followed by progression through meiosis I (MI oocyte). First meiosis is completed when the oocyte emits the first polar body and forms the MII spindle, producing a mature MII oocyte. GV = germinal vesicle, PB = polar body.

During this period, oocyte-granulosa gap junctional communication ceases, initiating oocyte independence from the follicular environment (Gilula *et al.*, 1978; Norris *et al.*, 2008). Cumulus expansion requires activation of cumulus cell dependent signaling pathways by the oocyte secreted factor(s) known as cumulus enabling expansion factor (CEEF) (Su *et al.*, 2002; Ochsner *et al.*, 2003). This is followed by an elevation in specific cumulus transcripts like *Hyaluronan synthase 2 (Has2)* (Richards, 2005), and ultimately production of hyaluronic acid. In recent years, the oocyte-secreted GDF9 was identified as the most likely candidate for CEEF (Salustri *et al.*, 2004).

Another area still under investigation in the field regards the cumulus cells' and oocyte's ability to undergo major changes in response to LH, despite the absence of LH receptor expression in oocytes (Amsterdam *et al.*, 1975; Lawrence *et al.*, 1980; Peng *et al.*, 1991). It was proposed that the ovulatory stimulus is transduced from theca and mural granulosa, cells rich in functional LH receptors, to cumulus granulosa and the oocyte in a paracrine or/and gap-junction mediated manner (Salustri *et al.*, 2004; Norris *et al.*, 2008). The EGF-like peptides epiregulin, amphiregulin and betacellulin are likely to play important roles in this transduction (Park *et al.*, 2004).

Both meiotic maturation and cumulus expansion facilitate the ovulation of the matured MII oocyte surrounded by mucified cumulus cells matrix. Stimulation of mural granulosa and theca cell LH receptors also triggers an inflammatory and pro-inflammatory gene expression cascade. This process translates into a local inflammatory reaction associated with proteolytic degradation of apical follicular tissue and follicle rupture accompanied by small hemorrhage on the ovarian surface (Espey *et al.*, 2004), enabling the expulsion of the mucified cumulus-oocyte complex. The remaining

granulosa and theca cells form the corpus luteum, which secretes progesterone and is important in the maintenance of early pregnancy.

1. 4. Oocyte maturation

Oocyte maturation is the main ovarian developmental process allowing the female gamete to mature into a mature MII oocyte that is competent to undergo fertilization and embryogenesis. Two major programs seem to play critical roles during oocyte maturation: (1) nuclear maturation, characterized by completion of first meiosis and maintenance of second meiosis metaphase arrest and (2) cytoplasmic maturation, including all cytoplasmic changes taking place during oocyte growth and meiotic maturation, critical for fertilization and early embryo development (Eppig *et al.*, 2004).

1. 4. 1. Oocyte nuclear (meiotic) maturation

Oocyte nuclear maturation encompasses two consecutive rounds of meiosis, a gamete-specific type of cell division, resulting in a reduction to a haploid number of chromosomes (Cooper, 1999). This process starts for the female gamete late in the fetal period with a single round of DNA replication followed by entrance into the prophase of first meiosis. Before birth, the oocyte advances through the initial prophase stages and arrests at the diplotene stage (McLaren and Southee, 1997; Cooper, 1999). Prophase arrest is maintained throughout oocyte growth. Fully-grown prophase I-arrested oocytes are termed Germinal Vesicle (GV) stage oocytes, after the prominent nucleus with a single nucleolus (GV) that is clearly visible at their centre. Upon stimulation of ovulation, the oocyte resumes meiosis I (MI), beginning with the loss of the GV nuclear membrane

in a process termed Germinal Vesicle Breakdown (GVBD), followed by chromosome condensation and assembly of the metaphase I spindle. Meiosis I ends with an asymmetric division resulting in the loss of half the chromosomes in a small polar body (first polar body). Following cytokinesis, the oocyte immediately enters meiosis II (MII) without any intervening DNA synthesis, and progresses directly into metaphase II. The chromosomes align on the spindle, and the oocyte re-arrests in metaphase II. Meiosis II terminates after fertilization with extrusion of the second polar body (Cooper, 1999).

The process of meiotic maturation is controlled by oscillations in the activity of CDK1, a protein kinase that forms an active complex with Cyclin B, known as maturation-promoting factor or M-phase promoting factor (MPF) (Fig. 3). At the beginning of meiotic maturation, an increase in Cyclin B synthesis (Polanski *et al.*, 1998; Ledan *et al.*, 2001) and dephosphorylation of CDK1 by the CDC25 phosphatase ensures CDK1 activation and progression through MI metaphase (Eppig *et al.*, 2004). Entry into metaphase is mainly influenced by Cyclin B synthesis, while entry into anaphase is driven by ubiquitin-mediated Cyclin B degradation by anaphase-promoting complex B (Cooper, 1999).

Once the first polar body is extruded, re-synthesis of Cyclin B permits entrance into meiosis II, where the oocyte re-arrests. In a series of experiments trying to elucidate what controls this arrest, Masui and Market proved that MPF inactivation (now known to be due mainly to cyclin B proteolysis) was prevented by a factor they named cytostatic factor (CSF) (Masui and Market, 1971). Later work uncovered that CSF is a signaling complex of protein kinases including Mos, an oocyte-specific activator of MEK, which targets MAPK (Cooper, 1999; Eppig *et al.*, 2004). MAPK becomes active only during the

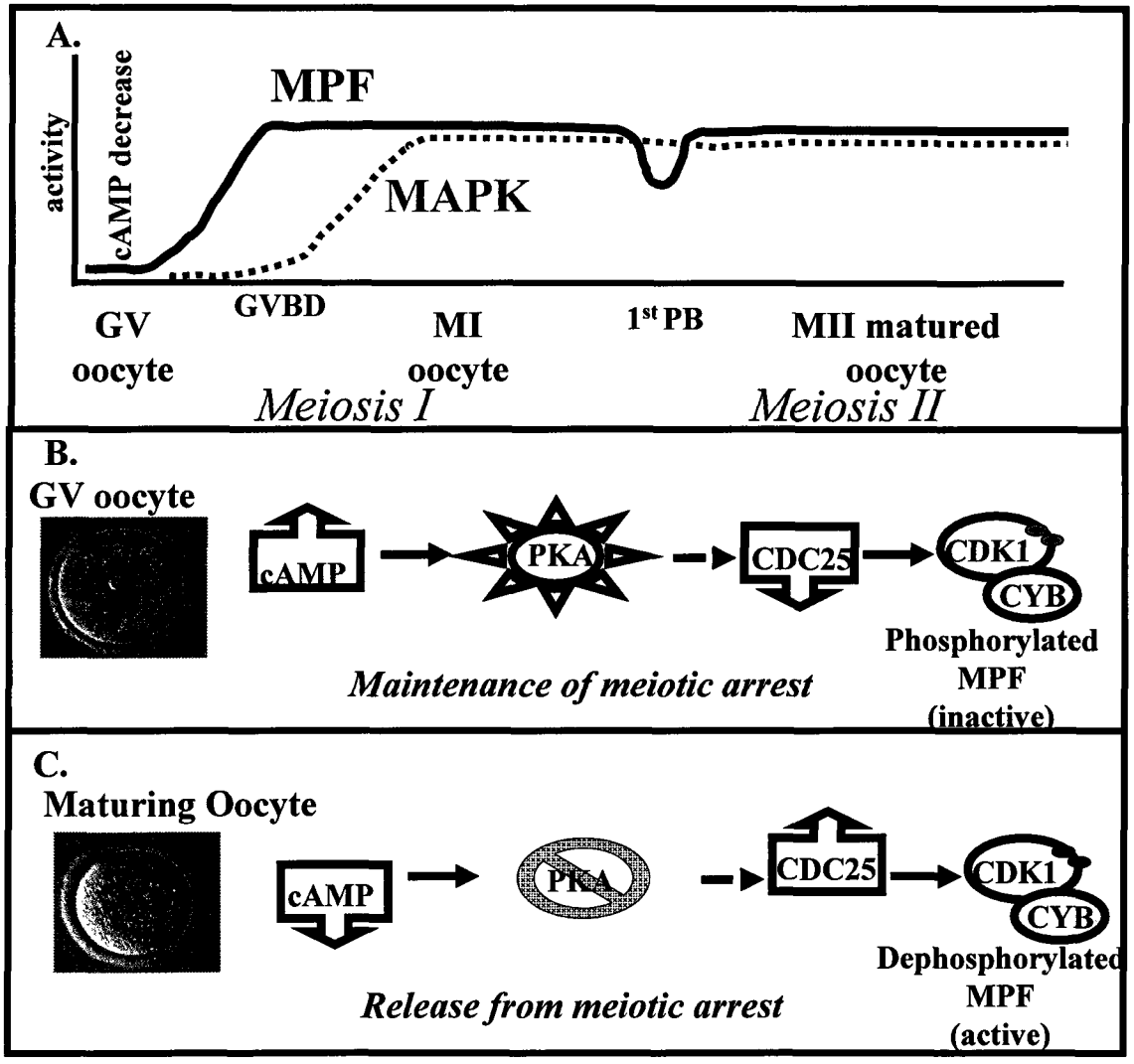

A.
activity
cAMP decrease
MPF
MAPK
GV oocyte
GVBD
MI oocyte
1st PB
MII matured oocyte
Meiosis I
Meiosis II
B.
GV oocyte
cAMP
PKA
CDC25
CDK1
CYB
Phosphorylated MPF (inactive)
Maintenance of meiotic arrest
C.
Maturing Oocyte
cAMP
PKA
CDC25
CDK1
CYB
Dephosphorylated MPF (active)
Release from meiotic arrest

Figure 3. Key molecules controlling the meiotic arrest or the progression through meiotic maturation. ***(A) Schematic of time-course of MPF and MAPK activity during oocyte meiotic maturation.*** In the fully grown prophase I-arrested GV oocyte, both MPF and MAPK are inactive. Following the stimulation for ovulation, the intracellular cAMP decrease results in MPF activation. The oocyte undergoes germinal vesicle breakdown (GVBD) and enters metaphase I, becoming a MI oocyte. A transient decrease in MPF takes place at the end of meiosis I, coincident with emission of first polar body (PB). MPF is reactivated during meiosis II and the oocyte is rearrested in metaphase II, becoming a matured MII oocyte. MAPK, part of CSF, is activated slowly following GVBD, becoming fully activated in the MI oocyte. Its activity remains high throughout meiosis I and II. ***(B) Schematics of events allowing maintenance of meiotic arrest.*** A high steady-state level of cAMP within the GV oocyte modulates PKA activity. Through steps unidentified to date, PKA activity mediates inactivation of CDC25 phosphatase, preventing dephosphorylation-dependent activation of CDK1, a component of MPF complex, resulting in maintenance of meiotic arrest. ***(C) Schematics of major steps allowing oocyte release from meiotic arrest.*** At the beginning of meiotic maturation, the decrease in oocyte intracellular cAMP level is followed by PKA inactivation. CDC25 phosphatase activity results in CDK1 dephosphorylation and ultimately activation of CDK1, allowing progression to metaphase I.

progression through MI, and remains high throughout MII metaphase arrest. Although in recent years a few of the MAPK targets have been identified (Sun and Fan, 2004; Nishiyama *et al.*, 2007), the exact mechanism allowing CSF to maintain the metaphase II arrest remains under investigation.

Following fertilization, the sperm-specific phospholipase C zeta triggers Ca^{2+} oscillations inside the egg as a result of inositol trisphosphate production (Swann *et al.*, 2006). Ca^{2+}-dependent activation of anaphase-promoting complex leads to MPF inactivation, and in the end asymmetric cytokinesis with extrusion of second polar body and completion of meiosis II (Cooper, 1999).

1. 4. 2. Maintenance of prophase arrest in GV oocytes

Before antral follicle formation, oocytes are unable to progress beyond prophase of meiosis I. These oocytes are therefore meiotically incompetent. Meiotic competence is acquired progressively during oocyte growth in direct relation to follicular and oocyte size (Eppig *et al.*, 2004). Meiotic competence acquisition is associated with an increase and activation of regulators controlling meiotic maturation Cyclin B, Cyclin dependent kinase 1 (CDK1) (Kanatasu-Shinohara *et al.*, 2000) and phosphatase CDC25 (Mitra and Schultz, 1996).

Within the antral follicle, although containing all the required cell cycle regulatory molecules to progress through meiosis, the fully-grown GV oocyte is maintained arrested in the prophase I until ovulation. Interestingly, if removed from the follicle, the fully-grown oocyte spontaneously undergoes meiotic maturation *in vitro* (Pincus and Enzmann, 1935; Edwards, 1965). This is suggestive of factors within the

follicular environment controlling meiotic arrest (Eppig *et al.*, 2004).

It is well established that the meiotic arrest depends on high levels of cyclic adenosine monophosphate (cAMP) within the oocyte (Dekel and Beers, 1978; Conti *et al.*, 2002). A high steady-state level of cAMP induces PKA activity followed by CDC25 phosphatase inactivation, preventing dephosphorylation-dependent activation of CDK1 and maintenance of meiotic arrest (Eppig *et al.*, 2004) (Fig. 3 B). Various agents increasing the intracellular level of cAMP are frequently utilized to maintain the prophase arrest *in vitro*. Some of these compounds include membrane permeable cAMP analogs (e.g., dibutyryl cAMP, dbcAMP), agonists of adenylate cyclase (enzyme responsible for intracellular cAMP synthesis), or phosphodiesterase (PDE) inhibitors (targeting cAMP degradation) (Eppig *et al.*, 2004).

It has been postulated that in the antral follicle, gap junctions modulate meiotic arrest by mediating the transfer of cAMP-dependent signals or cAMP itself from different follicular compartments (cumulus, mural granulosa) to the oocyte (Dekel, 1988; Downs, 1989; Eppig, 1991; Webb *et al.*, 2002; Eppig *et al.*, 2004). Work conducted in the rat proved that upon antral follicle culture in the presence of a gap junction inhibitor, the oocyte within was unable to maintain its meiotic arrest. Moreover, this was associated with a decrease in oocyte cAMP (Sela-Abramovich *et al.*, 2006). Interestingly, although gap junctions seem to play an important role in modulating meiotic arrest prior to ovulation, the oocyte itself is able to synthesize most of the cAMP needed for its arrest by adenylate cyclase activity stimulated by the G_s-linked G-protein coupled receptor GPR3 (Mehlmann *et al.*, 2004). Inhibition of PDE3, exclusively expressed in the oocyte, likely participates in the meiotic arrest as well. Hypoxanthine, a purine with PDE inhibitory

activity, measured to be at high concentrations in the antral follicle, is able to maintain the GV arrest *in vitro* (Downs *et al.*, 1986; Downs *et al.*, 1989; Downs, 1993). Moreover, injection of purine metabolism inhibitors into live mice triggered maturation of most GV oocytes (Downs and Eppig, 1987). Another molecule thought to have important roles in maintenance of meiotic arrest is cGMP. It has been proposed that production of cGMP by mural granulosa, followed by its passage through gap junctions into the oocyte results in inhibition of PDE3 and prevention of cAMP degradation (Tornell *et al.*, 1991; Norris *et al.*, 2008). Experiments in which meiotic arrest was released by an inhibitor of soluble guanylate cyclase (Sela-Abramovich, 2008) or maintained by cGMP injection into isolated oocytes (Tornell *et al.*, 1991), support this hypothesis. The mechanisms ensuring maintenance of high cAMP steady-state levels during meiotic arrest seem to be complex and intricate. Both oocyte intrinsic and extrinsic factors appear to be involved in controlling the prophase arrest. Future work will have to clarify the exact role of these factors.

1. 4. 3. Meiotic maturation induction

Meiotic maturation is triggered i*n vivo* by the ovulatory LH surge. The exact mechanism(s) allowing transduction of the LH signal from the outer follicular layers to the oocyte, in the absence of oocyte-expressed LH receptors, is not fully understood. The observation of oocyte spontaneous nuclear maturation upon removal from the follicular environment (Edwards, 1965) supports the hypothesis of a release from maturation inhibitory signal(s) generated by other follicular compartments once ovulation is triggered (Eppig *et al.*, 2004). Based on this model, interruption of gap junctional

communication in somatic cells upon LH stimulation leads to cessation of meiotic maturation inhibition, a decrease in oocyte cAMP and through downstream steps (Fig. 3 C), meiosis resumption.

Functional changes in granulosa gap junctions in response to LH, like phosphorylation of Cx43, have been reported to coordinate with GVBD (Larsen *et al.*, 1987; Granot and Dekel, 2002; Sela-Abramovich *et al.*, 2005). Recently, microinjection of fluorescent tracers into live antral follicle-enclosed mouse oocytes, revealed a MAPK-dependent phosphorylation and transient closure of the gap junctions (Cx43) between somatic cells following LH stimulation (Norris *et al.*, 2008). A more complex model supports the idea of meiotic maturation-inducing signals generated in mural and/or cumulus granulosa cells in parallel with closure of follicular gap junctions. In this model, LH *in vivo,* or alternatively FSH *in vitro*, causes an elevation in granulosa cAMP through unknown pathway(s) and activation of MAPK. This triggers generation of a not yet elucidated "meiosis-inducing signal" in granulosa, a signal proposed to be transferred to the oocyte via cumulus-oocyte gap junctions. Activation of oocyte PDE3, followed by a decrease in oocyte cAMP would thus induce meiosis (Tsafriri *et al.*, 1996; Eppig *et al.*, 2004). Recent studies confirmed that, although the gap junctional communication between granulosa cells is transiently in response to LH, the gap junctions at the interface between oocyte and cumulus cells are maintained in a functional state (Norris *et al.*, 2008).

1. 4. 4. Cytoplasmic maturation

The concept of cytoplasmic maturation originates from the observation that fully

grown oocytes, able to undergo nuclear maturation, will fertilize and progress through embryogenesis at low rates if collected from improperly matured follicles (Pavlok *et al.*, 1992). Factors that had accumulated during oocyte growth and maturation, allowing proper fertilization, correct progression through early embryogenesis, and implantation, must underlie the process of cytoplasmic maturation.

One such factor is the ability of the oocyte to produce normal Ca^{2+} waves following fertilization, enabling completion of meiosis II, oocyte cortical granule exocytosis (important for the polyspermy block), and recruitment of maternal mRNA during embryonic genome activation (Ducibella *et al.*, 2006). Recent investigations demonstrated that while a low number of Ca^{2+} transients are associated with impaired embryo implantation (Toth *et al.*, 2006), an excessive amount interferes with embryo post-implantation development (Ozil *et al.*, 2006). Cytoplasmic changes occurring in parallel with meiotic maturation, and ensuring proper oocyte Ca^{2+} wave release during fertilization, include accurate smooth endoplasmic reticulum reorganization (Mehlmann *et al.*, 1995) and an increase in the number and localization of IP3-receptors (Mehlmann, 1996).

Sperm incorporation during fertilization is followed by a series of reactions that transform certain sperm structures into embryo cellular components. This is dependent on molecules within the oocyte cytoplasm generated and/or accumulated during cytoplasmic maturation. Factors accumulated during cytoplasmic maturation are involved in protamine dissociation on sperm chromatin during 1-cell embryo pronuclei formation. This process is mediated by the maternal glutathione within the egg cytoplasm (Perreault, 1992) renders the paternal DNA into an accessible template for transcription (Cooper,

1999). Although some aspects of cytoplasmic maturation have been elucidated in the last two decades, many facets of this process remain undetermined.

1. 5. Preimplantation embryo development

Following fertilization, the 1-cell embryo consists of oocyte-inherited cell structures and two pronuclei containing maternal and paternal genetic material. One of the first major events the embryo undertakes is the activation of its own, distinct genome, allowing the transition from a maternal to a zygotic developmental program. This process, that in mice is completed during the 2-cell stage, is termed embryonic genome activation (Schultz, 1993).

While transiting the oviduct toward uterus, the embryo undergoes major changes. Progress through early steps of embryogenesis is characterized by consecutive rounds of mitosis, period during which the embryo undergoes reductive cleavages becoming a 2-, 4-, and 8-cell embryo (Fig. 4). Any of the cleavage stage embryo cells, known as blastomeres, are characterized by totipotency, being able to give rise to a whole new organism if separated from its sister blastomeres (Hogan *et al.*, 1994). Around the 16- to 32-cell stage, the blastomeres become flattened out, maximizing the contact area with neighboring cells. At this stage, called the morula stage, the embryo cells start to express cell surface adhesion molecules (E-cadherin) and to develop tight and gap junctions between blastomeres, ensuring intercellular communication (Hogan *et al.*, 1994). Late in the morula stage, the embryo undergoes a process called cavitation, with formation of a fluid filled-cavity, and segregation of embryo cells into different lineages. By the blastocyst stage, the outer cells differentiate into trophectoderm while the inner

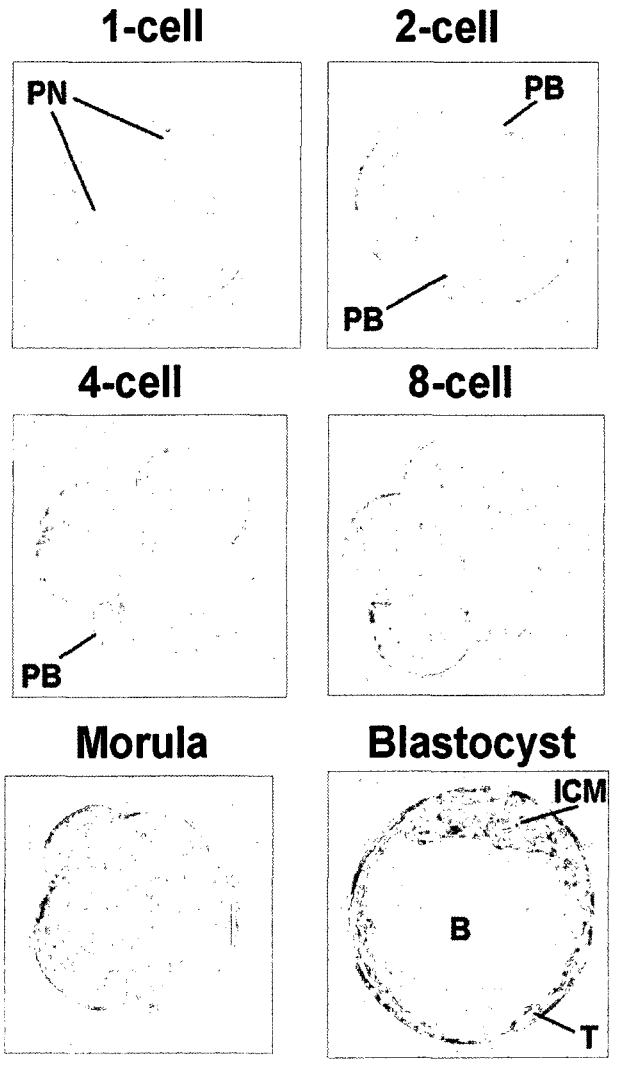


Figure 4. Preimplantation embryos. Photomicrographs of mouse preimplantation embryos removed from oviducts as 1-cell embryos and then cultured *in vitro* for up to 4 days. Shown: 1-cell, 2-cell, 4-cell, 8-cell, morula and blastocyst-stage embryos. PN = pronucleus, PB = polar body, ICM = inner cell mass, T = trophectoderm, B = blastocele.

embryonic cells become the inner cell mass. In mice, during the fifth day of development, the blastocyst digests its extracellular matrix, the zona pellucida, by making use of trypsin-like enzymes. This process is called hatching, and is a required step before implantation (Hogan *et al.*, 1994).

1. 6. Zona pellucida

The zona pellucida (ZP) is the extracellular matrix surrounding the mammalian oocyte and the early embryo. This porous extracellular matrix, allowing passage of various molecules including immunoglobulins and enzymes (Wassarman *et al.*, 2004) has important roles during oogenesis, fertilization and embryogenesis. In mice, three sulfated and heavily glycosylated proteins secreted by the oocyte, ZP1, ZP2 and ZP3, assemble to form the ZP (Wassarman, 1988). Based on electron microscopy and biochemical studies it has been proposed that mouse ZP is constructed out of ZP2-ZP3 polymeric filaments assembled into a double helical structure and cross-linked by the ZP1 glycoprotein (Greve and Wassarman, 1985; Green, 1997; Wassarman, 2008).

Although the ZP coat is retained until implantation of the embryo, expression of the three zona glycoproteins is oocyte-specific and is completed by the time the oocyte is fully grown (Epifano *et al.*, 1995). Synthesis of the ZP is initiated during oocyte growth, when glycoproteic material starts to be deposited between the growing oocyte membrane and the surrounding granulosa cells (Phillips and Shalgi, 1980). The ZP increases in thickness during the first 2-3 weeks of oocyte growth (Bleil and Wassarman, 1980; Epifano *et al.*, 1995), is fully assembled by the time the oocyte completes its growth, and ceases to be secreted once ovulation occurs (Qi *et al.*, 2002; Wassarman *et al.*, 2004). The

continuous steady-state level of ZP secretion in the oocyte was proved by a series of experiments in which epitope-tagged ZP2 or ZP3 cDNA was injected into growing oocytes. Observation by confocal microscopy determined that nascent ZP glycoproteins were deposited as a very thin layer in very close proximity to the oocyte membrane, suggesting that the ZP thickens from the inside, while the outer layers, synthesized earlier during growth, are gradually displaced towards the periphery (Qi *et al.*, 2002).

The molecular mechanisms controlling ZP assembly continue to remain under investigation. Once synthesized within the oocyte, post-translational modifications in the endoplasmic reticulum and Golgi apparatus ensure the N- and O-glycosylation of the ZP nascent proteins. Following these events, the ZP glycoprotein precursors are packed into large secretory vesicles in the *trans* Golgi network and shipped to the plasma membrane. Vesicle fusion with the oocyte membrane is thought to mediate ZP glycoprotein release onto the oocyte surface (Wassarman *et al.*, 2004). All three ZP polypeptides delivered to the oocyte membrane have a similar structure consisting of a ZP domain (ZPD) and a consensus furin cleavage site (CFCS) followed by a C-terminal propeptide containing a transmembrane domain (TMD) and a short cytoplasmic tail (Wassarman, 2008) (Fig. 5). It has been proposed that during ZP glycoprotein secretion, before incorporation into the ZP, a furin-like enzyme excises the C-terminal propeptide from ZP precursors by cleavage at the CFCS (Litscher *et al.*, 1999; Qi *et al.*, 2002). Supporting this is the observation that CFCS mutations hinder ZP secretion, causing an enhanced retention of ZP glycoprotein within growing oocytes (Jovine *et al.*, 2004). Analysis of mouse ZP3 mutant incorporation into ZP, suggested that ZP assemblage depends on the functional

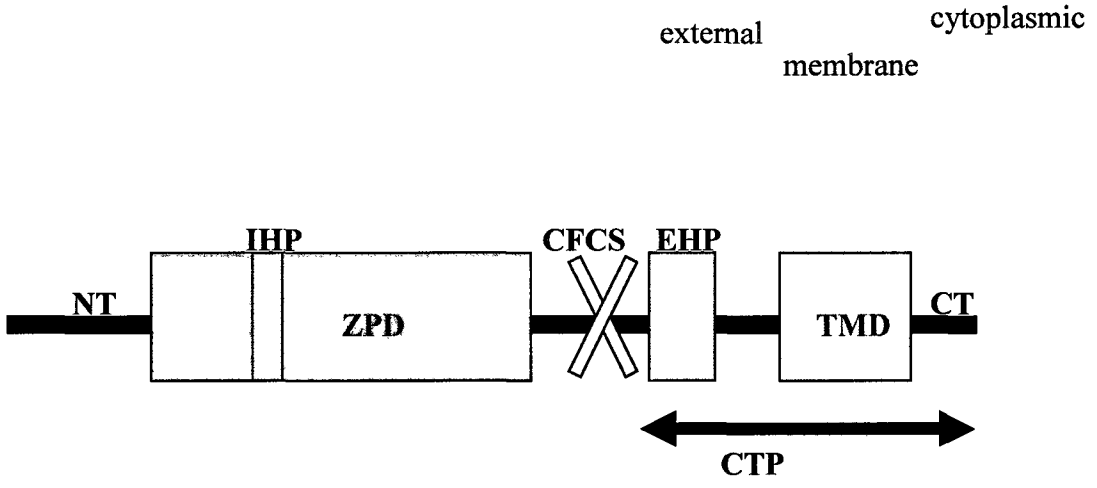


Figure 5. Schematic representation of a mouse ZP precursor polypeptide. The ZP precursors include a ZP domain (ZPD) and a consensus furin cleavage site (CFCS) followed by a C-terminal propeptide (CTP) containing a transmembrane domain (TMD) and a short cytoplasmic tail (CT). During the process of ZP protein secretion and assembly into the zona pellucida, the proteolytic cleavage at the CFCS allows separation from the CTP, followed by release of activated mature ZP protein. NT = N-terminus, IHP = internal hydrophobic patch, EHP = external hydrophobic patch.

interaction between an internal hydrophobic patch (IHP) located within the ZPD and an external hydrophobic patch (EHP) found in the C-terminal propeptide. Based on this model, nascent ZP glycoproteins, anchored to the oocyte plasma membrane by their TMD, are not competent for assembly due to interaction between EHP and IHP. Proteolytic excision at the CFCS leads to dissociation of the EHP and IHP followed by disassembly of C-terminal propetide (including EHP) and release of activated mature ZP protein. Moreover, the proteolytic processing is proposed to drive polymerization of proteins into ZP filaments by mediating interaction of IHP from different ZP glycoproteins (Williams and Wassarman, 2001; Jovine *et al.*, 2004; Wassarman, 2008). Based on this model, the proteases responsible for C-terminal propetide cleavage would play major roles in secretion and assembly of ZP. Although a few reports indicated that furin, a member of proprotein convertase family, is involved in ZP2 and ZP3 proteolytic cleavage and secretion (Litscher *et al.*, 1999; Williams and Wassarman, 2001), the exact identity of the protease involved in C-terminal propetide excision remains surrounded by controversy in the literature (Zhao *et al.*, 2002).

Important roles have been attributed to the ZP. During oogenesis and embryo progression through the oviduct, it is thought that the ZP protects and contains the oocyte and the developing embryo (Yanagimachi, 1994). Extensive research demonstrated that during fertilization the ZP not only modulates sperm-oocyte binding, with the ZP3 glycoprotein acting as a sperm primary receptor (Bleil and Wassarman, 1980; Wassarman, 2005), but once sperm penetration takes place ZP also acts as a barrier preventing polyspermy. This process, known as "zona hardening", is characterized by structural changes affecting ZP2 and ZP3 (Wassarman, 2005).

1. 7. The perivitelline space

The perivitelline space (PVS) is defined as the space between the ZP and plasma membrane (vitellus) of the oocyte or embryo. The PVS undergoes dynamic changes during oocyte and embryo development. To date, the precise moment of PVS formation and the mechanism(s) leading to its appearance have not been closely studied. Several reports investigating the ultrastructural configuration (by electron microscopy) of rodent oocyte cortex throughout meiotic maturation and fertilization indicated a lack of space (Kaufman *et al.*, 1989), or alternatively a very narrow space (Okada *et al.*, 1986) between the ZP and oocyte membrane when the oocyte was at GV stage. Although not discernible at the light microscopic level (Okada *et al.*, 1986), before ovulation the PVS is presumed to be very small and contains follicular fluid (Talbot and Dandekar, 2003). Once meiosis I terminates, a PVS is clearly visible and enlarged around the extruded polar body. A matrix rich in hyaluronic acid, similar to that present between cumulus cells, was found in the PVS only following stimulation of ovulation (Dandekar and Talbot, 1992; Talbot and Dandekar, 2003), and it is thought to contribute to the enlargement of the PVS (Okada *et al.*, 1986).

The assumption in the literature appears to be that the PVS largely results from the extrusion of the first polar body, and the consequent loss of oocyte volume coupled with the presence of the polar body between the ZP and oocyte. Following fertilization and throughout preimplantation development, the PVS remains large and takes different shapes accommodating embryogenesis (Talbot and Dandekar, 2003) until the blastocyst forms and expands, causing the outer trophectoderm to be pressed against the inner ZP surface.

The role of the PVS has been studied mainly during fertilization. Once in the oviduct and during fertilization, the content of PVS undergoes major changes. It is thought that following expulsion of the matured MII oocyte into the oviduct, the PVS sequesters oviductal proteins, like the oviduct secretory glycoprotein, supporting higher fertilization rates through unknown mechanisms (Kouba *et al.*, 2000; Talbot and Dandekar, 2003). The presence of the PVS prevents polyspermy as well, by concentrating in a very small space the hydrolytic enzymes released during fertilization from the granules positioned beneath oocyte membrane (egg cortical reaction), allowing in the end "zona hardening" (Hogan *et al.*, 1994; Talbot and Dandekar, 2003).

It is possible that both ZP and PVS play other important roles during early development. Future work will have to determine such possibilities.

2. Oocyte and preimplantation embryo culture

2. 1. Membrane transport requirements during oocyte and preimplantation embryo development

The unique changes the oocyte and early embryo undertake to properly progress through development are accompanied by major adjustments in cellular metabolic and homeostatic requirements. Although oocyte and early embryo physiology has been extensively studied in the last few decades, there is little information regarding changes in membrane transport during oocyte growth and maturation, with most of the research pertaining to early embryo ability to transport various molecules.

2. 1. 1. Oocyte-granulosa metabolic cooperativity

To support its metabolic and homeostatic needs during growth and maturation, the oocyte utilizes either its own plasma membrane transport systems or gap junction-mediated metabolite transfer from surrounding granulosa cells. Metabolic cooperativity defines the net movement of metabolizable substances from one cell to another, even if each cell can acquire the substance by itself (Haghighat and Van Winkle, 1990). Metabolites like uridine, guanosine, inositol, choline and various amino acids have been shown to be transferred from the granulosa cells to the growing oocyte (Heller *et al.*, 1981; Colonna and Mangia, 1983). Oocyte-granulosa metabolic cooperativity is utilized for the transfer of pyruvate, the main energy source employed by the maturing oocyte (Downs, 2002). It is thought that a low hexokinase activity (Brinster, 1968; Tsutsumi *et al.*, 1990) restricts the oocyte in utilizing glucose. Experimental evidence support the hypothesis that surrounding cumulus cells metabolize glucose to pyruvate and transfer it to the oocyte by gap junctions (Donahue and Stern, 1968; Downs and Mastropolo, 1994; Eppig *et al.*, 2004). The preference for pyruvate rather than glucose *in vitro* is maintained until the early stages of preimplantation embryo development (Brinster, 1969; Lane and Gardner, 2000).

Amino acids are also required by the oocyte. In the mature antral follicle, transport systems present on the oocyte plasma membrane ensure transport of amino acids like valine, leucine or phenylalanine. For proline, alanine, serine, tyrosine and lysine the transport into oocyte seems to be enhanced by granulosa cells due to the metabolic cooperativity (Colonna and Mangia, 1983; Haghighat and Van Winkle, 1990).

2. 1. 2. Amino acid transport during oocyte and preimplantation embryo development

The study of amino acid transport during oocyte and preimplantation embryo development was elicited by the observation that addition of specific amino acids *in vitro* improves embryo development (Van Winkle, 1996; Baltz, 2001). A recent survey investigating amino acid transport in maturing mouse oocytes indicated that systems like ASC (alanine transporter) or $b^{0,+}$ (arginine, lysine, glutamine transporter) have constant levels of activity throughout maturation. Conversely, system L (leucine-preferring) activity decreased between MI and MII stages, system x_c^- (cystine transporter) increased between MI and MII, while system β (β-amino acid transporter like taurine and β-alanine) appeared only at MI stage (Pelland, A. and Baltz, J.M. unpublished data). These changes may be a reflection of the oocyte acquiring independence from supporting granulosa cells following stimulation of ovulation. Alternatively, it is also possible for the oocyte and the embryo to adjust their membrane transport based on substrate availability in their environment. Supporting this is the observation that compared with blood, the murine follicular and oviductal fluid is low in glucose but high in pyruvate, whereas amino acids like glycine, taurine or lysine are found in blood-like concentrations in the follicular fluid but in higher amounts in the oviduct (Harris *et al.*, 2005).

While, within the follicle, the oocyte relies mainly on its surrounding granulosa cells for metabolic support, ovulated oocytes and the early embryos seem to have developed the required transport systems. Amino acids like glutamine, glycine and taurine were found to stimulate *in vitro* preimplantation embryo development (McKiernan *et al.*, 1995; Baltz, 2001). A better rate of human embryo development,

implantation and clinical pregnancy was associated with a metabolic profile in which glycine, leucine and asparagine are utilized at high levels (Brison *et al.*, 2004). Transport of such amino acids is ensured by activity of various amino acid transporters described to be present throughout preimplantation development, or restricted to certain embryonic stages: system β and L expressed at all stages; GLYT1 (glycine transporter) active until the morula stage; system $b^{0,+}$, $B^{0,+}$ (transporter of cationic amino acids like leucine and tryptophan), or System A (transporter of alanine, serine, and glutamine), active only in the inner cell mass at the blastocyst stage (Van Winkle, 2001). Utilization of these transporters at specific stages of embryo development is probably a reflection of homeostatic or metabolic requirements, since glycine can be used as an organic osmolyte (Baltz, 2001) or energy substrate (Khatchadourian *et al.*, 1994), taurine to increase resistance to oxidant stress, arginine and lysine for protein synthesis and nitrogen metabolism, and glutamine for synthesis of purines and pyrimidines (Van Winkle, 2001).

2. 1. 3. Influence of membrane transport on oocyte and embryo cell homeostasis

Oocyte and preimplantation embryo development are accompanied by major changes in the extracellular environment. Events like oocyte transition from the follicular to the oviductal environment at ovulation, or preimplantation embryo progression through the oviduct until arrival at the uterine implantation site, require cell adaptation to variations in pH, osmolarity, and composition of the external fluid.

Maintenance of cell volume constancy is one of the homeostatic mechanisms employed by all mammalian cells if changes in osmolarity of their environment occur. Little is known about such mechanisms in the oocyte, most of the studies concentrating

on the unique features of cell volume regulation in preimplantation embryos. The interest in developing an *in vitro* embryo culture system revealed that early embryos develop *in vitro* only at osmolarities much lower than those thought to be present *in vivo* (Brinster, 1965; Baltz, 2001). Regulation of embryo volume, ensuring survival and development, was shown to rely on accumulation of organic molecules, known as organic osmolytes. Amino acids like glycine, glutamine, betaine, proline, or β-alanine were demonstrated to have osmoprotective effects *in vitro,* acting as organic osmolytes, (Van Winkle *et al.*, 1990; Dawson and Baltz, 1997). Since glycine, glutamine or alanine are found at very high concentrations in the oviductal fluid (Harris *et al.*, 2005), it is thought that a similar mechanisms are utilized *in vivo* as well. Therefore, transport of these amino acids from the surrounding environment may be critical for embryo survival *in vivo* or *in vitro*. So far, three transport systems have been described to be involved in maintenance of a constant embryo volume: GLYT1 and system β, responsible for transport within the embryo of glycine, and β-amino acids, respectively, and VSOAC, an organic osmolyte exporting system (Baltz, 2001; Steeves and Baltz, 2005)(see Cell-volume regulation).

2. 2. *In vitro* culture of growing, maturing oocytes and early embryos

More than half a century ago, the possibility of studying female gamete and embryo physiology *in vitro* spurred a great deal of interest in developing media that would support oocyte and early embryo development (Summers and Biggers, 2003). In later years, development of Assisted Reproductive Technologies (ART) and their application to research and domestic animals as well as humans, together with expansion of genetic engineering, amplified the efforts in improving a culture system that spans a

period of development from oocyte growth to embryo implantation.

Major adjustments have taken place since Whitten utilized a simple chemically defined medium to culture successfully mouse embryos for the first time (Whitten, 1956). Over time, an embryo culture system with strict control of the temperature (37°C) and external pH (pH ≈ 7.2 using a HCO_3^-/CO_2 system) was developed. Two major approaches were utilized to improve embryo culture. The first one, known as "Let the embryos choose" principle led to development of KSOM (K^+ supplemented optimized medium), medium highly utilized nowadays (Lawitts and Biggers, 1993). Based on this approach, specific concentrations of individual ingredients were defined by the ability of 1-cell embryos to progress through "the 2-cell block" developmental blockade, and develop into blastocysts. The second method used the "back to nature" principle, in which various substances were incorporated in the media based on their concentrations in the female genital tract (Summers and Biggers, 2003). This approach, trying to mimic an environment to which embryo is normally exposed, gave rise to human tubal fluid (Quinn *et al.*, 1985) and mouse tubal fluid media (Gardner and Leese, 1990). The major observation that glucose inhibits oocyte and preimplantation development (Schini and Bavister, 1988), while pyruvate is the preferred energy source (see Oocyte-granulosa metabolic cooperativity), led to pyruvate inclusion in most media (Summers and Biggers, 2003). Amino acids proved to be beneficial for early development.Various groups described their multiple functions as precursors for biosynthesis, energy sources, osmolytes, buffers of intracellular pH, and chelators of heavy metals (Gardner *et al.*, 2000).

In recent years, stepwise protocols adapted for embryo stage-specific developmental requirements, have been formulated. These protocols, mainly applied to human embryo culture, utilize an initial medium low in glucose but high in pyruvate and nonessential amino acids, followed by 8-cell stage embryo transfer to a media high in glucose and containing all amino acids (Gardner and Lane, 1997).

Over time, various research groups have observed that certain strains of mice are prone to better develop embryos *in vitro*. The genetic background of the mouse influences not only the embryo development (Suzuki *et al.*, 1996), but also the pattern of DNA methylation and imprinting of specific genes (Doherty *et al.*, 2000; Summers and Biggers, 2003). One such example is the susceptibility of certain inbred mouse strains, like CF1, to exhibit the "2-cell block" when faced with a suboptimal culture environment (Summers and Biggers, 2003; Hadi *et al.*, 2005).

Efforts to develop an artificial system for *in vitro* oocyte development took place in parallel with embryo culture advancement. A system successfully supporting *in vitro* oocyte growth followed by meiotic maturation to produce a mature oocyte was described for the first time in late 1970s (Eppig, 1977; Eppig, 1979). These initial studies uncovered that proper oocyte growth and acquisition of meiotic competence require the presence of supporting follicular cells. In more recent years, this observation was clarified by multiple reports establishing the oocyte-somatic cell metabolic and developmental interdependence (Eppig, 2001; Matzuk *et al.*, 2002). Live births of mouse pups from oocytes grown and fertilized *in vitro* were reported for the first time in late 1980s (Eppig and Schroeder, 1989). Subsequent reports described a two-step culture system allowing complete oocyte development from primordial follicles, followed by

fertilization, progress through embryogenesis and live birth (Eppig and O'Brien, 1996; O'Brien *et al.*, 2003). These studies also uncovered that high glucose levels as well as the co-presence of insulin and FSH have deleterious effects over oocyte development *in vitro* (O'Brien *et al.*, 2003).

Although initially it was thought that gonadotropins are not essential for oocyte growth *in vitro* (Eppig, 1977; Eppig, 1979), later studies emphasized that addition of FSH to the culture system is beneficial allowing a more physiological oocyte development. The correct concentration of FSH (lower than expected) was shown to modulate paracrine signals, endorsing the oocyte-granulosa cell bidirectional communication, proper oocyte growth and somatic cell proliferation (Thomas *et al.*, 2005). Utilized in the past decades, FBS or BSA supplemented Wayemouth or MEM media remain valuable tools allowing oocyte culture. Although the mouse model suggests that healthy, fertilizable mammalian oocytes can be obtained in an artificial setting, *in vitro* maturation (IVM) of human oocytes remains a challenge in the field. Even if these oocytes spontaneously mature once removed from the follicles as their mouse counterparts, they will have reduced fertilization ability and impaired developmental competence with low progression to blastocyst stage (Banwell and Thompson, 2008). Defining the proper conditions of a culture system allowing not only nuclear but also cytoplasmic maturation, as well as enabling the correct timing of all the changes an oocyte undertakes during maturation, might improve the IVM system.

In later years, it was understood that defining the right conditions for *in vitro* oocyte and embryo development had to take into account not only physiological requirements specific to each developmental stage but also compensatory or adaptive

mechanisms employed upon exposure to an artificial environment such as regulation of cell volume or of intracellular pH. Initially observed more than 40 years ago (Cole and Paul, 1965), the mouse embryo "2-cell block", defined as an inability of mouse 1-cell embryos to develop *in vitro* further than the 2-cell stage, was later resolved by a decrease in culture medium osmolarity and addition of glutamine, thought to act as an organic osmolyte (Lawitts and Biggers, 1992; Dawson and Baltz, 1997), along with a reduction in glucose. The alleviation of similar blocks by low osmolarity for embryos of other species indicates that the sensitivity of cleavage-stage embryos to the osmolarity of their environment is a common characteristic across mammals (Baltz, 2001). The implications of *in vitro* gamete and embryo manipulation on the health of a newly created individual were highlighted by a series of studies examining disruption of gene expression (Ho *et al.*, 1995) or genomic imprinting (Rycke *et al.*, 2002). Although the literature abounds in studies supporting or disagreeing with a relationship between human oocyte and embryo *in vitro* culture and possible subsequent abnormalities in development, health, behavior or predisposition to disease (Summers and Biggers, 2003; Banwell and Thompson, 2008), future studies will have to pinpoint which steps, if any, still need to be corrected or further optimized. The mouse oocyte and embryo culture system remains an excellent experimental tool to uncover such subtle molecular changes triggered by culture and/or manipulation in an artificial setting.

3. Cell-volume regulation

3. 1. Osmolarity and tonicity

Osmolarity is defined as a measure of osmotic pressure exerted by a solution

across a semi-permeable membrane, and is dependent on the number 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. Since the cell membrane allows free passage of water but prevents free movement of most molecules, acting as a semi-permeable membrane, osmotic pressure is an important parameter for cells. Osmolality (osmoles per kilogram), easier to be measured, is almost identical with osmolarity (osmoles per liter) in solutions that are physiologically relevant (Baltz, 2001). Many authors utilize the terms interchangeably. Osmolarity and osmolality are conceptually different from tonicity, which is defined as the osmotic effect of a solution on a cell, compared with blood plasma, so is context-specific (Baltz, 2001). In mammalian organisms, blood plasma osmolarity is known to be around 300 mOsM. Therefore, a salt solution, to which cell membrane is impermeable, will be isotonic at 300 mOsM total dissolved species. On the other hand, a solution of urea or glycerol at 300 mOsM will be isosmotic with blood plasma but not isotonic since these compounds can freely pass and rapidly equilibrate across cell membranes (Woo *et al.*, 2002). Traditionally, osmolarity of the cell environment can be altered *in vitro* by varying the amount of NaCl or of an inert compound like manitol, raffinose or sucrose.

3. 2. Cell volume regulation and homeostatic mechanisms

Regulation of cell volume is an important homeostatic mechanism employed by all mammalian cells. In an animal organism, cell function is challenged by a multitude of factors, including nutrient availability, oxygenation, temperature, pH, and osmolarity. Under physiologic conditions, mammalian cells appear to be exposed to an internal

environment with very well controlled temperature and osmolarity. Kidney function and the thirst mechanism ensure an internal environment where water and electrolytes are maintained in homeostatic range (Ho, 2006; Burg and Ferraris, 2008). Although the internal osmolarity of mammalian organisms is overall well controlled, in recent years it was understood that basic cell functions like nutrient uptake, activation of membrane ion channels or transport systems and different metabolic events (Lang *et al.*, 1998), can create changes in the osmolarity of the cell microenvironment and the cell cytoplasm, leading to osmotic perturbations. Mammalian cells are prone to volume changes due to their lack of rigid walls and their cell membrane's high water permeability (Mongin and Orlov, 2001). Therefore, small differences between the intracellular and extracellular level of solutes can create an osmotic pressure differential resulting in cell stress and death (Baltz, 2001). Most mammalian cells are able to respond to osmotic variations in their surroundings by an immediate change in their volume needed to equalize osmotic pressure across the membrane (Baltz, 2001). Subsequently, highly active cellular mechanisms, known as "cell volume regulatory mechanisms", allow recovery from the volume loss or gain, ensuring maintenance of cell physiologic size under the new conditions (Fig. 6) (Lang *et al.*, 1998; Baltz, 2001). Although for a long time it was believed that osmotic stress responses in mammals are mainly important for the kidney medulla, tissue exposed to osmotic stress under physiological conditions during antidiuresis (Garcia-Perez and Burg, 1991), cell volume regulation seems to be a homeostatic mechanism commonly utilized by the majority of mammalian cells.

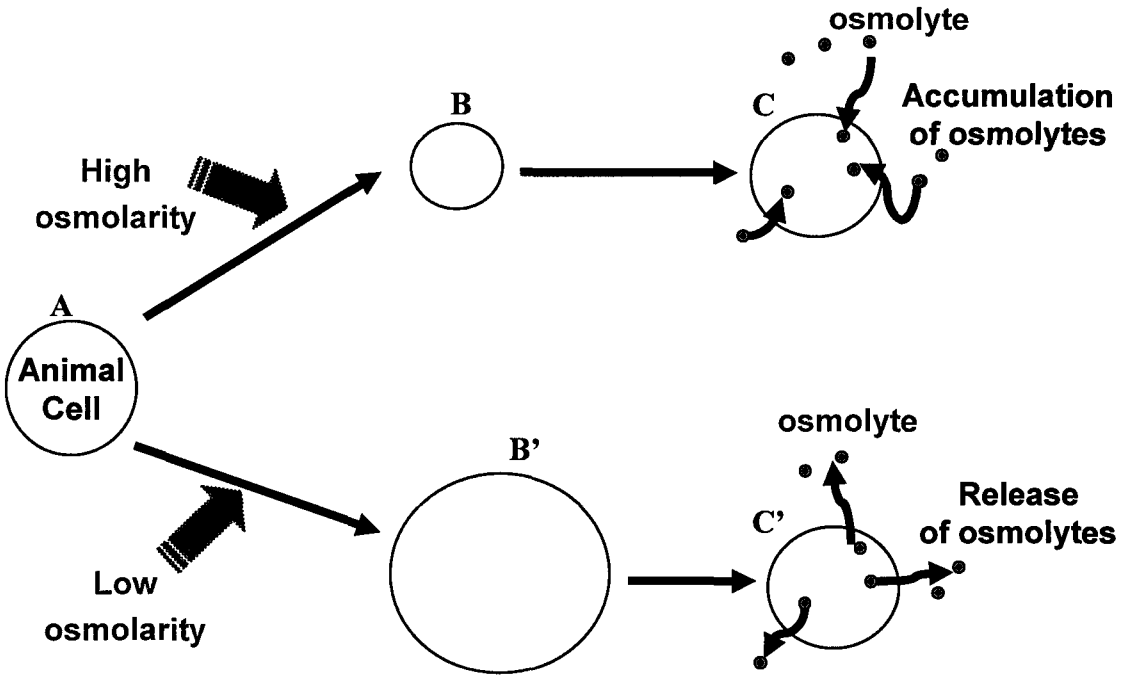


Figure 6. Schematic representation of volume regulation in animal cells. Animal cells respond to an increase or decrease in the osmolarity of their environment by altering their volume (shrinking (B) or swelling (B'), respectively). The general response once deviation from the cell's preferred size occurs is to import or to export osmolytes. This results in the adjustment of intracellular osmotic pressure until it is equalized with the extracellular environment at the preferred volume, so that volume is restored (C, C').

3. 3. Cell volume responses to changes in tonicity

In animal cells, water flux across the cell membrane is driven by the osmotic gradient between the extracellular and intracellular environment. Therefore, any variation in the external solute concentration leading to an osmotic differential causes cells to lose or gain water, and in the end to alter volume (Lang *et al.*, 1998). A cell exposed to high tonicity will shrink due to water efflux. The opposite, cell swelling, occurs upon exposure to low tonicity. Alterations in cell volume result not only in a change of cell surface to cell volume ratio, but also in disruption of optimal intracellular enzyme and metabolite concentration, interfering with cell function (Mongin and Orlov, 2001). Illustrating this, is the association between extensive swelling and necrosis (Lang *et al.*, 2000; Okada *et al.*, 2001), or cell shrinkage and initiation of apoptosis in a variety of cell types (Bortner and Cidlowski, 1996; Mongin and Orlov, 2001; Okada *et al.*, 2001). To survive such perturbations, cells have developed mechanisms allowing fast cell volume recovery as well as mechanisms allowing volume preservation endurance when tonicity alteration persists.

3. 3. 1. Acute responses to changes in tonicity

The acute (fast) cell volume regulatory mechanisms allowing recovery from shrinkage or swelling seem to be highly conserved between evolutionarily distant species (Lang *et al.*, 1998; Mongin and Orlov, 2001). The first cellular response of volume loss upon exposure to increased tonicity is rapidly compensated by accumulation of inorganic osmolytes and osmotically driven water, followed by cell volume gain until attainment of initial size through the "regulatory volume increase" (RVI) mechanism. The opposite

acute response, "regulatory volume decrease" (RVD), with ion efflux, osmotically driven water efflux, and cell volume loss, takes place following cell swelling at low tonicity (Lang *et al.*, 1998). Ion channels and transporters are the preferred mean of inorganic osmolyte transfer across the cell membrane during both RVI and RVD, ensuring recovery of volume in a matter of seconds to minutes. Activation of sodium channels, Na^+, K^+, $2Cl^-$ cotransporters of the NKCC family, or coupled activation of Na^+/H^+ antiporters and HCO_3^-/Cl^- exchangers mediate the ionic influx during RVI (Lang *et al.*, 1998; Baltz, 2001). Activation of K^+ channels, Cl^- (anion) channels or K^+, Cl^- cotransporters ensures ionic efflux during RVD (Lang *et al.*, 1998; Mongin and Orlov, 2001). The mechanism(s) allowing a cell to sense a change in volume and to generate and transfer signals to the ion transport system ensuring RVI or RVD, although subjects of intense investigation, are not elucidated to date.

3. 3. 2. Mechanisms ensuring long-term adaptation to hypertonic conditions

Although RVI acts as an immediate rescue mechanism allowing cell survival, ion accumulation can be deleterious over time for cell homeostasis and metabolism. High intracellular concentration of electrolytes is known to interfere with protein function and structure, membrane potential, and overall cell function (Lang *et al.*, 1998). Therefore, cells that have to adapt to chronic hypertonic conditions developed the ability to accumulate organic osmolytes. Osmolytes are small osmotic effector molecules, able to accumulate or be released from a cell, accounting for cell isosmotic balance with the surrounding environment (Yancey, 1994). The main characteristic of inorganic or organic osmolytes is their ability to protect the cell against deleterious effects of increased or

decreased osmolarity. Organic osmolytes are organic compounds able to provide intracellular osmotic support and to concentrate within the cytoplasm without interfering with cell biochemistry (Burg, 1995).

Accumulation of organic osmolytes has been found to be utilized for adaptation to hyperosmotic conditions by a variety of organisms from bacteria to plants and mammals (Yancey, 1994; Burg and Ferraris, 2008), suggesting an evolutionary conservation of this mechanism. Two complementary attributes confer protective qualities to all compounds used as organic osmolytes. The first, known as the "compatible" versus "perturbing" solute concept, refers to organic osmolytes' ability to accumulate over wide range of concentrations without compromising cell biochemistry, in contrast with disturbance of proteins and cell metabolism by increased intracellular electrolyte levels (Yancey *et al.*, 1982; Burg and Ferraris, 2008). The second major attribute of organic osmolytes is their ability to stabilize native protein structure. In recent years it has been shown that organic osmolytes presence protect the folding of proteins, preserving the native protein backbone configuration (Street *et al.*, 2006; Burg and Ferraris, 2008). Organic osmolytes in mammalian cells include: polyalcohols such as sorbitol and inositol, the methylamine glycerophosphorylcholine (GPC), and amino acids or their derivates such as glycine, glutamine, taurine, proline or betaine (Lang *et al.*, 1998; Baltz, 2001).

The mechanisms controlling organic osmolyte accumulation were extensively studied in kidney medulla, tissue exposed to hyperosmotic conditions during urine concentration. These studies uncovered that some organic osmolytes like GPC or sorbitol are produced within the kidney cells, while others are imported by specialized transporters from the extracellular environment (Yancey *et al.*, 1982; Kwon and Handler,

1995; Lang *et al.*, 1998). Four organic osmolyte transporters were identified in the kidney: Na^+/myo-inositol transporter (SMIT), betaine transporter (BGT1), taurine/β-amino acid transporter (TAUT) and system A amino acid transporter (Garcia-Perez and Burg, 1991; Kwon and Handler, 1995). Other tissues have been shown to utilize organic osmolytes as well. Brain tissue utilizes mainly taurine in addition to choline, creatine and inositol (Law, 1994), whereas liver cells are able to employ the osmoprotective effects of taurine, betaine and inositol (Haussinger, 1998; Burg and Ferraris, 2008). The same four organic osmolyte transporters used by the kidney medulla (or subsets of them) are also employed in all other mammalian tissues thus far studied.

Different organic osmolytes accumulate within the kidney cells simultaneously. So far, research of cell volume regulation suggests that protection provided by osmolytes is independent of the osmolyte specific chemical properties (Burg and Ferraris, 2008). The advantage of having more than one route allowing organic osmolyte accumulation might reside in the necessity of back-up systems if variations in environmental substrate availability are encountered. The widespread utilization of organic osmolytes in most mammalian cells suggests that they are generally employed to provide osmotic support, even in tissues that normally do not encounter major variations in tonicity (Lang *et al.*, 1998).

3. 3. 3. Regulation of organic osmolyte accumulation by tonicity

Accumulation of organic osmolytes under hypertonic conditions requires hours to days (Garcia-Perez and Burg, 1991) and can be achieved by stimulated uptake, synthesis or/and decreased degradation. Organic osmolyte accumulation in kidney cells is

controlled by tonicity enhancer binding protein (TonEBP), the only known osmo-sensitive mammalian transcription factor (Miyakawa *et al.*, 1998; Miyakawa *et al.*, 1999; Woo *et al.*, 2002). Exposure of kidney cells to hypertonic conditions induces TonEBP synthesis over several hours, phosphorylation and nuclear translocation (Woo *et al.*, 2000). Once in the nucleus, TonEBP binds to TonE (tonicity responsive enhancer) elements in several genes. First described in 1994 as a hypertonic stress responsive element in the 5'-flanking region of the BGT1 gene (Takenaka *et al.*, 1994), TonE is responsible for the transcriptional stimulation of genes encoding organic osmolyte transporters (BGT1, SMIT, TAUT) or key enzymes involved in organic osmolyte synthesis (aldose reductase and phospholipase B involved in sorbitol and GPC synthesis, respectively) (Woo *et al.*, 2002; Burg and Ferraris, 2008). Ultimately, increased transcription and translation of these genes, ensures high levels of proteins mediating accumulation of organic osmolytes and preservation of cell volume. TonEBP seems to be active under isotonic conditions (Miyakawa *et al.*, 1999) and able to respond to changes in tonicity in both directions. Exposure to hypotonic conditions results in its down-regulation followed by decreased transcription of genes mediating organic osmolytes accumulation (Woo *et al.*, 2000). The tonicity-dependent shuttling of TonEBP between nucleus and cytoplasm seems to be the main mechanism allowing bidirectional regulation of TonEBP and adaptation of organic osmolyte accumulation to cell volume necessities (Burg and Ferraris, 2008). A continuous TonEBP activation in cells that had already adapted to hypertonicity was shown recently to be controlled by lesions that in other settings are regarded as pathological. Cytoskeletal rearrangements, DNA strand breaks or

oxidation of proteins and DNA bases provide signaling and maintenance of an active TonEBP (Burg *et al.*, 2007; Burg and Ferraris, 2008).

TonEBP is present and active in a variety of cells, although its exact function in non-renal tissues is not fully understood. TonEBP transcripts are ubiquitously present in mammalian cells, organs like brain and thymus expressing a higher level of protein than kidney (Trama *et al.*, 2000; Woo *et al.*, 2002). Recent reports have suggested that TonEBP functions not only as a tonicity responsive transcription factor but also modulates expression of genes involved in protein folding, acting overall as a sensor of available intracellular volume, supporting optimal cell function and proliferation (Ho, 2003).

Cells are able not only to accumulate but also to release organic osmolytes if swelling occurs. This process is thought to involve two steps. The first one, very rapid (seconds) is characterized by an increase in passive organic and inorganic osmolyte efflux through swelling-activated channels (Strange, 2004). The molecular identity of the channel mediating osmolyte efflux, also known as volume sensitive organic osmolyte and anion channel (VSOAC), remains unresolved (Yamamoto-Mizuma *et al.*, 2004). Based on their electrophysiological, permeability and pharmacological properties, these channels seem to resemble Cl^- channels (Strange *et al.*, 1996; Okada, 1997). It is generally accepted that the swelling-activated Cl^- current, the swelling-activated organic osmolyte efflux and the organic anion currents measured in response to cell swelling in a variety of cells (Strange *et al.*, 1996; Okada, 1997), including early embryos (Kolajova and Baltz, 1999), are an indicator of VSOAC activity (Baltz, 2001). The second step

involves the down-regulation of organic osmolyte synthesis and uptake over a period of many hours to days (Strange, 2004), controlled by TonEBP (see above).

3. 4. Cell volume regulatory mechanisms involving organic osmolyte accumulation in mammalian preimplantation embryos

3. 4. 1. Preimplantation embryo sensitivity to the osmolarity of their environment

The mammalian preimplantation embryo regulates its size precisely and responds to changes in tonicity (Baltz, 2001). An increased embryo sensitivity to the osmolarity of its environment was first reported almost half a century ago by Brinster (Brinster, 1965), who demonstrated that mouse two-cell embryos will develop *in vitro* to blastocysts only at a specific range of osmolarities, around 275 mOsM. Later studies described similar findings in different mammalian species (Baltz, 2001). Later studies also demonstrated the influence of media composition, method of altering osmolarity (salt or inert compound) or mouse strain over the "permissive range" of osmolarities (Baltz, 2001). These suggested that solute availability and genetic background influenced embryo developmental response to osmolarity. The observation that 1-cell embryos develop successfully *in vitro* at osmolarities lower than those present *in vivo* triggered interest in studying the mechanism allowing the mammalian embryo to control its volume.

3. 4. 2. Oviductal fluid osmolarity

The necessity of low osmolarity (≈ 250-275 mOsM) for successful *in vitro* embryo culture implies that the early embryo may perceive solutions with osmolarities similar to that of oviductal fluid as hypertonic. This could be explained in a few different

ways: 1) the oviductal environment has a much lower osmolarity compared with blood (≈ 300 mOsM); 2) the oviductal fluid contains osmolytes able to protect embryo cell volume; or, 3) the preference for low osmolarity is specific to the *in vitro* culture setting. Early measurements of oviductal osmolarity indicated great variability between species and between different measurements (from 290 to 360 mOsM) and within species (Baltz, 2001). This could be a reflection of difficulties in measuring osmolarity in very small volumes or of technique variation between different groups. A more original approach employed living 1-cell and 2-cell embryos as osmo-sensors. By monitoring, immediately after collection, embryo swelling or shrinking in media of known osmolarities, it was estimated that the osmolarity that produced no net change in volume was around 290 – 300 mOsM (Collins and Baltz, 1999). Overall, most of the research supports an oviductal osmolarity very close to blood plasma.

3. 4. 3. Accumulation of organic osmolytes by preimplantation embryos

In vivo, early preimplantation embryo development occurs at an oviductal osmolarity of ≈ 300 mOsM (Collins and Baltz, 1999). In contrast, *in vitro*, an osmolarity equivalent to that of oviductal fluid in simple culture media (containing salts and metabolic substrates) has been shown to be deleterious, with progression through early development occurring only under conditions of low osmolarity (≈ 250 – 270 mOsM) (Lawitts and Biggers, 1992; Dawson and Baltz, 1997; Baltz, 2001). This implied that preimplantation embryos perceive oviductal osmolarity as detrimental *in vitro*. In the early 1990s it was proposed for the first time that early embryos normally utilize organic osmolytes to counteract the effects of high electrolyte concentrations in their environment

(Van Winkle *et al.*, 1990; Biggers *et al.*, 1993). Subsequent work showed that addition of any one of several organic compounds to the culture environment rescued *in vitro* development in simple culture media at oviductal osmolarities, suggesting that these molecules might function as organic osmolytes (Van Winkle *et al.*, 1990; Dawson and Baltz, 1997; Baltz, 2001). Such compounds, mainly amino acids and their derivatives, included glycine, glutamine, betaine, proline, β-alanine, and hypotaurine (Dawson and Baltz, 1997).

Glycine was one of the most effective molecules, conferring osmoprotection for early embryonic development *in vitro* at osmolarities equal to or higher than those of oviductal fluid (Dawson and Baltz, 1997; Dawson *et al.*, 1998). Eggs and early embryos freshly removed from the oviduct were shown to contain a high intracellular concentration of glycine relative to other amino acids (Schultz *et al.*, 1981; Van Winkle and Dickinson, 1995). These attributes, characteristic for a molecule acting as an organic osmolyte, were indicative of glycine being utilized as an organic osmolyte by early embryos. Moreover, high amounts of glycine were found to be present in the oviductal fluid of various mammalian species, suggesting its utilization *in vivo* (Baltz, 2001). In mice, oviductal fluid glycine was measured in millimolar range concentrations (~ 3 mM), ten times higher than in blood plasma (Harris *et al.*, 2005), sufficient to ensure embryo osmoprotection (Dawson and Baltz, 1997; Baltz, 2001).

A series of experiments conducted in recent years conclusively support glycine utilization as an organic osmolyte by mouse early preimplantation embryos. First, 1-cell embryo intracellular glycine accumulation *in vitro* has been shown to increase linearly as the environmental osmolarity was raised (Dawson *et al.*, 1998; Steeves *et al.*, 2003).

Second, the accumulated glycine has been shown to be free in the cytoplasm rather than incorporated into macromolecules and therefore able to contribute to intracellular osmotic pressure. This was demonstrated by direct cytosolic glycine measurements using two different methods: measurement of accumulated radio-labeled glycine, and amino acid microanalysis by high performance liquid chromatography. Both approaches confirmed the increase in total intracellular soluble glycine with osmolarity (Steeves *et al.*, 2003). Third, glycine accumulation improved the one-cell embryo's ability to maintain its cell volume when the osmolarity of its environment was raised (Dawson *et al.*, 1998; Steeves *et al.*, 2003). The forth line of evidence is indirect and refers to embryo access to glycine *in vivo*, where this amino acid was measured to be present in very high amounts (Harris *et al.*, 2005), suggesting its utilization as an organic osmolyte in a physiological setting.

Although early embryos were known to be able to transport glycine via one of the Neurotransmitter Transporter family (NTT) members, GLYT1 (Hobbs and Kaye, 1985; Van Winkle *et al.*, 1988; Van Winkle *et al.*, 1990; Dawson and Baltz, 1997), this glycine transporter was only recently fully characterized as a novel organic osmolyte transporter in cleavage-stage embryos (Steeves *et al.*, 2003) (discussed more extensively below). Altogether, this novel cell volume regulatory mechanism involving GLYT1 activity accounted for glycine utilization as an organic osmolyte in early preimplantation embryos. The osmosensitive accumulation of glycine by this mechanism seems to be a characteristic only of early cleavage-stage embryos (1-cell and 2-cell stage), with the ability to accumulate high amounts of glycine in response to high tonicity being lost by the 4-cell stage (Hammer *et al.*, 2000). The possible utilization of glycine as an organic

osmolyte and the role of GLYT1 prior to fertilization has not been addressed by previous studies.

A recent study suggested the presence of a second organic osmolyte transport pathway involving betaine and proline in 1-cell mouse embryos (Anas *et al.*, 2007). The osmoprotective roles of betaine during embryo development *in vitro* has been proposed previously (Biggers *et al.*, 1993; Hammer and Baltz, 2002). Both proline and betaine are likely to be utilized by 1-cell embryos as organic osmolytes since their accumulation increased with external osmolarity, and both compounds, once accumulated, remained at high concentrations in a soluble form within the cytoplasm (Anas *et al.*, 2007). The authors of this recent study suggest that although both betaine and proline have organic osmolyte attributes, glycine accumulation at higher levels and showing a stronger dependence on osmolarity, remains the main mechanism utilized for osmotic support by early embryos. The activity of the newly identified betaine/proline transporter (Anas *et al.*, 2007) might thus be utilized as a back-up mechanism for the organic osmolyte transporter GLYT1 in cleavage-stage embryos.

3. 4. 4. GLYT1 is a novel organic osmolyte transporter utilized by early preimplantation embryos

As already discussed, glycine has been shown to have the attributes of an organic osmolyte, being utilized for osmotic support by early embryos *in vitro* and *in vivo*. On the basis of kinetics and substrate specificity, studies conducted more than two decades ago reported that in mouse eggs and early cleavage stage embryos, glycine transport was mediated by the NTT family member, GLYT1 (Hobbs and Kaye, 1985; Van Winkle *et*

al., 1988). Among the other known organic osmolyte transporters, only the TAUT β-amino acid transporter is expressed in cleavage-stage embryos (Van Winkle *et al.*, 1994), but glycine is not a substrate for this system. Together, this led to the premise of GLYT1 functioning as an organic osmolyte transporter in early embryos, mediating the osmosensitive glycine accumulation.

Several lines of experimental evidence published recently indicated the utilization of GLYT1 as an organic osmolyte transporter in 1-cell embryos (Steeves *et al.*, 2003). First, its activity was shown to be stimulated in 1-cell embryos by increased osmolarity with the maximal transport rate (V_{max}) becoming somewhat increased without changes in substrate affinity (K_m). Second, GLYT1 activity was required for osmoprotection by glycine in 1-cell embryos. This was demonstrated by utilizing a pharmacological agent, the specific GLYT1 inhibitor ORG23798. The specific inhibition of GLYT1 had several effects: 1) it abolished glycine ability to rescue embryo development *in vitro* at oviductal osmolarity; 2) it blocked glycine accumulation in response to osmolarity and 3) it reversed the ability of external glycine to maintain embryo volume (Steeves *et al.*, 2003). Thus, GLYT1 activity mediates the osmosensitive accumulation of glycine in 1-cell embryos, being required for maintenance of normal cell volume and allowing development at the external osmolarity of oviductal fluid. Together, these results demonstrated a novel function for GLYT1: its utilization as an organic osmolyte transporter in early preimplantation embryos, mediating glycine accumulation to support maintenance of embryo cell volume.

Intriguingly, protein synthesis is not necessary for the increased GLYT1 activity under osmotic stimulation, suggesting a different regulation from the four previously-

known mammalian organic osmolyte transporters (Steeves *et al.*, 2003). While organic osmolyte transporters described in kidney medulla ensure long-term adaptation to hypertonic stress, GLYT1 seems to be utilized by early embryos under conditions of normal tonicity. Therefore, GLYT1 represents the first identified organic osmolyte transporter ensuring volume homeostasis exclusively in near-isotonic circumstances.

GLYT1 activity and expression has been described only in cleavage-stage embryos from 1-cell to 8-cell (Hobbs and Kaye, 1985; Van Winkle *et al.*, 1988). Moreover, the osmosensitive accumulation of glycine seems to cease around 4-cell stage (Hammer *et al.*, 2000). These studies suggest that GLYT1 function as an organic osmolyte transporter is restricted to cleavage-stage embryos. Several reports have indicated glycine transport to be present in mouse oocytes (Colonna and Mangia, 1983; Haghighat and Van Winkle, 1990), although GLYT1 activity and its possible function as an organic osmolyte transporter before fertilization remained to be investigated.

3. 4. 5. Osmotic regulation of the intracellular glycine concentration in early embryos

If osmoregulated glycine accumulation within early mouse embryos is under GLYT1 control, glycine efflux, most likely done through the VSOAC, is also under osmotic regulation. The partnership between influx (GLYT1) and efflux (VSOAC) pathways in maintaining the steady-state level of glycine within the embryo was established through a series of experiments showing: 1) dependence of both glycine influx and efflux on osmolarity; 2) balance attainment between the rates of influx and efflux once necessary accumulation of glycine took place; and 3) a decrease in efflux

pathway activity when the rate of influx was reduced (Steeves and Baltz, 2005). Together, GLYT1 and the efflux pathway activity allow maintenance of a controlled steady-state concentration of glycine, ensuring intracellular osmotic pressure and embryo volume preservation at isotonic conditions.

Based on such a model, an increased accumulation of glycine via GLYT1 would mediate increased embryo volume. Once the increase of cell volume exceeds a specific threshold, the VSOAC efflux pathway becomes activated, resulting in glycine release and maintenance of volume near threshold (Baltz, 2001). VSOAC has been hypothesized to mediate maintenance of steady-state concentrations of organic osmolytes like betaine and proline in 1-cell embryos, as well (Anas *et al.*, 2007). GLYT1 and VSOAC-like channel have been described to function in a similar manner in human embryos and eggs, suggesting a general role for glycine utilization in mammalian early embryos (Hammer *et al.*, 2000).

4. The glycine transporter GLYT1

4. 1. GLYT1 gene, structure and tissue expression

The glycine transporter GLYT1 corresponds to the classical glycine transport designated System GLY, originally described in pigeon erythrocytes (Vidaver, 1964; Eavenson and Christensen, 1967). GLYT1 activity was first partially isolated as a preparation enriched in glycine transport activity from rat spinal cord in the late 1980s (Lopez-Corcuera and Aragon, 1989). Subsequently, the gene encoding GLYT1 was cloned from different sources including human, rat and mouse (Guastella *et al.*, 1992; Liu *et al.*, 1992; Smith *et al.*, 1992; Kim *et al.*, 1994b; Lopez-Corcuera *et al.*, 2001). Studies

done previous to its cloning had established its ability to transport sarcosine (N-methyl glycine), Na^+ and Cl^- dependence, stoichiometry, electrogenicity, and pharmacology of glycine transport in brain-derived preparations (Aragon *et al.*, 1987; Lopez-Corcuera *et al.*, 2001). Based on these characteristics, GLYT1 was included in the Na^+,Cl^--dependent Neurotransmitter Transporters (NTT) family, a group of glycoproteins with major roles in neurotransmission and containing transporters for GABA, monoamine (dopamine, serotonine), and amino acids like proline or taurine. GLYT1 together with GLYT2 and PROT (proline transporter) is a member of Na^+,Cl^--dependent amino acid transporters NTT subfamily. The other neuronal glycine transporter, GLYT2, encoded by a distinct gene, has a different structure (50% homology with GLYT1), tissue specificity and pharmacological properties (Nelson, 1998). Key differences between GLYT1 and GLYT2 include the ability of GLYT1 but not GLYT2 to transport sarcosine (Lopez-Corcuera *et al.*, 1991) and GLYT2 expression restricted to neurons (Luque *et al.*, 1995), while GLYT1 is expressed in glia and widely in cells outside the central nervous system (CNS) (Borowsky *et al.*, 1993).

GLYT1 protein is encoded by the *Glyt1* (*Slc6a9*) gene (Borowsky *et al.*, 1993). Cloning of the GLYT1 transporter led to identification of several isoforms with the same core protein but different N-terminal domains (Borowsky *et al.*, 1993) due to transcription initiated at alternate promoters (Borowsky and Hoffman, 1998), or different C-terminus as a result of alternative splicing (Hanley *et al.*, 2000). So far, in mice, only two isoforms, GLYT1a and GLYT1b, which differ in their N-terminus, have been described, (Borowsky *et al.*, 1993). The transport kinetics and pharmacology of the various isoforms seem to be indistinguishable.

GLYT1 variants have tissue specific distributions. While GLYT1a was found to be expressed in mammalian gray matter of the CNS and in macrophages and mast cells of peripheral tissues, GLYT1b was shown to be restricted to the CNS white matter (Borowsky *et al.*, 1993). GLYT1c is specifically expressed in human brain (Kim *et al.*, 1994a) whereas several other isoforms have been described to be restricted to bovine retina (Lopez-Corcuera *et al.*, 2001). The tissue-specific isoform distribution was proposed to be related with variations in requirements of glycine transport in different regions or at different times during development (Borowsky and Hoffman, 1998).

Like all other NTT members thus far examined, GLYT1 protein consists of 12 transmembrane domains (TMD) connected by six extracellular and five intracellular loops, with both C- and N-terminal ends cytoplasmic, and a large second external loop (Nelson, 1998) (Fig. 7). Site-directed mutagenesis confirmed the presence of four N-glycosylation sites on the second external loop. They seem to be essential not only for transport activity itself but for proper GLYT1 protein trafficking to the plasma membrane (Olivares *et al.*, 1995). Analysis of the GLYT1 cDNA sequence led to identification of two putative phosphorylation sites on the GLYT1a C-terminal extension and two additional sites on the N-terminus of GLYT1b, possible targets for PKA, PKC or calmodulin-dependent kinase (Liu *et al.*, 1993).

4. 2. GLYT1 mechanism of transport

Substrate transport via the GLYT1 transporter is dependent on both Na^+ and Cl^- (Aragon *et al.*, 1987). Glycine and sarcosine are classic high-affinity substrates while glutamine and proline are transported with lower affinity (Baltz, 2001). GLYT1 transport

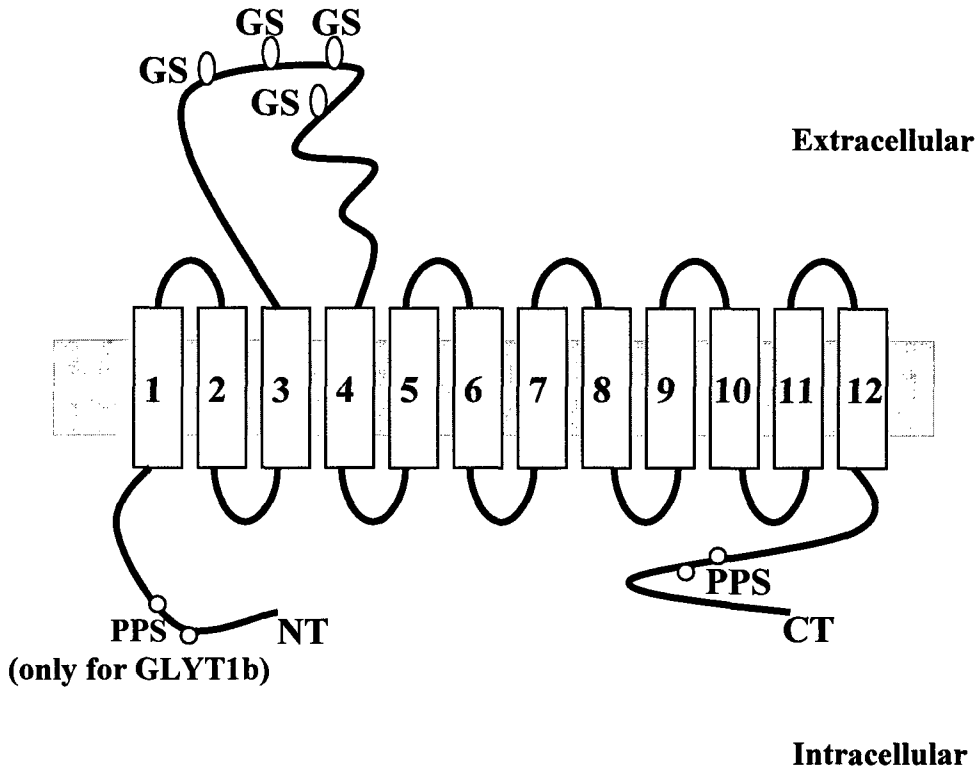


Figure 7. Schematic representation of GLYT1 transporter structure. GLYT1 is characterized by 12 transmembrane domains, connected by six extracellular and five intracellular loops. The N- (NT) and C- (CT) termini are located intracellularly. The second extracellular loop is large and heavily glycosylated (GS = glycosylation site). Two putative phosphorylation sites have been identified on the C-terminal extension of both mouse isoforms GLYT1a and b, whereas two additional sites on the N-terminus are characteristic only for the GLYT1b variant (PPS = putative phosphorylation site).

stoichiometry is proposed to be $2Na^+$ and $1Cl^-$ together with glycine. Transporter activity is driven by the transmembrane Na^+ electrochemical gradient maintained by Na^+/K^+-ATPase activity (Nelson, 1998). GLYT1 is thought to utilize a transport model common for most NTT family members. The first step involves extracellular Na^+ and Cl^- binding, followed by transporter conformational change allowing substrate binding. Consequently, a switch from an "outward" to an "inward" facing state exposes the glycine binding site to the cytosol allowing release into the cytoplasm of glycine and Na^+ first, followed by Cl^-. GLYT1 returns spontaneously to the initial conformation ("outward" state), becoming competent for a new cycle (Nelson, 1998; Eulenburg *et al.*, 2005). The recent determination of the crystal structure of the bacterial leucine transporter from *Aquifex aeolicus*, a member of the NTT family, revealed an architecture proposed to apply to all NTT transporters. Based on this crystal structure, the speculative transport mechanism involves TM1 and TM6 movement relative to the TM3 and TM8 domains, a shift responsible for opening and closing the "outward" and "inward" gates. The authors also infer that the binding and release of substrate and ions stabilizes the transporter in different conformational states (Yamashita *et al.*, 2005). In brain, GLYT1 has been proposed to operate in a reverse uptake mode as well, allowing release of its substrate from the cytosol into the extracellular space (Eulenburg *et al.*, 2005).

Since GLYT1 has important roles in modulation of excitatory neurotransmission, with possible involvement in the pathophysiology of psychiatric disorders like schizophrenia (Eulenburg *et al.*, 2005), its pharmacology is an area of research under continuous investigation. Glycine transport by GLYT1 is competitively inhibited by sarcosine (N-methyl glycine) (Lopez-Corcuera *et al.*, 1989). Synthetic derivates like

NFPS (N-[3-(4'-fluorophenyl)-3-(4'phenylphenoxy) propyl] sarcosine) and ORG24461 (N-methyl-N-[(4-trifluoromehtyl)phenoxy]-3-phenyl-propyl-glycine) have been shown to act as GLYT1 selective high-affinity inhibitors on brain preparations (Harsing *et al.*, 2003). Another inhibitor, ORG23798 [bis(4-fluorophenyl)methylenepiperidineacetic acid, lithium salt] has been proven to exert specific inhibition of GLYT1 activity in preimplantation embryos (Steeves *et al.*, 2003).

4. 3. GLYT1 function and activity regulation in brain

Glycine is known to be one of the major inhibitory neurotransmitters in the mammalian CNS (Werman *et al.*, 1967). In the glycinergic synapse, binding of glycine to specific receptors on the post-synaptic membrane results in an increased influx of chloride ions into cytoplasm, followed by hyperpolarization and inhibition of post-synaptic neuron signaling (Eulenburg *et al.*, 2005). Although initially it was thought that GLYT2 is the only glycine transporter responsible for the clearance of presynaptically released glycine (Jursky and Nelson, 1995), recent studies indicate complementary roles for GLYT1 and GLYT2 (Eulenburg *et al.*, 2005). Electrophysiological studies of brain stem motorneurons from GLYT1-knockout mice, revealed a tonic activation of glycine receptors associated with high extracellular level of glycine. The phenotype of GLYT1-knockout mice indicates a glycinergic over-inhibition due to sustained activation of glycine receptors. These mice die on the day of birth as a result of a severe motor deficit and respiratory depression (Gomeza *et al.*, 2003). Altogether, these findings are supportive of a role for GLYT1 in glycine clearance from glycinergic synaptic cleft and termination of glycine neurotransmission.

Glycine has also been shown to be involved in mediating the excitatory glutamatergic neurotransmission acting as a co-agonist of glutamate on N-methyl-D-aspartate (NMDA) receptors (Johnson and Ascher, 1987). The presence of both glycine and glutamate binding sites on the same protein (Moriyoshi *et al.*, 1991), and the lack of NMDA receptor activity in the absence of glycine (Kleckner and Dingledine, 1988), support the role played by glycine. GLYT1, mainly expressed in glia cells surrounding the synapse, is thought to remove glycine from the synaptic cleft, preventing the saturation of the glycine-binding site on the NMDA receptor (Eulenburg *et al.*, 2005). This is supported by inhibition studies showing that upon blockade of GLYT1, an increase in extracellular glycine concentration augments NMDA currents, resulting in prolonged long-term neuronal communication (Bergeron *et al.*, 1998; Martina *et al.*, 2004). Moreover, analysis of brain slices from heterozygous mice with only one functional GLYT1 allele, showed an increase in NMDA receptor function associated with a reduction in GLYT1 expression (Gabernet *et al.*, 2005).

Although GLYT1 has important roles in both inhibitory and excitatory neurotransmission pathways, the regulatory mechanisms controlling its function remain uncertain to date. The presence of putative phosphorylation sites (see GLYT1 gene, structure and tissue expression) suggests that GLYT1 protein phosphorylation state might influence its activity. Studies in which phorbol esters (potent activators of PKC) were utilized confirmed a decrease in GLYT1 activity upon PKC activation (Gomeza *et al.*, 1995). A different approach in which all predicted phosphorylation sites for PKC were removed by site-directed mutagenesis demonstrated that down-regulation of glycine transport was still induced by phorbol ester, indicating that an intermediate substrate

phosphorylation is involved in regulation of GLYT1 activity (Sato *et al.*, 1995). Other factors like arachidonic acid (Zafra *et al.*, 1990) or Zn^{2+} (Laube, 2002) have been shown to down-regulate GLYT1 activity through unelucidated mechanisms. The SNARE protein Syntaxin A, which has an important role in vesicle fusion during the exocytosis process, has been described also as one of the possible regulators of GLYT1 activity in brain. Co-transfection and immunoprecipitation studies in both COS cells and the brain tissue indicated a functional interaction between GLYT1 and Syntaxin A resulting in partial removal of glycine transporters from the plasma membrane (Geerlings *et al.*, 2000). This is suggestive of vesicle trafficking involvement in modulating GLYT1 activity. The level of transporter oligomerization could be critical for its functionality. Although increasing evidence suggests that most of the NTT members form active dimeric transport complexes (Eulenburg *et al.*, 2005), gel-electrophoretic analysis of Xenopus oocyte-expressed mouse GLYT1, indicated the presence of functional monomeric GLYT1 protein in plasma membrane, associated with an oligomeric cytoplasmic GLYT1 fraction (Horiuchi *et al.*, 2001), making it unlikely for GLYT1 activity to be regulated by oligomerization state.

4. 4. Novel function as an organic osmolyte transporter in early preimplantation embryos

As already discussed (see previous chapter), GLYT1 has been shown to function as an organic osmolyte transporter in cleavage-stage embryos, responsible for mediating osmotically regulated accumulation of glycine. Several attributes showed that GLYT1 functions as an organic osmolyte transporter in early preimplantation embryos: 1)

GLYT1 activity protects early embryo development against increased osmolarity; 2) GLYT1 activity is required for osmosensitive glycine accumulation; 3) GLYT1 supports the maintenance of normal embryo volume. As already discussed, GLYT1 activity is regulated by unknown mechanism(s), different from those controlling the other known mammalian organic osmolyte transporter (Steeves *et al.*, 2003).

5. Objective and specific aims

5. 1. Objective and significance of thesis subject

The interest in studying oocytes and early embryos has increased in the last decades in parallel with development of ART and their utilization as part of human infertility treatment or for domestic and research animal husbandry. The ability to grow, manipulate and maintain healthy oocytes and embryos *in vitro* requires a high level of understanding of gamete and early embryo physiology, especially of those processes critical for allowing their development *in vivo* and *in vitro*. Early embryos have been shown to be very sensitive to perturbation of their cell size (Baltz, 2001), implying that only an appropriate osmolarity and solute content of their environment allows proper preimplantation development.

Recently, the discovery of a novel cell volume-regulatory mechanism involving glycine accumulation via GLYT1 enhanced the understanding of how embryos control their volume at oviductal osmolarities (Steeves *et al.*, 2003). Osmoregulation in early human embryos occurs similarly as in the mouse, indicating that utilization of glycine and GLYT1 is a general cell-volume control mechanism employed by mammalian embryos (Hammer *et al.*, 2000). Glycine accumulation via GLYT1 probably represents a

major requirement for maintaining embryo viability under *in vivo*-like conditions. Although important improvements in female gamete culture have been accomplished in recent years, the ability to mature and obtain fertilizable oocytes *in vitro* at high rates, especially human oocytes, has had minimal success so far. Such competence is highly desirable, since it would avoid the risks of hyper-stimulation protocols in women undergoing infertility treatment. Once removed from its protective follicular environment, the immature GV oocyte not only has to adapt to an artificial environment with most likely sub-optimal conditions, but also has to progress correctly through meiotic and cytoplasmic maturation. Defining the key processes that maintain an oocyte healthy, including its behavior when interacting with the external environment *in vitro*, would nevertheless advance the oocyte culture. Similar mechanisms like those employed by early embryos, including cell volume regulatory mechanisms, might be critical for allowing oocyte survival and development *in vitro*. Although GLYT1 activity has been demonstrated to be present in oocytes by previous studies (Haghighat and Van Winkle, 1990), the exact moment of GLYT1 activation during development, as well as its possible utilization as an organic osmolyte transporter in oocytes was not known. Moreover, the manner in which the oocyte controls its volume before fertilization and before ovulation has never been addressed in previous studies.

Therefore, **the overall objective of my thesis was to determine the mechanisms of cell volume regulation in oocytes prior to fertilization.** By using the mouse as an animal model and a combination of functional studies and molecular biology techniques, I have investigated the exact moment of glycine transport initiation in oocytes, its relationship with the ovulatory process, and possible mechanisms controlling this

activation. Since this part of my project provided intriguing results, demonstrating that cell-volume regulatory glycine transport via GLYT1 is initiated only following stimulation of ovulation, I have also studied the manner in which the oocyte controls its volume before activation of GLYT1.

Information provided in my thesis revealed important aspects of cell volume regulation before fertilization, allowing a better understanding of oocyte physiology. Also, it clarified the requirements for maturing and maintaining healthy oocytes *in vitro*, a necessary step in the treatment of infertility and preservation of oocytes for women undergoing chemotherapy.

5. 2. Specific Aims and hypotheses

Specific Aim 1: Determine the presence, role and regulation of glycine transport mediated by GLYT1 in oocytes before fertilization. Recent studies suggest that the early preimplantation embryo regulates its volume by a novel mechanism involving the accumulation of glycine as an organic osmolyte, via the glycine transporter GLYT1 (Steeves *et al.*, 2003). Moreover, glycine transport seems to be critical for the fate of the embryo cultured at oviductal osmolarity (Dawson and Baltz, 1997). Although several reports have indicated glycine transport to be present in mouse oocytes at various stages of growth, including in fully-grown oocytes (Colonna and Mangia, 1983; Haghighat and Van Winkle, 1990), little is known about GLYT1 activity and its role before fertilization. *Therefore, I hypothesized that glycine transport and accumulation via GLYT1 are present before fertilization, in oocytes, within the ovary. I also hypothesized, that similarly with early embryos, the oocyte glycine accumulation via GLYT1 supports the maintenance of*

normal oocyte cell volume.

Specific Aim #2: Determine if the oocyte can regulate its volume independently before initiation of glycine transport.

My data obtained trying to address the above hypotheses generated intriguing results indicating that the osmosensitive glycine transport via GLYT1 is initiated in the oocyte only after stimulation of ovulation. Since cell volume-regulatory glycine accumulation mediated by GLYT1 allows the early embryo to control its volume, and this mechanism is absent before stimulation of ovulation, the possibility of a different mechanism regulating GV oocyte volume within the ovary arose, leading to my second specific aim.

The close physical contact between the oocyte and surrounding somatic cells, allowing bidirectional communication, is of critical importance for oocyte development and function. Because these connections must be maintained, and independent movement of the oocyte within the ZP would make this difficult, it is possible that the oocyte must remain in contact with the ZP rather than independently regulating its volume. *Therefore, I hypothesized that, prior to ovulation, the oocyte cannot independently regulate its volume, but rather relies on the ZP to determine its size.*

MATERIAL AND METHODS

1. Chemicals and solutions

1. 1. Chemicals and pharmaceutical agents

Chemicals and various pharmaceutical agents were purchased from Sigma (St. Louis, MO, USA) unless otherwise noted (Table 1, Table 2). All chemicals and solutions were embryo tested or cell culture grade.

1. 2. Solutions

1. 2. 1. Oocyte and embryo culture and handling media

Oocyte and embryo culture media were based on KSOM (K^+ supplemented Simplex Optimized Medium) embryo culture medium (Lawitts and Biggers, 1993), with glutamine omitted and polyvinyl alcohol (1mg/ml) substituted for BSA. 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), Bovine serum albumin (BSA, 1 mg/ml).

Hepes-KSOM containing 21 mM Hepes and 4 mM $NaHCO_3$ (pH adjusted with NaOH to 7.4) was used for oocyte, follicle and embryo collection and handling. The osmolarities of KSOM and Hepes-KSOM were 250 and 240 mOsM (± 5 mOsM), respectively. Osmolarity was increased with D(+)-raffinose or decreased to 150 mOsM by lowering NaCl to 45 mM. Osmolarities were measured and confirmed by osmometer (Vapro 5520, Wescor, Logan, UT, USA).

Table 1. List of chemicals or compounds utilized.

Name of chemical or compound	Source
4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES)	Sigma (St. Louis, MO, USA)
Agar	Sigma (St. Louis, MO, USA)
Ascorbic acid	Sigma (St. Louis, MO, USA)
Bovine serum albumin (BSA)	Sigma (St. Louis, MO, USA)
$CaCl_2$	Sigma (St. Louis, MO, USA)
Cacodylic acid	Sigma (St. Louis, MO, USA)
D(+)-raffinose	Sigma (St. Louis, MO, USA)
Dimethyl sulfoxide (DMSO)	Calbiochem (La Jolla, CA, USA)
EDTA (tetra Na)	Sigma (St. Louis, MO, USA)
Ethanol	Sigma (St. Louis, MO, USA)
Fetal bovine serum (FBS)	Invitrogen (Carlsbad, CA, USA)
Formaldehyde (37 % solution)	Sigma (St. Louis, MO, USA)
Glucose	Sigma (St. Louis, MO, USA)
Glutaraldehyde	Sigma (St. Louis, MO, USA)
Glycine	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)
Methanol	Sigma (St. Louis, MO, USA)
Methionine	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)
Osmium tetroxide	Sigma (St. Louis, MO, USA)
Phenylmethylsulphonyl fluoride (PMSF)	Sigma (St. Louis, MO, USA)
Potassium Hydroxide (KOH)	Sigma (St. Louis, MO, USA)
Selenium (Na salt)	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)
Trichloroacetic Acid	Sigma (St. Louis, MO, USA)
Tris	Sigma (St. Louis, MO, USA)
Triton X	Sigma (St. Louis, MO, USA)
Tween20	Sigma (St. Louis, MO, USA)

Table 2. List of pharmaceutical agents (see also Tables 4, 5, 6).

Name of pharmaceutical agent	**Action**	**Source**
18-α glycyrrhetinic acid (AGA)	Disruptor of gap junctional communication	Sigma (St. Louis, MO, USA)
Brefeldin A (BFA)	Golgi apparatus disruptor	Sigma (St. Louis, MO, USA)
Collagenase Type I	Enzyme used to digest neonatal ovaries	Sigma (St. Louis, MO, USA)
Cytochalasin D	Disruptor of F-actin filaments	Sigma (St. Louis, MO, USA)
Demecolcine	Microtubule depolymerizer	Sigma (St. Louis, MO, USA)
Dibutyryl cAMP (dbcAMP)	cAMP membrane permeable analog, used to maintain GV-arrest	Sigma (St. Louis, MO, USA)
DNAse	Enzyme used to digest neonatal ovaries	Sigma (St. Louis, MO, USA)
Forskolin	Agonist of adenylate cyclase, used to maintain GV-arrest	Sigma (St. Louis, MO, USA)
FSH (Follicle stimulating hormone)	Hormone used to support antral follicle culture *in vitro*	National hormone and peptide program (Harbor-UCLA Medical Center, CA)
hCG (human chorionic gonadotropin)	Hormone used to trigger ovulation in mice	Sigma (St. Louis, MO, USA)
Hyaluronidase	Enzyme used to disperse cumulus cells	Sigma (St. Louis, MO, USA)
Hypoxanthine	Purine with phosphodiesterase inhibitory activity, used to maintain GV-arrest	Sigma (St. Louis, MO, USA)
IBMX	Phosphodiesterase inhibitor, used to maintain GV-arrest	Sigma (St. Louis, MO, USA)
ORG23798 [bis(4-fluorophenyl) methylenepiperidineacetic acid, lithium salt]	GLYT1 specific inhibitor	Organon Laboratories (Scotland, UK)
PMSG (pregnant mares' serum gonadotropin)	Hormone used to stimulate follicle development	Sigma (St. Louis, MO, USA)
Protease inhibitor cocktail III	General inhibitor of proteases	Calbiochem (La Jolla, CA, USA)
Roscovitine	Inhibitor of CDK1, used to maintain GV-arrest	Sigma (St. Louis, MO, USA)
Tyrode's solution	Solution used to remove zona pellucida	Sigma (St. Louis, MO, USA)

1. 2. 2. Antral follicle culture media

For antral follicle culture (Sheng *et al.*, 2005), the following ingredients were added to commercial MEM with essential amino acids, without L-glutamine (Invitrogen, Carlsbad, CA): 10% FBS, 0.01 mM EDTA, 1% Penicillin, 50 μg/ml ascorbic acid, 2 ng/ml Selenium (Na salt), 100 ng/ml FSH (ovine pituitary, National hormone and peptide program, Harbor-UCLA Medical Center, CA).

2. Animals, oocytes, antral follicles and embryo manipulations

2. 1. Animals

Animal protocols were approved by the Animal Care Committee of the Ottawa Health Research Institute. All animals were procured from Charles River, St-Constant, PQ, Canada. CF1 strain of mouse was used to obtain growing, fully grown oocytes, and preimplantation embryos. This strain produces matured oocytes and embryos that are sensitive to *in vitro* conditions, including osmolarity (Biggers, 1998; Hadi *et al.*, 2005), and thus were chosen as the model system in which to investigate culture effects. To achieve fertilization, females were mated with B6D2F1 males (age 8-24 weeks) overnight. Animals were maintained on a 12 hr light : 12 hr dark cycle. All animals were sacrificed by cervical dislocation.

2. 2. Superovulation

In order to obtain a higher number of oocytes or preimplantation embryos, a standard superovulation protocol was utilized (Hogan *et al.*, 1994; Dawson and Baltz, 1997). Stimulation of ovarian folliculogenesis was achieved by using pregnant mares'

serum gonadotropin (PMSG), a FSH-like hormone, while ovulation was triggered with human chorionic gonadotropin (hCG), a LH-like hormone. Female mice, between 4 and 7 weeks old, were injected intraperitoneally (i. p.) with 5 IU PMSG. For GV oocytes, the injected female mice were kept in their cages until the time of oocyte collection (see below for timing of collection). To obtain *in vivo* matured MI and MII oocytes or embryos, mice were injected intraperitoneally with 5 IU hCG 47 hr after PMSG. For embryos, the female mice were mated with the males overnight immediately after hCG injection.

2. 3. Oocyte collection

Fully grown GV oocytes and unexpanded COCs were obtained 43-46 hr after PMSG injection as previously described (Phillips *et al.*, 2002) (Fig. 8). At the time of collection, the ovaries were surgically removed and mechanically minced with a razor blade in Hepes-KSOM solution. COCs were retrieved from the minced ovarian tissue and GV oocytes were isolated by gentle, repeated pipetting of COCs with a flame-pulled pipette, allowing removal of cumulus cells. In one experiment, COCs were released by puncturing the antral follicles visible on the ovary surface with a fine needle.

To obtain ***in vitro*** **MI and matured MII oocytes**, GV oocytes were cultured for 1.5 to 4 hr or overnight (22 hr), respectively in KSOM microdrop cultures (Wassarman *et al.*, 1979).

In order to maintain the **oocytes in GV stage arrest**, 100 μM dbcAMP was added to the collection and culture media (see below).

MI oocytes that developed *in vivo* and expanded COCs were obtained between

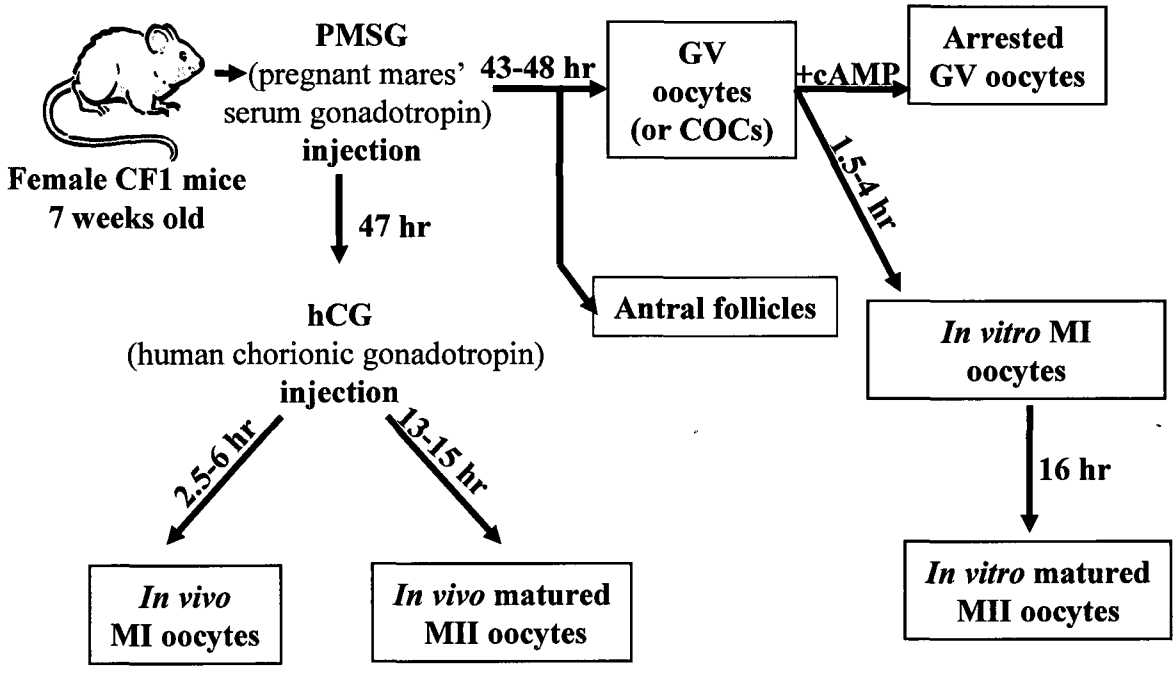


Figure 8. Schematic representation of steps utilized to collect oocytes at different stages of maturation *in vivo* or *in vitro*. Female mice, approximately 7 weeks old, were injected intraperitoneally with PMSG (pregnant mares' serum gonadotropin) to stimulate ovarian follicle growth. Fully grown GV oocytes and unexpanded COCs were obtained 43-48 hours later. GV oocytes were isolated by gentle, repeated pipetting of COCs. To maintain the oocytes in GV-arrest, cAMP was added to the culture media. To obtain MI oocytes that developed *in vitro*, the GV oocytes were cultured between 1.5 and 4 hr. In order to obtain matured MII oocytes *in vitro*, the GV oocytes were cultured for 16-20 hr. *In vivo* MI and matured MII oocytes were obtained 2.5-6 hr and 13-15 hr, respectively, subsequent to ovulation stimulation by intraperitoneal injection with hCG (human chorionic gonadotropin). Antral follicles were obtained 43-48 hr after PMSG injection.

2 and 6 hours after hCG injection. An identical method as described for fully grown oocytes was utilized. When needed (between 4-6 hr after hCG, when the cumulus had expanded), Hepes-KSOM containing 300μg/ml hyaluronidase and mechanical pipetting were used to remove the expanded cumulus cells surrounding the MI oocytes.

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

Growing oocytes were obtained from female mice on days 9 – 21 postnatally. During the immediate postnatal period, a synchronized wave of primordial follicles initiates follicular development simultaneously. Thus, oocyte and follicle size can be directly related to postnatal age (Dissen *et al.*, 2004) and a relatively homogeneous populations of growing oocytes at a particular stage can be obtained from neonatal mice. At the appropriate neonatal age, ovaries were digested enzymatically for 30 minutes by collagenase (Type I, 2 mg/ml) and DNAse (0.01 mg/ml) in Ca^{2+} and Mg^{2+}-free KSOM (Eppig, 1976). Alternatively (where specified), neonatal ovaries were finely minced in Hepes-KSOM with a razor blade. Following release from ovaries, oocytes were isolated from the minced ovaries, gently cleaned from the surrounding cumulus cells with a flame-pulled pipette, and washed in 3 consecutive drops of Hepes-KSOM. When needed, all oocytes from juvenile females of a given age were measured immediately after collection by using an eyepiece reticule on an inverted Nikon Diaphot microscope with a 40X objective or digital images (see Volume measurements).

2. 4. Preimplantation embryo collection

One-cell embryos were collected approximately 24 hr after ovulation was triggered by hCG. Embryos were flushed from surgically removed oviducts with Hepes-KSOM. Hyaluronidase (300 μg/ml) was added to the Hepes-KSOM to facilitate dispersion of cumulus cells. After embryos were transferred through 5 successive wash drops of Hepes-KSOM to remove any residual hyaluronidase, fertilization was confirmed by observing the presence of two pronuclei (Hogan *et al.*, 1994).

Two-cell, eight-cell, and morula embryos were obtained by oviduct flushing with Hepes-KSOM, 42-44 hr, 66-68 hr and 76 hr, respectively following hCG (Hogan *et al.*, 1994).

Blastocysts were retrieved 93-94 hr subsequent to hCG injection, by flushing excised uterine horns (Hogan *et al.*, 1994).

2. 5. Embryo and oocyte culture

Oocytes or embryos were cultured according to standard techniques routinely utilized in our laboratory (Dawson and Baltz, 1997). In each replicate, oocytes or embryos obtained from several female mice were pooled. Droplets of 50 μl of culture medium were placed in 35 mm tissue culture dishes (Falcon # 3001, Fisher, Pittsburgh, PA, USA) and overlaid with KSOM-washed mineral oil. These microdrop cultures were pre-equilibrated in an incubator with 5% CO_2 in air at 37°C and 100% humidity (Lawitts and Biggers, 1993), for minimum 2 hr prior to utilization. Oocytes and embryos were placed into drops and dishes were returned and retained in the incubator for various amounts of time, according to experimental requirements.

All oocytes and embryos were manipulated and moved by using flame-pulled, mouth operated Pasteur pipettes. Before placement in culture, they were washed in a minimum of 3 drops of media containing the same treatment as the final microdrop to eliminate any carryover of media from collection or previous treatment steps.

2. 6. Antral follicle collection and culture

Antral follicles were obtained 43-48 hr after PMSG injection. Individual antral follicles were dissected from intact ovaries in Hepes-KSOM by carefully removing the ovarian tissue surrounding the follicle with two fine tweezers. Antral follicles were considered healthy and selected for culture if they exhibit a clear antrum containing a visible COC. In order to remove any left-over debris, follicles were thoroughly washed through 2 ml of antral follicle media. Subsequently, they were transferred to organ dishes (Falcon # 3037, Fisher, Pittsburgh, PA, USA) (between 10 and 15 follicles per dish) containing antral follicle media (1 ml/dish) with or without test treatment, based on experiment requirements. Antral follicles were incubated in 5% CO_2 in air at 37°C and 100% humidity for the amount of time specified. At the end of culture, follicles were punctured, oocytes retrieved and utilized for subsequent experimental steps (Sheng *et al.*, 2005).

A very small number of follicles exhibited a dark, opaque antrum while the oocyte inside was dead or were not fully enclosed by multiple layers of cumulus cells at the end of culture. These follicles were considered atretic or otherwise unhealthy and were excluded from experiments (Downs, 2000). Throughout antral follicle culture, culture media was pre-equilibrated in organ dishes in an incubator with 5% CO_2 in air at

37°C and 100% humidity for a minimum of 2 hours prior to utilization. All follicles were carefully manipulated and transferred one by one by using fine tweezers, when needed.

2. 7. Removal of zona pellucida

Several experiments required that the extracellular matrix of the oocyte, the zona pellucida, be removed. Groups of 10-15 oocytes were briefly exposed (30 seconds) to 100 µl of warm acid Tyrode's solution (Sigma St. Louis, MO, USA) (Hogan *et al.*, 1994). Oocytes that had the zona successfully dissolved, as assessed visually, were transferred through 5 successive drops of Hepes-KSOM to wash away any excess Tyrode's solution and neutralize the acidity. Subsequently they were incubated for 5-10 minutes in KSOM (incubation conditions as above) to allow recovery before utilization for the next experimental steps.

2. 8. Oocyte microinjections

Oocyte microinjection was performed using Narishige micromanipulators mounted on a Zeiss Axiovert microscope fitted with Hoffmann optics. Injections were performed in a drop of Hepes-KSOM under oil. Oocytes were immobilized using a holding pipette (Humagen Fertility Diagnostics, Charlottesville, VA) while the injection solution was delivered through a preloaded injection pipette. Holding and injection pipette pressure, and delivery of the injection volume were achieved by pressure-pulse controlled by a microinjection apparatus (Harvard Apparatus PLI-100, Holliston, MA, USA). For intracytoplasmic injections, injection volumes were an estimated 5% of oocyte volume based upon cytoplasmic displacement. When mineral oil was microinjected

between oocyte membrane and zona pellucida, the microinjection pipette tip was aligned at oocyte-zona interface and the injection volume was visually estimated between 5-10% of oocyte volume.

3. Radiolabeled compound measurements

3. 1. Rate of transport measurements in oocytes and embryos using ^{3}H- labeled amino acids

[^{3}H]Glycine ([2-^{3}H]glycine; ~20 Ci/mmol; Amersham Pharmacia, Arlington Heights, IL, USA) was added directly to the culture media. Since previous studies indicated that glycine transport was linear for at least one hour (Dawson *et al.*, 1998; Steeves and Baltz, 2005), I chose to measure 10 minutes uptake rates. Thus, **the rate of transport of [^{3}H]glycine** was measured by incubating oocytes or embryos for a period of 10 minutes in the presence of 10 μM [^{3}H]glycine. Following oocyte or embryo culture in ^{3}H- labeled amino acid containing medium under conditions specific to each experiment, 5-10 oocytes or embryos were removed and washed through 5 successive drops of ice-cold Hepes-KSOM. Subsequently, the oocytes or embryos were transferred to scintillation vials together with 4 ml of scintillation fluid (Scintiverse BD, Fisher Scientific, Pittsburgh, PA, USA). ^{3}H content of oocytes or embryos was quantified using a scintillation counter (2200CA TriCarb, Packard Instrument Co., Downer's Grove, IL, USA) with a 5 minute measurement 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 oocyte or embryo samples. To allow

conversion from counts per minute to glycine concentrations, standard curves were constructed monthly by counting serial dilutions of the ^{3}H- labeled amino acid. The rate of glycine transport was calculated as the molar amount of glycine per oocyte or embryo per minute per micromolar.

In one experiment, I have measured **the rate of transport of [^{3}H]leucine** ([4,5-^{3}H]leucine; ~250 μCi/mmol; Amersham Pharmacia, Arlington Heights, IL, USA) by using a similar method as for rate of transport of [^{3}H]glycine, with the exception that 1μM instead of 10 μM of [^{3}H]leucine was used. The rate of leucine transport was calculated as for glycine.

3. 2. ^{3}H- labeled glycine accumulation measurements in oocytes and embryos

In one set of experiments, **[^{3}H]glycine accumulation measurements** were made by incubating the oocytes or embryos for longer periods of time (up to 3 hr) in 1 mM total glycine. To avoid the toxic effects of accumulated radioactivity, a mixture of [^{3}H]glycine and glycine was used at a fixed ratio of 1:1000, since [^{3}H]glycine and unlabeled glycine are similarly used for transport and cell metabolism (Dawson *et al.*, 1998). After similar washing steps in ice-cold Hepes-KSOM as the ones described above, ^{3}H in oocytes or embryos was measured by liquid scintillation counter (see above), with 5–10 oocytes or embryos pooled for each replicate. The total molar amount of accumulated glycine was expressed per oocyte or embryo.

3. 3. Measurements of ^{35}S-labeled methionine incorporation rate into proteins in oocytes

[^{35}S]methionine (L-[^{35}S]methionine; ~1000 Ci/mmol; Amersham Pharmacia, Arlington Heights, IL, USA) was added directly to the culture media. Standard curves from serial dilutions for conversion of cpm to concentration were generated every time ^{35}S was measured, in a similar manner as for [^{3}H]glycine conversion. ^{35}S was detected for 5 minutes by using a scintillation counter (2200CA TriCarb, Packard Instrument Co., Downer's Grove, IL, USA). Oocytes were manipulated by using pressure-guided (pressure controlled by a plastic bulb inserted at the end of the glass pipette) instead of mouth-operated pipettes, and all manipulations were carried out behind a Plexiglas shield and monitored with a Geiger counter.

Immediately after collection, freshly-collected GV oocytes were cultured in the presence of 1 μM [^{35}S]methionine. Since this experiment was designed to measure the rate of [^{35}S]methionine incorporation when protein synthesis is inhibited, the oocytes were maintained in GV-arrest (by 100 μM dbcAMP, see below) in the presence or absence of the protein synthesis inhibitor Cycloheximide (50μg/ml, see below). Subsequent to 1 hr or 4 hr culture in these conditions, groups of 10 oocytes were washed through 5 drops of ice-cold Hepes-KSOM and transferred to a tube containing 20 μl distilled water, 1 mg cold methionine and 1 mg BSA [adapted from (Borland and Tasca, 1974)]. The oocytes were lysed using 4 freeze-thaw cycles (tubes containing oocytes were dipped in liquid nitrogen until frozen, followed by thawing with warm tap water). To precipitate proteins, ice-cold 10% Trichloroacetic acid (TCA) was added to the samples, followed by robust vortexing. Tubes were stored on ice for 10 min to allow

precipitation, and then centrifuged for 10 min at 5000×g followed by supernatant removal. After the last step was repeated one more time, the pellet containing the precipitated proteins was dissolved in 100 µl of 10% KOH for 30 min, transferred to a vial containing 4 ml of scintillation fluid and utilized for ^{35}S measurements. A small amount of medium from the last washing drop was treated similarly and used for background count measurements that subsequently were subtracted from the counts obtained from the oocyte samples. The total amount of incorporated methionine was expressed as fmol per oocyte.

4. Endogenous cytosolic glycine measurements by amino acid microanalysis

In order to measure the TCA-soluble glycine fraction, oocytes and embryos were processed as previously described (Steeves *et al.*, 2003). Groups of oocytes or embryos were rinsed immediately after collection on watch glasses coated with gelatin. To remove any collection media carryover, the 2 rinse drops contained Hepes-KSOM, no glutamine, no glucose, no PVA. Subsequently, groups of 90 oocytes or embryos were pooled and transferred to 20 µl of distilled water. A background sample was collected from the last rinse drop in a separate tube. After 4 cycles of freeze-thaw, ensuring cell lysis, protein precipitation was achieved by adding 100 µl of ice-cold 10% TCA. Following centrifugation at 5000×g for 20 min, 120 µl of lysate supernatant (TCA-soluble fraction) was removed, placed in a new tube and kept at -20°C until quantification.

TCA-soluble glycine in each sample was measured in collaboration with Dr. Ajoy Basak (Ottawa Health Research Institute), by using a BioLC system equipped with an AminoPac PA10 anion-exchange analytical and guard columns (Dionex Co., Sunnyvale,

CA, USA) at 30°C. The column was prewashed with three eluents: water, NaOH (250 mM) and sodium acetate (1 M), employed to separate common amino acids. Standards were prepared by dissolving a known amount of glycine in 8% TCA. Peak analysis was performed using the PeakNet v6.4 software.

5. Molecular biology techniques

5. 1. Western-blot

The following solutions were used for Western Blot analysis: Lysis buffer containing 10 mg/ml SDS (Sigma, St. Louis, MO, USA) and 1mM PMSF (Sigma, St. Louis, MO, USA) in PBS; Protease inhibitor cocktail III (Calbiochem, La Jolla, CA, USA); 2XLaemmli Buffer (Sigma, St. Louis, MO, USA); 10X SDS PAGE Buffer (AMRESCO Inc, Solon, OH, USA); Transfer Buffer containing Tris 25mM Sigma (St. Louis, MO, USA) and 192mM glycine (Sigma, St. Louis, MO, USA) 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 (Sigma, St. Louis, MO, USA) in TBS buffer; Blocking solution containing 1% BSA in TBS; Antibody solution containing 1% BSA in TTBS; Prestained Protein Molecular Weight Marker (Fermentas, Hanover, MD, USA).

Approximately 400 freshly-collected GV oocytes and an equal number of MI oocytes that developed *in vitro* were each lysed by repeated freeze-thaw in 15 µl of lysis buffer containing protease inhibitor cocktail (cocktail:lysis buffer 1:100). Brain homogenates, whose protein amount was measured by Bradford assay (Bio-Rad DC protein assay), were adjusted to approximate the estimated protein concentration in

oocyte samples, assuming ~23 ng total protein in one mouse oocyte (Sternlicht and Schultz, 1981). A brain sample was loaded in an adjacent well to the embryo samples and used as a positive control.

Samples mixed with equal amounts of Laemlli buffer and 1% 2-mercaptoethanol were boiled for 10 min at 100°C, separated by SDS-PAGE on 4-15% Tris-HCl precast gels (BioRad, Life Science Group, Mississauga, ON, Canada) (1 hr at 150V), and subsequently transferred to Protran nitrocellulose (0.45μm) (Whatman, Florham Park, NJ, USA) (for 1 hr at 100V, Bio-Rad electroblotting system). After blocking nonspecific protein binding with 1% BSA in TBS (1 hr at room temperature), each blot was incubated with affinity-purified antiserum (1:500) raised against the carboxy terminus of rabbit GLYT1 (Zafra et al., 1995) overnight and then with goat anti-rabbit Alexa 488 secondary antibody (Invitrogen, Carlsbad, CA, USA). After visualizing bands, blots were re-probed with rabbit anti-β actin antiserum (Abcam, Cambridge, MA, USA) which was used to confirm equal loading. Bands were visualized and quantified with an Image Quant 5.2 Typhoon 8600 Scanner (blot excited with 532 nm wave length and signal collected through a 562 nm emission filter) and Image Quant 5.2 software for analysis (Molecular Dynamics, Sunnyvale, CA, USA).

5. 2. RT-PCR

The following materials were utilized to extract RNA and conduct RT-PCR: RNA extraction kit, RNAeasy Micro (Qiagen, Mississauga, ON, Canada); Reverse transcription kit : Retroscript (Ambion, Austin, TX, Canada); PCR kit: HotStarTaq (Qiagen, Mississauga, ON, Canada); DNA agarose gel extraction kit: QIAquick gel

extraction (Qiagen, Mississauga, ON, Canada).

Groups of 30 oocytes or embryos were used for RNA extraction (RNAeasy Micro, Qiagen) and reverse transcribed (Retroscript, Ambion), according to manufacturers' instructions. Liver (GLYT1a and GLYT1b) and brain (GLYT1b) were used as positive controls, using an amount of cDNA approximately corresponding with that of three oocytes based upon spectrophotometric measurement (Ultraspec 2000, Pharmacia Biotech, Cambridge, England) of RNA content of control tissue preparations and the known RNA content of one oocyte (~0.6 ng) (Sternlicht and Schultz, 1981). PCR (40 cycles, 60°C annealing temperature) (HotStarTaq, Qiagen) was performed on cDNA aliquots equivalent to three oocytes or embryos. Samples of the final drop of medium in which the oocytes or embryos were washed, treated identically, served as negative controls.

PCR was repeated on two different oocyte and embryo preparations. Amplicons were visualized on a 3% agarose gel containing 0.13 μg/ml ethidium bromide. Primers for peptidylprolyl isomerase A (PPIA), a housekeeping gene expressed throughout early developmental stages (from oocyte to blastocyst) (Mamo *et al.*, 2007), encoding an enzyme catalyzing the cis-trans isomerization of peptide bonds N-terminal to proline residues, were utilized to confirm correct RNA extraction, reverse transcription, PCR and loading.

To distinguish between the GLYT1 isoforms, GLYT1a (consensus sequence NM_008135) and GLYT1b (M88595), a common 3' primer coupled with isoform-specific 5' primers were used (Table 3). Primers were obtained from Invitrogen (Carlsbad, CA, USA). All products were of the predicted size based on their position on

the gel: 108 bp for GLYT1a, 105 bp for GLYT1b and 150 bp for PPIA. After DNA agarose gel extraction (QIAquick gel extraction, Qiagen), the identity of GLYT1a products was confirmed by direct sequencing (100% identity to predicted sequence).

Table 3. Primers used for isoform-specific detection of GLYT1 mRNA by RT-PCR

Primer	**sequence (5'→3')**	**mRNA sequence source**	**amplicon**
Glyt1a	aaaaggtgccaaagggatgtt	GenBank NM_008135 mouse Glyt1a	108 bp
Glyt1b	cacctcttccccagaacaga	GenBank M88595 rat Glyt1b	105 bp
Glyt1 common	gtcagtacaaactcgatctggtt	both	
PPIA	cgcgtctccttcgagctgtttg tgtaaagtcaccaccctggcacat	GenBank NM_008907 mouse PPIA	150 bp

The GLYT1a and common primer sequences were obtained from PrimerBank (ID 6680029a1;http://pga.mgh.harvard.edu/primerbank/).
Primer 3 (http://frodo.wi.mit.edu/cgi-bin /primer3/primer3_www.cgi) was then used to design a 5' primer to rat GLYT1b compatible with the common primer. These portions of the rat and mouse gene sequence are 100% identical, and so the deposited rat Glyt1b (M88595) was used. The source of PPIA primers was Mamo et al., 2007.

5. 3. Immunocytochemistry

The following solutions were utilized for immunocytochemistry: Rinse solution: Phosphate-buffered saline (PBS) (Invitrogen, Carlsbad, CA, USA); Fixing solution: 50% Methanol (Sigma, St. Louis, MO, USA) plus 50% DMSO (Calbiochem, La Jolla, CA, USA), or alternatively 2% formaldehyde solution (Sigma, St. Louis, MO, USA) in PBS; Permeabilizing solution: 0.1% Triton X (Sigma, St. Louis, MO, USA) in PBS; Blocking solution: 2% FBS, 2% BSA in PBS; Mounting solution: SlowFade Antifade (Invitrogen, Carlsbad, CA, USA).

Groups of 5-10 oocytes were fixed on coverslips for 2.5 minutes in 50% Methanol, 50% DMSO. Following fixation and 3 rounds of washing in PBS, nonspecific protein binding was blocked by incubating oocytes at 37°C for 1 hour in 2% FBS, 2% BSA. Subsequently, the oocytes were rinsed in PBS and incubated overnight at 4° C with primary antiserum followed by 3 hr with secondary antibody. Antisera used were affinity-purified antiserum (1:200) raised against the carboxy terminus of rabbit GLYT1 (Zafra *et al.*, 1995) with goat anti-rabbit Alexa 488 secondary (1:500; Invitrogen, Carlsbad, CA, USA), or goat anti-GLYT1 carboxy terminal peptide antiserum (1:1000; Chemicon, Millipore, Billerica, MA, USA) with donkey anti-goat Alexa 488 secondary antibody (1:750; Invitrogen).

For the anti-GLYT1 peptide antiserum, co-incubation with the immunogenic peptide (Chemicon, Millipore, Billerica, MA, USA) or an irrelevant peptide (from mouse anion exchanger 2; Santa Cruz Biothechnology, Santa Cruz, CA, USA) served as controls. After the oocytes were individually mounted in 5 µl of SlowFade mounting solution on glass slides, immunofluorescence was examined by confocal microscopy

(Nikon Diaphot 200 with MRC-1024 Laser Sharp, BioRad, Life Science Group, Mississauga, ON, Canada). Oocyte samples were excited with the 488 nm line of the laser of the confocal microscope and emission was collected using a 522-535 nm bandpass filter. The optic sections' thickness measured 2 μm and 2.5 μm for samples exposed to Chemicon anti-GLYT1 antiserum and Zafra's anti-GLYT1 antiserum, respectively, with a 2 μm distance between each section.

Alternatively, a milder fixation protocol was tested as well. For this, groups of 15 oocytes were exposed to 2% formaldehyde, washed in PBS, permeabilized with 0.1% Triton X, washed in PBS and blocked in 2% FBS and 2% BSA (37°C, 1 hour). Subsequent labeling and confocal microscopy steps were identical to the ones described above. Unfortunately, this method of fixing oocytes proved to be ineffective when Zafra anti-GLYT1 antiserum was utilized. Moreover, a weaker, inconsistent signal was visualized when Chemicon anti-GLYT1 antiserum was used.

5. 4. Electron microscopy

The following solutions were utilized throughout oocyte preparation for electron microscopy: Rinse solution: 0.1 M cacodylate buffer (Sigma, St. Louis, MO, USA) (pH 7.4) in distilled water; Fixing solution: 3% glutaraldehyde (Sigma, St. Louis, MO, USA) in 0.1 M cacodylate buffer; Embedding solution: 1% agar (Sigma, St. Louis, MO, USA) in 0.1 M cacodylate buffer (gelling temperature 32-35°C); Osmicating solution: 1% osmium tetroxide (Sigma, St. Louis, MO, USA) in 0.1 M cacodylate buffer; Dehydrating solutions: ethanol (Sigma, St. Louis, MO, USA) dilutions of 30%, 50%, 75%, 95% and 100% in distilled water; Infiltration solution: 100% ethanol:Spurr's plastic (SPI Supplies,

West Chester, PA, USA) (1:1); Plasticizing material: low viscosity "Spurr" Kit (SPI Supplies, West Chester, PA, USA).

For transmission electron microscopy (TEM), oocytes were fixed, embedded and processed as previously described (Dandekar *et al.*, 1995). After being exposed to various experimental conditions based on experimental requirements, oocytes were fixed in 3% glutaraldehyde at room temperature for 2 hr. After 2 consecutive rinses through 1 ml of 0.1 M cacodylate buffer, individual oocytes were embedded in small drops of melted 1% agar. Following solidification, the excess agar was trimmed away in a cubical shape as close as possible to the oocyte membrane. The oocytes were osmicated by transferring the agar cubes for 45 min to 1% osmium tetroxide solution. After thorough washing in distilled water, the agar blocks were dehydrated by exposure to a graded series of ethanol dilutions (30%, 50%, 75%, 95% and 100%, 15 min each). The dehydrated agar blocks were infiltrated in 1:1 100% ethanol:Spurr's plastic for 24 hr at 4°C, followed by transfer into embedding molds containing pure Spurr's plastic and polymerization at 70°C for 16 hr. They were then shipped to our collaborator, Dr. Prue Talbot, in the Biology Department at the University of California, Riverside. Thin sections were cut on a Sorvall microtome using a diamond knife, prepared as previously described (Dandekar *et al.*, 1995), and TEM images obtained with a Phillips 500 Transmission Electron Microscope at the University of California, Riverside.

6. Pharmacological manipulations

6. 1. GLYT1 inhibition by ORG23798

ORG23798 [bis(4-fluorophenyl)methylenepiperidineacetic acid, lithium salt] was

synthesized and provided by Organon Laboratories, Scotland, UK. Its specific inhibitory action on GLYT1 transport has been previously demonstrated in cleavage stage embryos and Chinese hamster ovary cells expressing GLYT1 and GLYT2 (Steeves *et al.*, 2003). ORG23798 5mM stocks were prepared in DMSO from dry powder and stored at -20°C. When used, the stock was diluted directly into the media (1:1000) to reach a final concentration of 5 μM as previously described (Steeves *et al.*, 2003). A control group containing 0.1% DMSO (vehicle) was always run in parallel and used to compare any experimental outcome of the ORG23798 group.

6. 2. Maintenance of meiotic arrest

Maintenance of prophase I arrest (GV-arrest) depends on maintenance of a high intracellular level of cAMP (see Introduction). Throughout this project, GV-arrest was normally maintained when needed by elevating the intracellular cAMP with the membrane permeable analog **dibutyryl cAMP (dbcAMP)** (Sigma, St. Louis, MO, USA) (Cho *et al.*, 1974). After preliminary experiments to determine the percentage of oocytes maintained in GV arrest at different dbcAMP concentrations (from 0 to 120 μM) at different time points (from 1 to 24 hr) following oocyte removal from the ovarian follicle (not shown) 100 μM was chosen for all further work as the minimum concentration at which 100% oocytes maintained GV arrest indefinitely. dbcAMP 25 mM stocks were prepared in water from dry powder and stored at -20°C for a maximum of 1 month.

In one experiment, other agents known to maintained the GV-arrest were utilized: **hypoxanthine** (Sigma, St. Louis, MO, USA) (Eppig *et al.*, 1985; Downs, 1993) (4 mM), a purine with phosphodiesterase inhibitory activity; **forskolin** (Sigma, St. Louis, MO,

USA) (Dekel *et al.*, 1984) (100 μM), agonist of adenylate cyclase; **IBMX** (Sigma, St. Louis, MO, USA) (Downs *et al.*, 1989) (200 μM), a phosphodiesterase inhibitor; **Roscovitine** (Sigma, St. Louis, MO, USA) (Meijer *et al.*, 1997) (50 μM), an inhibitor of CDK1 activity. Hypoxanthine was added as a dry powder directly to culture media to a final concentration of 4 mM. Forskolin, IBMX and Roscovitine were prepared as stocks of 100 mM, 200 mM and 50 mM, respectively, stored at -20°C and diluted into the media when needed (1:1000). For Roscovitine, utilization of a mineral oil overlay for the culture drops was avoided since it interfered with drug efficacy (Kolajova *et al.*, 2001). Therefore, microdrop culture was replaced, and oocytes were instead cultured in organ dishes containing 1 ml of culture media with Roscovitine. Maintenance of GV-arrest was confirmed for all drugs at 4 hr following oocyte exposure by the presence of an intact germinal vesicle.

6. 3. Disruption of protein synthesis

Protein synthesis in oocytes was inhibited by exposure to the classic protein synthesis inhibitor Cycloheximide (DeSousa *et al.*, 1993). Stocks of 50 μg/μl were prepared by dissolving the dry powder in distilled water. Stocks were stored at - 20°C. The final concentration of 50 μg/ml was obtained by dissolving the stock directly to media (1:1000). The efficiency of inhibition was tested by measuring the ^{35}S-labeled methionine incorporation rate into proteins (see above).

6. 4. Disruption of cytoskeleton, Golgi apparatus, and vesicle fusion

Many modes of protein trafficking to the membrane require an intact cell

cytoskeleton, with functional microtubules and microfilaments. Disruption of cytoskeletal elements was achieved by oocyte exposure to **Demecolcine** (Sigma, St. Louis, MO, USA) (Schatten *et al.*, 1989) (1 μg/ml) to induce microtubule depolymerization or **Cytochalasin D** (Sigma, St. Louis, MO, USA) (Schatten *et al.*, 1989) (10 μM) to block F-actin polymerization. **Brefeldin A** (Sigma, St. Louis, MO, USA) (BFA, 5 μg/ml) (Dinter and Berger, 1998) was used to cause disturbance of intracellular protein traffic from the endoplasmic reticulum to the Golgi apparatus and the disruption of Golgi apparatus morphology. Stocks for all drugs were prepared from dry powders in DMSO (Demecolcine 1 μg/μl; Cytochalasin D 10 mM; BFA 5 μg/μl), stored at -20°C until use, and added directly to the media 1:1000 when needed. The efficiency of the working drug concentration was confirmed by the inability of oocyte progression through first meiosis (microtubules are required to form the meiotic spindle) for Demecolcine, or by the specific phenotype (collapsed cell structure) and oocyte inability to extrude first polar body (Wassarman *et al.*, 1979) for Cytochalasin. The efficacy of brefeldin A was confirmed in our lab previously (Anas *et al.*, 2008; Wang *et al.*, 2008).

To attempt to disrupt vesicle fusion with the plasma membrane in experiments designed to assess a requirement for protein insertion into the membrane, various Clostridial neurotoxins targeting SNARE (soluble N-ethylmaleimid-sensitive factor attachment protein receptors) were utilized (Humeau *et al.*, 2000) (Table 4). **Tetanus toxin** stock aliquots of 1 mg/ml were kept at -80°C and resuspended in media when needed (final concentration 10 μg/ml) or utilized for oocyte microinjection. The effect of

Table 4. The effect of vesicle fusion inhibitors on glycine transport in oocytes

Inhibitor	**Concentration**	**General action**	**Effect on development of glycine transport in oocytes after 4hr of exposure or microinjection**	**Source**
Tetanus Toxin (exposure or microinjection)	1 mg/ml	Inhibitor of vesicle fusion by cleaving VAMP3 SNARE	No effect	Sigma (St. Louis, MO, USA)
Tetanus Toxin light chain recombinant (microinjection)	1 mg/ml	Inhibitor of vesicle fusion by cleaving VAMP3 SNARE	No effect	Cedarlane Laboratories (Burlington, ON, Canada)
Botulinum Toxin A light chain recombinant (microinjection)	0.5 mg/ml	Inhibitor of vesicle fusion by cleaving SNAP23 SNARE	No effect	Cedarlane Laboratories (Burlington, ON, Canada)
Botulinum Toxin C light chain recombinant (microinjection)	1 mg/ml	Inhibitor of vesicle fusion by cleaving Stx2, Stx3 SNARE	No effect	Cedarlane Laboratories (Burlington, ON, Canada)
Tetanus Toxin+ Botulinum Toxin A+ Botulinum Toxin C (all light chain recombinant) (microinjection)	As above	See above	No effect	Cedarlane Laboratories (Burlington, ON, Canada)

Tetanus toxin either following oocyte exposure in culture or upon oocyte microinjection was followed. In neurons, the heavy chain of the Clostridial neurotoxins binds to specific receptors allowing penetration of the light chain, which in turn cleaves SNARE preventing fusion of docked synaptic vesicles (Humeau *et al.*, 2000). Oocytes were microinjected with the light chain recombinant of various neurotoxins: **Tetanus toxin light chain recombinant** (1 mg/ml); **Botulinum Toxin A light chain recombinant** (0.5 mg/ml); **Botulinum Toxin C light chain recombinant** (1 mg/ml); the light chains of Tetanus toxin plus Botulinum Toxin A plus Botulinum Toxin C at the concentrations mentioned above. The microinjected volume of the toxin stocks was approximately equal with 5% oocyte volume, leading to a final toxin dilution of 1:20 within oocyte cytoplasm.

6. 5. Disruption of different signaling pathways

The potential involvement of phosphorylation/dephosphorylation processes in various aspects of this project was studied by using a series of kinase inhibitors, kinase activators or phosphatase inhibitors (Table 5). All stocks were stored at -20°C and added directly to media when needed. Efficiency of PMA (Cuthbertson and Cobbold, 1985) and Okadaic acid (Phillips *et al.*, 2002) was confirmed by their ability to parthenogenetically activate matured MII oocytes, revealed by the extrusion of second polar body.

Membrane cholesterol depletion and intracellular Ca^{2+} chelation were accomplished by exposure to **Methyl-Beta-Cyclodextrin** (Sadler and Jacobs, 2004) and **BAPTA**-AM (Strayer *et al.*, 1999), respectively (Table 5).

Table 5. List of pharmaceutical agents targeting various signaling pathways

Inhibitor or activator	Vehicle	Stock	Final concentration	General action	Source	Reference
Staurosporine	DMSO	100 mM	100 μM	General kinase inhibitor	Sigma (St. Louis, MO, USA)	(Tamakoi, 1991)
PMA (phorbol 12-myristate 13-acetate)	DMSO	50 μg/ml	50 ng/ml	PKC activator	Sigma (St. Louis, MO, USA)	(Gomeza *et al.*, 1995)
Okadaic acid	DMSO	2.5 mM	2.5 μM	Inhibitor of serine/threonine phosphatases	Sigma (St. Louis, MO, USA)	(Moos *et al.*, 1995)
β-glycerophosphate	Water	10 M	10 mM	Serine/threonine phosphatases inhibitor	Sigma (St. Louis, MO, USA)	(Huang *et al.*, 2003)
Sodium Orthovanadate	Water	1 M	1 mM	Protein Tyrosine phosphatase inhibitor	Sigma (St. Louis, MO, USA)	(Kim *et al.*, 1999)
Methyl-Beta-Cyclodextrin	Media	-	4 mM	Induces membrane cholesterol depletion and disruption of lipid rafts	Sigma (St. Louis, MO, USA)	(Sadler and Jacobs, 2004)
BAPTA-AM N, N'[1,2-ethanediyl bis(oxy-2,1-phenylene)]bis[N-[2-](acetyloxy)-methoxy]2-oxoethyl]]-,bis[(acetyloxy)methyl]ester	DMSO	50 mM	50 μM	Intracellular Ca^{2+} chelator	Sigma (St. Louis, MO, USA)	(Strayer *et al.*, 1999)

6. 6. Protease activity inhibition

In one set of experiments trying to elucidate the nature of adherence between oocyte and ZP, I attempted to disrupt various protease activities. Various protease inhibitors were tested (Table 6). All stocks were stored at -20°C and added directly to media when needed. If a biological effect on oocyte-ZP adherence was observed, the inhibitor's possible toxicity was tested by following the ability of fresh GV oocytes to survive and undergo meiotic maturation following drug exposure.

6. 7. Disruption of gap junction communication

Gap junctional communication was disrupted by 18-α glycyrrhetinic acid (100 μM, AGA), a drug inducing connexin (the structural unit of a gap junction) phosphorylation, followed by disruption of gap junction structure (Evans and Boitano, 2001). At this concentration, AGA has been shown previously by our laboratory to interfere with dye transfer from the oocyte to surrounding granulosa cells when fluorescein was injected into follicle-enclosed oocytes, proving its efficacy in interrupting the functionality of gap junctions in ovarian follicles (Fitzharris and Baltz, 2006). AGA stock was made fresh every time from dry powder dissolved in DMSO at a concentration of 100 mM. The final working concentration of 100 μM was obtained by adding the stock directly to culture media at a dilution of 1:1000.

Table 6. List of pharmaceutical agents targeting various proteases

Inhibitor	Vehicle	Stock	Final concentration	General action	Source	Reference	Toxic effect (effect on meiotic maturation)
Decanoyl -Arg-Val-Lys-Arg-chloromethylketone (RVKR)	DMSO	50 mM	50 μM	General convertase inhibitor	Sigma (St. Louis, MO, USA)	(McLeskey Kiefer and Saling, 2002)	Not tested
Alpha1-PDX	Media	-	20 μM	Selective inhibitor of Furin	donated by Dr. Basak	(Basak and Lotfipour, 2005)	Not tested
Calbiochem Protease Inhibitor Cocktail Set I (AEBSF hydrochloride+ Aprotinin + E64 protease inhibitor+ EDTA disodium+ Leupeptin hemisulfate)	Water	-	AEBSF hydrochloride 500 μM + Aprotinin 150 nM + E64 protease inhibitor 1 μM + EDTA disodium 0.5 mM + Leupeptin hemisulfate 1 μM)	Serine proteases, Esterases, Cysteine proteases, Metalloproteases, Trypsin-like proteases inhibitor	Calbiochem (La Jolla, CA, USA)		Partial toxicity after 45 min exposure (only 60% oocytes extruded first polar body)
4-(2-Aminoethyl)benzene sulfonylfluoride (AEBSF) hydrochloride	Water	500 mM	500 μM	Serine proteases inhibitor	Sigma (St. Louis, MO, USA)	(Ryu *et al.*, 2005)	No toxic effect

Aprotinin	Water	150 µM	150 nM	Serine proteases and Esterases inhibitor	Sigma (St. Louis, MO, USA)	(Togo and Morisawa, 1997)	Not tested
E64 protease inhibitor	Water	1 mM	1 µM	Cysteine proteases inhibitor	Sigma (St. Louis, MO, USA)	(Ryu *et al.*, 2005)	Not tested
EDTA disodium	Water	0.5 M	0.5 mM	Metalloproteases inhibitor	Sigma (St. Louis, MO, USA)	(Ryu *et al.*, 2005)	Not tested
Leupeptin hemisulfate	Water	1 mM	1 µM	Cysteine proteases and Trypsin-like proteases inhibitor	Sigma (St. Louis, MO, USA)	(Ryu *et al.*, 2005)	Not tested
Calbiochem Protease Inhibitor Cocktail Set III (AEBSF hydrochloride+ Aprotinin+ E64 protease inhibitor+ Bestatin+ Leupeptin hemisulfate+ Pepstatin A)	DMSO	-	Calbiochem Protease Inhibitor Cocktail Set III (AEBSF hydrochloride 100 µM + Aprotinin 80 nM + E64 protease inhibitor 1.5 µM + Bestatin 5 µM + Leupeptin hemisulfate 1 µM + Pepstatin A 1 µM)	Serine proteases, Aminopeptidase B, Leucine Aminopeptidase, Cysteine proteases, Trypsin-like proteases, Aspartic proteases inhibitor	Calbiochem (La Jolla, CA, USA)		Not tested

7. Oocyte volume and surface area measurements

Oocyte dimensions were measured in microdrop cultures under oil immediately after collection or immediately after removal from the incubator, as specified for each experiment. Images of oocytes were obtained using a Nikon Coolpix 4500 digital camera on a Zeiss IM35 microscope fitted with Hoffman optics. The focal plane was placed at the center of the oocyte, to encompass the largest diameter. For each oocyte, two orthogonal diameters perpendicular to each other in the plane of focus were measured on the image, and their average was used as the effective oocyte radius for volume and surface area calculations. Oocytes were assumed to be approximately spherical, with a volume equal with $4/3\pi r^3$, and a surface equal with $4\pi r^2$, where r represents the radius.

8. Statistics

Data were plotted using SigmaPlot 8.2 (SPSS, Chicago, IL). 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, using Instat (GraphPad, San Diego, CA). Regression analysis was performed to calculate standard curve coefficients when needed using SigmaPlot 8.2.

RESULTS

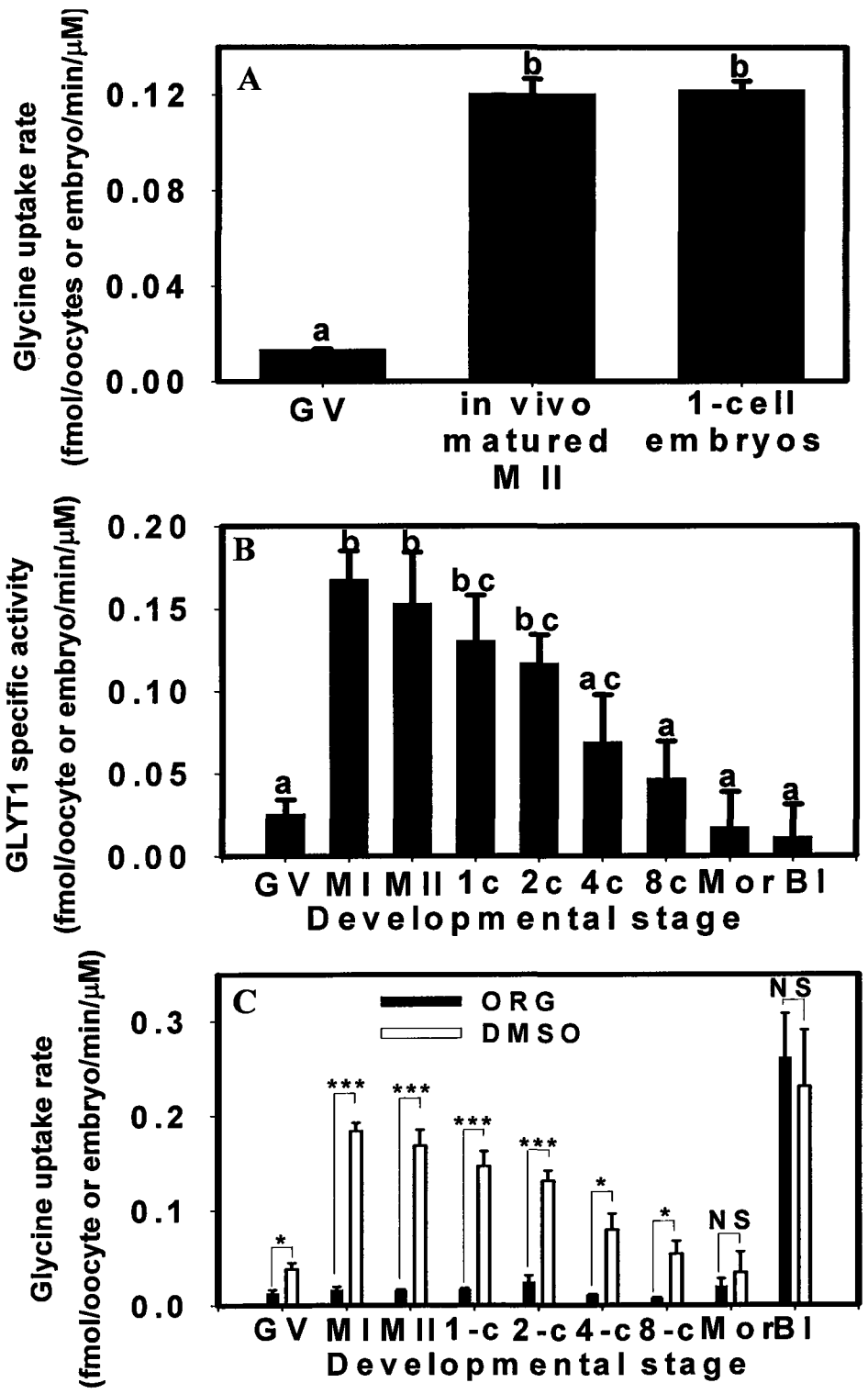
1. Cell-volume regulatory glycine transport is initiated in oocytes upon ovulation

Glycine transport via GLYT1 is the major volume-regulatory mechanism employed by the early preimplantation embryo at the 1-cell stage (see Introduction), but whether it is present before fertilization was not known. A few reports have indicated glycine transport to be present in mouse oocytes at various stages (Colonna and Mangia, 1983; Haghighat and Van Winkle, 1990) although detailed information about glycine transport during oocyte development and maturation was not available, and the exact moment during development when GLYT1 activity is initiated was not known. Therefore, I first investigated glycine transport by oocytes starting with fully-grown oocytes from the ovarian follicle through meiotic maturation.

1. 1. Glycine transport in oocytes is activated following stimulation of ovulation

To determine whether there was glycine transport present prior to fertilization, I measured the ability to take up ^{3}H-labeled glycine by freshly-isolated GV stage oocytes, unfertilized ovulated MII oocytes, and 1-cell embryos. Surprisingly, I found that, while matured MII oocytes before fertilization possessed robust transport comparable to that of 1-cell embryos after fertilization, the rate of glycine transport by GV oocytes before meiotic maturation was negligible (Fig. 9 A).

Previous studies had demonstrated that the NTT family member GLYT1 was responsible for glycine transport in 1-cell embryos (Van Winkle *et al.*, 1990; Steeves *et al.*, 2003). To further investigate the initial findings as well to determine whether glycine


A
Glycine uptake rate
(fmol/oocytes or embryo/min/μM)
0.12
0.08
0.04
0.00
a
b
b
GV
in vivo matured M II
1-cell embryos
B
GLYT1 specific activity
(fmol/oocyte or embryo/min/μM)
0.20
0.15
0.10
0.05
0.00
a
b
b
bc
bc
ac
a
a
a
GV MI MII 1c 2c 4c 8c MorBl
Developmental stage
C
Glycine uptake rate
(fmol/oocyte or embryo/min/μM)
ORG
DMSO
0.3
0.2
0.1
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NS
NS
GV MI MII 1-c 2-c 4-c 8-c MorBl
Developmental stage

Figure 9. Initiation of glycine transport in oocytes. ***(A) Glycine transport measurements in fresh GV, matured MII oocytes and 1-cell embryos immediately after collection.*** Glycine transport is essentially negligible in fresh GV oocytes but robust in matured MII oocytes and 1-cell embryos. Different letters indicate significance ($p<0.001$; ANOVA and Tukey-Kramer test). ***(B) Specific glycine transport by GLYT1 in oocytes at different stages of meiotic maturation and in preimplantation embryos.*** Specific glycine transport by GLYT1 appears during meiotic maturation with highest activity during meiosis and the earliest stages of preimplantation embryogenesis. MI oocytes were obtained by culturing GV oocytes for 4 hr *in vitro*. Specific transport was calculated by subtracting the rate of glycine transport in the presence of 5μM ORG23798 (specific GLYT1 inhibitor) from the rate of glycine transport in DMSO 0.1% (vehicle). Different letters indicate significance ($p<0.01$; ANOVA and Tukey-Kramer test). ***(C) Glycine transport inhibition by ORG23798 at different stages of meiotic maturation and in preimplantation embryos.*** Measurements of glycine transport in the presence or absence of ORG23798 indicates GLYT1 activity during meiotic maturation and initial stages of preimplantation embryo development, until the 8-cell stage. Comparisons were done between rates within each stage in the presence of ORG23798 or vehicle alone (DMSO 0.1%). (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, by Student's *t*-test). For (B) and (C) GV = freshly collected GV oocytes, MI = *in vitro* obtained MI oocyte, MII = *in vivo* matured MII oocyte, 1c to 8c = 1- to 8-cell embryos as indicated, Mor = morulae and Bl = Blastocysts. Throughout, each bar or point represents mean±SEM of 3-5 independent measurements.

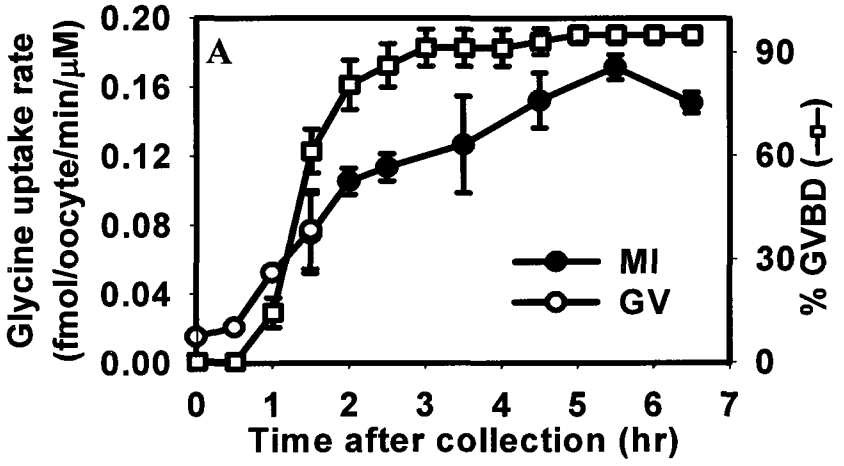
transport was via GLYT1, I have studied the presence of GLYT1 activity over the period of development spanning from the GV oocyte, through meiotic maturation, fertilization, and through preimplantation embryo development to the blastocyst stage (Fig. 9 B). Since essentially complete inhibition of glycine transport via GLYT1 by ORG23798 with a high specificity was previously established (Steeves *et al.*, 2003), I used this pharmacological agent to uncover at what stages during early development GLYT1 was active in mediating glycine transport. Specific GLYT1 glycine transport at various stages of oocyte and embryo development was calculated by subtracting the rate of glycine transport in the presence of ORG23798 (5 μM) from the rate of glycine transport in the presence of vehicle alone (DMSO 0.1%) (data at each stage in the presence and absence of ORG23798 is shown in Fig. 9 C, and the difference between the two rates at each stage in Fig. 9 B).

GLYT1 was found to be quiescent prior to meiotic maturation in growing and GV oocytes, and highly active during meiotic maturation in MI, matured MII oocytes as well as in early embryo developmental stages (1-, and 2-cell embryos; the measurements in embryos shown for the 2-cell to blastocyst stage were collected by Tiffany Richards, MSc candidate, and Mary-Anne Hammer, Research assistant in Dr. Jay Baltz's laboratory). GLYT1 activity gradually decreased after the 2-cell stage and was almost completely lost by the morula stage as previously reported (Van Winkle *et al.*, 1988) (Fig. 9 B). Taken together, these findings indicated that the ability to transport glycine via GLYT1 develops in oocytes sometime during meiotic maturation so that it is fully developed in MI and MII oocytes.

Since these results implied that GLYT1 is activated between the GV and MI

stages, I studied glycine transport as a function of time over the course of meiotic maturation. Since the mammalian oocyte spontaneously enters meiotic maturation if removed from the antral follicle (see Introduction), I measured the rate of glycine transport in oocytes cultured *in vitro* between 0 and 6.5 hr after retrieval from the ovary, during their spontaneous progression through germinal vesicle breakdown (GVBD) and the initial hours of first meiotic metaphase. Very little transport was evident during the first 0.5 hr after collection, but robust activity developed by 2 – 3 hr (Fig. 10 A). This development of glycine transport activity took place in parallel with oocyte meiotic maturation resumption, with GVBD also reaching a maximum between 2 and 3 hours subsequent to oocyte removal from the follicle (Fig. 10 A).

To validate that glycine transport was similarly initiated *in vivo*, oocytes were retrieved from mice that were stimulated to ovulate by hCG injection. Oocytes collected at different time points (between 0 and 6.5 hr) after hCG, were immediately utilized for measuring the rate of glycine transport, thus providing a time-course for glycine transport activity as a function of time after ovulation was induced by a physiological trigger *in vivo*. Oocytes retrieved in the first 1.5 after stimulation of ovulation were virtually all at the GV stage as expected (Fig. 10 B). These oocytes exhibited minimal glycine transport activity (Fig. 10 B). Starting at 2 hr after hCG, oocytes that had undergone GVBD were found, and robust glycine transport was measured in these MI oocytes that developed *in vivo*. Interestingly, those GV oocytes on the threshold of GVBD (1.5-2 hr after hCG) seemed to have started to develop the ability to transport glycine, since glycine transport activity was increasing at this time, and both GV and MI oocytes exhibited transport activity (Fig. 10 B). Oocytes that failed to be stimulated to enter meiosis (~50-60% of


A
Glycine uptake rate (fmol/oocyte/min/μM)
0.20
0.16
0.12
0.08
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0.00
90
60
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MI
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0 1 2 3 4 5 6 7
Time after collection (hr)

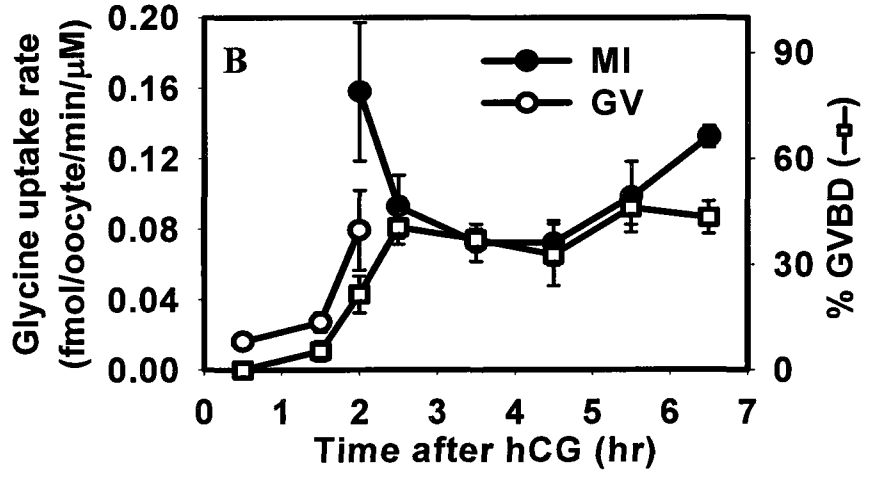

B
Glycine uptake rate (fmol/oocyte/min/μM)
0.20
0.16
0.12
0.08
0.04
0.00
90
60
30
0
% GVBD (—□—)
MI
GV
0 1 2 3 4 5 6 7
Time after hCG (hr)

Figure 10. Time-course of glycine transport development. ***(A) Time-course of glycine transport development in vitro.*** Glycine transport develops during the first 2-3 hr of oocyte meiotic progression *in vitro* (circles, left axis). The timing of glycine transport development coincides with entry into MI, as indicated by right axis (% GVBD, squares). At 1.5 hr, there was a mixture of GV (open circles) and MI (closed circles) oocytes, but rates were similar and symbols nearly coincide. ***(B) Time-course of glycine transport development in vivo.*** Glycine transport (circles, left axis) develops during oocyte meiotic maturation *in vivo* around 2 hr after hCG-induced triggering of ovulation, in parallel with oocyte entry into MI (right axis, squares). Oocytes that failed to be stimulated to enter meiosis by hCG *in vivo* , a cohort that presumably would not be ovulated, also did not develop GLYT1 activity (not shown). There was a mixture of GV and MI at 2 hr post-hCG, both exhibiting transport activity. Symbols are as in (A). Throughout, each point represents mean±SEM of 3-5 independent measurements.

total after 2.5 hr post-hCG) (Fig. 10 B) did not develop GLYT1 activity (measurements done at 2 – 6.5 hr; not shown). Together, these data confirm a similar timing for initiation of GLYT1 activity both *in vivo* and *in vitro*, and development of glycine transport activity in parallel with meiotic maturation, shortly after release from meiotic arrest either by removal from the ovarian follicle or by physiological stimulation of ovulation.

1. 2. Glycine transport develops independently of meiotic maturation

The above data indicated that GLYT1 activity developed with a similar time course to meiotic maturation both *in vitro* and *in vivo*. Therefore, I determined whether activation of glycine transport required release from GV arrest to test the hypothesis that meiotic progression is required for activation of GLYT1-mediated glycine transport by oocytes. Since oocytes can be kept in GV arrest by maintaining high intracellular cAMP concentrations, the cell-permeant cAMP analog, dibutyryl cAMP (dbcAMP, 100 μM), was used to prevent resumption of meiosis. Oocytes maintained in GV arrest in culture were utilized at different time points following removal from ovaries (spanning between 0 and 6 hr) to measure the rate of ^{3}H-labeled glycine transport.

Surprisingly, although no oocytes entered MI in the presence of dbcAMP, they nevertheless exhibited a similar development of glycine transport, with initiation of transport at about 1 hr after removal from the ovary, and robust activity having developed by ~ 2 – 3 hr (Fig. 11), similar to *in vitro* maturing MI oocytes in the absence of dbcAMP (Fig. 10 A). Thus, although the timing of resumption of meiosis and glycine transport activation were essentially identical, they were occurring independently.

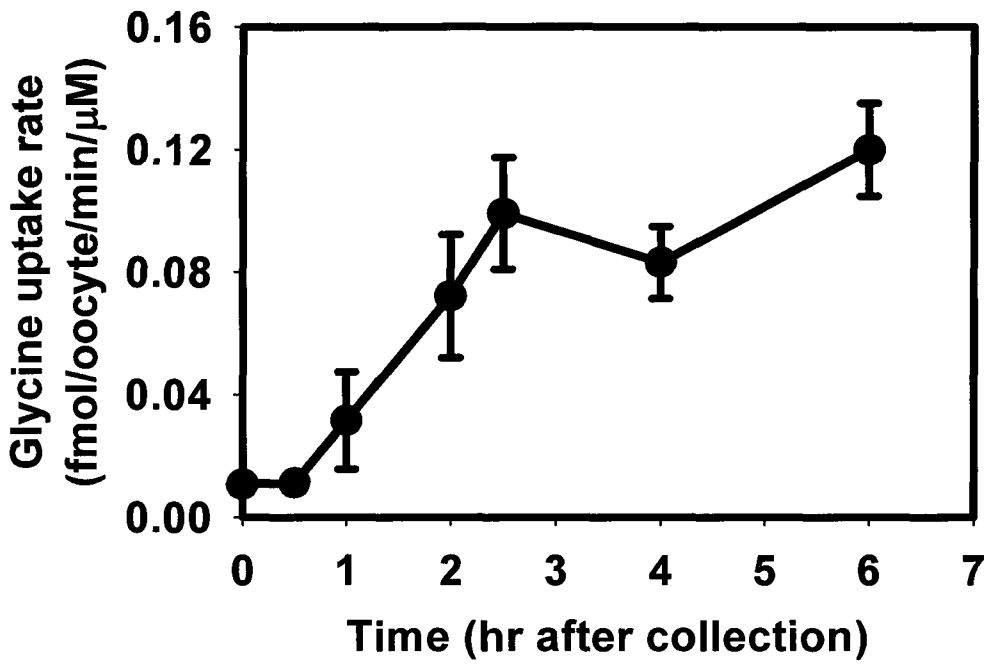


Figure 11. Time-course of glycine transport development in GV-arrested oocytes. Glycine transport rates were measured at different time points after collection for oocytes maintained GV-arrested in the presence of cAMP. Glycine transport appears around 2 hr after collection. Throughout, each point represents mean±SEM of 3 independent measurements.

Similarly, glycine transport developed by 4 hr after collection in the presence of other compounds that maintain GV arrest (Fig. 12) that included hypoxanthine (4 mM), a purine with phosphodiesterase inhibitory activity; forskolin (100 μM), an agonist of adenylate cyclase; IBMX (200 μM), a phosphodiesterase inhibitor; and Roscovitine (50 μM), an inhibitor of CDK1 activity.

The manner of oocyte collection did not influence development of glycine transport in GV-arrested oocytes since glycine transport similarly developed by 4 hr after collection when GV oocytes were retrieved by puncturing the antral follicles on the ovary surface instead of mincing the ovary (not shown). This provided at least a partial control to indicate that damage or transient stress from the mincing procedure was not responsible for the lack of glycine transport activity, and confirmed this behavior occurred in oocytes from confirmed large antral follicles.

Other amino acid transporters such as System A can be activated by their substrate starvation (Pastor-Anglada *et al.*, 1996). Since throughout the above experiments culture media lacked glycine, I have also tested the possibility of a starvation effect being involved in initiation of GLYT1 activity. Oocytes maintained in the GV arrest (by 100 μM dbcAMP) or let to undergo maturation (absence of dbcAMP) were cultured for 4 hr in the presence or absence of 1mM glycine. Subsequent measurements of glycine transport rates in these oocytes demonstrated development of GLYT1 activity in all situations (Fig. 13), providing evidence that oocyte glycine starvation did not influence initiation of glycine transport in oocytes.

The up-regulation of transport independent of meiotic maturation did not seem to be a general characteristic of amino acid transport in oocytes but rather specific to

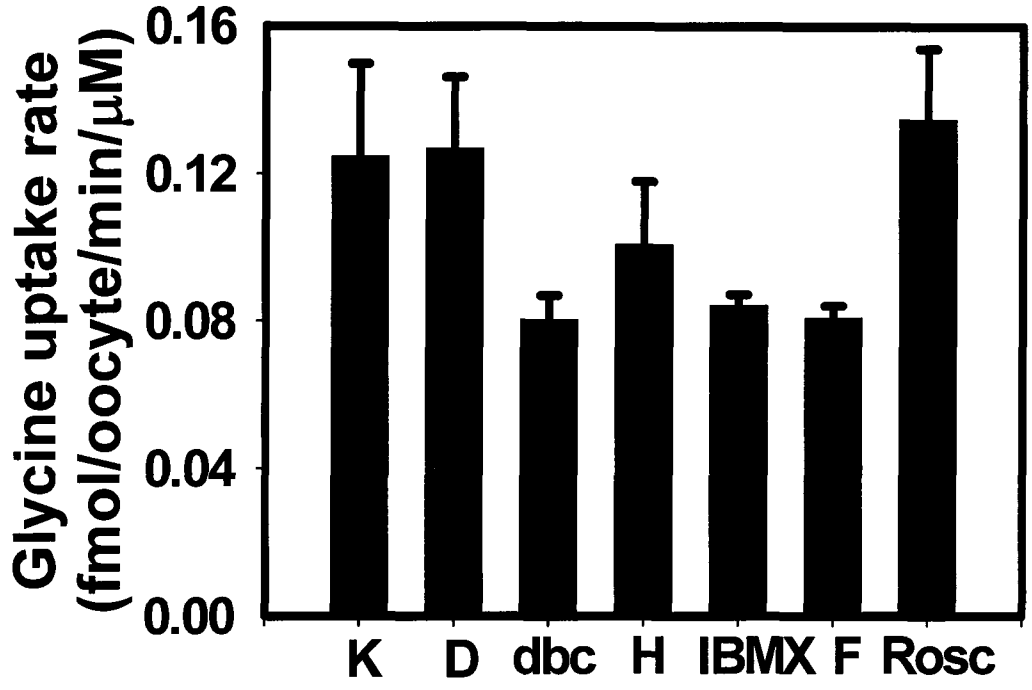


Figure 12. Glycine transport at 4 hr after collection for oocytes maintained in the GV-arrest by various compounds. Glycine transport in oocytes cultured in the presence of dbcAMP (dbc), hypoxanthine (H), IBMX, forskolin (F), or roscovitine (Rosc) developed similarly with the control groups KSOM (K) and DMSO 0.1% (D) ($p>0.05$; ANOVA and Tukey-Kramer test). Throughout, each bar represents mean±SEM of 3 independent measurements.

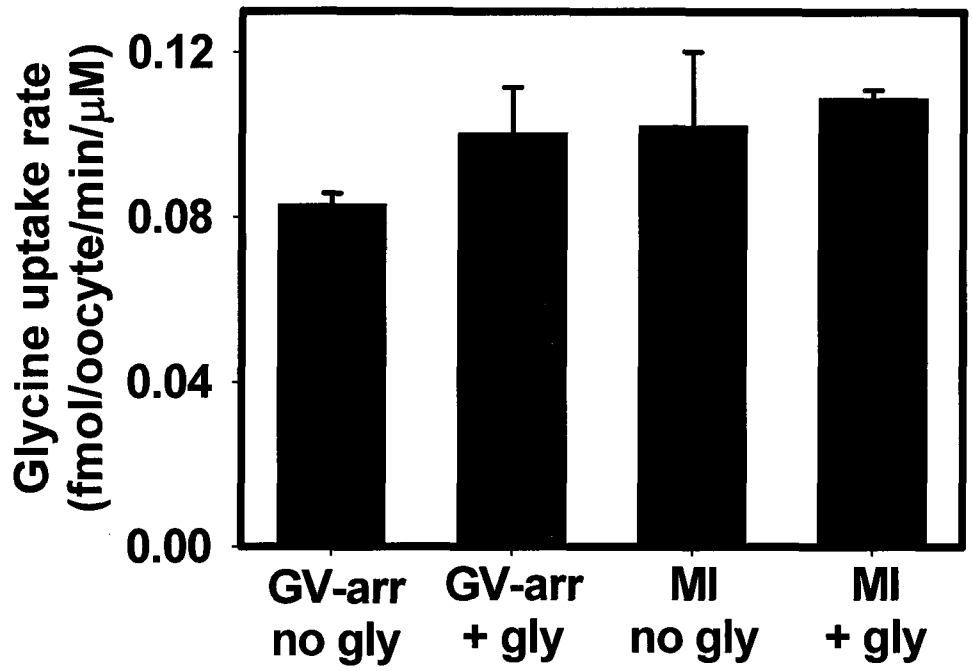


Figure 13. Influence of glycine starvation over development of glycine transport. Glycine transport measurements were done in GV-arrested (by dbcAMP) and *in vitro* obtained MI oocytes after 4 hr of culture in the absence (no gly) or presence of 1 mM glycine (+ gly). Starvation (absence of glycine) had no effect over GV-arrested or MI oocyte ability to develop glycine transport ($p>0.05$; ANOVA and Tukey-Kramer test). Throughout, each bar represents mean±SEM of 3 independent measurements.

glycine, since measurements of transport rates for another amino acid, leucine, indicated that transport measured in freshly-collected GV oocytes became partially down-regulated by 4 hr after collection (not shown).

1. 3. Growing, meiotically incompetent oocytes develop GLYT1 activity subsequent to their removal from the follicle

To examine further any possible relationship between development of GLYT1 and meiotic maturation, I tested the ability of meiotically incompetent oocytes to develop GLYT1 activity once released from the ovary. Previous studies have demonstrated that the oocyte will acquire the ability to undergo meiotic maturation (acquisition of meiotic competence) at a specific oocyte size (Eppig *et al.*, 2004). To investigate the presence of GLYT1 activity before acquisition of meiotic competence, I have chosen to use growing oocytes (~45 μm diameter) collected from 9 day-old mice that I previously have confirmed to be meiotically incompetent (Erdogan *et al.*, 2005) (not shown). By using the specific GLYT1 inhibitor ORG23798 (see above), I was able to evaluate glycine transport specific to GLYT1. To test if oocyte removal from follicles would trigger activation of GLYT1 in incompetent oocytes, the ability of growing oocytes to take up glycine was measured immediately after collection and 4 hr after culture *in vitro*. The rate of glycine transport was normalized to oocyte surface area to allow direct comparison with transport in fully-grown oocytes, since the rate of transport per unit surface area could be directly related to the density of active GLYT1 transporters in the membrane .

GLYT1 was quiescent in freshly-isolated growing oocytes, but glycine transport developed by 4 hr after collection (Fig. 14). Thus, activation of GLYT1 upon oocyte

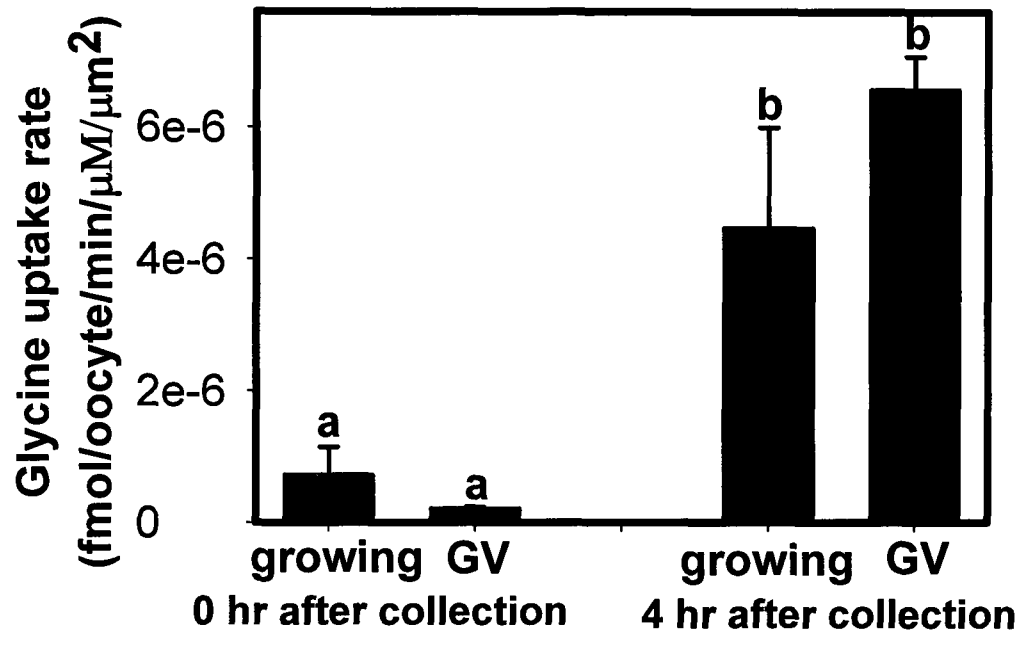


Figure 14. Glycine transport development in growing, meiotically incompetent oocytes. Glycine transport was measured immediately and 4 hr after collection in growing oocytes removed from neonatal ovaries of 9 days old mice and compared with glycine transport in fully-grown GV oocytes, maintained arrested by dbcAMP. The rate of glycine transport was normalized to oocyte surface area. Both growing and fully-grown GV-arrested oocytes developed glycine transport similarly by 4 hr after collection. Different letters indicate significance ($p<0.0001$; ANOVA and Tukey-Kramer test). Each bar represents mean±SEM of 3 independent measurements.

removal from follicle also occurs in meiotically incompetent growing oocytes. Overall, these data indicated activation of GLYT1 transport independence from competence to undergo meiotic maturation.

1. 4. The oocyte accumulates glycine after ovulation

Previous studies have demonstrated that GLYT1 activity mediates the accumulation of high levels of soluble glycine in cleavage-stage embryos (Dawson *et al.*, 1998; Steeves *et al.*, 2003). My findings regarding GLYT1 activity initiation only after stimulation of ovulation implied that glycine accumulation would begin around the same period. To investigate the onset of glycine accumulation, GV stage oocytes (maintained in arrest with dbcAMP) were cultured in the presence of glycine (1 mM ^{3}H-labeled glycine), beginning immediately after their removal from ovaries, to allow them to accumulate labeled glycine. In parallel, 1-cell embryos were similarly treated and used to compare the oocyte ability to accumulate glycine. Oocytes and embryos were removed from culture every 20 min for measurement of total glycine content up to 3 hr. Data shown for 1-cell embryos were collected by Mary-Anne Hammer. As previously shown (Steeves *et al.*, 2003; Steeves and Baltz, 2005), 1-cell embryos immediately began accumulating glycine (Fig. 15). However, GV oocytes revealed an initial lag period in accumulation of ~ 2 hr during which time accumulation was much slower than in 1-cell embryos, followed by accelerated rate only after about 2 hr subsequent to their removal from the ovary. Similar accumulation trends were obtained when oocytes and 1-cell embryos were exposed to high osmolarity (350 mOsM) (not shown), indicating that this was not an effect of osmotic stimulation or inhibition of the transporter. This timing of

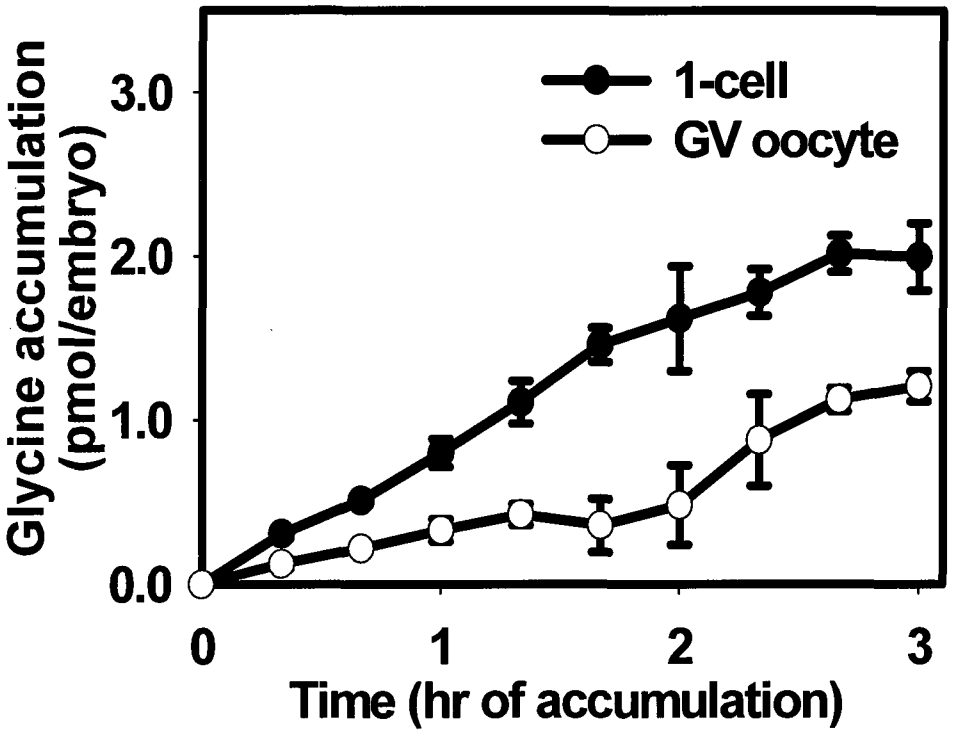


Figure 15. Initiation of glycine accumulation in GV oocytes and 1-cell embryos, *in vitro*. GV oocytes (maintained arrested by dbcAMP) or one-cell embryos were cultured in the presence of 1mM glycine. Total glycine accumulated by GV oocytes and one-cell embryos was measured as a function of time. Glycine accumulation began immediately in 1-cell embryos but was delayed in GV-arrested oocytes. Throughout, each point represents mean±SEM of 3 independent measurements.

accelerated glycine accumulation in oocytes was coincident with the onset of GLYT1 activity (above), demonstrating that initiation of both transport and accumulation of glycine occur concurrently.

Free intracellular glycine is accumulated *in vitro* at high levels by early preimplantation embryos, consistent with its role as an organic osmolyte (Steeves *et al.*, 2003). However, whether glycine is accumulated by oocytes or embryos *in vivo* has not been investigated. If GLYT1 activity and glycine accumulation *in vivo* is initiated only after stimulation of ovulation, then endogenous soluble glycine content would be expected to be low in GV oocytes prior to ovulation and high in those developmental stages succeeding ovulation. To test this, I measured the endogenous TCA-soluble glycine by amino acid microanalysis in GV oocytes, MI oocytes that developed *in vivo*, *in vivo* matured MII oocytes, and preimplantation embryos at several stages of development, immediately after they were isolated from the ovary or oviduct. The preimplantation embryos used for these measurements were collected by Mary-Anne Hammer. Soluble glycine in GV oocytes was nearly undetectable (Fig. 16). In contrast, a large amount of free glycine was present in ovulated MII oocytes, 1-cell and 2-cell embryos. Morulae contained very little free glycine, consistent with the loss of GLYT1 activity by this stage of development (see Fig 9 B). Taken together, these data collected from both *in vitro* and *in vivo* indicated that the ability to accumulate glycine via GLYT1 develops in oocytes only after stimulation of ovulation.

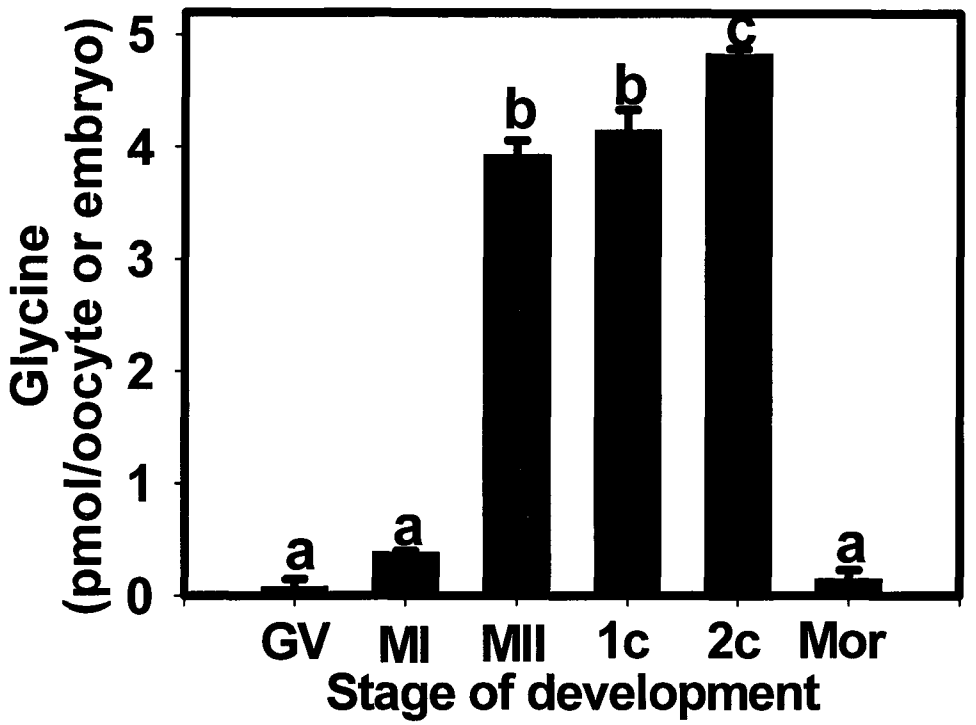
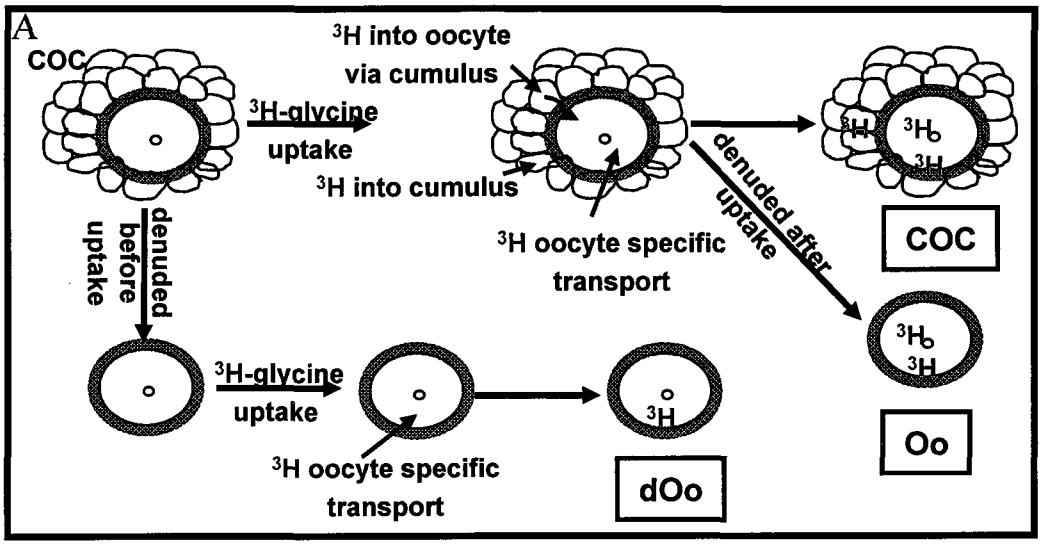


Figure 16. The increase of free cytosolic glycine accumulated in vivo at ovulation. Oocytes and embryos at different stages of development were utilized immediately after collection for amino acid microanalysis. The matured MI oocytes were surrounded by cumulus-granulosa shells. Measurements done in GV surrounded by cumulus-granulosa did not differ from those in freshly denuded GV oocytes (not shown). Endogenous soluble glycine was high in MII oocytes, 1- and 2-cell embryos, but low in GV and MI oocytes, and morulae. Different letters indicate significant difference ($p<0.01$; ANOVA and Tukey-Kramer test). Throughout, each bar represents mean±SEM of 3 independent measurements.

1. 5. Glycine transport into oocytes is not influenced by the surrounding cumulus cells

Metabolic cooperativity between oocyte and surrounding somatic cells has been shown to ensure supply of some nutrients, including some amino acids, by transfer from granulosa cells to the enclosed oocyte (Heller *et al.*, 1981; Colonna and Mangia, 1983) (see Introduction). To test if cumulus cells are able to supply the GV oocyte with glycine prior to GLYT1 activation and if any transfer persists following initiation of glycine transport in oocytes, I investigated glycine transport into different compartments of the cumulus-oocyte complex (COC) immediately after collection and 4 hr following COC culture. Three different groups were evaluated: cumulus-oocyte complexes (COCs), GV oocytes denuded before glycine uptake (dOo), and GV oocytes denuded after glycine uptake (Oo). This approach allowed assessment of different components of transport in COCs: 1) total transport (glycine transport into cumulus cells plus oocyte-specific glycine transport plus glycine transferred from surrounding cumulus cells into oocyte) for intact COCs; 2) oocyte-specific glycine transport for dOo; 3) oocyte-specific glycine transport plus glycine transferred from surrounding cumulus cells into oocyte for Oo (Fig. 17 A). To maintain GV arrest dbcAMP was present throughout experiments.

For each group specific GLYT1 activity was measured by utilizing the specific GLYT1 inhibitor ORG23798 and a similar approach as described above. Essentially no specific GLYT1 activity was detected immediately after collection (Fig. 17 B), indicating the absence of GLYT1 transporter activity (or any other significant component of glycine transport) in all COC compartments. Activity, however, developed by 4 hr in all three


A
COC
^{3}H into oocyte
via cumulus
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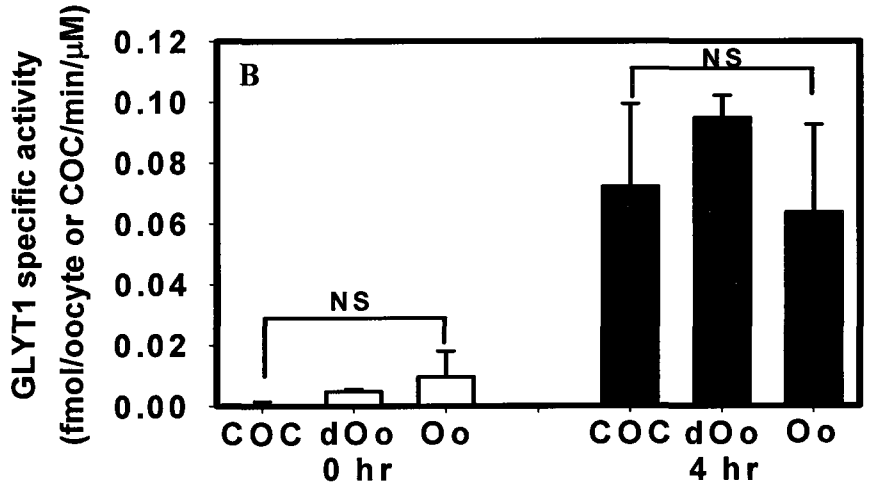
B
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NS
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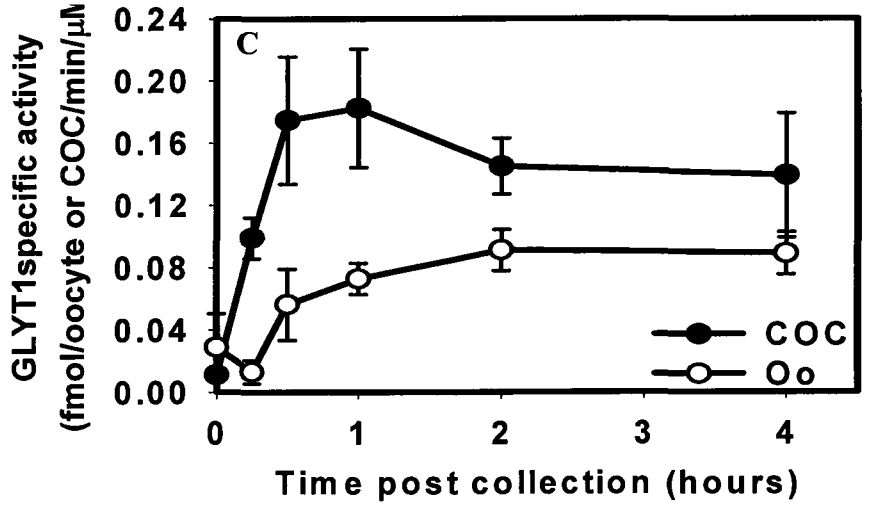
C
GLYT1specific activity
(fmol/oocyte or COC/min/μM
0.24
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Time post collection (hours)

Figure 17. GLYT1 activity in cumulus-oocyte complexes. ***(A) Schematic representation of experimental design utilized to evaluate glycine transport in different COC compartments.*** Measurement of glycine transport in the intact COC allowed assessment of total transport (glycine transport into cumulus cells plus oocyte-specific glycine transport plus glycine transferred from surrounding cumulus cells into oocyte). The oocyte-specific glycine transport was evaluated by utilizing oocytes denuded before glycine uptake (dOo). Utilization of oocytes denuded after uptake (Oo) allowed assessment of oocyte-specific glycine transport added to the amount of glycine transferred from surrounding cumulus to the oocyte. **(B) GLYT1 activation in cumulus-oocyte complexes.** Specific glycine transport by GLYT1 in intact COC, dOo, and Oo was measured immediately after collection and following 4hr of COC culture. dbcAMP was present in culture at all times to maintain the GV arrest. Specific GLYT1 transport was calculated as in Fig. 9 B. GLYT1 activity developed similarly by 4 hr in all 3 groups. Comparisons were done between rates within each time point. There was no statistical difference between COC, dOo and Oo at 0 or 4 hr ($p>0.05$; ANOVA and Tukey-Kramer test). ***(C) Time-course of GLYT1 activity initiation in intact COC and Oo.*** Specific transport by GLYT1 was measured similarly as in (B) at various times after collection. Initiation of GLYT1 activity took place for both COC and Oo in the first 0.5 hr after COCs were removed from follicles. Throughout, each bar or point represents mean±SEM of 3 independent measurements.

groups, as previously found for dOo alone, above.

To determine the exact timing of GLYT1 activity initiation in COC and oocytes denuded after uptake (Oo), the rate of glycine transport was measured (from 0 to 4hr) similar to the measurements described above for dOo. GLYT1 specific activity development followed a similar time course in both COC and Oo, with maximal activity achieved by 0.5 – 1 hr after collection (Fig. 17 C). Intriguingly, when compared with denuded oocytes where robust transport was detected only by ~ 2 hr (see Fig. 11), intact COCs seemed to have the ability to initiate glycine transport faster. Overall, however, these data indicated that glycine transport activity is only initiated after ovulation or removal form the follicle whether the oocyte is contained in an intact COC or isolated.

These findings were consistent with the free cytosolic glycine measurements by amino acid analysis (see above). Measurements done in freshly-collected intact COCs did not differ from those in denuded GV oocytes (not shown), indicating that, *in vivo*, the COCs contain essentially no free glycine fraction accumulated in the cytoplasm. Together these data support the idea of a quiescent GLYT1 not only in GV oocytes but also in intact COCs prior to removal from follicle or stimulation of ovulation.

1. 6. GLYT1 activity protects oocyte volume

Previous studies demonstrated that 1-cell mouse embryos require GLYT1 activity and glycine transport to regulate their volumes against osmotic shrinkage (Steeves *et al.*, 2003). I therefore hypothesized that oocytes would not be able to regulate their volumes until after glycine transport via GLYT1 was initiated. To test this, I utilized two different categories of oocytes: freshly-collected GV oocytes that had a quiescent GLYT1, and GV

oocytes maintained arrested in culture (with 100 μM dbcAMP) for 3 hr that had already developed GLYT1 activity (see Fig. 11). For each group, their ability to maintain cell volume against increased osmolarity was tested by exposure for 2 hr to media of lower (250 mOsM) or higher osmolarity (350 mOsM) in the presence or absence of glycine (1 mM).

When assessed immediately after removal from the ovary, before GLYT1 activity had appeared, oocytes were significantly smaller after 2 hr exposure to the higher osmolarity and the presence of glycine had no effect on their volumes (Fig. 18 A). In contrast, when GV oocytes that had already developed maximal GLYT1 activity were assessed, they were significantly better able to maintain their volumes in the presence of glycine at the higher osmolarity (Fig. 18 B). To further demonstrate that the enhanced ability of oocytes to maintain their volumes at higher osmolarity depended on GLYT1 activity, GV-arrested oocytes that had already developed GLYT1 activity (3 hr in culture) were exposed for 2 hr to high osmolarity (350 mOsM) in the presence or absence of glycine and the specific GLYT1 inhibitor ORG23798. Inhibition of GLYT1 with ORG23798 eliminated the protective effect of glycine on oocyte volume (Fig. 18 C), showing that GLYT1 activity enables the oocyte to maintain their volume at high osmolarity.

2. Regulation of GLYT1 activity initiation in oocytes

Overall, my data suggested that the volume-regulatory glycine transport mediated by GLYT1 is normally activated in oocytes only upon ovulation (or, *in vitro*, after removal from the ovary). This activity initiation did not require meiotic maturation, as it

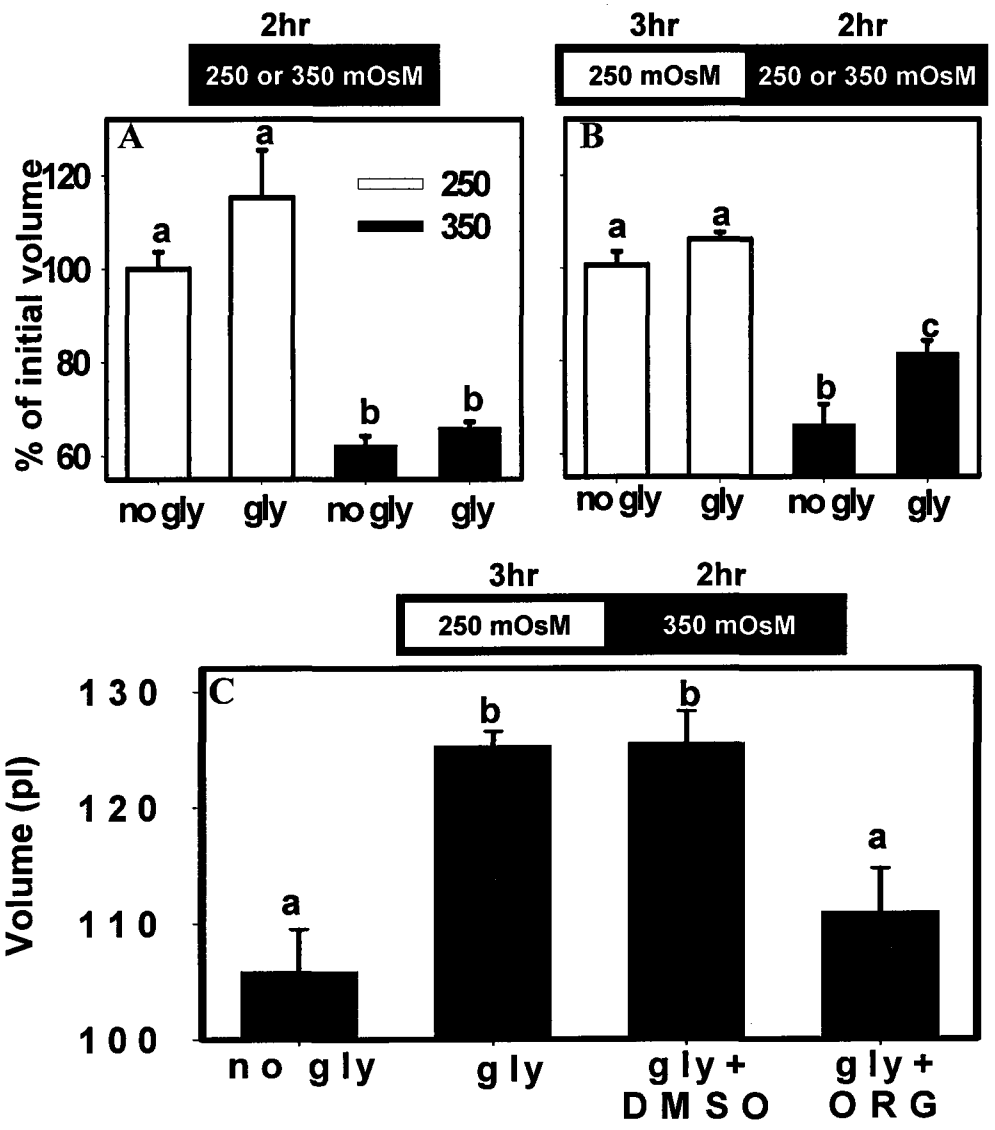

2hr
250 or 350 mOsM
3hr
2hr
250 mOsM
250 or 350 mOsM
A
B
% of initial volume
120
100
80
60
250
350
a
a
b
b
a
a
b
c
no gly
gly
no gly
gly
no gly
gly
no gly
gly
3hr
2hr
250 mOsM
350 mOsM
C
Volume (pl)
130
120
110
100
a
b
b
a
no gly
gly
gly + DMSO
gly + ORG

Figure 18. GLYT1 activity protects oocyte volume. ***(A) Lack of ability of oocyte to maintain cell volume before development of GLYT1 activity.*** GV oocytes maintained in GV arrest with dbcAMP were placed immediately after collection (before GLYT1 activity developed) in media of 250 or 350 mOsM in the absence (no gly) or presence (gly) of 1mM glycine. The mean oocyte volume was measured after 2 hr (experimental protocols indicated by diagrams above each panel). Glycine presence had no effect on oocyte volume. Oocyte volumes were normalized to the mean volume at 250 mOsM in the absence of glycine. ***(B) Ability of oocyte to maintain cell volume after GLYT1 activation.*** GV-arrested oocytes were cultured for 3 hr at 250 mOsM to allow GLYT1 activation, followed by additional 2 hr culture in condition similar with the ones described for (A). Oocyte volume was measured and normalized as for (A). The presence of glycine had no effect at low osmolarity, but resulted in significantly higher volume at 350 mOsM. ***(C) Maintenance of oocyte volume at high osmolarity requires GLYT1 activity.*** GV-arrested oocytes with fully active GLYT1, as for (B), were cultured for 2 hr at 350 mOsM in the absence (no gly) or presence of 1mM glycine (gly), with ORG23798 (gly+ORG) or without (gly+DMSO= vehicle alone). Oocyte volume was evaluated as for (A). Oocyte volume at 350 mOsM was higher in the presence of glycine than in its absence, and the glycine protective effect was eliminated when GLYT1 activity was inhibited by ORG23798. Throughout, different letters indicate significant differences ($p < 0.05$ by ANOVA and Tukey-Kramer test). Each bar represents mean ± SEM of 3-5 independent determinations.

occurred *in vitro* in isolated oocytes maintained in GV-arrest. Therefore, I have tried to elucidate the mechanisms controlling GLYT1 activation during oocyte development.

2. 1. Activation of GLYT1 activity occurs without *de novo* GLYT1 synthesis

The other known organic osmolyte transporters are controlled by changes in transcription upon variation in the environmental osmolarity (Garcia-Perez and Burg, 1991; Kwon and Handler, 1995) (see Introduction). To determine if *de novo* synthesis of GLYT1 mRNA is involved in its activation, I examined the presence of GLYT1 transcripts over oocyte and embryo development spanning from the growing oocyte (mid-growth phase) to the blastocyst, including stages of oocyte or embryo development where GLYT1 was quiescent or fully functional.

Two GLYT1 isoforms have been described in mouse: GLYT1a and GLYT1b. They are identical except for the N-terminus and their tissue distribution: GLYT1a mRNA was found to be expressed in brain and various peripheral tissues, whereas GLYT1b mRNA is reportedly restricted to the brain white matter (Borowsky *et al.*, 1993) (see Introduction). Although GLYT1 mRNA was previously detected in oocytes and preimplantation embryos (Van Winkle *et al.*, 1990), the isoform expressed has never been determined, nor was expression assessed immediately upon isolation of GV oocytes.

The transcripts identified using RT-PCR and isoform-specific primers (for primers, see Material and Methods) indicated that the GLYT1a isoform is expressed during oocyte development in mid-growth phase growing oocytes (from 9 day-old neonatal mice; oocytes are ~45 μm diameter and meiotically incompetent) (Erdogan *et al.*, 2005), fully-grown GV oocytes, mature MII oocytes, and at early stages of

preimplantation embryo development (1-cell through morula stage) (Fig. 19 A). Conversely GLYT1b was not detected in oocytes or preimplantation embryos at any stage (Fig 19 A). Positive control tissues showed the expected expression of GLYT1a in liver and brain, and GLYT1b in brain but not liver. Thus, GLYT1a is likely to be the isoform expressed in oocytes and preimplantation embryos responsible for GLYT1 activity, but its transcription is not likely to be involved in control of the appearance of glycine transport in oocytes since GLYT1 transcripts were identified well before its activation takes place.

Next, I examined the possible role of translation in initiating GLYT1 activity in oocytes. By using a GLYT1-specific antiserum (supplied by the Zafra laboratory) and Western blot analysis, I have evaluated the amount of GLYT1 protein present in freshly-isolated GV oocytes with quiescent GLYT1, and in MI oocytes that developed *in vitro*, and therefore with fully functional GLYT1.

The Western blot analysis of protein extracted from freshly-collected GV oocytes and *in vitro* (4 hr) matured MI oocytes (brain protein was used as a positive control) indicated the presence of GLYT1 protein in GV oocytes (Fig. 19 B). The amount of GLYT1 did not appear to increase from GV to MI, over the time when GLYT1 activity appears. Re-probing the blots for actin allowed a semi-quantitative assessment of GLYT1 expression using the GLYT1:actin ratio, which was found to be not significantly different between GV and MI (Fig. 19 C).

To determine whether GLYT1 activation required protein synthesis (perhaps of a regulator), oocytes maintained in GV-arrest (100 μM dbcAMP) were exposed immediately after collection to Cycloheximide (50 μg/ml), a classic protein synthesis

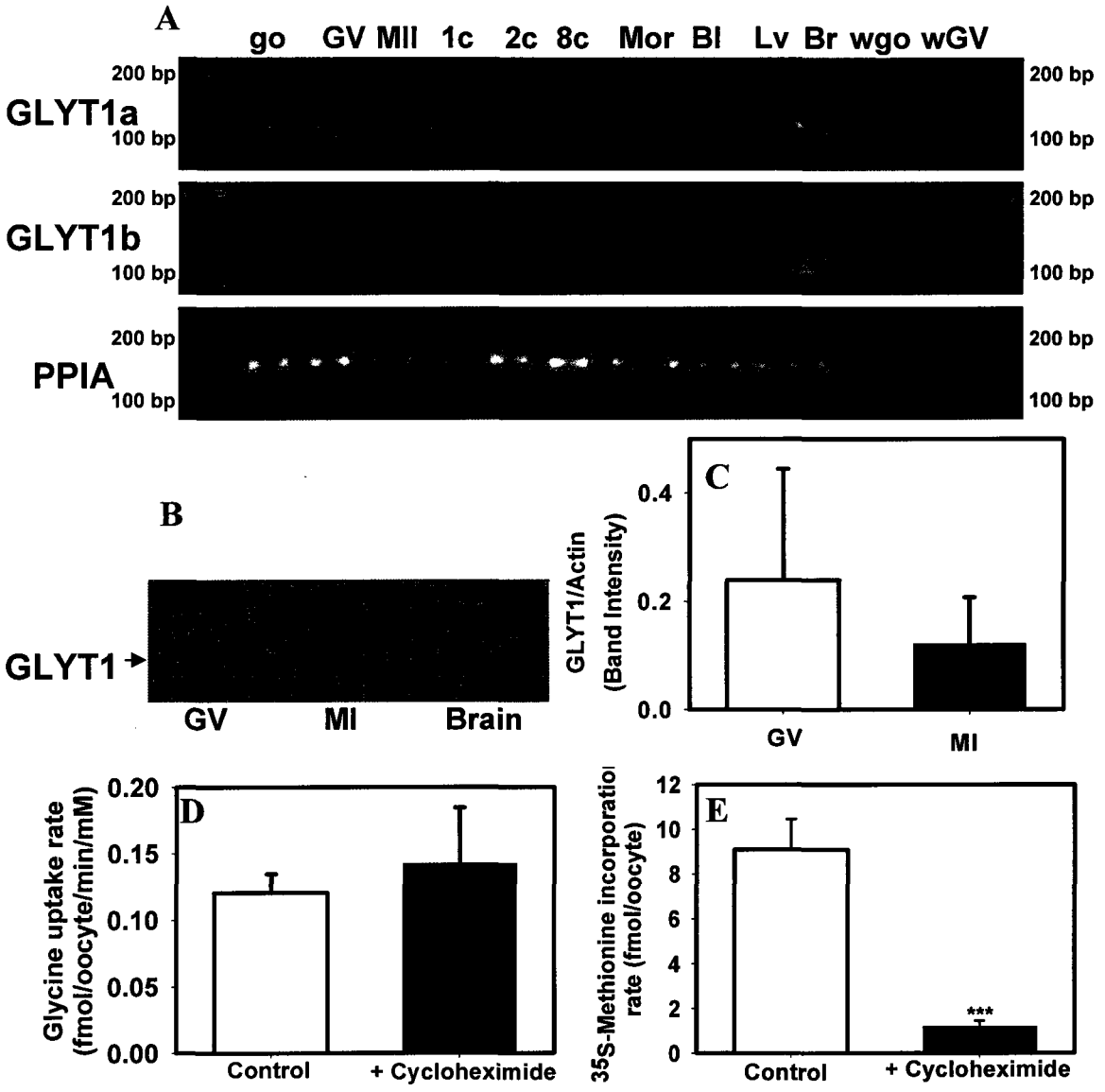


Figure 19. Activation of GLYT1 activity does not require *de novo* GLYT1 synthesis. ***(A) GLYT1a mRNA expression throughout oocyte and preimplantation embryo development.*** RT-PCR indicated GLYT1a mRNA expression in growing oocytes (go), fully-grown GV, matured MII oocytes, 1-, 2-, 8-cell and morulae (Mor) embryos and in liver (Lv) and brain (Br) controls. No bands were detected in blastocysts (Bl) or the wash

drops (wgo, wGV). GLYT1b mRNA was detected only in brain. The presence of PPIA mRNA at all stages confirmed correct loading. Gel shown is a representative of two. ***(B) GLYT1 protein expression at different stages of oocyte maturation.*** Western blot analysis of GLYT1 protein with GLYT1 affinity-purified antiserum (Zafra laboratory) and brain as positive control determined the presence of GLYT1 protein in freshly-collected GV and in *in vitro* obtained MI oocytes. MI oocytes were utilized 4 hr after collection. Arrowhead indicates GLYT1 protein band. Blot shown is a representative of three independent replicates. ***(C) Quantification of Western blot analysis.*** GLYT1 and actin bands from three independent Western blot analyses of GV and *in vitro* obtained MI oocytes were analyzed and quantified. The ratio of GLYT1/Actin band intensity showed no significant difference between groups (by Student's *t* test; $p = 0.41$). ***(D) Glycine transport development is not influenced by protein synthesis inhibition in oocytes.*** Glycine transport rates were measured in GV oocytes maintained arrested by dbcAMP after 4 hr of culture in the absence (control) or presence of Cycloheximide, a protein synthesis inhibitor. Inhibition of protein synthesis did not influence glycine transport development since no significant difference was found between groups (Student's *t* test, $p = 0.43$). ***(E) Inhibition of protein synthesis by Cycloheximide in GV-arrested oocytes.*** The ^{35}S-Methionine incorporation rate strongly decreased in GV-arrested oocytes exposed for 4 hr to Cycloheximide compared with the control group (no Cycloheximide), validating the effectiveness of protein synthesis inhibition ($p < 0.001$, by Student's *t*-test). Throughout, each bar represents mean ± SEM of 3 replicates.

inhibitor. Glycine transport developed during 4 hr following exposure to the inhibitor to the same level as in GV-arrested oocytes maintained in parallel culture without Cycloheximide (Fig. 19 D). In separate experiments, the ^{35}S-Methionine incorporation rate was shown to be strongly decreased (by 87%) in oocytes exposed to Cycloheximide, validating the effectiveness of protein synthesis inhibition (Fig 19 E).

Altogether, these data demonstrated that *de novo* transcription or translation of GLYT1 (or other proteins) is not required for its activation, indicating a different regulatory mechanism from the other known organic osmolyte transporters.

2. 2. GLYT1 protein appears to be membrane-localized prior to and following its activation in oocytes

The above data were highly suggestive of a post-translational event being involved in regulating GLYT1 activation in oocytes. One possible mechanism of regulation would be translocation of pre-synthesized GLYT1 to the plasma membrane. Since some mechanisms of membrane trafficking require the actin or microtubule cytoskeleton, I tested whether disruption of the cytoskeleton affected the development of GLYT1 activity. Disruption of microtubules with Demecolcine (1 μg/ml) or of microfilaments with Cytochalasin (10 μM) or of Golgi apparatus with Brefeldin A (5 μg/ml) did not interfere with GLYT1 activation in oocytes (Fig. 20), since oocytes exposed for 4 hr to these disruptors developed glycine transport similarly with the control group (oocytes exposed only to vehicle DMSO 0.1%).

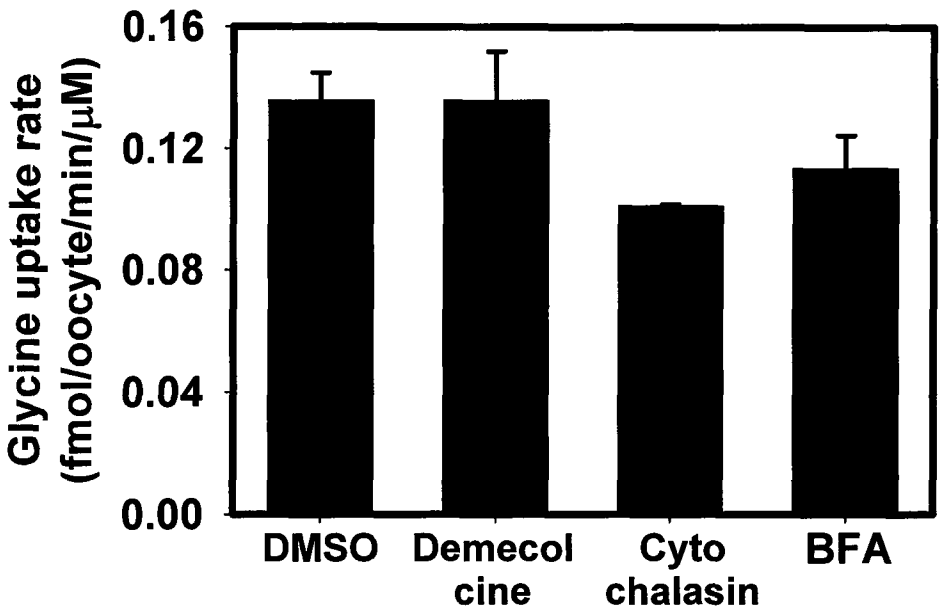


Figure 20. Influence of cytoskeleton and Golgi apparatus disruption on the development of glycine transport in oocytes. Glycine transport rates were measured in GV oocytes exposed for 4 hr to Demecolcine (microtubule disruptor) or Cytochalasin (microfilament disruptor) or Brefeldin A (Golgi apparatus disruptor) and DMSO (control). No statistical difference was found between groups ($p>0.05$; ANOVA and Tukey-Kramer test). Each bar represents mean ± SEM of 3 replicates.

I also investigated the possibility of vesicle trafficking and fusion in activation of GLYT1 activity in oocytes. The SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) protein Syntaxin A, has been shown to functionally interact and influence GLYT1 activity in cell lines and brain tissue (Geerlings *et al.*, 2000) (see Introduction). The SNARE model refers to the ability of specific vesicle and target membrane proteins to form a SNARE-complex during vesicle docking and fusion allowing release of the vesicle cargo molecules (Rothman, 2002). Although this process was originally described in neurotransmission, other tissues including oocytes express SNARE proteins. Some of these proteins have been shown to exhibit sensitivity to the Botulinum (BoNT) or Tetanus neurotoxins (TeNT) (Humeau *et al.*, 2000). Therefore, I have investigated the involvement of SNARE proteins in controlling activation of GLYT1 by direct oocyte exposure to toxin in culture or by microinjection of their recombinant light chains within the oocyte (see Table 4). Evaluation of glycine transport 4 hr later revealed no effect on development of transport in oocytes. The possibility of neurotoxin-insensitive SNARE involvement in GLYT1 activation in oocytes remains an open possibility, since a number of SNARE protein isoforms are not sensitive to these neurotoxins.

Some mechanisms of protein trafficking and insertion into the membrane do not depend on the cytoskeleton or toxin-sensitive SNARE proteins (Clayton *et al.*, 1995). Therefore, I tried to directly identify the localization of GLYT1 protein within the oocyte in freshly-collected GV oocytes before GLYT1 activity has developed and in GV-arrested oocytes or *in vitro* obtained MI oocytes maintained for 3 hr in culture to allow GLYT1 activation. Immunocytochemistry and two different anti-GLYT1 antiserums, one

generated in the Zafra laboratory (Zafra *et al.*, 1995), used for Western blotting, above, and the other a commercially available antiserum raised against a GLYT1-specific peptide (Chemicon), were utilized. Confocal microscopy was used to examine the pattern of GLYT1 protein localization.

The immunocytochemistry studies proved to be challenging and time consuming. One factor that constituted a major hurdle during these experiments was represented by the absence of a good, reliable anti-GLYT1 antibody. After several antibodies were tested I opted for 2 antisera, one commercially available and one generously donated by Dr. Francisco Zafra. An initial fixation protocol, successfully used in our laboratory for staining intracellular tubulin (Petrunewich *et al.*, 2008), utilizing a 2% formaldehyde solution to fix and a 0.1% Triton X solution to permeabilize oocytes permitted preservation of the ZP around the oocyte membrane. This method was chosen based on the need for a mild fixation protocol, allowing preservation of oocyte membrane, the cell structure where GLYT1 protein was expected to be localized when active. Unfortunately, this manner of processing oocytes generated a series of artifacts, appearing to indicate that GLYT1 protein was indeed membrane-localized only at those stages of meiotic maturation where GLYT1 was known to be active (MI and MII matured oocytes), but not before (fresh GV oocytes). However, after numerous controls were run to confirm this observation, I was unfortunately able to prove that the antibodies were precipitating between the oocyte membrane and ZP. The presence of a larger PVS at those stages at which GLYT1 was active (MI, MII matured oocytes), compared with freshly collected GV oocytes (with inactive GLYT1), allowed the precipitation, leading to the false impression of GLYT1 appearance in the membrane. When the ZP was removed, none of

the antibodies provided any staining at any stage of oocyte maturation with this fixation protocol. A different fixation protocol utilizing Methanol and DMSO was then subsequently tested. After determining the best working conditions, I have opted for a short fixation (2.5 min) in a fixation solution of 50% Methanol and 50% DMSO.

With this fixation protocol, confocal immunocytochemistry with both anti-GLYT1 antisera (Chemicon, Zafra) showed staining in the membranes of fresh GV oocytes as well as in the membrane of GV-arrested oocytes (Fig. 21) and *in vitro* obtained MI oocytes (not shown). The peptide against which one of the anti-GLYT1 antisera (Chemicon) was raised eliminated much of the membrane staining (Fig 21 , lower panel) although the amount of decrease of staining was variable, while the presence of an irrelevant peptide (Anion Exchanger 2) did not influence the staining (not shown).

Together, these results indicated similar GLYT1 membrane-localization prior to and subsequent to its activation implying activation of pre-existing GLYT1 through a mechanism not involving protein trafficking.

2. 3. Potential regulators of GLYT1 activity in brain do not control activation of GLYT1 in oocytes

Activity, regulation and function of GLYT1 in modulating inhibitory and excitatory neurotransmission have been studied in the last few decades, however the exact molecular regulatory mechanisms controlling its activity in brain have not yet been identified. Several factors are thought to contribute to this regulation, although most of the data rely on *in vitro* expression systems or tissue preparation (see Introduction). To determine if some of these potential regulators are involved in initiating GLYT1 activity

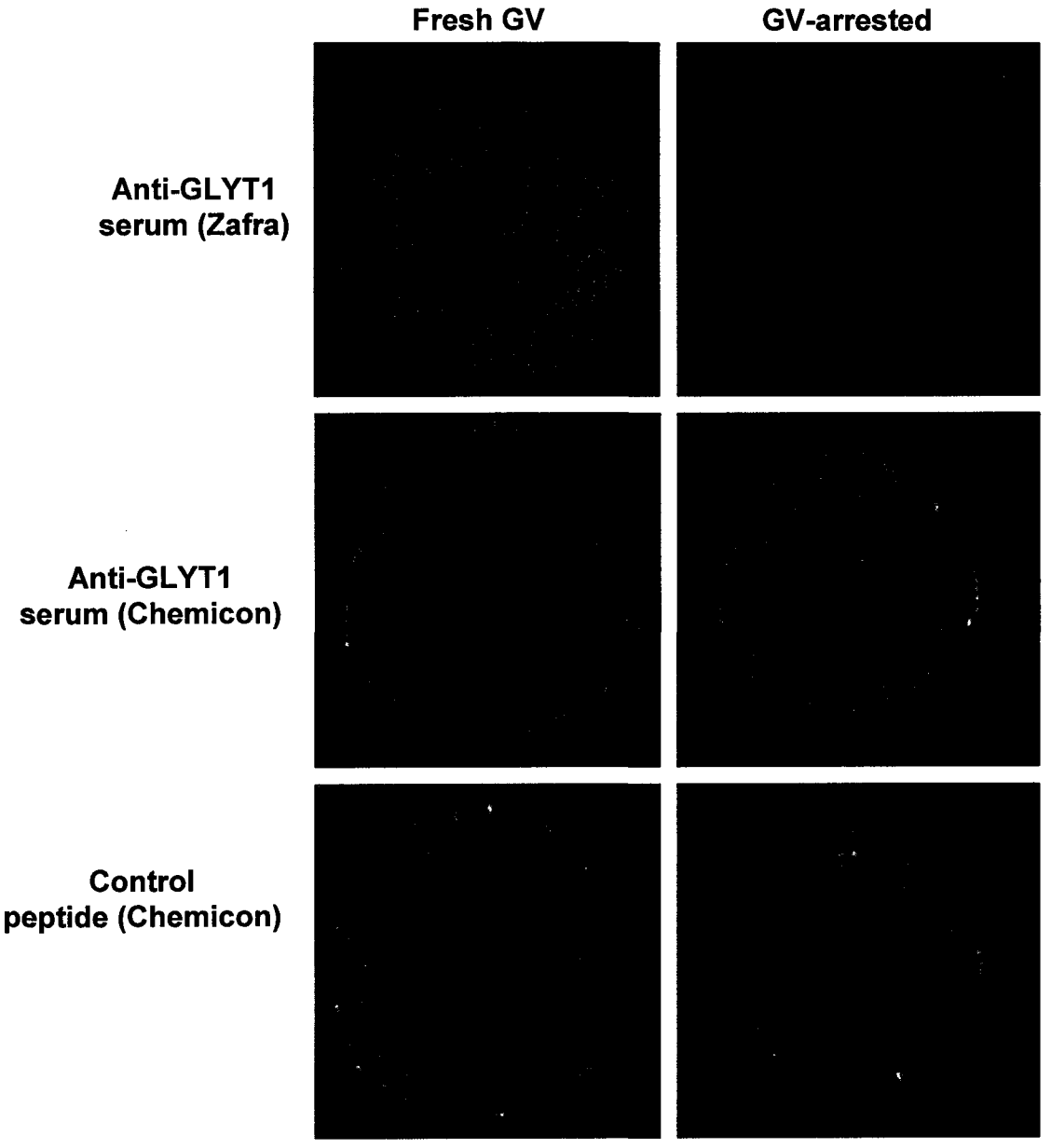

Fresh GV
GV-arrested
Anti-GLYT1 serum (Zafra)
Anti-GLYT1 serum (Chemicon)
Control peptide (Chemicon)

Figure 21. Localization of GLYT1 protein before and after its activation in oocytes. Immunocytochemistry was carried out with GLYT1 affinity-purified antiserum (Zafra laboratory) (upper panel) and a GLYT1-specific antiserum (Chemicon) (mid and lower panel). GLYT1 protein was detected near the plasma membrane in freshly-collected GV oocytes (left) as well as in GV-arrested oocytes after 4 hr in culture to allow development of maximal GLYT1 activity (right). Presence of the immunogenic GLYT1 peptide (lower panel) decreased membrane labeling, while an unrelated peptide (against mouse anion exchanger isoform 2) had no effect (not shown). GV oocytes after 4 hr culture had both decreased volume and exhibited greater shrinkage when fixed, accounting for the smaller size. Confocal micrographs are representative of 3-5 independent replicates.

in oocytes, I tested an array of inhibitors or activators of various signaling pathways proposed to be important in regulating GLYT1 activity in CNS. The general approach of these experiments aimed at inhibiting development of glycine transport in oocytes maintained in culture for a period of 4 hr. Therefore, freshly-collected GV oocytes cultured in the presence or absence of various inhibitors and activators (Table 7) for 4 hr were tested for their ability to transport glycine compared to a control group (freshly-collected GV oocytes cultured in the presence of vehicle, DMSO 0.1%).

The presence of putative phosphorylation sites in the GLYT1 polypeptide and the ability of phorbol esters, potent activators of PKC, to down-regulate GLYT1 activity in cell lines (Gomeza *et al.*, 1995; Sato *et al.*, 1995) (see Introduction), implied a possible regulatory mechanism of GLYT1 involving phosphorylation. To assess this possibility, I targeted various kinases or phosphatases by exposing the oocytes to a PKC activator (phorbol 12-myristate 13-acetate, PMA, 50 ng/ml), a general kinase inhibitor (Staurosporine, 100 μM), or serine/threonine phosphatase inhibitors (Okadaic acid, 2.5 μM or β-glycerophosphate, 10mM). However, none of these drugs impeded development of glycine transport after 4 hr of exposure (not shown). A protein tyrosine phosphatase inhibitor (Sodium Orthovanadate, 1mM), exerted a partial, reversible inhibitory effect on the development of glycine transport. However, when tested for any toxic effect, orthovanadate decreased the oocyte's ability to survive *in vitro* (even after short exposure), suggesting a toxic effect rather than a direct action on GLYT1 activity initiation.

Table 7. The effect of potential regulators of GLYT1 activity in brain on glycine transport in oocytes

Inhibitor or activator	**Concentration**	**General action**	**Effect on development of glycine transport in oocytes after 4hr of exposure or microinjection**
Staurosporine	100 μM	General kinase inhibitor	No effect
PMA (phorbol 12-myristate 13-acetate)	50 ng/ml	PKC activator	No effect
Okadaic acid	2.5 μM	Inhibitor of serine/threonine phosphatases	No effect
Sodium fluoride	5 mM	Acid phosphatases inhibitor	Unable to determine, the oocytes did not survive
Sodium pyrophosphate, decahydrate	1 mM	Serine/threonine phosphatases inhibitor	Unable to determine, the oocytes did not survive
β-glycerophosphate	10 mM	Serine/threonine phosphatases inhibitor	No effect
Sodium Orthovanadate	1 mM	Protein Tyrosine phosphatase inhibitor	Partial reversible inhibition of development of glycine transport in oocytes (by 4 hr of exposure only half transport developed); Toxic effect (many oocytes died).

In the last decade, it was suggested that the cellular membrane contains liquid-ordered microdomains, called lipid rafts, rich in cholesterol and saturated phospholipids. They are thought to act as platforms for cell adhesion and signaling molecules (Simons and Ehehalt, 2002). The inhibitory effect of arachidonic acid on GLYT1 activity in cell lines was proposed to be a consequence of disruption in the lipid domain surrounding the transporter (Zafra *et al.*, 1990). To investigate the involvement of lipid rafts in controlling GLYT1 activation, I made use of Methyl-Beta-Cyclodextrin (4 mM), a compound that is able to induce membrane cholesterol depletion causing disruption of lipid rafts.

Fresh GV oocytes exposed for 3 hr to Methyl-Beta-Cyclodextrin did not develop glycine transport (Fig. 22). I have also tried to dissociate between disruption of an unidentified signaling pathway(s) via lipid rafts necessary for GLYT1 activation, and the necessity of an intact membrane for GLYT1 functionality. For this, I assessed ability of MI oocytes that developed *in vitro* or 1-cell embryos (stages at which GLYT1 is fully active) to transport glycine following exposure to Methyl-Beta-Cyclodextrin for 3 hr. Unexpectedly, neither the oocyte or the 1-cell embryo (not shown) were able to transport glycine (Fig. 22), suggesting that functional lipid rafts or other cholesterol-dependent processes are necessary for maintaining GLYT1 activity.

Intracellular Ca^{2+} is known to be able to act as a second messenger, participating in numerous signaling pathways. By using BAPTA-AM (50 μM), an intracellular Ca^{2+} chelator, I have investigated intracellular Ca^{2+} involvement in the initiation of GLYT1 activity in oocytes. When exposed to BAPTA-AM for 3 hr (Fig. 23), the oocytes did not develop glycine transport. However, BAPTA-AM had a similar effect on 1-cell embryos, inhibiting GLYT1 activity. Together, these results suggested that intracellular Ca^{2+} is also

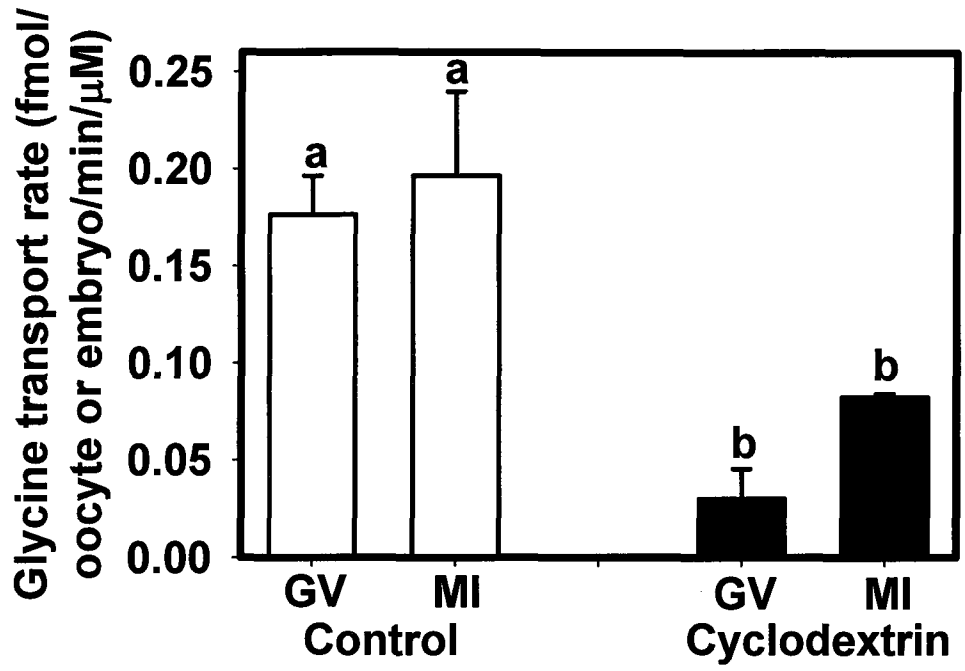


Figure 22. The influence of membrane cholesterol on glycine transport in oocytes. Oocytes were treated with Cyclodextrin, which should cause depletion of membrane cholesterol. Fresh GV oocytes (before GLYT1 activity developed) and *in vitro* obtained MI oocytes (3 hr in culture, with fully active GLYT1) were exposed to Cyclodextrin for an additional 3 hr. GV oocytes were maintained in GV arrest with dbcAMP. Glycine transport rates were low for both GV and MI oocyte groups exposed to Cyclodextrin, when compared with control groups (no Cyclodextrin). Different letters indicate significant differences ($p < 0.001$ by ANOVA and Tukey-Kramer test). Each bar represents mean ± SEM of 3 replicates.

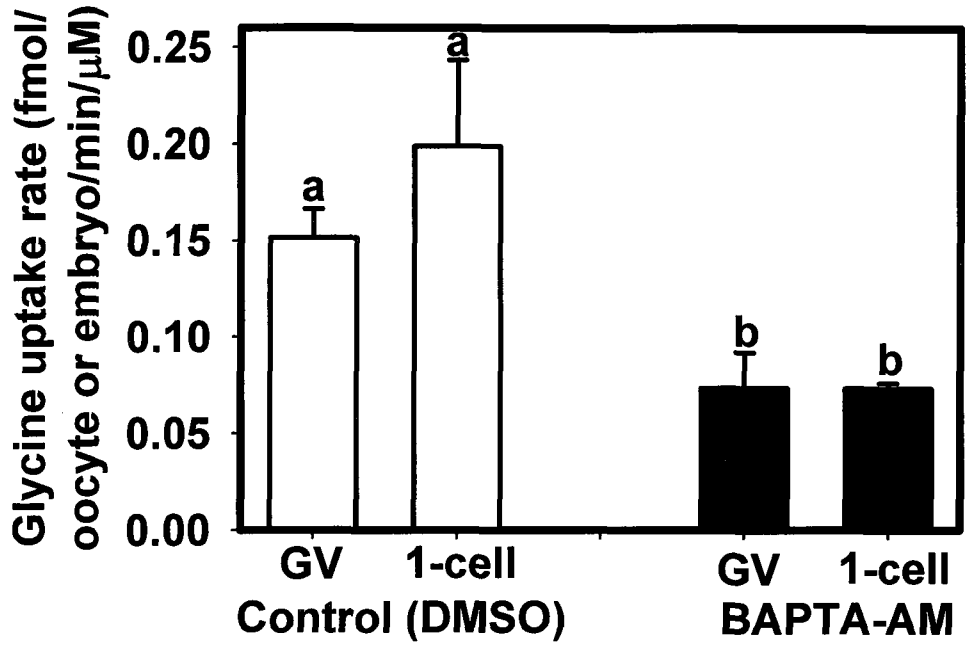


Figure 23. The influence of intracellular Ca^{2+} on glycine transport in oocytes. Intracellular Ca^{2+} was chelated with BAPTA-AM. Experimental groups were chosen as for Fig. 22 with the exception of MI oocytes being replaced by fresh 1-cell embryos (with fully active GLYT1). Glycine was transported at low rates in both groups compared with control groups (GV oocytes or 1-cell embryos exposed to DMSO = vehicle). Different letters indicate significant differences ($p < 0.05$ by ANOVA and Tukey-Kramer test). Each bar represents mean ± SEM of 3 replicates.

needed for maintaining GLYT1 function rather than being implicated only in activation of transport activity.

Overall, the results reported in this section indicate that potential regulators of GLYT1 activity in brain were not found to be clearly implicated in GLYT1 transport activation in oocytes.

2. 4. An intact antral follicle and functional gap junctions are required to maintain GV oocyte GLYT1 quiescence

During cumulus cell expansion, the connections between cumulus granulosa and oocyte become gradually lost by ~ 2 hr following stimulation of ovulation (Gilula *et al.*, 1978). Gap junctions are thought to constitute an important route for metabolite and signals transfer between oocyte and its surrounding somatic cells (Heller *et al.*, 1981; Kidder and Mhawi, 2002; Gittens *et al.*, 2004) (see Introduction). My data indicated that GLYT1 activity was initiated only following stimulation of ovulation in parallel with but independent of meiotic maturation (above). Furthermore, in both intact COC and denuded oocytes maintained in GV-arrest, GLYT1 transport began shortly after their removal from follicles and developed similarly to meiotically maturing oocytes. Together, these suggested that a loss of oocyte interaction with its follicular environment both *in vivo* and *in vitro* might lead to initiation of GLYT1 activity in oocytes. To test such a possibility, I studied GLYT1 activity in oocytes removed from *in vitro* cultured antral follicles with functional or disrupted gap junctional communication.

Since my data indicated that glycine transport development follows removal of the oocyte from the ovarian follicle (above), I have hypothesized that the follicular

environment inhibits GLYT1 activity in the oocyte. Furthermore, I hypothesized that functional gap junctions in the follicle are required to suppress GLYT1 activity. If these hypotheses are correct, intact antral follicles should be sufficient to maintain GLYT1 quiescence, but disrupting gap junctional communication should relieve the inhibition.

To test if an intact antral follicle is necessary to maintain GLYT1 quiescence in oocytes, glycine transport rates were measured in GV oocytes that were retrieved from *in vitro* cultured (for 4h) antral follicles and compared with glycine transport rates in denuded GV oocytes maintained arrested in culture (with 100 μM dbcAMP) for the same amount of time. Confirming my prediction, oocytes removed from *in vitro*-cultured antral follicles after 4 hr had significantly lower GLYT1 activity compared with denuded oocytes cultured for 4 hr (Fig. 24 A). All oocytes removed from the cultured antral follicles had maintained their GV arrest and were able to progress through meiotic maturation once removed from the follicle (not shown), confirming their viability.

To determine whether functional gap junctions were required for GLYT1 inhibition in the intact follicle, I cultured antral follicles in the presence or absence of a gap junction inhibitor (50 μM, 18-α-glycyrrhetinic acid, AGA) (Fitzharris and Baltz, 2006). Four hours later, glycine transport rates were measured in GV oocytes removed from these follicles. GLYT1 activity developed in the enclosed oocytes when the antral follicles were cultured in the presence of AGA (Fig. 24 B), in contrast to GV oocytes collected from follicles cultured in the presence of vehicle (DMSO 0.1%) alone or in the absence of both AGA and DMSO. All oocytes collected from the cultured antral follicles, including those exposed to AGA had maintained their GV-arrest.

Interestingly, when cultured *in vitro*, those oocytes retrieved from AGA-exposed

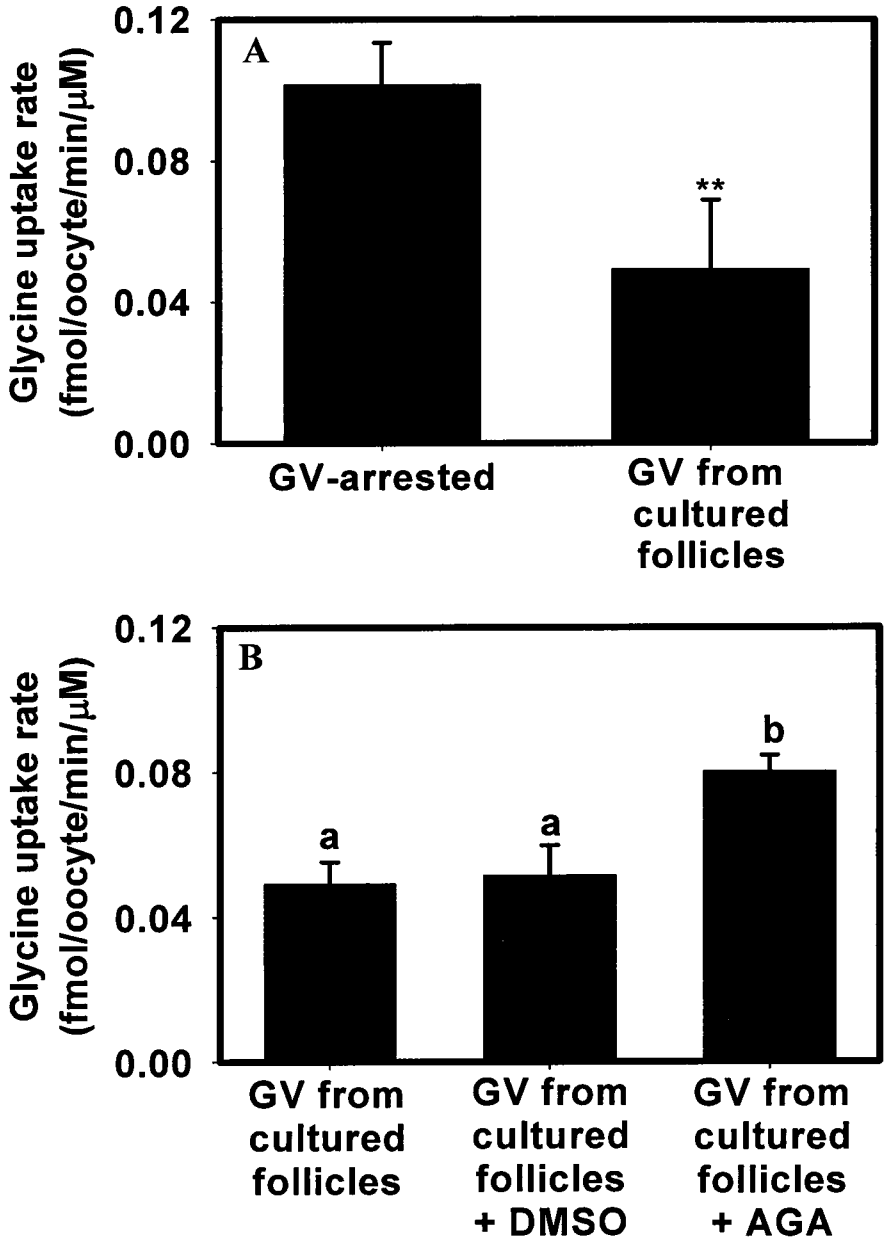

A
Glycine uptake rate (fmol/oocyte/min/μM)
0.12
0.08
0.04
0.00
**
GV-arrested
GV from cultured follicles
B
Glycine uptake rate (fmol/oocyte/min/μM)
0.12
0.08
0.04
0.00
a
a
b
GV from cultured follicles
GV from cultured follicles + DMSO
GV from cultured follicles + AGA

Figure 24. Influence of the antral follicle environment on GLYT1 activation in oocytes. ***(A) Lack of glycine transport development in oocytes within the antral follicle.*** Glycine transport rates were low in GV oocytes immediately after their removal from cultured antral follicles (4 hr in culture) compared with oocytes maintained in GV arrest by dbcAMP for 4hr (**, $p<0.01$, by Student's *t*-test). ***(B) Influence of follicular gap junction functionality on the development of glycine transport in GV oocytes.*** Functionality of antral follicle gap junctions was disrupted by the gap junction inhibitor 18-α-glycyrrhetinic acid, AGA. All antral follicles were maintained in culture (without or with AGA or with DMSO, vehicle) for 4 hr. GV oocytes freshly removed from antral follicle cultured in the presence of AGA exhibited higher glycine transport rates compared with controls (GV oocytes freshly removed from antral follicles cultured without AGA or with DMSO, vehicle) ($p < 0.05$ by ANOVA and Tukey-Kramer test). Throughout, each bar represents mean ± SEM of 4-6 replicates.

follicles were unable to progress through first meiosis (only 25% underwent GVBD) (not shown). This was most likely not due to AGA toxicity since freshly-denuded GV oocytes exposed to AGA survived and progressed through meiotic maturation (not shown), but rather a reflection of improper communication between different follicular compartments (including oocyte) in the absence of gap junctional communication prior to oocyte removal from follicle.

Overall, these experiments confirmed that the integrity of antral follicle and functional gap junctional communication were required to maintain GLYT1 quiescent in the oocyte within the follicle.

3. Prior to GLYT1 activity initiation oocyte size is controlled by the zona pellucida

Glycine accumulation via GLYT1 is the main cell-volume regulatory mechanism utilized by the 1-cell embryo (Steeves *et al.*, 2003). My findings, as reported above, indicated that this mechanism is initiated only after ovulation is triggered, around the time of the GV-to-MI transition. Together, these raised the possibility of a different mechanism controlling GV oocyte volume within the antral follicle. Therefore, I have investigated the manner in which the oocyte regulates its volume before ovulation was triggered and GLYT1 became activated, beginning by determining whether oocyte volume changes during this period.

3. 1. Oocyte volume changes after stimulation of ovulation or when released from follicle

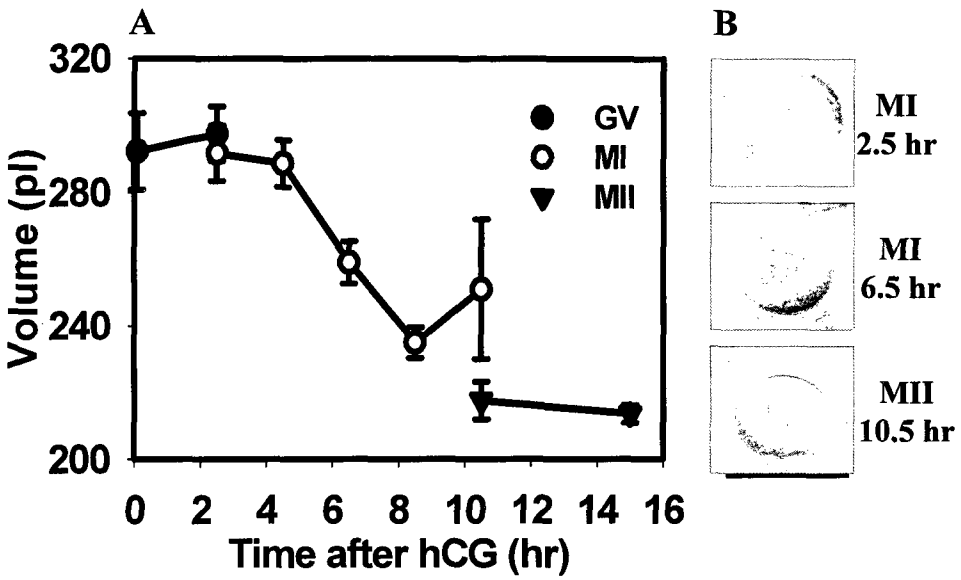
To investigate whether the oocyte controlled its volume differently before

ovulation, I first aimed to detect any changes in oocyte volume following stimulation of ovulation. To examine this, oocytes were retrieved at different time points 2-15 hr after ovulation was triggered *in vivo* by hCG injection. Oocytes were immediately placed into microdrops in culture dishes and micrographs were obtained as quickly as possible, with oocyte volume subsequently determined from the micrographs.

These volume measurements revealed a progressive decrease in oocyte volume (Fig. 25 A, B) with a loss of approximately 21% of initial volume that began at about 4 hr after ovulation was triggered by hCG stimulation and was completed by 8-10 hr. This reduction in oocyte volume was largely achieved before emission of the first polar body, which took place at approximately 10 hr post-hCG (Fig. 25 A).

A gap between the oocyte and its extracellular matrix zona pellucida (ZP), known as the perivitelline space (PVS) is not present in GV oocytes but is very apparent in MII oocytes (Talbot and Dandekar, 2003) (see Introduction). The exact timing and mechanism of PVS formation has not been previously investigated. The oocyte volume measurements I have collected following stimulation of ovulation indicated not only that the cell volume decrease was completed before first polar body emission, but also that PVS became apparent and formed in parallel with oocyte volume loss (Fig. 25 B).

Since the loss in oocyte volume following stimulation of ovulation occurred while the oocyte underwent meiotic maturation, I have tested the possibility of any association between these two events. Therefore, I have examined the decrease in oocyte volume *in vitro*, using oocytes that were arrested at the GV stage with dbcAMP (100 μM) up to 7 hr after collection. Despite the continued presence of the GV and prevention of polar body emission, oocyte volume again was found to decrease (Fig. 25 C, D).


A
B
320
280
240
200
Volume (pl)
GV
MI
MII
0 2 4 6 8 10 12 14 16
Time after hCG (hr)
MI
2.5 hr
MI
6.5 hr
MII
10.5 hr

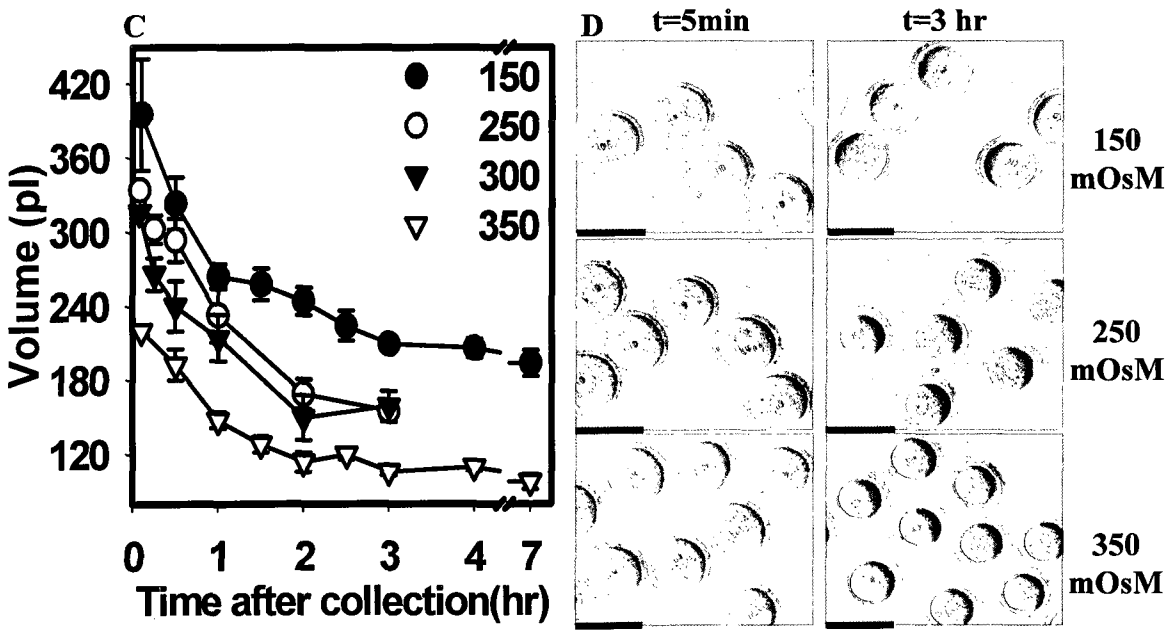

C
D
t=5min
t=3 hr
420
360
300
240
180
120
Volume (pl)
150
250
300
350
0 1 2 3 4 7
Time after collection(hr)
150
mOsM
250
mOsM
350
mOsM

Figure 25. Change in oocyte volume after ovulation or after release from follicle. ***(A) Oocyte volume changes in vivo after stimulation of ovulation.*** Oocytes collected at different time points after ovulation was triggered with hCG, were measured immediately after removal from ovaries. Oocyte volumes decreased with time after stimulation of ovulation, with the major decrease occurring during MI (meiotic stage indicated by key). ***(B) Examples of oocytes at several time points after ovulation was triggered with hCG.*** The times post-hCG and meiotic stages are indicated at right. Scale bar = 100μm. ***(C) Oocyte volume changes in vitro after oocyte removal from follicle.*** Oocytes maintained arrested at GV stage by dbcAMP were cultured at various osmolarities from hypotonic (150 mOsM) to osmolarity of follicular fluid (isotonic, 300 mOsM) to hypertonic (350 mOsM) (osmolarities indicated by key) and used for measurements at different time points after collection. A decrease in GV oocyte volume was evident *in vitro* after removal from the ovary at all osmolarities. ***(D) Examples of oocytes at various osmolarities at several time points after oocyte removal from follicle.*** The times after collection and osmolarities are indicated at top and at right, respectively. Scale bar = 100μm. Throughout, each point represents mean ± SEM of 3 replicates.

The loss of volume *in vitro* occurred similarly in media with osmolarities ranging from 150 to 350 mOsM (Fig. 25 C, D). Surprisingly, even in substantially hypotonic medium (150 mOsM) or in medium isotonic with follicular or oviductal fluid (300 mOsM) the oocyte volume decreased. This excluded the possibility that the decrease in volume was simply the result of a change in tonicity following transfer from the ovary to culture. These data also confirm that PVS development with time *in vitro* resembles its formation *in vivo* following stimulation of ovulation (Fig. 25 D). Thus, the decrease in oocyte volume took place both *in vivo* following stimulation of ovulation while the oocyte was transitioning to MI stage, and *in vitro* in oocytes maintained in GV-arrest, confirming independence of volume loss and PVS formation from meiotic maturation.

3. 2. The PVS forms only after oocyte removal from the follicle

The above observations suggested that a PVS forms only after ovulation is triggered *in vivo* or following oocyte removal from the follicle *in vitro*. To examine this at the ultrastructural level, GV-arrested oocytes were fixed, embedded, and thin-sectioned for transmission electron microscopy (TEM) 0-3 hr after removal from the ovary. Sections of freshly-collected GV oocytes observed by TEM showed a lack of space between oocyte and ZP, with the oocyte plasma membrane and surface microvilli closely apposed to the inner surface of the ZP (Fig. 26). Sections of oocytes fixed at later time points indicated that the oocyte progressively lost its contact with the ZP material, allowing the time-dependent formation of a PVS. By 2-3 hr after removal from follicle, the oocyte had completely lost its physical interaction with ZP, a PVS had formed, and the oocyte microvilli lay free in the newly formed space (Fig. 26). These observations

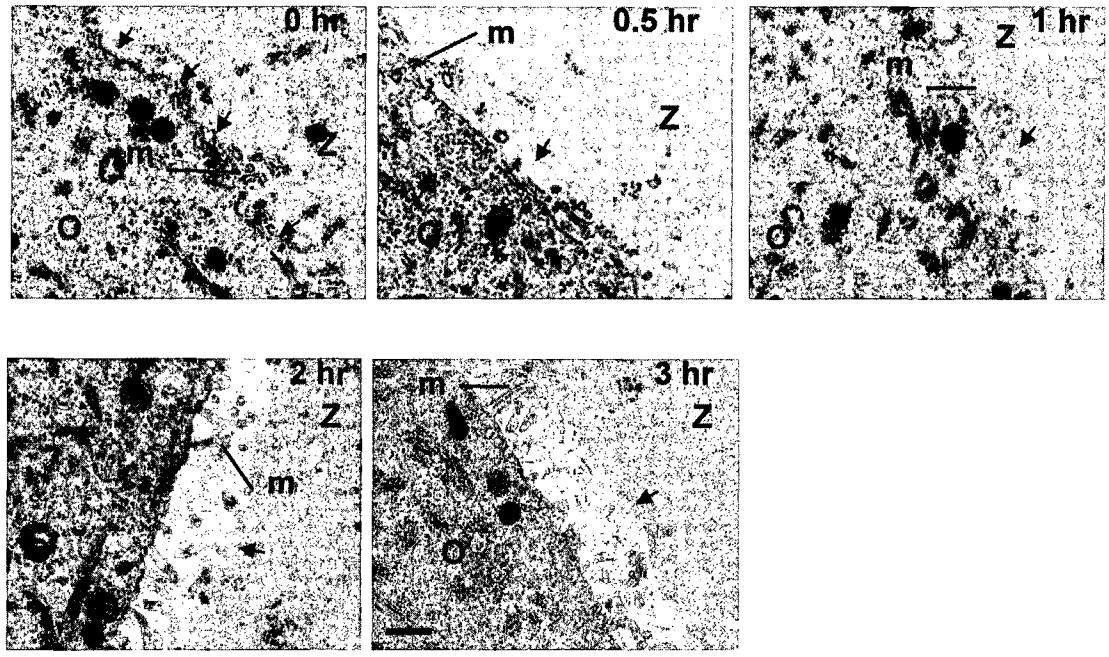


Figure 26. Appearance of PVS *in vitro*. Electromicrographs (TEM) of GV-arrested oocytes (by dbcAMP) at various times after collection (indicated in panels) revealed the appearance of a gap between oocyte (O) and ZP (Z) beginning around 0.5 hr and full formation of a space by 2hr. Arrowheads indicate inner ZP surface; m indicates microvilli. Scale bar in lower right panel = 0.5 μm. Electromicrographs are representative of more than 3 independent sets of replicates.

strongly suggested the absence of PVS in fresh GV oocytes removed from the antral follicles. They also indicated that the physical interaction between oocyte and ZP became lost by 2 hr after collection allowing PVS formation.

3. 3. The ZP determines oocyte volume before ovulation

3. 3. 1. Before the PVS forms, oocyte size is constrained by a physical oocyte-ZP interaction

Together, the above data suggested either that the oocyte is passively maintained in contact against the inner ZP surface due to a large size relative to the ZP cavity, or that a physical interaction between the oocyte and inner surface of the ZP constrains the size of the enclosed oocyte and maintains oocyte-ZP contact. To differentiate between these possibilities, I exposed freshly-isolated GV oocytes to a substantial hypertonic shock (450 mOsM). The hypothesis was that, in the absence of oocyte-ZP adhesion, hypertonically-shocked oocytes should shrink into small spheres with a large space between the oocyte membrane and ZP, whereas, if there was a strong adhesion, the oocyte would not be able to respond freely but should remain in contact with the ZP.

When GV oocytes were exposed immediately after collection to hypertonic shock and examined by light microscopy, they were found to have become deformed (Fig. 27 B), assuming a shape with a concave surface that also deformed the ZP (Fig. 27 C), or to have developed concave cavities between oocyte and ZP that I interpreted as local adhesion failures (Fig. 27 D).

Conversely, if the oocytes were exposed for more than 30 min to hypertonic shock (Fig. 27 H) or kept for 30 min at normal osmolarity and then exposed to hypertonic

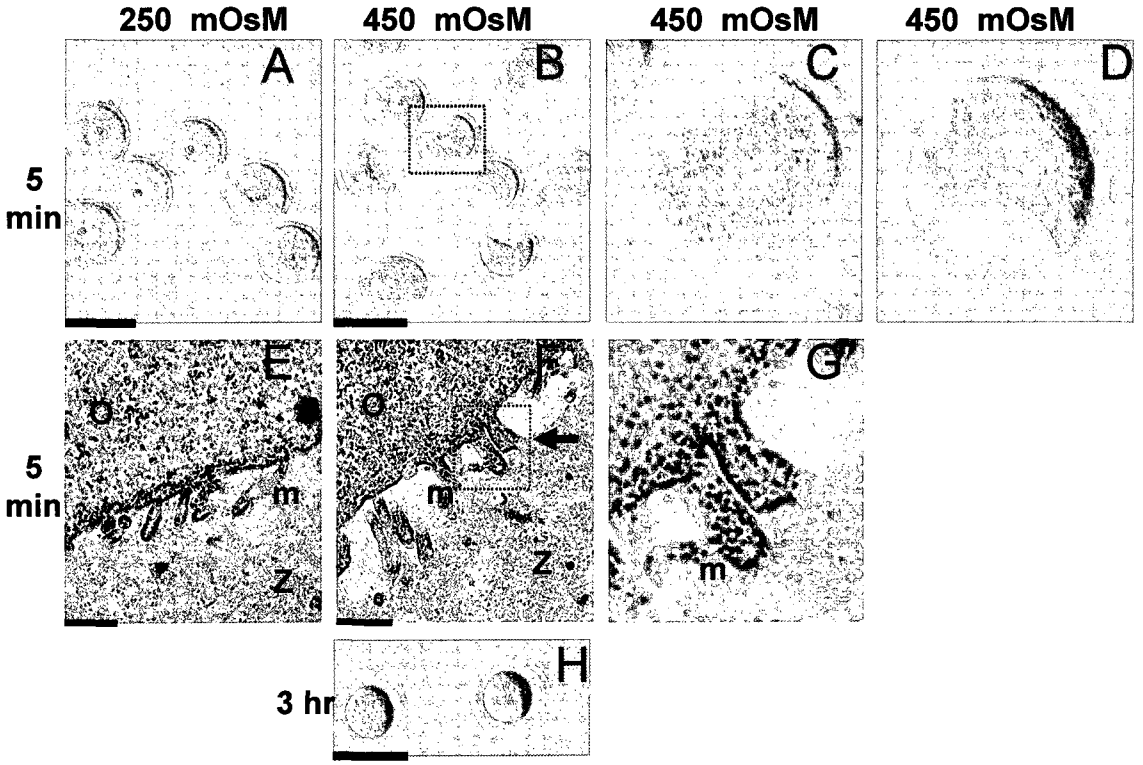

250 mOsM
450 mOsM
450 mOsM
450 mOsM
A
B
C
D
5 min
E
F
G
5 min
o
m
z
H
3 hr

Figure 27. Effect of hypertonic shock on oocyte-zona pellucida contact. Light microscopy pictures of oocytes exposed to osmolarity of normal culture medium, 250 mOsM (A) or hypertonic shock, 450 mOsM (B, C, D, H) (as indicated at the top) were taken at 5 minutes after collection (as indicated at left) (A, B, C, D) or 3 hr after collection (H). Hypertonic shock of freshly-collected oocytes (B) resulted in deformation of ZP (magnified in C) or formation of concavities (exemplified in D) indicating the apparent adhesion between oocyte and ZP. The oocyte within the box in B is shown at higher magnification in C. Hypertonic shock of oocytes following culture for 3 hr resulted in uniform shrinkage revealing lack of adhesion (H). Middle panel shows TEM of oocyte-zona contact regions at 250 mOsM (E) or 450 mOsM (F, G). For oocytes exposed to hypertonic shock adhesion between microvilli and ZP (arrow) was evident (F, boxed area is magnified in G). For A, B, H, scale bar = 100μm; for E, F, scale bar = 0.5 μm. Photo- and electromicrographs are representative of more than 3 independent replicates.

shock (not shown), they were able to respond by shrinking substantially in a uniform spherical shape away from the ZP, as expected for an independent, osmotically-responsive cell.

To examine the possible adhesion between oocyte and ZP in more detail, fresh GV oocytes were fixed immediately after hypertonic (450 mOsM) shock and examined at the ultrastructural level by TEM. This revealed that, in regions of the oocyte in which the ZP had become deformed and concave, the oocyte surface and the inner surface of the ZP were still closely associated (Fig. 27 F). Furthermore, the oocyte microvilli had clearly become stretched and appeared to adhere to the inner ZP surface at their tips (Fig 27 F, G) In contrast, oocytes that had not been subjected to hypertonic shock displayed microvilli mainly lying flat against the oocyte surface and embedded within the ZP material (Fig 27 E). Taken together, these results implied that the GV oocyte from an antral follicle strongly adhered to the inner ZP surface, probably through the microvillar tips.

By using a microinjection technique I developed, I was able to obtain additional evidence indicating the absence of a space and strong adhesion between the oocyte and ZP immediately after collection. Freshly-collected GV oocytes and oocytes left to develop the PVS *in vitro* (either maintained in the GV-arrest or maturing) were microinjected with a drop of mineral oil at the interface between oocyte and ZP. The oil drop clearly was deposited in a space between the oocyte plasma membrane and the inner surface of the ZP in GV-arrested oocytes (Fig. 28 B) or *in vitro* obtained MI oocytes (not shown) that had been removed from the ovary 3 hr before microinjection. However, in fresh GV oocytes (Fig. 28 A), the oil remained trapped in a restricted cavity, and was

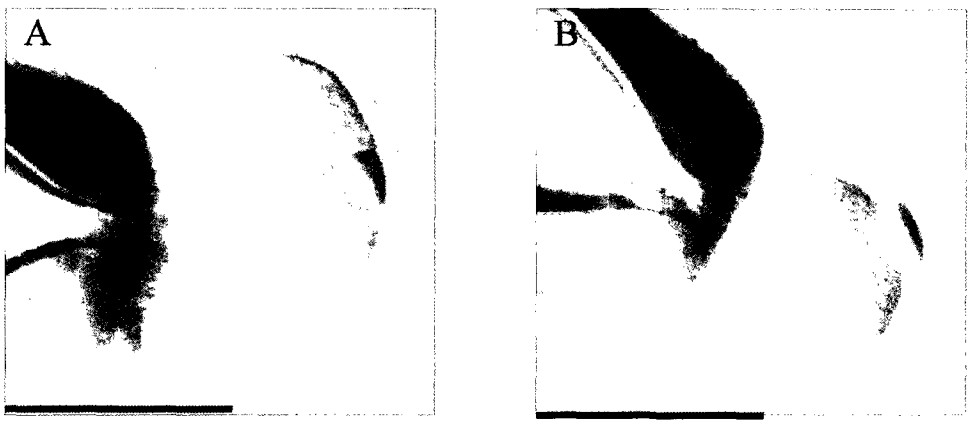


Figure 28. Differences in oocyte-ZP interaction in fresh and GV-arrested oocytes revealed by oil microinjections. Freshly collected GV oocyte (A) or oocytes arrested at GV stage by dbcAMP (4 hr in culture) (B) were microinjected with mineral oil between oocyte membrane and ZP. Oil microdrop revealed that oocyte surface could be displaced from the ZP only in arrested but not freshly-collected GV oocytes. Scale bar = 100 μm. Photomicrographs are representative of more than 3 independent replicates.

unable to displace the oocyte surface from the ZP, indicating a strong adhesion.

3. 3. 2. In the absence of the ZP, oocyte volume decrease occurs faster

If oocyte size is determined by a strong adhesion to ZP within the follicle, this would predict that removal of the ZP might accelerate the volume decrease that occurs after removal of GV oocytes from the follicle (described above; Fig 25). To test this, I immediately removed the ZP from fresh GV oocytes using acid Tyrode's solution. Subsequently, oocyte volume was measured over time (0 to 3 hr) upon exposure to a range of different osmolarities from 150 mOsM to 350 mOsM and compared to oocytes treated similarly but with an intact ZP. Oocytes without a ZP decreased in volume more quickly compared with those with intact ZP (Fig. 29 A, B). This supported a role of oocyte-ZP adhesion in determining GV oocyte volume.

Overall, my data indicated that, within the ovarian follicle, a strong adhesion between oocyte and ZP determines oocyte volume at the GV stage before ovulation is triggered. Following ovulation or oocyte release from the follicle, this physical interaction is gradually lost, and a resetting in oocyte volume takes place.

3. 4. Possible mechanisms controlling oocyte-ZP adhesion

An issue I tried to address concerned the nature of the adhesion between the oocyte and ZP. The three glycoproteins that assemble to form the ZP (ZP1, ZP2 and ZP3) are synthesized by the oocyte and released by proteolytic cleavage at the oocyte surface. The nascent ZP glycoproteins are thought to be deposited in a thin layer in very close proximity to the oocyte membrane allowing ZP thickening from inside out. Interestingly,

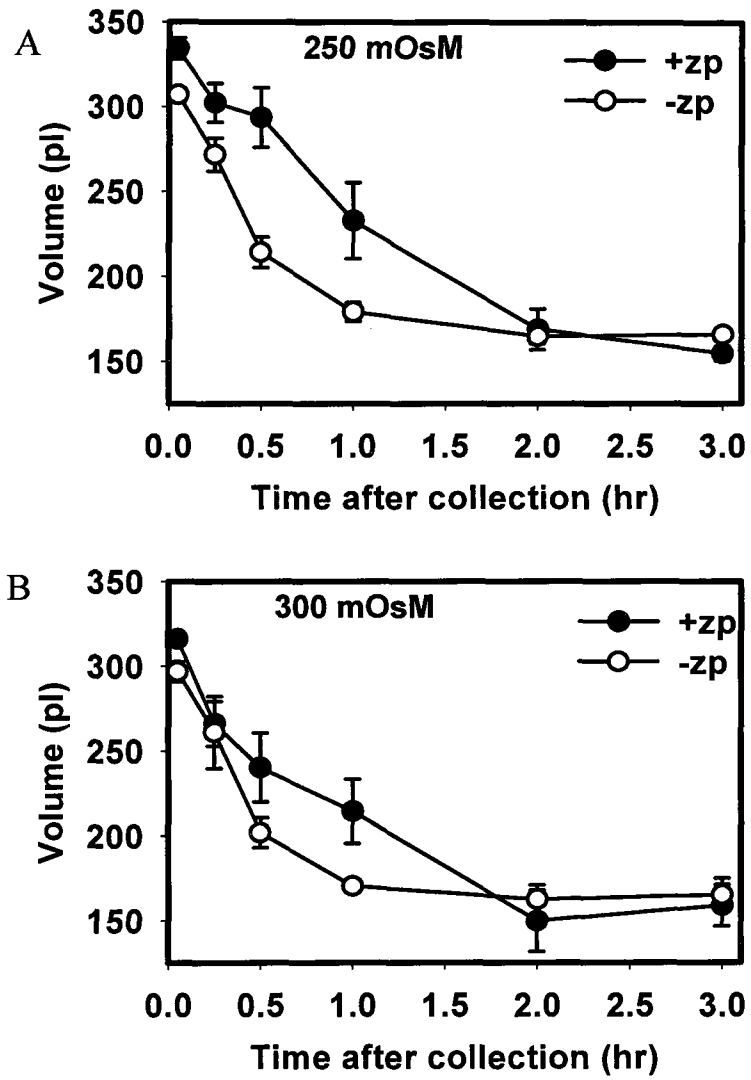


Figure 29. Volume decrease in oocytes with ZP removed. GV oocytes without or with ZP removed by acid Tyrode's solution (as indicated by key) were exposed to 250 mOsM (A), 300 mOsM (B), 150 mOsM and 350 mOsM (not shown) and volumes measured at various time points after collection. Throughout experiments oocytes were maintained arrested by dbcAMP. Removal of the ZP accelerated the initial volume decrease in GV oocytes by ~0.5 hr. Throughout, each point represents mean±SEM of 3 independent measurements.

this process stops at ovulation (Qi *et al.*, 2002) (see Introduction). Therefore, I have hypothesized that the continuous cleavage of ZP glycoproteins at the oocyte surface and their immediate assembly into the ZP constitutes the basis of oocyte-ZP adhesion before ovulation. Based on this hypothesis, the protease involved in ZP cleavage might be relevant for release of ZP adhesion from the microvilli and formation of PVS. If my hypothesis was correct inhibition of the protease responsible for ZP cleavage would result in oocyte-ZP adherence maintenance. Several studies suggested that furin, a member of proprotein convertase family, is the main protease candidate thought to mediate the ZP glycoprotein cleavage (Litscher *et al.*, 1999; Williams and Wassarman, 2001) although the involvement of unidentified trypsin-like proteases has been hypothesized as well (Zhao *et al.*, 2002) (see Introduction).

To test if furin is involved in the release of oocyte-ZP adhesion, I assessed the effects of two different furin inhibitors: α1-PDX (20 μM) a specific furin inhibitor and Decanoyl-Arg-Val-Lys-Arg-chloromethylketone (RVKR) (50 μM) a general convertase inhibitor. Two different approaches were utilized: 1) assess the behavior of freshly-collected GV oocytes cultured in the presence of one of the furin inhibitors for 30-45 min (sufficient time to allow release of adherence, but before large PVS forms) and subsequently expose to hypertonic shock (450 mOsM + furin inhibitor). In this assay, if the adherence was maintained, the oocyte was expected to develop the specific deformed, concave shape observed after exposure of freshly-obtained GV oocytes to hypertonicity (Fig. 30 A), whereas if adherence was lost the oocyte should uniformly shrink away from ZP. 2) Assess the freshly-collected oocyte's ability to decrease in volume over a period of 3 hr subsequent to their culture in the presence of one of the furin inhibitors compared

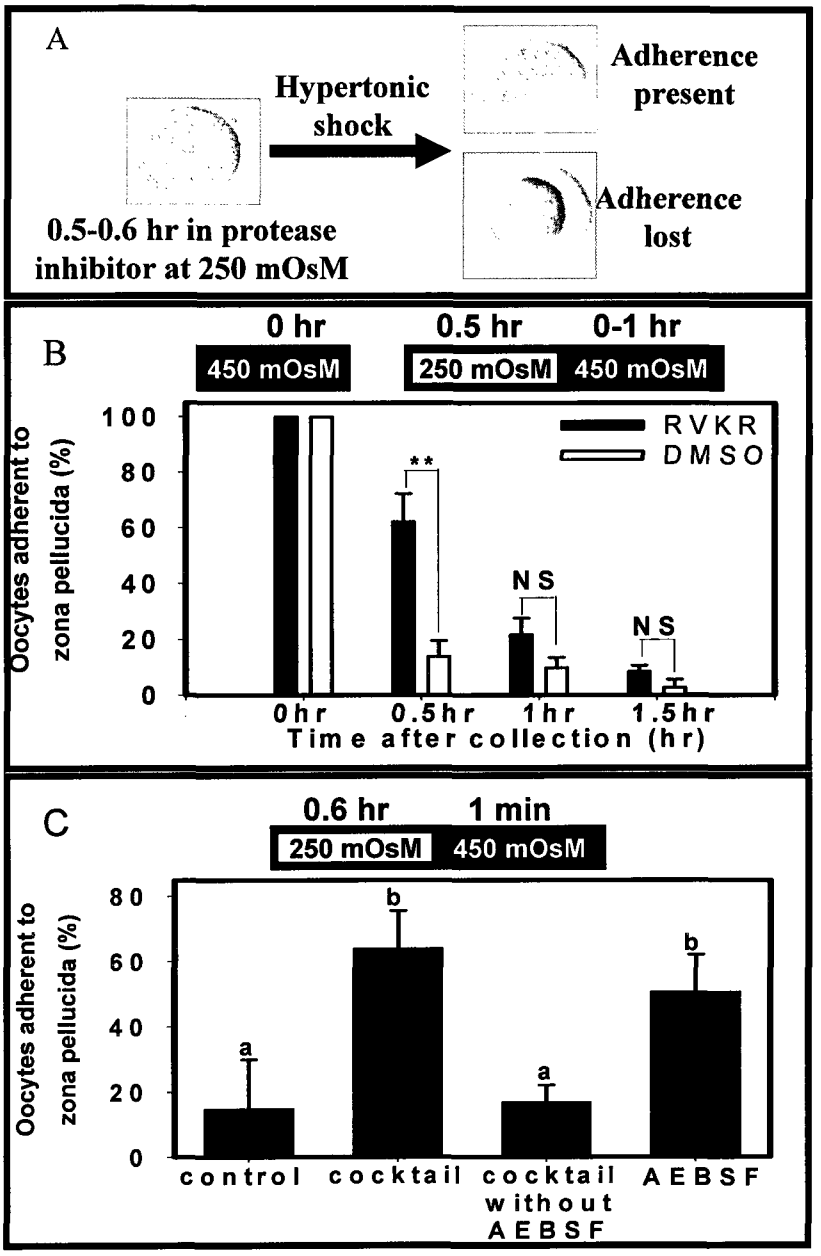


Figure 30. Effect of protease inhibition on oocyte-ZP adherence release. ***(A) Schematic representation of experimental design utilized to evaluate the effect of protease inhibitors on oocyte-ZP adherence.*** Freshly collected oocytes were exposed for 0.5-0.6 hr (time sufficient for oocyte-ZP adherence release) to various protease inhibitors. Upon exposure to hypertonic shock, if the protease inhibitor exerted an

inhibitory effect over oocyte-ZP adherence release, the oocyte should become deformed. Conversely, in the absence of an effect, the adherence should be lost and the oocyte should uniformly shrink away from the ZP. ***(B) Effect of RVKR on oocyte-ZP adherence release.*** Subsequent to their culture for 0.5 hr in the presence of RVKR, a general convertase inhibitor, or DMSO (vehicle), GV oocytes were transferred to hypertonic shock. Oocyte-ZP adherence presence was followed immediately after transfer (0.5 hr after collection) and every 0.5 hr thereafter (experimental protocol indicated by diagram above panel). For 0 hr group, immediately after collection oocytes were exposed to hypertonic shock. RVKR or DMSO were present in the media throughout all experimental steps. Upon exposure to hypertonic shock the oocytes exposed to RVKR maintained their adherence and developed the deformed phenotype, compared with the control group (DMSO). This effect was lost after 0.5 hr of exposure to hypertonicity. Comparisons between oocytes exposed to RVKR and DMSO were done within each time point (**, $p < 0.01$, by Student's *t*-test). ***(C) Effect of AEBSF on oocyte-ZP adherence release.*** Freshly collected GV oocytes were cultured immediately after collection for 0.6 hr in the absence or presence of a protease cocktail inhibitor containing AEBSF, or the same cocktail with AEBSF omitted, or to AEBSF alone (experimental protocol indicated by diagram above panel). Exposure to hypertonic shock at the end of culture revealed maintenance of oocyte-ZP adherence only for those groups exposed to AEBSF. Different letters indicate significant differences ($p < 0.05$ by ANOVA and Tukey-Kramer test). Throughout, each bar represents mean±SEM of 3 independent measurements.

with a control group (cultured without furin inhibitor). This assay was expected to determine if furin activity is necessary for the normal appearance of the PVS.

α1-PDX had no effect on oocyte-ZP adherence (not shown), whereas ~ 60% of oocytes exposed to RVKR maintained their adherence and developed the deformed phenotype when exposed to hypertonic shock (Fig. 30 B). This effect was lost after 30 min of exposure to hypertonicity, suggesting a delay in oocyte-ZP adherence release. Culture in the presence of RVKR, however, did not hinder oocyte ability to form a PVS, which was puzzling.

Since these data proved to be inconclusive, I tested a battery of protease inhibitors (Table 8), trying to identify a possible protease candidate involved in oocyte-ZP adhesion release. A similar approach as above, in which the oocytes were exposed for 45 min to an inhibitor, and than transferred to 450 mOsM, was utilized. Since one of the broad-specificity protease inhibitor cocktails tested seemed to influence oocyte ability to release adhesion (Fig. 30 C), its specific ingredients were examined. Among all the tested inhibitors, culture in the presence of the serine protease inhibitor AEBSF hydrochloride (500 μM) allowed maintenance of adhesion for ~60% of oocytes when exposed to hypertonic shock. This effect was lost by 30 min of additional hypertonic exposure, again suggesting a delay rather than complete inhibition of release of oocyte-ZP adhesion. Overall, although I was unable to show conclusively a role of a specific protease in oocyte-ZP release or PVS formation, involvement of such a protease (possibly from the convertase family) still remains a likely possibility.

Table 8. Influence of different protease inhibitors on oocyte-ZP adherence release

Inhibitor (concentration)	**General action**	**Action over oocyte-zona adherence release**	**Toxic effect on oocyte (effect on meiotic maturation)**
Decanoyl -Arg-Val-Lys-Arg-chloromethylketone (RVKR) (50 μM)	General convertase inhibitor	Partial effect after 30 min exposure (~ 60 % of oocytes maintained the adherence when exposed to hypertonic shock)	Not tested
Alpha1-PDX (20 μM)	Selective inhibitor of Furin	No effect	Not tested
Calbiochem Protease Inhibitor Cocktail Set I (AEBSF hydrochloride 500 μM + Aprotinin 150 nM + E64 protease inhibitor 1 μM + EDTA disodium 0.5 mM + Leupeptin hemisulfate 1 μM)	Serine proteases, Esterases, Cysteine proteases, Metalloproteases, Trypsin-like proteases inhibitor	Partial effect after 45 min exposure (~ 60 % of oocytes maintained the adherence when exposed to hypertonic shock)	Partial toxicity after 45 min exposure (only 60% oocytes extruded first polar body)
Calbiochem Protease Inhibitor Cocktail Set III (AEBSF hydrochloride 100 μM + Aprotinin 80 nM + E64 protease inhibitor 1.5 μM + Bestatin 5 μM + Leupeptin hemisulfate 1 μM + Pepstatin A 1 μM)	Serine proteases, Aminopeptidase B, Leucine Aminopeptidase, Cysteine proteases, Trypsin-like proteases, Aspartic proteases inhibitor	No effect	Not tested
AEBSF hydrochloride (500 μM)	Serine proteases inhibitor	Partial effect after 45 min exposure (~ 60 % of oocytes maintained the adherence when exposed to hypertonic shock), inconsistent effect	No toxic effect

Aprotinin(150 nM)	Serine proteases and Esterases inhibitor	No effect	Not tested
E64 protease inhibitor (1 μM)	Cysteine proteases inhibitor	No effect	Not tested
EDTA disodium (0.5mM)	Metalloproteases inhibitor	No effect	Not tested
Leupeptin hemisulfate (1 μM)	Cysteine proteases and Trypsin-like proteases inhibitor	No effect	Not tested
AEBSF hydrochloride (500 μM) + Aprotinin (150 nM)	Serine proteases, Esterases inhibitor	Partial effect after 45 min exposure (~ 50 % of oocytes maintained the adherence when exposed to hypertonic shock), inconsistent effect	Not tested
AEBSF hydrochloride (500 μM) + E64 protease inhibitor (1 μM)	Serine proteases, Cysteine proteases	Partial effect after 45 min exposure (~ 60 % of oocytes maintained the adherence when exposed to hypertonic shock)	Partial toxicity after 45 min exposure (only 70% oocytes extruded first polar body)
AEBSF hydrochloride (500 μM) + EDTA disodium 0.5 mM	Serine proteases, Metalloproteases	Partial effect after 45 min exposure (~ 60 % of oocytes maintained the adherence when exposed to hypertonic shock)	No toxic effect
AEBSF hydrochloride (500 μM) + Leupeptin hemisulfate (1 μM)	Serine proteases, Cysteine proteases, Trypsin-like proteases inhibitor	Partial effect after 45 min exposure (~ 50 % of oocytes maintained the adherence when exposed to hypertonic shock)	Partial toxicity after 45 min exposure (only 60% oocytes extruded first polar body)
Alpha 1 antitrypsin (129mg/ml)	Trypsin inhibitor	No effect	Not tested
Bikunin (1μM)	Ovarian plasminogen inhibitor	No effect	Not tested

DISCUSSION

1. Cell-volume regulatory glycine transport via GLYT1 is initiated in oocytes at ovulation

Utilization of GLYT1 as an organic osmolyte transporter by early preimplantation embryos to resist osmotically-induced volume decreases at oviductal osmolarities has been established in recent years (Steeves *et al.*, 2003). This novel cell volume regulatory mechanism involving glycine accumulation via GLYT1 allows maintenance of 1-cell embryo volume at physiological osmolarities enabling proper transition through preimplantation embryonic development both *in vivo* and *in vitro*. The ability to osmosensitively accumulate glycine and to utilize it as an organic osmolyte (Van Winkle *et al.*, 1990; Dawson and Baltz, 1997; Dawson *et al.*, 1998; Steeves *et al.*, 2003) seems to be characteristic only of early preimplantation embryonic stages, ceasing around 4-cell stage (Hammer *et al.*, 2000). Although, oocytes have been reported to transport glycine (Colonna and Mangia, 1983; Haghighat and Van Winkle, 1990), the exact moment, before fertilization, when GLYT1 activity and osmosensitive accumulation of glycine is initiated had not been investigated.

1. 1. The glycine transporter GLYT1 is activated in oocytes following stimulation of ovulation or oocyte release from follicle

My thesis work demonstrated that GLYT1 activity is initiated upon ovulatory stimulation *in vivo* or alternatively following oocyte removal from follicle *in vitro*. There was no measurable transport of radiolabeled glycine at stages of development preceding

ovulation, including freshly-isolated fully-grown GV oocytes (Fig 9 A, B). Measurements of specific GLYT1 activity at developmental stages spanning from GV oocyte to blastocyst indicated the presence of a quiescent GLYT1 transporter prior to ovulation (GV stage) and an active GLYT1 at stages succeeding the ovulatory stimulation, including MI, matured MII oocytes and cleavage stage embryos until around the 4-cell stage (Fig. 9 B, C).

Robust glycine transport via GLYT1 developed in oocytes *in vitro* within a few hours following removal from the follicle, or *in vivo* with a similar time course after hormonal stimulation of ovulation (Fig. 10). Moreover, my data also indicated that GLYT1 activity is only initiated after removal from the follicle whether the oocyte is contained within an intact COC or isolated (Fig. 17). This supports the idea of a quiescent GLYT1 prior to ovulation or removal from follicles not only in oocytes but also in COCs. Intriguingly, glycine transport development in COC (Fig. 17 C) was faster (GLYT1 active ~ 0.5 hr after collection) than in denuded oocytes (Fig. 11) (GLYT1 active ~ 2 hr), suggesting that the presence of cumulus cells accelerates oocyte GLYT1 activation. Since both groups - intact COC and oocytes denuded after uptake - behaved similarly, the possibility of glycine transfer from cumulus cells into oocyte in the first 2 hr after removal from the follicle remains open. Interestingly, *in vivo*, closure of gap junctions, which would be the route of such transfer, has been reported to occur around 2 hr subsequent to ovulatory stimulation (Gilula *et al.*, 1978). Future studies looking more closely at the development of glycine transport in COCs and isolated cumulus shells might elucidate whether such a transfer mechanism exists immediately after ovulation induction.

Thus, together these data indicated that activity of GLYT1, a glycine transporter utilized as an organic osmolyte transporter by early preimplantation embryos, is initiated before fertilization, shortly (~ 2 hr) after stimulation of ovulation.

Two studies previously reported the presence of glycine transport in fully-grown GV oocytes (Colonna and Mangia, 1983; Haghighat and Van Winkle, 1990), which would appear to contradict my results here. However, these groups' methods of oocyte manipulation and glycine transport measurement included a 1-2 hr incubation step prior to ^{3}H-glycine transport assay (Dr. Van Winkle personal communication to Dr. Baltz) or a 1 hr ^{14}C-glycine uptake assay (Colonna and Mangia, 1983). Based on my data (Fig 10 A), these intervals of time between oocyte removal from the follicle and the actual transport measurements are sufficient for GLYT1 to initiate its activity. Thus, the initiation of glycine transport activity that I discovered here would not likely have been detected in these previous studies.

1. 2. The cell volume-regulatory accumulation of glycine is initiated in oocytes following stimulation of ovulation

Several attributes characterize glycine as a molecule utilized for osmotic support by the 1-cell embryos: 1) glycine accumulates within embryos and is present free in the cytoplasm rather than incorporated into macromolecules and therefore able to contribute to intracellular osmotic pressure (Steeves *et al.*, 2003); 2) glycine accumulation improves 1-cell embryo ability to maintain its cell volume when the osmolarity of its environment is raised (Dawson *et al.*, 1998; Steeves *et al.*, 2003); and 3) glycine accumulation is osmosensitive, increasing linearly as the environmental osmolarity is elevated (Dawson

et al., 1998; Steeves *et al.*, 2003).

My data indicating GLYT1 activation following stimulation of ovulation, suggested that glycine accumulation, and therefore the cell-volume regulatory mechanism involving GLYT1-mediated glycine accumulation described in early embryos, would begin around the same period. Indeed, following an initial lag period of ~ 2 hr, GV oocytes started to accumulate glycine rapidly (Fig. 15). Thus, the timing of glycine transport activation coincides with the onset of glycine accumulation. My measurements of total free endogenous glycine, the intracellular glycine fraction able to act as an organic osmolyte, revealed that GV oocytes contain almost no detectable free glycine, whereas ovulated MII oocytes have very high levels that were comparable to those in 1-cell embryos (Fig. 16). Since utilization of any compound as an organic osmolyte *in vivo* implies its intracellular accumulation at high concentrations in a soluble form (Baltz, 2001), these data demonstrated that accumulation of glycine as an organic osmolyte in ovulated MII oocytes and cleavage stage embryos occurs *in vivo*, and that glycine accumulation *in vivo* is initiated only following stimulation of ovulation. Furthermore, glycine had no effect on oocyte volume at stages where GLYT1 was not active, but, after initiation of GLYT1 activity, glycine allowed oocytes to maintain better their volumes against hypertonic stress (Fig. 18). Data collected by other members of our laboratory indicated that, although GLYT1 is active over a period of development spanning from the mature MI oocyte to the embryonic 8-cell stage, glycine accumulation was only stimulated by hypertonicity during a very restricted interval of development, starting in the mature MII oocyte and ending at 2-cell stage (Fig. 31, Rudraraju, N. and Baltz, J.M., unpublished).

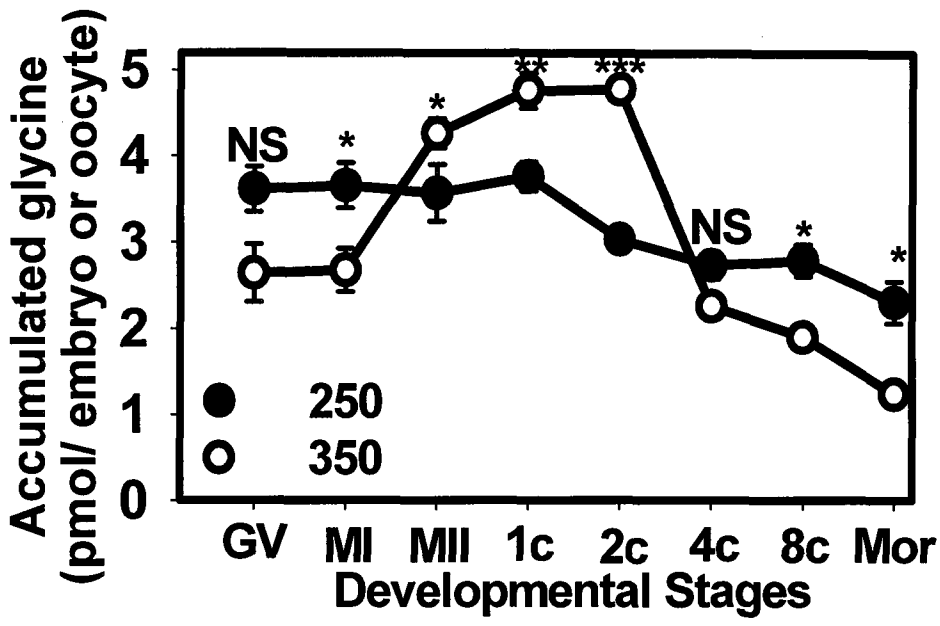


Figure 31. Osmotic regulation of glycine accumulation in oocytes and preimplantation embryos. The amount of glycine accumulated by oocytes at different stages of meiotic maturation and preimplantation embryos was measured at low (250 mOsM) and high (350 mOsM) osmolarity (as indicated by key). Oocytes or embryos were cultured for 8 hours in the presence of 1 mM glycine. GV oocytes were maintained arrested at the GV stage by dbcAMP. MI oocytes were cultured in the absence of dbcAMP, and transitioned from GV oocytes within the first 2-4 hours. MII oocytes and preimplantation embryos were collected at the stage indicated and remained at that stage for the entire incubation. Osmotic stimulation of glycine accumulation was found only for MII eggs and 1- and 2-cell embryos. Comparisons were done between osmolarities within each stage (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, by Student's *t*-test). Each bar represents mean ± SEM of 3-5 independent determinations. (These measurements were performed by Nirmala Rudraraju).

Together, initiation of glycine accumulation coincidently with activation of GLYT1 activity, presence of a considerable free cytosolic glycine fraction only at stages subsequent to ovulation, maintenance of oocyte volume in the presence of glycine at high osmolarity only when GLYT1 is active, and the osmosensitive accumulation of glycine only in ovulated oocytes and early embryos, demonstrated that the cell-volume regulatory accumulation of glycine via GLYT1 is initiated in oocytes at ovulation.

1. 3. Activation of GLYT1 at ovulation might be accompanied by increased glycine availability in the oviductal environment

My data indicated that development of GLYT1 activity occurs subsequent to the hormonal initiation of ovulation (Fig 10 B). Moreover, the osmosensitive glycine accumulation, enabling maintenance of cell volume, is initiated in oocytes around the time of ovulation as well (see above). Thus, the initiation of a glycine-dependent cell-volume regulatory mechanism characteristic of early embryonic stages takes place during ovulation, close to the transition from the follicular to oviductal environment. Measurements of various amino acids in mouse reproductive tract fluids reported a 10 fold increase in glycine concentration in oviductal fluid compared with follicular fluid (Harris *et al.*, 2005). This might be suggestive that GLYT1 activation upon stimulation of ovulation is accompanied by an increase in substrate availability as the oocyte enters the oviduct. Since glycine is present in millimolar range (~ 3 mM) in the oviductal fluid (Harris *et al.*, 2005), this would be sufficient to ensure maximal osmoprotection of MII oocytes and early embryos, which requires 0.5-1 mM external glycine (Dawson and Baltz, 1997; Baltz, 2001). Thus, utilization of glycine as the favorite osmolyte by the

matured MII oocyte and the early embryo could be facilitated by higher glycine oviductal availability compared with the other potential osmoprotectant amino acids or derivatives. Betaine is thought to be present at high concentrations in oviductal fluid (Anas *et al.*, 2007) as well, and its accumulation may be a possible back-up mechanism.

Utilization of glycine accumulation via GLYT1 to regulate cell volume seems to be restricted to matured MII oocytes and the very initial stages of preimplantation embryo development, until around 4-cell stage (Fig. 31) (Hammer *et al.*, 2000). The advantage of utilizing GLYT1 as an organic osmolyte transporter by matured MII oocytes and cleavage-stage embryos, rather than a different glycine transporter, could reside with the characteristic kinetic properties of members of the NTT family. Interestingly, two other organic osmolyte transporters, TAUT and BGT1, are members of the same family (Nelson, 1998). Many NTT members co-transport Na^+ and Cl^- together with their substrate. This stoichiometry (GLYT1 co-transports $2Na^+$ and $1Cl^-$ together with glycine) permits intracellular accumulation of the substrate to high levels due to the steep inward gradients of both Na^+ and Cl^-, a desirable attribute for an organic osmolyte transporter that must concentrate its substrate intracellularly.

Taken together, data discussed in this chapter showed that cell-volume regulatory glycine transport via GLYT1 is initiated in oocytes following ovulatory stimulation. These findings partially confirmed my first set of hypotheses, indicating the presence of volume-regulatory glycine accumulation via GLYT1 in oocytes before fertilization, but only after stimulation of ovulation.

2. Unidentified factors within follicular environment appear to control GLYT1 activation in oocytes

2. 1. GLYT1 activation in oocytes occurs independently of meiotic maturation

Upon LH stimulation, meiotic maturation of the oocyte occurs within the fully grown ovarian follicle. During this process, the GV oocyte progresses through two rounds of meiosis, becoming a MI oocyte (during meiosis I), and in the end a mature MII oocyte (arrested at metaphase of meiosis II) awaiting fertilization. *In vitro*, meiotic maturation is spontaneously initiated following oocyte removal from the follicular environment (Wassarman *et al.*, 1979) (see Introduction).

My data indicated that GLYT1 activity is initiated in oocytes ~ 2 hr subsequent to ovulatory stimulation *in vivo* or oocyte removal from follicle *in vitro*. Thus, activation of GLYT1 occurred at the same time as meiotic maturation initiation, both *in vivo* and *in vitro* (Fig 10). Surprisingly, my data indicate that GLYT1 activity initiation does not require meiotic maturation, as it also occurred *in vitro* in isolated oocytes that were maintained in arrest at GV stage (Fig. 12) with a similar time course (Fig 11) as oocytes that underwent meiotic maturation. Moreover, meiotically incompetent growing oocytes initiated GLYT1 activity by 4 hr after collection, to a similar level as fully-grown GV oocytes (Fig 14), supporting a lack of correlation between meiotic maturation resumption and activation of GLYT1. Thus, although the timing of resumption of meiosis and glycine transport activation is identical, they seem to occur independently.

Activity of other amino acid transporters seems to change during meiotic maturation, perhaps as a requirement for subsequent embryo development. A cysteine transporter, x_c^-, is reportedly activated late during meiotic maturation (Van Winkle *et al.*,

1992), while an unpublished study conducted in our laboratory indicated that system β turns on at the MI stage and confirmed that x_c^- is activated at MII (Pelland, A. and Baltz, J.M. unpublished data). My measurements of leucine transport into GV-arrested oocyte indicated, conversely, a partial down-regulation of system L activity subsequent to oocyte collection, also confirmed by independent experiments (Pelland, A. and Baltz, J.M. unpublished data). Future studies will have to determine if independence of amino acid transporter activation (or downregulation) from meiotic maturation is an attribute specific to GLYT1 or applies to other transport systems as well.

2. 2. Activation of GLYT1 in oocytes appears to be controlled by a mechanism different from other organic osmolyte transporters

Transport of organic osmolytes has been extensively studied in kidney medulla, a tissue exposed to osmotic stress under physiological conditions during antidiuresis (Garcia-Perez and Burg, 1991). In kidney, *de novo* synthesis under the control of the only known osmosensitive mammalian transcription factor, TonEBP (Miyakawa *et al.*, 1999; Woo *et al.*, 2002) ensures an increased activity of the four known mammalian organic osmolyte transporters (SMIT, TAUT, BGT1 and system A) (see Introduction). In 1-cell embryos, osmotic stimulation of GLYT1 has been shown to be undiminished when protein synthesis was suppressed (Steeves *et al.*, 2003), suggesting a different regulation from the other mammalian organic osmolyte transporters.

My data indicated that the initial activation of GLYT1 in oocytes at ovulation also does not require *de novo* synthesis. First, I was able to identify expression of GLYT1a isoform transcripts throughout oocyte development (growing, GV or matured oocytes)

and at those stages of preimplantation embryo development at which GLYT1 was known to be active (1-cell through morula stage) (Fig 19 A). Thus, GLYT1 transcripts are present in oocytes well before GLYT1 activation takes place, indicating that transcription is less likely to control the appearance of GLYT1 activity in oocytes. Second, GLYT1 activation did not require *de novo* protein synthesis since Western blot analysis of protein extracted from freshly collected GV oocytes and MI oocytes that developed *in vitro* indicated the presence of a comparable amount of GLYT1 protein at stages with an inactive (GV) or fully functional (MI) transporter (Fig. 19 B, C). Moreover, confirmed protein synthesis inhibition with cycloheximide did not hinder the oocyte's ability to activate GLYT1 and develop glycine transport 4 hr subsequent to its collection (Fig. 19 D). Taken together, these data support the idea of GLYT1 activation in oocytes through a mechanism not involving *de novo* synthesis.

A different GLYT1 regulation and activation from the other organic osmolyte transporters might be a reflection of differences in their roles. GLYT1 is the only known organic osmolyte transporter functioning at isotonic conditions, whereas the kidney transporters secure accumulation of their substrates at high osmolarity, over a period of hours to days. A membrane-localized, readily functional GLYT1 would allow rapid activation or increase in activity, ensuring a quick response upon deviations from the preferred cell volume or intracellular glycine concentration. Moreover, in the absence of massive osmolarity variations (follicular and oviductal fluid are isotonic), a GLYT1 regulatory mechanism involving a tonicity-responsive transcription factor would be redundant. Thus, while the other known organic osmolyte transporters are utilized for long-term adaptation to conditions of sustained hypertonicity, GLYT1 is part of a

mechanism maintaining the steady-state intracellular organic osmolyte concentration. Future studies will have to determine if such a mechanism is strictly restricted to matured MII oocytes and cleavage embryos or if it applies to other tissues as well.

2. 3. Initiation of GLYT1 activity in oocytes appears to involve activation of pre-existent membrane-localized GLYT1 protein

The above findings were suggestive of involvement of post-translational events in GLYT1 activation in oocytes. Intracellular trafficking depends on active transport along the cytoskeleton, and thus can require an intact microtubule and microfilament network (Etienne-Manneville, 2004; Caviston and Holzbaur, 2006). Such trafficking could have been responsible for activation of GLYT1 activity in oocytes. My data indicated, however, that disruption of microtubules, or actin filaments, or the Golgi apparatus (Fig. 20), or inhibition of SNARE-sensitive vesicle fusion by various Clostridial neurotoxins, did not interfere with GLYT1 activity development. Moreover, my data demonstrated that even if GLYT1 was inactive in GV oocytes freshly retrieved from the ovary, GLYT1 protein appeared to be already membrane-localized (Fig. 21). A similar membrane localization was displayed at stages were GLYT1 had acquired full activity (arrested GV or MI oocytes). Together, these data suggest initiation of GLYT1 activity in oocytes through a mechanism involving activation of pre-existent GLYT1 proteins.

Insertion of pre-synthesized proteins into plasma membranes through a mechanism insensitive to disruption of F-actin or microtubules but sensitive to Golgi apparatus disruption, has been described before. One such example is the appearance of the cell adhesion molecule uvomorulin (E-cadherin) in the plasma membrane of one-cell

embryos, following fertilization (Clayton *et al.*, 1995). This mechanism is less probable to be involved in activation of GLYT1 at ovulation, since glycine transport developed when oocytes were cultured in the presence of BFA (Fig. 20), a Golgi-based membrane vesicle fusion disruptor (Dinter and Berger, 1998) that was found to prevent uvomorulin insertion into the membrane in 1-cell embryos (Clayton *et al.*, 1995). Future studies involving immunogold electron microscopy to allow quantification of GLYT1 protein at the plasma membrane level in oocytes during glycine transport, and activation will determine more exactly any possible involvement of GLYT1 protein trafficking to the oocyte plasma membrane.

Regulation of GLYT1 activity in brain has been studied over the last two decades, mainly due to its implications in the pathophysiology of psychiatric diseases like schizophrenia. A few regulatory factors, most of them involved in post-translational modifications of GLYT1 protein, have been described to influence GLYT1 activity (see Introduction), although the exact molecular mechanisms controlling activity of this transporter in brain remain elusive. GLYT1, similarly with all other NTT transporters, has predicted intracellular consensus sites for phosphorylation by PKC (Liu *et al.*, 1993). Neuronal GLYT1 activity was shown to be down-regulated by PKC activation (Gomeza *et al.*, 1995), although not directly but rather through an intermediate substrate phosphorylation (Sato *et al.*, 1995). One of the approaches utilized in my thesis to probe the manner in which GLYT1 is activated in oocytes surveyed the involvement of several factors described to control GLYT1 activity in CNS, mainly targeting the posphorylation/dephosphorylation process. None of the pharmaceutical inhibitors or activators tested in my thesis had an effect on GLYT1 activation in oocytes. Activation of

GLYT1 in oocytes by other post-transcriptional modifications like oligomerization (Horiuchi *et al.*, 2001) glycosylation (Olivares *et al.*, 1995) and/or by involvement of other factors like Zn^{2+} (Laube, 2002) or arachidonic acid (Zafra *et al.*, 1990), remain open possibilities.

2. 4. The follicular environment appears to control activation of GLYT1 in oocytes

A recent study demonstrated granulosa-granulosa gap junction closure in mural or cumulus granulosa populations over the period of 0.5 – 2 hr subsequent to stimulation of ovulation (Norris *et al.*, 2008). Moreover, previous reports have indicated that during cumulus expansion, the connections between cumulus granulosa and oocyte become gradually lost by ~ 2 hr following stimulation of ovulation (Gilula *et al.*, 1978). 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 raised the possibility of a constitutively active GLYT1 on the oocyte membrane being maintained in a quiescent state before ovulation by factor(s) within the follicular environment, particularly if they must pass through gap junctions to affect the oocyte. In such a model, before ovulation, when the GV oocyte is strongly coupled via gap junctions with its surrounding somatic cells, a potential follicular inhibitory signal(s) would be able to maintain GLYT1 quiescence (Fig. 27). Following ovulation, during cumulus expansion, when the oocyte-granulosa interactions are gradually lost, causing the oocyte's functional isolation from the follicular environment, the hypothesized inhibition would also be removed, allowing thus activation of GLYT1 in parallel with meiotic maturation.

Several lines of evidence support this model. First, physiological events or experimental manipulations that separated the oocyte from its follicular environment *in vivo* or *in vitro* resulted in development of GLYT1 activity. *In vivo*, a fully active GLYT1 was present in matured MI oocytes by ~2 hr after ovulation was stimulated (Fig. 10 B), coincident with the loss of cumulus-granulosa gap junctional communication (Gilula *et al.*, 1978). *In vitro*, GLYT1 activity developed in growing oocytes (Fig. 14), intact COCs (Fig. 17), MI (Fig. 10 A) or GV-arrested (Fig. 11) fully-grown oocytes after retrieval from the ovarian follicle. Moreover, inhibition of meiotic maturation or disruption of events intrinsic to the oocyte (various signaling pathways) did not influence the oocyte's ability to activate GLYT1 following its removal from the follicle (see above). Second, experiments in which cultured antral follicles were utilized indicated that the follicle-enclosed oocyte exhibited very low GLYT1 activity (Fig. 24 A), whereas the oocytes enclosed by follicles with pharmacologically-disrupted gap junctions initiated GLYT1 activity (Fig. 24 B).

Together, my results support the hypothesis of a gap junction-mediated inhibitory signal that suppressed the activity of a pre-existing, oocyte membrane-localized GLYT1 transporter within the antral follicle (Fig. 32). Corroborating this model is my finding that GLYT1 activity is initiated *in vivo* only following hormonal stimulation of ovulation (Fig. 10 B). A GLYT1 gap-junction mediated inhibitory signaling would cease subsequent to ovulatory stimulation as a consequence of gap junction breakdown. Future studies, using a more direct approach (e.g., connexin knock-out mice; oocytes cultured in the presence of follicular fluid; use of pharmacological agents with intact antral follicles *in vitro*) might help uncover the exact nature and mechanism of the GLYT1 inhibitory

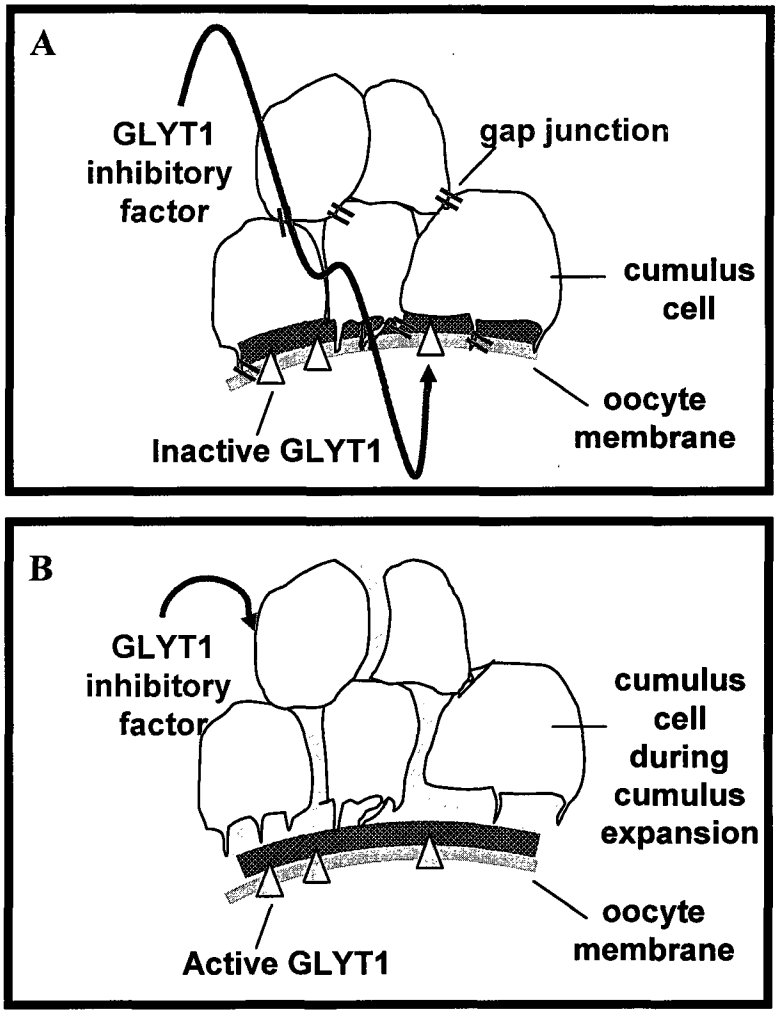


Figure 32. Schematic representation of hypothesized model of GLYT1 inhibition within the antral follicle. **(A)** Before ovulation, within the antral follicle an unidentified factor(s) is transferred to the oocyte via gap junctions, and exerts an inhibitory effect over a constitutively active GLYT1, maintaining its quiescence. **(B)** Following ovulation, during cumulus expansion, the gap junctional communication between oocyte and cumulus cells is gradually lost, allowing removal of the hypothesized inhibition and thus activation of GLYT1.

follicular signal.

The advantage of such a mechanism controlling GLYT1 activation might be found in the manner the ovarian follicle functions. Throughout folliculogenesis, the strong functional interdependence of different follicular compartments allows the ovarian follicle to act as a unit, with the final purpose of producing and ovulating a healthy, fertilizable female gamete, the oocyte. Before ovulatory stimulation, the oocyte relies on its surrounding granulosa cells for metabolic support and transfer of signals via gap junctions (Biggers *et al.*, 1967; Donahue and Stern, 1968; Heller *et al.*, 1981; Kidder and Mhawi, 2002; Senbon *et al.*, 2003; Gittens *et al.*, 2004), whereas around the time of ovulation the maturing oocyte acquires the ability to function independently (see Introduction). In this context, prior to ovulation, inhibition of independent cell volume regulation would guarantee undisrupted follicular function by preventing physical disruption of oocyte-follicle connections. If instead the oocyte could freely alter its volume within the ovarian follicle, the physical relations between its surface and neighboring cumulus-granulosa cells mediated by trans-ZP processes would be altered by the movement of the oocyte surface during volume regulation. This could result in breakage of oocyte-cumulus gap junctions and disruption of follicular functionality. Therefore, I propose that suppression of independent cell volume regulation by the oocyte is necessary to prevent stress and breakage of vital oocyte-follicle cell connectivity. The presence of a membrane-localized GLYT1 protein in the fully-grown oocyte would also allow rapid activation once the oocyte acquires independence from the follicular support.

Studies conducted in recent years support the idea of a bidirectional oocyte-

granulosa cell communication controlled mainly by the oocyte (Matzuk *et al.*, 2002), involving both gap junctions and paracrine factors. If the identity and function of several oocyte- and granulosa-derived factors involved in early folliculogenesis have been well defined, the nature of signals transferred via gap junctions or in a paracrine manner between fully-grown oocyte and granulosa during the periovulatory period remains under investigation. One such example is the oocyte-secreted GDF9, thought to act as the cumulus enabling expansion factor (CEEF) and modulating cumulus expansion in response to ovulatory stimulation (Matzuk *et al.*, 2002; Salustri *et al.*, 2004). If the mechanism proposed in my thesis exists, involving follicular inhibition of an oocyte membrane-localized constitutively active GLYT1, it is one of the very few examples of direct action of follicular signals over a specific oocyte activity before ovulation in the fully-grown GV. The possibility of a more intricate pathway maintaining GLYT1 quiescent before ovulation and involving both gap junctional and paracrine mediated signaling remains to be investigated.

Taken together, data discussed in this chapter indicated that GLYT1 activation in oocytes occurs through a mechanism not involving *de novo* synthesis or signalling pathways known to influence GLYT1 activity in brain. Although, this activation might be controlled by an unidentified post-translational event, several lines of evidence support the involvement of an inhibitory follicular environment over an already membrane-localized and constitutively active GLYT1 transporter in the oocyte within the antral follicle.

3. Oocyte volume resets following stimulation of ovulation

Data collected throughout my thesis and discussed above strongly indicated that GLYT1 mediated cell volume-regulatory glycine transport is initiated in oocytes only after stimulation of ovulation. This volume regulatory mechanism is thought to be the main process allowing early embryos to resist the osmotically-induced volume decrease at physiological osmolarities (Steeves *et al.*, 2003). This suggested that the independent cell volume regulation involving utilization of organic osmolytes is initiated only at ovulation.

3. 1. Oocyte assumes a smaller volume in response to ovulation or removal from follicle

My investigations of a change in oocyte volume at ovulation, over the same period in development when independent GLYT1-mediated cell volume regulation is activated, revealed a resetting of oocyte volume following ovulatory stimulation or upon oocyte removal from the follicle. *In vivo*, an approximately 20% loss of initial volume occurred after hCG stimulation (Fig. 25 A, B). This reduction in oocyte volume was largely achieved before the emission of the first polar body, suggesting an active change in oocyte volume following stimulation of ovulation, which took place by approximately 8 – 10 hr post-hCG. A similar change occurred *in vitro*, when the oocyte was arrested at the GV stage and kept in culture for 4 – 7 hr (Fig. 25 C, D). The loss of volume *in vitro* occurred whether the oocyte was exposed to isotonic, hypertonic, or, surprisingly, hypotonic media. Thus, the oocyte decrease in volume appeared not to be osmotically induced but rather an expression of an active preference for smaller volume after

ovulation. Such volume resetting could be reflective of a switch between different cell-volume regulatory mechanisms around ovulation time, when the oocyte begins autonomous volume regulation.

3. 2. The PVS forms in parallel with oocyte volume resetting

The exact timing and mechanism of PVS formation has not been previously investigated. Several electron microscopy studies from the late 1980s support the lack of a space (Kaufman *et al.*, 1989), or possibly the presence of a very narrow PVS (Okada *et al.*, 1986) at the GV stage. Since the PVS is not discernable by light microscopy at the GV stage (Okada *et al.*, 1986) but is easily visualized after extrusion of first polar body, the general assumption in the literature has been that PVS formation mainly results from the loss of oocyte volume during first polar body extrusion.

My thesis indicated, instead, that the PVS forms in the first hours following stimulation of ovulation or oocyte removal from the follicle. First, *in vivo*, the oocyte volume measurements I have collected following stimulation of ovulation indicated not only that the cell volume decrease was completed before first polar body emission, but also that the PVS became apparent and formed in parallel with oocyte volume loss (Fig 25 B). Second, *in vitro*, PVS formation was observed to occur in GV-arrested oocytes, demonstrating independence of PVS appearance from meiotic maturation (Fig. 25 D). Third, electron microscopy (TEM) studies allowed me to follow the time dependent formation of PVS *in vitro* at the ultrastructural level for arrested GV oocytes exposed to isotonic conditions (Fig. 26). These data indicated that PVS appears by ~ 0.5 – 1 hr after release from the follicle. They also suggested the absence of PVS in GV oocytes freshly

removed from the antral follicle. Moreover, before a PVS forms, the oocyte volume seems to be determined by a very strong physical interaction with the ZP (Fig. 26, first panel). A similar finding of PVS appearance at 1 hr subsequent to fully-grown oocyte collection has very recently been described by a different group (Inoue *et al.*, 2007).

Thus, *in vitro*, the appearance of the PVS occurs in the first hour after oocyte collection, at the same time with the resetting of oocyte volume. The presence of a PVS at 6.5 hr following stimulation of ovulation (Fig 25 B), and the similarity between oocyte volume decrease *in vitro* and *in vivo*, although with a slower time course *in vivo* (Fig. 25), support the idea of a similar PVS formation in the physiological setting of ovulation. Overall, data discussed in this chapter support a physiological resetting in oocyte volume that occurs around the time of ovulation and release from the follicle. Once ovulation is stimulated or if the GV oocyte is removed from the follicle, the strong interaction present between oocyte and ZP appears to be gradually lost, and oocyte volume is reset so that the oocyte assumes a smaller volume. My thesis is the first study reporting such volume resetting and closely looking at the exact timing of PVS formation.

4. The GV oocyte controls its volume in a unique way within the ovarian follicle

The extracellular glycoprotein matrix, the ZP, is secreted by the oocyte and deposited as a thin layer from inside out over a period of development spanning from oocyte growth to ovulation (Qi *et al.*, 2002). Besides its important roles during sperm-egg binding and fertilization (see Introduction), the ZP is thought to contain and ensure protection of the oocyte and embryo during oogenesis and embryo progression through the oviduct (Yanagimachi, 1994).

My data indicated that GLYT1-mediated cell volume-regulatory glycine transport, critical for early embryo development, is initiated in oocytes only after stimulation of ovulation. Moreover, a resetting in oocyte volume occurs around ovulation time. This implied a different cell volume regulatory mechanism being utilized by the oocyte prior to GLYT1 activation. The manner in which oocytes control their volume within the antral follicle had not been addressed by other studies.

4. 1. Oocyte volume is determined by oocyte-ZP adhesion before ovulation

My investigations regarding the cell volume-regulatory mechanism utilized by oocytes prior to GLYT1 activation revealed that the GV oocyte volume within antral follicles is determined by the ZP. First, electron microscopy data indicated a strong physical interaction between the oocyte and ZP immediately after collection (Fig. 26, first panel). Second, when the GV oocyte freshly removed from an antral follicle was exposed to hypertonic shock, the oocyte-ZP contact was maintained and the oocyte assumed a distorted shape that also deformed the ZP, indicating the oocyte's inability to shrink independently and strong attachment to the ZP (Fig. 27 B, C). Moreover, if the oocytes were exposed for more than 30 min to hypertonic shock or kept for more than 30 min at normal osmolarity and then exposed to hypertonic shock, they were able to respond osmotically, by shrinking in a uniform spherical shape away from the ZP (Fig. 27 H). This indicated that the dependence of oocyte volume on ZP is characteristic only of those GV oocytes that were freshly removed from the antral follicle. Third, TEM studies revealed that when freshly-obtained GV oocytes were exposed to hypertonic shock, the oocyte microvilli became stretched and seemed to adhere to the inner zona surface at

their tips (Fig. 27 F, G). In contrast, at normal osmolarity, they remained embedded within zona material (Fig. 27 E). This suggested the presence of some form of adherence between the GV oocyte and ZP. This adherence seemed to occur mainly at the tip of the oocyte microvilli. Fourth, following injection of an oil microdrop at the interface between oocyte and ZP, the fresh GV oocyte surface could not be displaced from the ZP and the oil remained trap in a small cavity, further supporting oocyte attachment to ZP (Fig. 28 A). Conversely, the oil microdrop clearly accentuated the presence of a space after oocytes had been maintained in culture (Fig. 28 B). Fifth, removal of the ZP from fresh GV oocytes accelerated the resetting of oocyte volume, consistent with oocyte size being constrained by the ZP before the oocyte-ZP adhesion is released (Fig. 29 A, B). Based on the above data I am proposing that before ovulation, within the antral follicle, oocyte volume is determined by its adherence to ZP.

4. 2. Unidentified mechanism(s) controls oocyte-ZP adhesion before ovulation

The nature of oocyte-ZP adherence might be found in the manner of ZP secretion and assembly. The three glycoproteins that assemble to form the ZP are synthesized by the oocyte and released by proteolytic cleavage from the oocyte surface through a complex mechanism not fully elucidated but likely involving a furin-like protease (Williams and Wassarman, 2001; Jovine *et al.*, 2004; Wassarman, 2008) (see Introduction). Thus, the protease involved in ZP cleavage might be relevant for release of ZP adhesion from the oocyte microvilli and formation of PVS. Although a battery of protease inhibitors were tested (Table 8), including furin inhibitors, I was not able to pinpoint decisively the involvement of any protease in the adherence release. The delay

in oocyte-ZP release upon exposure to general convertase inhibitors (Fig. 25) supports the participation of one of the members of this family in the mechanism of adherence release, and may possibly indicate some functional redundancy between more than one protease.

The mammalian oocyte and its neighboring cumulus granulosa cells are strongly interconnected within the ovarian follicle by granulosa processes that traverse the ZP (Fig 33) and terminate on the oocyte cell surface (Anderson and Albertini, 1976; Motta *et al.*, 1994). There, the granulosa cell process becomes flattened and thickened, and impinges upon the oocyte surface to form both adhesive and gap junctional connections with the oocyte plasma membrane (Motta *et al.*, 1994; Albertini *et al.*, 2001). Work done on human ovarian follicles indicated that these processes dynamically vary throughout folliculogenesis, being present with a higher density at preantral stages, and actively retracting at ovulation, ensuring disconnection between granulosa and the oocyte during cumulus expansion (Motta *et al.*, 1994). The same study reported an increase in attachment zones (zonula adherens and desmosomes) at the interface between oocyte and granulosa processes in large antral follicles prior to ovulatory stimulation.

It still remains possible for the granulosa cell processes that traverse the ZP to play a role in controlling oocyte size, most likely through their adhesive contacts. Future studies would be needed to examine such a possibility. My ultrastructural observations suggested that the much more numerous oocyte microvilli adhere to the ZP at their tips and provide a mechanical connection between the oocyte and ZP. However, a mechanism involving both oocyte microvilli adherence to ZP and granulosa process adherence to the oocyte plasma membrane cannot be excluded. This possibility could explain the lack of

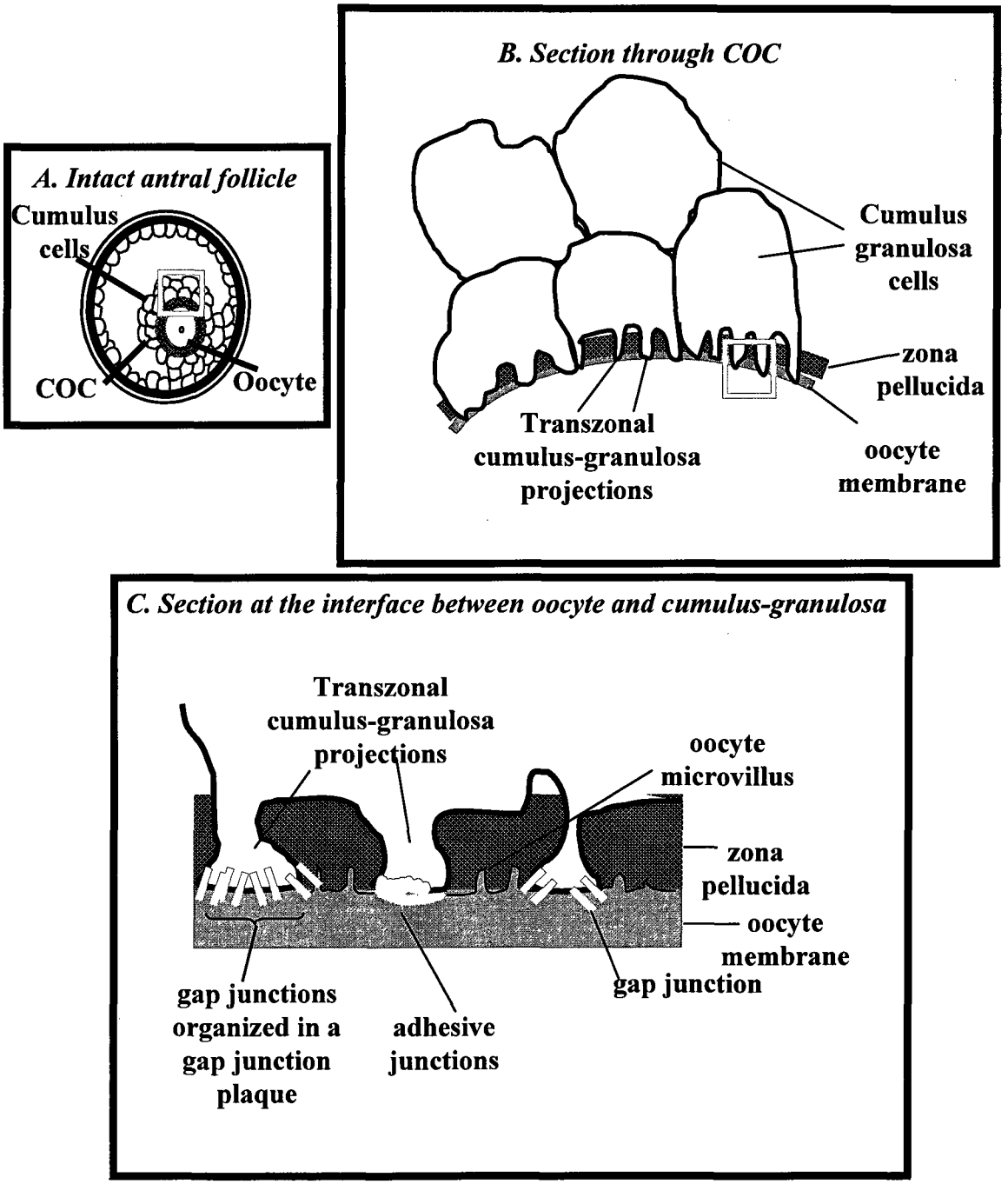

A. Intact antral follicle
Cumulus cells
COC
Oocyte
B. Section through COC
Cumulus granulosa cells
zona pellucida
Transzonal cumulus-granulosa projections
oocyte membrane
C. Section at the interface between oocyte and cumulus-granulosa
Transzonal cumulus-granulosa projections
oocyte microvillus
zona pellucida
oocyte membrane
gap junctions organized in a gap junction plaque
adhesive junctions
gap junction

Figure 33. Within the antral follicle the oocyte and the cumulus cells are strongly interconnected through cumulus transzonal processes. ***(A)*** Within the antral follicle, the oocyte and neighboring cumulus cells, closely associate forming the cumulus-oocyte complex (COC). Area highlighted is magnified in (B). ***(B)*** Cumulus granulosa cells are strongly interconnected with the oocyte through processes that traverse the ZP and terminate on the oocyte plasma membrane (also known as transzonal processes). Area highlighted is magnified in (C). **(C)** At the area of contact between cumulus projection and oocyte membrane, the process becomes flattened and impinges upon the oocyte surface. Both gap junctions and adhesive junctions form at the interface between oocyte and cumulus-granulosa process, ensuring a strong connectivity between cells.

complete inhibition of ZP-oocyte adherence by convertase inhibitors, since in the context of a mechanism such as the one proposed immediately above, protease inhibition would target only one aspect of a complex process with several parallel components.

4. 3. The oocyte-ZP adhesion is released following ovulation or oocyte removal from the follicle, allowing formation of the PVS

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. Data discussed above revealed that, before ovulation, the oocyte contained in the antral follicle has a fixed volume that is determined by the rigid cavity delimitated by the ZP. This is mediated by a strong adherence between the oocyte membrane and the ZP. My data indicated that, following removal from follicle, the oocyte releases its adherence to ZP in the ~ 0.5 hr subsequent to its collection (Fig. 26), allowing appearance of a PVS. Around the same time, the oocyte initiates resetting of its volume (Fig. 25 C). Thus, *in vitro*, the PVS seems to form as a consequence of oocyte release from its adherence to the ZP. A similar mechanism most likely occurs *in vivo* upon ovulatory stimulation as well, since my data indicated the appearance of PVS concomitant with initiation of oocyte volume resetting by 6.5 hr after hormonal stimulation (Fig. 25 B).

My thesis proposes a new model of PVS formation (Fig. 34). Within the antral follicle, the GV oocyte lacks the PVS, its membrane tightly adhering to ZP. Following oocyte removal from the follicle or subsequent to ovulatory stimulation, this adherence becomes lost through a mechanism that remains to be identified, most likely involving a

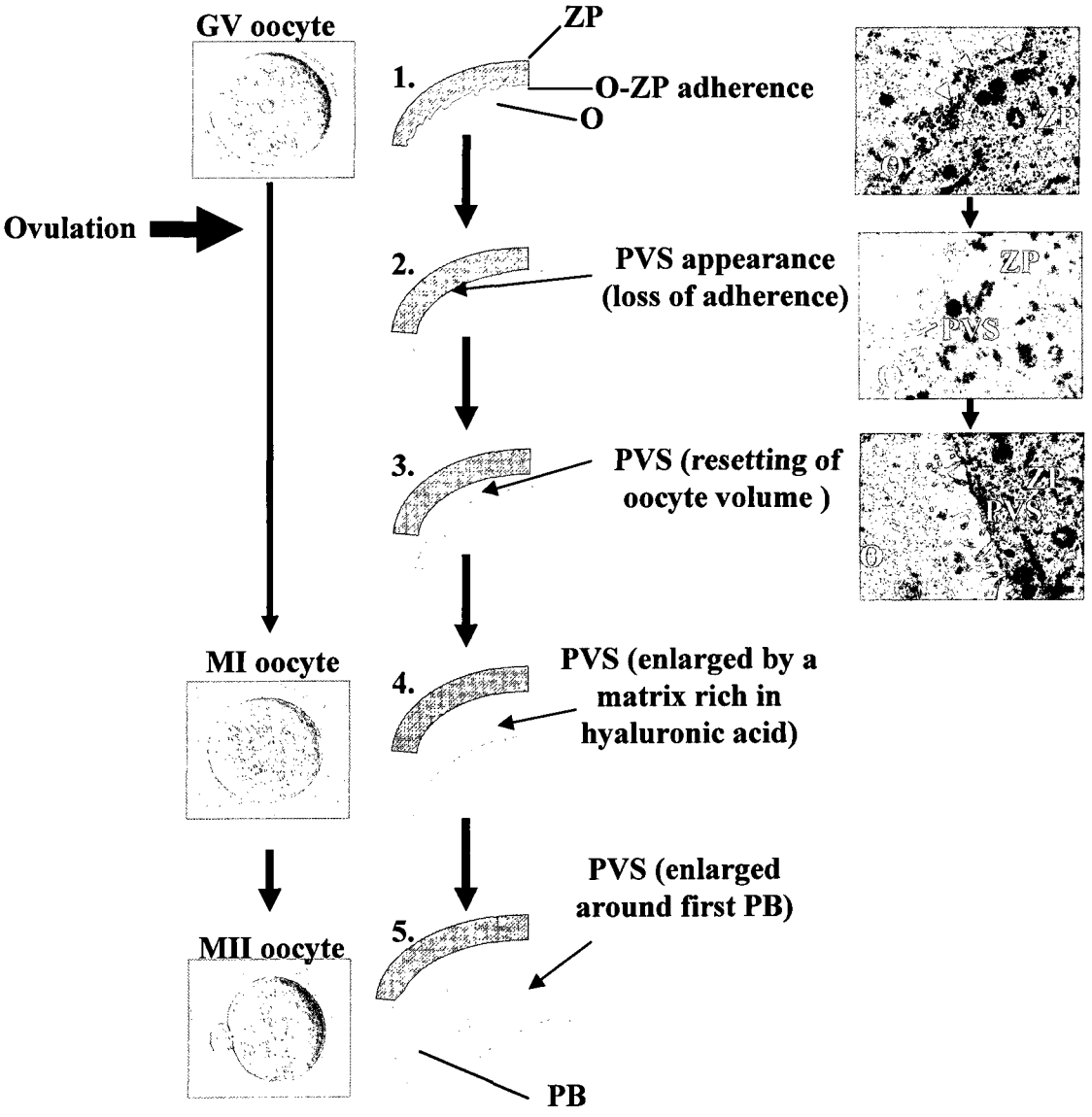

GV oocyte
Ovulation
MI oocyte
MII oocyte
1.
ZP
O-ZP adherence
O
2.
PVS appearance
(loss of adherence)
3.
PVS (resetting of
oocyte volume)
4.
PVS (enlarged by a
matrix rich in
hyaluronic acid)
5.
PVS (enlarged
around first PB)
PB
ZP
PVS
O

Figure 34. Proposed model for PVS formation. The proposed steps leading to PVS formation are schematically represented in the middle column. **(1.)** Within the antral follicle, the GV oocyte lacks a PVS, and its membrane tightly adheres to the ZP. The absence of a PVS at this stage is noticeable by light microscopy (left panel) and by electron microscopy (right panel). **(2.)** Following ovulatory stimulation, the adherence between oocyte and ZP becomes lost, allowing appearance of the PVS. At this stage a small gap between the oocyte and ZP is visible by electron microscopy. **(3.)** The PVS becomes larger (visible by electron microscopy, right), while the oocyte resets its volume. **(4.)** During cumulus expansion and in parallel with meiotic maturation the PVS becomes enlarged and possibly fills up with a matrix rich in hyaluronic acid, similar to that present between cumulus cells. Now, the PVS becomes visible by light microscopy (left). **(5.)** Following first polar body extrusion, the PVS is large and easily observable by light microscopy. O = oocyte, ZP = zona pellucida, PVS = perivitelline space, PB = polar body.

protease, allowing appearance of the PVS. *In vivo*, while the oocyte undergoes meiotic maturation and resets its volume, the PVS becomes enlarged and possibly fills up with a matrix rich in hyaluronic acid, similar to that present between cumulus cells during cumulus expansion (Dandekar and Talbot, 1992; Talbot and Dandekar, 2003). Once the first polar body is extruded, both *in vivo* and *in vitro*, the PVS becomes large and easily observable by light microscopy.

Data discussed in this chapter indicated the presence of a unique manner of oocyte volume control within the antral follicle before ovulation, through a mechanism involving oocyte-ZP adherence. This adherence is released subsequent to ovulatory stimulation or upon oocyte release from the follicle, allowing PVS formation and oocyte volume resetting towards a new, much lower preferred cell volume. Together, these confirmed my second set of hypotheses, indicating that prior to ovulation, the oocyte cannot independently regulate its volume, but rather relies on its extracellular matrix, ZP.

5. Initiation of independent cell volume regulation occurs at ovulation

Together, my thesis supports a model in which the oocyte switches from a fixed ZP-dependent volume to an active osmolyte-dependent volume regulation at ovulation (Fig. 35).

5. 1. Resetting of oocyte volume occurs at the transition between a ZP-dependent to GLYT1 mediated independent cell-volume regulation

The data collected throughout my thesis project indicates that independent cell volume regulation is acquired only after ovulation, in a period of development that

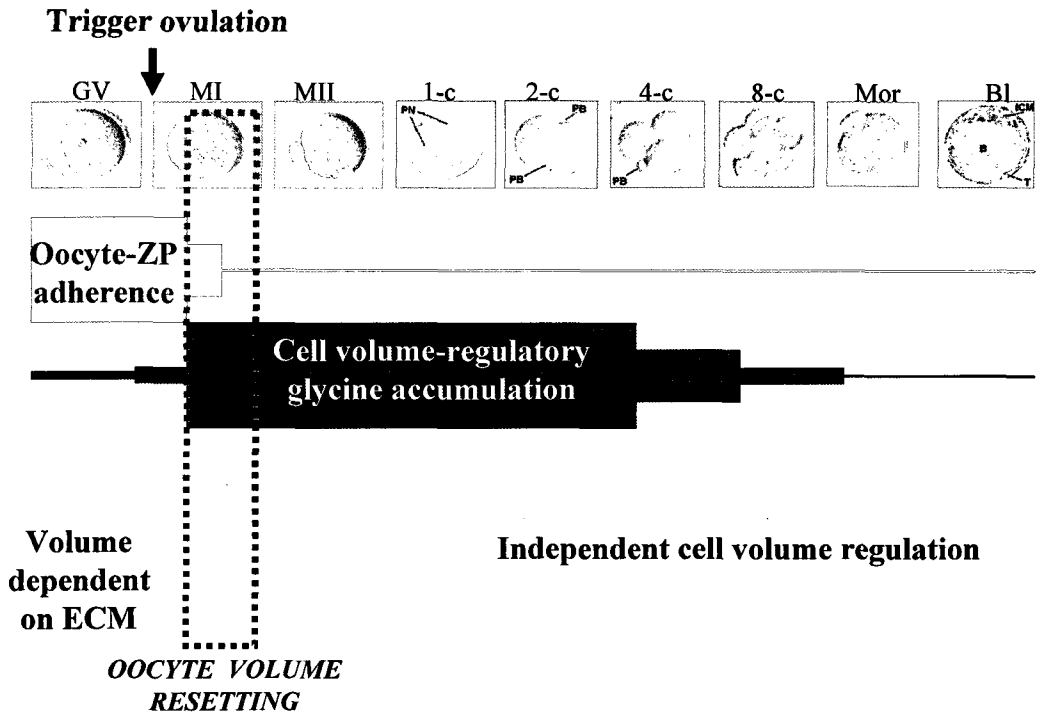


Figure 35. Initiation of cell volume-regulation in the nascent organism at ovulation. Before ovulation, oocyte volume is determined by a strong adherence to the ZP (red). This manner of volume determination is lost subsequent to ovulatory stimulation. Cell volume-regulatory glycine transport via GLYT1 (dark blue) is initiated within several hours after ovulation is stimulated, beginning oocyte independence in controlling its volume. This ability to accumulate glycine is lost around the 4-cell embryonic stage. The oocyte resets its volume at the transition between a ZP-dependent to GLYT1-mediated independent cell volume regulation (highlighted area). Thus, at ovulation, the mammalian cell switches from a form of volume control dependent on an extracellular matrix (ECM) (light red) to independent cell volume regulation involving accumulation or release of osmolytes (light blue). The transition of the oocyte and the preimplantation embryo through early development is summarized in the upper panel.

closely precedes the conception of a new organism. Glycine transport and accumulation via GLYT1 are initiated within several hours after ovulation is stimulated, supporting oocyte independence in controlling its volume. Before ovulation, within the ovarian follicle, the GV oocyte volume is determined by a strong adherence between the oocyte and its extracellular matrix, ZP. Once ovulation is triggered, the strong physical interaction between oocyte and ZP is lost gradually, allowing oocyte resetting towards a smaller volume (Fig. 35) .

Release of oocyte-ZP adherence and resetting in oocyte volume seem to occur over the same period of time during which GLYT1 is activated. *In vitro*, the timing of GLYT1 activity initiation at ~ 1 hr (Fig. 10 A) slightly lags behind the release of ZP-adherence (Fig. 26) and volume resetting (Fig. 25 C), both shown to initiate ~ 0.5 hr subsequent to oocyte removal from antral follicle, although the resolution of the experiments may not be precise enough to rule out the possibility that these processes occur simultaneously in a given oocyte. By ~ 2 hr after collection, GLYT1 activation (Fig. 10 A), release of adherence (Fig. 26) and volume resetting (Fig. 25 C) are entirely achieved. *In vivo*, oocyte volume resetting seem to occur more slowly, being initiated ~ 6 hr following hormonal stimulation (Fig. 25 A), by which stage GLYT1 is fully activated (Fig. 10 B). Although the *in vitro* timings could be interpreted as reflective of a mechanism in which GLYT1 activation is triggered somehow by the loss of oocyte-ZP adherence, the disconnection between the timing of GLYT1 activation and resetting of oocyte volume *in vivo* suggest independence of initiation of GLYT1 activity from release of adherence. Nevertheless, activation of cell-volume regulatory glycine transport via GLYT1 and release of oocyte volume-dependence on adherence to ZP occurs over

essentially the same period of time, in response to ovulatory stimulation or release of the oocyte from the follicle. Resetting of oocyte volume seems to occur at the transition between these two very different mechanisms of cell volume control, most likely to support an independent osmotically-induced manner of oocyte volume regulation, characteristic to animal cells.

Thus, the switch from a fixed volume determined by the ZP cavity, to an active glycine-dependent volume regulation occurs upon ovulatory stimulation, before oocyte expulsion into the oviduct and fertilization. This constitutes, in my opinion, the initiation of independent cell volume regulation in the new mammalian organism (Fig. 35).

5. 2. The switch from a ZP-dependent to independent cell-volume regulation might occur in response to physiological requirements at various stages of oocyte development

The need for different cell-volume regulatory mechanisms prior to and following the ovulatory process might reside in functional differences characterizing the follicle-enclosed GV oocyte and the ovulated MII oocyte, respectively.

5. 2. 1. The dependence of GV oocyte volume on ZP allows proper communication between oocyte and other follicular compartments within the ovarian follicle

Before ovulation, the ovarian follicle functions as a unit, with all its compartments synchronously responding to endocrine or paracrine signals. This is achieved through a complex bidirectional communication between oocyte and its surrounding somatic cells that includes both paracrine signaling and direct connections between oocytes and

granulosa cells via gap junctions (Matzuk *et al.*, 2002).

Within the ovarian follicle, the oocyte and its neighboring cumulus granulosa cells are strongly interconnected through granulosa processes that traverse the ZP (Anderson and Albertini, 1976). These granulosa cell processes are thin and microtubule-rich, and are embedded in the ZP matrix until reaching the inner ZP surface. There, the process becomes flattened and thickened, and impinges upon the oocyte surface and is in close apposition. At the terminus of the cumulus process, adhesive connections and gap junction plaques are located and mediate communication between the granulosa cell and the oocyte (Motta *et al.*, 1994; Albertini *et al.*, 2001; Kidder and Mhawi, 2002). These gap junctions are essential in allowing transfer not only of various signals but also of nutrients and metabolic precursors to support oocyte activities (Heller *et al.*, 1981; Simon *et al.*, 1997; Kidder and Mhawi, 2002; Senbon *et al.*, 2003; Gittens *et al.*, 2004). Indeed, disruption of these gap junctions prevents oocyte development and results in complete infertility, as proven by connexin knock-out studies (Simon *et al.*, 1997; Ackert *et al.*, 2001; Li *et al.*, 2007).

The requirement to maintain the granulosa-oocyte gap junctions may be an important factor in the apparent lack of independent cell volume regulation by oocytes before ovulation. An oocyte that is able to control its volume independently would inevitably result in size alterations in response to, for example, environmental changes, metabolic generation of intracellular osmotically-active species, and cytoskeletal dynamics. If the oocyte surface could freely move away from the inner ZP surface, as it is able to do after ovulation, then strain put on the contact areas between granulosa processes and oolema surface could result in disruption of these vital connections. Thus,

the unique situation described in my thesis, with GV oocyte size being determined by the rigid ZP cavity through oocyte-ZP adhesion rather than by independent cell volume regulation, may exist to allow the maintenance of gap junctional communication and the s

This manner of volume control where an extracellular matrix determines cell volume is uncommon among mammalian cells, and is reminiscent instead of volume control employed by organisms like bacteria or plants. Animal cells are normally in osmotic equilibrium with their environment, being able to change their shape to adapt to any variation in their surroundings (see Introduction), while in contrast plant cells are contained in an extracellular matrix (polysaccharide cell wall) able to resist to large osmotic gradients between the intracellular and extracellular milieu (Peters *et al.*, 2000). In plants, the rigid wall ensures not only osmotic protection but also constrains cell size and shape. The GV oocyte preference for a fixed size might reside, as already mentioned, in the architecture and functional characteristics of the ovarian follicle, to enable in the end synchronized follicular behavior.

5. 2. 2. Initiation of independent cell volume regulation occurs in preparation for early embryo developmental requirements

Once ovulation is stimulated, gap junction-mediated communication between oocyte and cumulus cells has been previously shown to be lost (Gilula *et al.*, 1978), and the maturing oocyte becomes independent from its surrounding follicular cells. Therefore, a strong physical interaction between oocyte and ZP is not needed anymore. Moreover, the oocyte has to acquire specific traits in preparation to becoming a future embryo. Cell-volume regulatory glycine accumulation has been shown to be critical in

allowing very early embryo survival and development at oviductal osmolarity (Steeves *et al.*, 2003). Thus, initiation of GLYT1 activity before fertilization would equip the oocyte with one of the mechanisms needed for the subsequent development in the context of an oviductal environment rich in glycine.

Accumulation of proteins and RNA during the oocyte growth period supports completion of meiosis, fertilization and early preimplantation development (Eppig *et al.*, 2004). GLYT1 is presumably one of these proteins made by the oocyte during its growth but only used after ovulation. The exact stage at which GLYT1 is synthesized and transferred to the membrane, however, remains to be determined by future studies. This event probably takes place early in development since growing oocytes from 9 day old neonatal mice were able to develop rapidly glycine transport when removed from the follicle (Fig. 14). A GLYT1 transporter already synthesized during growth, maintained quiescent prior to ovulation, and subsequently activated to support survival and development in the oviductal environment resembles other processes thought to be part of cytoplasmic maturation (see Introduction), and may be one component of this process.

5. 3. The independent cell volume regulation of the mammalian organism is initiated at ovulation

The results presented throughout my thesis indicated that independent cell volume regulation in the nascent mammalian organism begins once ovulation is triggered. Before ovulation the oocyte control its volume by relying on its extracellular matrix, the ZP, reminiscent of mechanisms utilized by plants. Once ovulation is stimulated, the oocyte resets its volume and acquires ability to control independently its size in response to

changes in its environment, through a mechanism involving accumulation and release of an organic osmolyte, glycine (Fig. 35). Such a mechanism utilizing accumulation and release of inorganic or organic osmolytes to provide intracellular osmotic support is generally utilized by mammalian cells (Lang *et al.*, 1998). Thus, initiation of mammalian cell volume regulation occurs at ovulation (Fig. 35).

Utilization of glycine accumulation via GLYT1 to regulate cell volume seems to be restricted to matured MII oocytes and the initial stages of preimplantation embryo development, ceasing around 4-cell stage (Fig. 31) (Hammer *et al.*, 2000). GLYT1 expression reappears during organogenesis, mainly in the CNS (Adams *et al.*, 1995). Future work will have to determine if GLYT1 is generally utilized as an organic osmolyte transporter in other mammalian tissues.

CONCLUSIONS

The results presented in my thesis indicated that independent cell volume regulation of the emerging mammalian organism starts only after ovulation. My work determined that GLYT1-mediated cell volume-regulatory glycine transport is initiated in oocytes only after ovulation, independent of meiotic maturation. Before ovulation, within the ovarian follicle, a strong adhesion between the oocyte and ZP determines preovulatory oocyte volume. Following ovulation, this physical interaction is gradually lost, and a resetting in oocyte volume takes place in parallel with PVS formation. Thus, subsequent to ovulatory stimulation, the oocyte switches from a fixed, ZP-dependent volume to an active glycine-dependent volume regulation, critical for subsequent preimplantation development. My data also suggests that GLYT1 activation requires a mechanism different from the other known organic osmolyte transporters, not involving *de novo* synthesis. Release of the oocyte from the inhibitory action of unidentified follicular factor(s) following ovulation, is most likely responsible for this initiation.

SIGNIFICANCE

My thesis revealed new information in the area of reproductive physiology and cell volume regulation, uncovering physiologically important aspects of oocyte behavior over the period of development when the female gamete attains independence from its protective follicular environment. Whilst still dependent on follicular support, oocyte volume is controlled in a unique way not previously described for a mammalian cell, possibly to support oocyte-follicular cell connections through the ZP. While attaining independence at ovulation, the oocyte also switches from a fixed, extracellular matrix-

dependent volume control to an osmo-responsive size-adaptable manner of volume regulation. This intriguing transition seems to occur just before the conception of a new organism. Thus, my thesis describes the beginning of independent cell volume regulation in the mammalian organism. This is acquired at ovulation and from then on this capacity will be present in every cell of the newly-created organism.

Besides answering several basic physiological questions, my findings could also be utilized in improving conditions for culturing and maturing oocytes in a clinical or experimental setting. My data make evident the need for glycine inclusion in culture media as the oocyte matures and for subsequent embryonic development. The presence of GLYT1 activity in human embryos (Hammer *et al.*, 2000) makes it more likely that initiation of its activity occurs in human oocytes as well. Therefore, data collected throughout my thesis could found application in developing media and culture conditions for maturing human oocytes in ART laboratories.

The two different mechanisms of volume control prior to and following ovulation could also have implications in the cryopreservation techniques needed to be utilized for oocytes at different stages of maturation. While the matured MII oocyte and early embryos can be predicted to endure volume changes more easily in response to cryopreservants, the freshly-isolated GV oocyte will probably not be able to respond independently of the ZP and could thus be damaged. Moreover, the physiological ability of matured oocyte and embryo to control their volume by maintaining a steady-state intracellular glycine level could be utilized during freezing and thawing processes, and may point to a role for glycine as a benign intracellular osmoprotectant in cryopreservation. Overall, the findings in my thesis could improve the ability of

culturing, maturing and preserving female gametes, highly applicable in ART centers for treatment of human infertility or cryopreservation of oocytes for women undergoing chemotherapy.

LITERATURE CITED

Ackert, C. L., Gittens, J. E. I., O'Brien, M. J., Eppig, J. J. and Kidder, G. M. (2001) Intercellular communication via Connexin43 gap junctions is required for ovarian folliculogenesis in the mouse. Dev. Biol., 233, 258-270.

Adams, R. H., Sato, K., Shimada, S., Tohyama, M., Puschel, A. W. and Betz, H. (1995) Gene structure and glial expression of the glycine transporter GlyT1 in embryonic and adult rodents. J. Neurosci., 15, 2524-2532.

Adashi, E. Y. (1994) Endocrinology of the ovary. Hum. Reprod., 9, 815-827.

Adashi, E. Y. and Hsueh, A. J. W. (1982) Estrogens augment the stimulation of ovarian aromatase activity by follicle-stimulating hormone in cultured rat granulosa cells. J. Biol. Chem., 257, 6077-6083.

Albertini, D. F., Combelles, M. H., Benecchi, E. and Carabastos, M. J. (2001) Cellular basis for paracrine regulation of ovarian follicle development. Reproduction, 121, 647-653.

Amsterdam, A., Koch, Y., Lieberman, M. E. and Linder, H. R. (1975) Distribution of binding sites for human chorionic gonadotropin in the preovulatory follicle of rat. J. Cell. Biol. , 67, 894-900.

Anas, M. K., Hammer, M. A., Lever, M., Stanton, J. A. and Baltz, J. M. (2007) The organic osmolytes betaine and proline are transported by a shared system in early preimplantation mouse embryos. J. Cell. Physiol., 210, 266-277.

Anas, M. K., Lee, M. B., Zhou, C., Hammer, M. A., Slow, S., Karmouch, J., Liu, X. J., Broer, S., Lever, M. and Baltz, J. M. (2008) SIT1 is a betaine/proline transporter that is activated in mouse eggs after fertilization and functions until 2-cell stage. Development, 135, 4123-4130.

Anderson, E. and Albertini, D. F. (1976) Gap junctions between the oocytes and companion follicle cells in the mammalian ovary. J. Cell. Biol., 71, 680-686.

Aragon, C., Gimenez, C. and Mayor, F. (1987) Stoichiometry of sodium- and chloride-coupled glycine transport in synaptic plasma membrane vesicles derived from rat brain. FEBS Letters, 212, 87-90.

Baltz, J. M. (2001) Osmoregulation and cell volume regulation in the preimplantation embryo. Curr. Top. Dev. Biol., 52, 55-106.

Banwell, K. M. and Thompson, J. G. (2008) In vitro maturation of Mammalian oocytes: outcomes and consequences. Semin. Reprod. Med., 26, 162-174.

Basak, A. and Lotfipour, F. (2005) Modulating furin activity with designed mini-PDX peptides: Synthesis and in vitro kinetic evaluation. FEBS Lett., 579, 4813-4821.

Bergeron, R., Meyer, T. M., Coyle, J. T. and Greene, R. W. (1998) Modulation of N-methyl-D-aspartate receptor function by glycine transport. Proc. Natl. Acad. Sci. U. S. A., 95, 15730-15734.

Biggers, J. D. (1998) Reflections on the culture of the preimplantation embryo. Int. J. Dev. Biol., 42, 879-884.

Biggers, J. D., Lawitts, J. A. and Lechene, C. P. (1993) The protective action of betaine on the deleterious effects of NaCl on preimplantation mouse embryos in vitro. Mol.Reprod. Dev., 34, 380-390.

Biggers, J. D., Whittingham, D. G. and Donahue, R. P. (1967) The pattern of energy metabolism in the mouse oocyte and zygote. Proc. Nat. Acad. Sci. USA, 58, 560-567.

Bleil, J. D. and Wassarman, P. M. (1980) Structure and function of the zona pellucida: identification and characterization of the proteins of the mouse oocyte's zona pellucida. Dev. Biol., 76, 185-202.

Borland, R. M. and Tasca, R. J. (1974) Activation of a Na+-dependent amino acid transport system in preimplantation mouse embryos. Dev. Biol. , 36, 169-182.

Borowsky, B. and Hoffman, B. J. (1998) Analysis of a gene encoding two glycine transporter variants reveals alternative promoter usage and a novel gene structure. J. Biol. Chem., 273, 29077-29085.

Borowsky, B., Mezey, E. and Hoffman, B. (1993) Two glycine transporters variants with distinct localization in the CNS and peripheral tissues are encoded by a common gene. Neuron, 10, 851- 863.

Bortner, C. D. and Cidlowski, J. A. (1996) Absence of volume regulatory mechanisms contributes to the rapid activation of apoptosis in thymocytes. Am. J. Physiol. Cell Physiol., 271, C950-C961.

Brinster, R. L. (1965) Studies on the development of mouse embryo *in vitro*. The effect of osmolarity and hydrogen ion concentration. J. Exp. Zool., 158, 49-58.

Brinster, R. L. (1968) Hexokinase activity in the preimplantation mouse embryo. Enzymologia, 34, 304-308.

Brinster, R. L. (1969) Radioactive carbon dioxide production from pyruvate and lactate by the preimplantation rabbit embryo. Exp. Cell Res., 54.

Brison, D. R., Houghton, F. D., Falconer, D., Roberts, S. A., Hawkhead, J., Humpherson, P. G., Lieberman, B. A. and Leese, H. J. (2004) Identification of viable embryos in IVF by non-invasive measurement of amino acid turnover. Hum. Reprod., 19, 2319-2324.

Brower, P. T. and Schultz, R. M. (1982) Intercellular communication between granulosa cells and mouse oocytes: existence and possible nutritional role during oocyte growth. Dev. Biol., 90, 144-153.

Burg, M. B. (1995) Molecular basis of osmotic regulation. Am. J. Physiol., 268, F983-F996.

Burg, M. B. and Ferraris, J. D. (2008) Intracellular organic osmolytes: function and regulation. J. Biol. Chem., 283, 7309-7313.

Burg, M. B., Ferraris, J. D. and Dimitrieva, N. I. (2007) Cellular response to hyperosmotic stresses. Physiol. Rev., 87, 1441-1474.

Byskov, A. G. and Hoyer, P. E. (1988) Embryology of mammalian gonads and ducts. In The Physiology of Reproduction(Ed, Knobil, E., Neill, J) Raven Press, New York, pp. 265-302.

Caviston, J. P. and Holzbaur, E. L. (2006) Microtubule motors at the intersection of trafficking and transport. Trends Cell Biol. , 16, 530-537.

Chesnel, F., Wigglesworth, K. and Eppig, J. J. (1994) Acquisition of meiotic competence by denuded mouse oocytes: participation of somatic-cell product(s) and cAMP. Dev. Biol., 161, 285-295.

Cho, W. K., Stern, S. and Biggers, J. D. (1974) Inhibitory effect of dibutyryl cAMP on mouse oocyte maturation *in vitro*. J. Exp. Zool., 187, 383-386.

Clayton, L., McConnell, J. M. and Johnson, M. H. (1995) Control of the surface expression of uvomorulin after activation of mouse oocytes. Zygote, 3, 177-189.

Cole, R. J. and Paul, J. (1965) Properties of cultured preimplantation mouse and rabbit embryos, and cell strains derived from them. In Preimplantation Stages of Pregnancy(Eds, Wolstenholm, G. E. W. and O'Connor, M.) Churchill, London, pp. 82-112.

Collins, J. L. and Baltz, J. M. (1999) Estimates of mouse oviductal fluid tonicity based on osmotic responses of embryos. Biol. Reprod., 60, 1188-1193.

Colonna, R. and Mangia, F. (1983) Mechanism of amino acid uptake in cumulus-enclosed mouse oocytes. Biol. Reprod., 28, 797-803.

Conti, M., Andersen, C. B., Richard, F., Mehats, C., Chun, S. Y., Horner, K., Jin, C. and Tsafriri, A. (2002) Role of cyclic nucleotide signalling in oocyte maturation. Mol. Cell. Endocrinol., 187, 153-159.

Cooper, G. (1999) The cell cycle. In The cell. A molecular approachASM Press, Washington, pp. 571-607.

Cuthbertson, K. S. R. and Cobbold, P. H. (1985) Phorbol ester and sperm activate mouse oocyte by inducing sustained oscillations in cell Ca^{2+}. Nature, 316, 541-542.

Dandekar, P., Mate, K. E. and Talbot, P. (1995) Perivitelline space of marsupial oocytes: extracellular matrix of the unfertilized oocyte and formation of a cortical granule envelope following the cortical reaction. Mol. Reprod. Dev., 41, 368-373.

Dandekar, P. and Talbot, P. (1992) Perivitelline space of mammalian oocytes: extracellular matrix of unfertilized oocytes and formation of a cortical granule envelope following fertilization. Mol. Reprod. Dev., 31, 135-143.

Dawson, K. M. and Baltz, J. M. (1997) Organic osmolytes and embryos: substrates of the Gly and β transport system protect mouse zygotes against raised osmolarity. Biol. Reprod., 56, 1550-1558

Dawson, K. M., Collins, J. L. and Baltz, J. M. (1998) Osmolarity-dependent glycine accumulation indicates a role for glycine as and organic osmolyte in early preimplantation mouse embryos. Biol. Reprod., 59, 225-232.

Dekel, N. (1988) Regulation of oocyte maturation. The role of cAMP. Ann. NY Acad. Sci., 541, 211-216.

Dekel, N., Aberdam, E. and Sherizly, I. (1984) Spontaneous maturation in vitro of cumulus-enclosed rat oocytes is inhibited by forskolin. Biol. Reprod., 31, 224-250.

Dekel, N. and Beers, W. H. (1978) Rat oocyte maturation in vitro: relief of cyclic AMP inhibition by gonadotropins. Proc. Natl. Acad. Sci. USA, 75, 4369-4373.

DeSousa, P., Vladimarsson, G., Nicholson, B. J. and Kidder, G. M. (1993) Connexin trafficking and the control of gap junction assembly in mouse preimplantation embryos. Development, 117, 1355-1367.

Dierich, A., Sairam, M. R., Monaco, L., Fimia, G. M., Gansmuller, A., LeMeur, M. and Sassone-Corsi, P. (1998) Impairing follicle-stimulating hormone (FSH) signaling in vivo: Targeted disruption of the FSH receptor leads to aberrant gametogenesis and hormonal imbalance. Proc. Natl. Acad. Sci. U S A., 95, 13612-13617.

Dinter, A. and Berger, E. G. (1998) Golgi-disturbing agents. Histochem. Cell Biol., 109,

571-590.

Dissen, G. A., Paredes, A., Romero, C., Dees, L. W. and Ojeda, S. R. (2004) Neural and neurotrophic control of ovarian development. In The ovary(Ed, Leung, P. C. K., Adashi E. Y.), pp. 3-23.

Doherty, A. S., Mann, M. R., Tremblay, K.D., Bartolomei, M. S. and Schultz, R. M. (2000) Differential effects of culture on imprinted H19 expression in the preimplantation mouse embryo. Biol. Reprod., 62, 1526-1535.

Donahue, R. P. and Stern, S. (1968) Follicular cells support of oocyte maturation: production of pyruvate in vitro. J. Reprod. Fertil. , 17, 395-398.

Dong, J., Albertini, D. F., Nishimori, K., Kumar, T. R., Lu, N. and Matzuk, M. M. (1996) Growth differentiation factor-9 is required during early ovarian folliculogenesis. Nature, 383, 531-535.

Downs, S. M. (1993) Purine control of mouse oocyte maturation: evidence that nonmetabolized hypoxanthine maintains meiotic arrest. Mol. Reprod. Dev., 35, 82-94.

Downs, S. M. (2000) Adenosine blocks hormone-induced meiotic maturation by suppressing purine de novo synthesis. Mol. Reprod. Dev, 56, 172-179.

Downs, S. M., Coleman, D. L. and Eppig, J. J. (1986) Maintenance of murine oocyte meiotic arrest: uptake and metabolism of hypoxanthine and adenosine by cumulus cell-enclosed and denuded oocytes. Dev. Biol., 117, 174-183.

Downs, S. M., Daniel, S. A., Bornslaeger, E. A., Hoppe, P. C. and Eppig, J. J. (1989) Maintenance of meiotic arrest in mouse oocytes by purines: modulation of cAMP levels and cAMP phosphodiesterase activity. Gamete Res., 23, 323-334.

Downs, S. M., Daniel, S.A., Bornslaeger, E.A., Hoppe, P.C., and Eppig, J.J. (1989) Maintenance of meiotic arrest in mouse oocytes by purines: modulation of cAMP levels and cAMP phosphodiesterase activity. Gamete Res., 23, 323-334.

Downs, S. M. and Eppig, J. J. (1987) Induction of mouse oocyte maturation in vivo by perturbants of purine metabolism. Biol. Reprod., 36, 431-437.

Downs, S. M., Humpherson, P.G., and Leese, H.J. (2002) Pyruvate utilization by mouse oocyte is influenced by meiotic status and the cumulus oophorus. Mol. Reprod. Dev., 62, 113-123.

Downs, S. M. and Mastropolo, A. M. (1994) The participation of energy substrates in the control of meiotic maturation in murine oocytes. Dev. Biol., 162, 154-168.

Ducibella, T., Schultz, R. M. and Ozil, J. P. (2006) Role of calcium signals in early development. Semin. Cell Dev. Biol. , 17, 324-332.

Eavenson, E. and Christensen, H. N. (1967) Transport systems for neutral amino acids in the pigeon erythrocyte. J. Biol. Chem., 242, 5386-5396.

Edwards, R. G. (1965) Maturation in vitro of mouse, sheep, cow, pig, rhesus monkey, and human ovarian oocytes. Nature, 208, 349-351.

Epifano, O., Liang, L. F., Familari, M., Moos, M. C. and Dean, J. (1995) Coordinate expression of the three zona pellucida genes during mouse oogenesis. Development, 121, 1947-1956.

Eppig, J. J. (1976) Analysis of mouse oogenesis in vitro. Oocyte isolation and the utilization of exogenous energy sources by growing oocytes. J. Exp. Zool., 198, 375-382.

Eppig, J. J. (1977) Mouse oocyte development in vitro with various culture systems. Dev. Biol. Reprod., 60, 371-388.

Eppig, J. J. (1979) A comparison between oocyte growth in coculture with granulosa cells and oocytes with granulosa cell-oocyte junctional contact maintained in vitro. J. Exp. Zool., 209, 345-353.

Eppig, J. J. (1991) Maintenance of meiotic arrest and the induction of oocyte maturation in mouse oocyte-granulosa cell complexes developed in vitro from preantral follicles. Biol. Reprod., 45, 824-830.

Eppig, J. J. (1996) Coordination of nuclear and cytoplasmic oocyte maturation in eutherian mammals. Reprod. Fertil. Dev., 8, 485-489.

Eppig, J. J. (2001) Oocyte control of ovarian follicular development and function in mammals. Reproduction, 122, 829-838.

Eppig, J. J. and O'Brien, M. J. (1996) Development in vitro of mouse oocytes from primordial follicles. Biol. Reprod., 54, 197-207.

Eppig, J. J. and Schroeder, A. C. (1989) Capacity of mouse oocytes from preantral follicles to undergo embryogenesis and development to live young after growth, maturation and fertilization in vitro. Biol. Reprod., 41, 268-276.

Eppig, J. J., Viveiros, M. M., Bivens, C. M. and De la Fuente, R. (2004) Regulation of mammalian oocyte maturation. In The ovary(Eds, Leung, P. C. K. and Adashi, E. Y.), pp. 113-129.

Eppig, J. J., Ward-Bailey, P. F. and Coleman, D. L. (1985) Hypoxanthine and adenosine

in murine ovarian follicular fluid: concentrations and activity in maintaining oocyte meiotic arrest. Biol. Reprod., 33, 1041-1049.

Eppig, J. J., Wigglesworth, K. and Pendola, F. L. (2002) The mammalian oocyte orchestrates the rate of ovarian follicular development. Proc. Natl. Acad. Sci. U.S.A., 99, 2890-2894.

Erdogan, S., FitzHarris, G., Tartia, A. P. and Baltz, J. M. (2005) Mechanisms regulating intracellular pH are activated during growth of the mouse oocyte coincident with acquisition of meiotic competence. Dev Biol, 286, 352-360.

Erickson, G. F. (1983) Primary cultures of ovarian cells in serum-free medium as models of hormone-dependent differentiation. Mol. Cell. Endocrinol., 29, 21-49.

Espey, L. L., Bellinger, A. S. and Healy, J. A. (2004) Ovulation: An inflammatory cascade of gene expression. In The ovary(Eds, Leung, P. C. K. and Adashi, E. Y.), pp. 145-165.

Etienne-Manneville, S. (2004) Actin and Microtubules in cell motility: Which one is in control? Traffic, 5, 470-477.

Eulenburg, V., Armsen, W., Betz, H. and Gomeza, J. (2005) Glycine transporters: essential regulators of neurotransmission. Trends Bichem. Sci., 30, 325-333.

Evans, W. H. and Boitano, S. (2001) Connexin mimetic peptides: specific inhibitors of gap-junctional intercellular communication. Biochem. Society Trans., 29, 606-612.

Falconer, D. S. and Avery, P. J. (1978) Variability of chimaeras and mosaics. J. Embryol. Exp. Morphol., 43, 195-219.

Fitzharris, G. and Baltz, J. M. (2006) Granulosa cells regulate intracellular pH of the murine growing oocyte via gap junctions: development of independent homeostasis during oocyte growth. Development, 133, 591-599.

Gabernet, L., Pauly-Evers, M., Schwerdel, C., Lentz, M., Bluethmann, H., Vogt, K., Alberati, D., Mohler, H. and Boison, D. (2005) Enhancement of the NMDA receptor function by reduction of glycine transporter-1 expression. Neurosci. Lett., 373, 79-84.

Galloways, S. M., McNatty, K. P., Cambridge, L. M., Laitinen, M. P., Juengel, J. L., Jokiranta, T. S., McLaren, R. J., Luiro, K., Dodds, K. G., Montgomery, G. W., Beattie, A. E., Davis, G. H. and Ritvos, O. (2000) Mutations in an oocyte derived growth factor gene (BMP-15) cause increased ovulation rate and infertility in a dosage-sensitive manner. Nat. Genet., 25, 279-283.

Garcia-Perez, A. and Burg, M. B. (1991) Role of organic osmolytes in adaptation of renal cells to high osmolarity. J. Membrane Biol., 119, 1-13.

Gardner, D. K. and Lane, M. (1997) Culture and selection of viable human blastocysts: a feasible proposition for human IVF. Hum. Reprod. Update, 3, 367-382.

Gardner, D. K. and Leese, H. J. (1990) Concentrations of nutrients in mouse oviduct fluid and their effects on embryo development and metabolism in vitro. J. Reprod. Fertil., 88, 361-368.

Gardner, D. K., Pool, T. B. and Lane, M. (2000) Embryo nutrition and energy metabolism and its relationship to embryo growth, differentiation, and viability. Semin. Reprod. Med., 18, 205-218.

Geerlings, A., Lopez-Corcuera, B. and Aragon, C. (2000) Characterization of the interactions between the glycine transporters GLYT1 and GLYT2 and the SNARE protein syntaxin 1A. FEBS Lett., 470, 51-54.

Gilula, N. B., Epstein, M. L. and Beers, W. H. (1978) Cell-to-cell communication and ovulation. A study of the cumulus-oocyte complex. J.Cell Biol., 78, 58-75.

Gittens, J. E. I., Barr, K. J., Vanderhyden, B. C. and Kidder, G. M. (2004) Interplay between paracrine signaling and gap junctional communication in ovarian follicles. J. Cell. Sci., 118, 113-122.

Gomeza, J., Hulsmann, S., Ohno, K., Eulenburg, V., Szoke, K., Richter, D. and Betz, H. (2003) Inactivation of the glycine transporter 1 gene discloses vital role of glial glycine uptake in glycinergic inhibition. Neuron, 40, 785-796.

Gomeza, J., Zafra, F., Olivares, L., Gimenez, C. and Aragon, C. (1995) Regulation by phorbol esters of the glycine transporter (GLYT1) in glioblastoma cells. Biochim. Biophys. Acta, 1233, 41-46.

Gougeon, A. (2004) Dynamics of human follicular growth: Morphologic, dynamic, and functional aspects. In The ovary(Ed, Leung, P. C. K., Adashi E. Y.), pp. 25-43.

Granot, I. and Dekel, N. (2002) The ovarian gap junction protein connexin-43: Regulation by gonadotropins. Trends Endocrinol. Metab., 13, 310-313.

Green, D. P. L. (1997) Three-dimensional structure of the zona pellucida. Rev. Reprod., 2, 147-156.

Greve, J. M. and Wassarman, P. M. (1985) Mouse egg extracellular coat is a matrix of interconnected filaments possessing a structural repeat. J. Mol. Biol., 181, 253-264.

Guastella, J., Brecha, N., Weigmann, C., Lester, H. A. and Davidson, N. (1992) Cloning, expression, and localization of a rat brain high-affinity glycine transporter. Proc. Natl. Acad. Sci. U. S. A., 89, 7189-7193.

Hadi, T., Hammer, M. A., Algire, C., Richards, T. and Baltz, J. M. (2005) Similar effects of osmolarity, glucose, and phosphate on cleavage past the 2-cell stage in mouse embryos from outbred and F1 hybrid females. Biol. Reprod., 72, 179-187.

Haghighat, N. and Van Winkle, L. J. (1990) Developmental change in follicular cell-enhanced amino acid uptake into mouse oocytes that depends on intact gap junctions and transport system Gly. J. Exp. Zool., 253, 71-82.

Hammer, M. A. and Baltz, J. M. (2002) Betaine is a highly effective organic osmolyte but does not appear to be transported by established organic osmolyte transporters in mouse embryos. Mol. Reprod. Dev., 62, 195-202.

Hammer, M. A., Kolajova, M., Leveille, M.-C., Claman, P. and Baltz, J. M. (2000) Glycine transport by single human and mouse embryos. Hum. Reprod., 15, 419-426.

Hanley, J. G., Jones, E. M. and Moss, S. J. (2000) GABA receptor rho1 subunit interacts with a novel splice variant of the glycine transporter, GLYT-1. J. Biol. Chem., 275, 840-846.

Harris, S. E., Gopichandran, N., Picton, H. M., Leese, H. J. and Orsi, N. M. (2005) Nutrient concentrations in murine follicular fluid and the female reproductive tract. Theriogeneology, 64, 992-1006.

Harsing, L. G., Jr., Gacsalyi, I., Szabo, G., Schmidt, E., Sziray, N., Sebban, C., Tesolin-Decros, B., Matyus, P., Egyed, A., Spedding, M. and Levay, G. (2003) The glycine transporter-1 inhibitors NFPS and Org 24461: a pharmacological study. Pharmacol. Biochem. Behav., 74, 811-825.

Haussinger, D. (1998) Osmoregulation of liver cell function: signalling, osmolytes and cell heterogeneity. Contrib. Nephrol., 123, 185-204.

Heller, D. T., Cahill, D. M. and Schultz, R. M. (1981) Biochemical studies of mammalian oogenesis: metabolic cooperativity between granulosa cells and growing mouse oocytes. Dev. Biol., 84, 455-464.

Hirshfield, A. N. (1991) Development of follicles in the mammalian ovary. Int. Rev. Cytol., 124, 43-101.

Ho, S. N. (2003) The role of NFAT5/TonEBP in establishing an optimal intracellular environment. Arch. Biochem. Biophys., 413, 151-157.

Ho, S. N. (2006) Intracellular water homeostasis and the mammalian cellular osmotic stress response. J. Cell. Physiol., 206, 9-15.

Ho, Y., Wigglesworth, K., Eppig, J. J. and Schultz, R. M. (1995) Preimplantation development of mouse embryos in KSOM: augmentation by amino acids and analysis of gene expression. Mol. Reprod. Dev, 41, 232-238.

Hobbs, J. G. and Kaye, P. L. (1985) Glycine transport in mouse eggs and preimplantation embryos. J. Reprod. Fertil., 74, 77-86.

Hogan, B., Beddington, R., Constantini, F. and Lacy, E. (1994) In Manipulating the mouse embryo(Ed, Press, G. S. H. L.), pp. 28-56.

Horiuchi, M., Nicke, A., Gomeza, J., Aschrafi, A., Schmalzing, G. and Betz, H. (2001) Surface-localized glycine transporters 1 and 2 function as monomeric proteins in Xenopus oocytes. Proc. Natl. Acad. Sci. U.S.A., 98, 1448-1453.

Huang, Z., Li, J., Jiang, Z., Qi, Y., Tang, C. and Du, J. (2003) Effects of adrenomedullin, C-type natriuretic peptide, and parathyroid hormone-related peptide on calcification in cultured rat vascular smooth muscle cells. J. Cardiovasc. Pharmacol., 42, 89-97.

Humeau, Y., Doussau, F., Grant, N. and Poulain, B. (2000) How botulinum and tetanus neurotoxins block neurotransmitter release. Biochim Biophys Acta, 82, 427-446.

Inoue, A., Akiyama, T., Nagata, M. and Aoki, F. (2007) The perivitelline space-forming capacity of mouse oocytes is associated with meiotic competence. J. Reprod. Dev., 53, 1043-1052.

Jamnongjit, M. and Hammes, S. R. (2006) Ovarian steroids: the good, the bad, and the signals that raise them. Cell Cycle, 5, 1178-1183.

Johnson, J. W. and Ascher, P. (1987) Glycine potentiates the NMDA response in cultured mouse brain neurons. Nature, 325, 529-531.

Jovine, L., Qi, H., Williams, Z., Litscher, E. S. and Wassarman, P. M. (2004) A duplicated motif controls assembly of zona pellucida domain proteins. Proc. Natl. Acad. Sci. U.S.A., 101, 5922-5927.

Jursky, F. and Nelson, N. (1995) Localization of glycine neurotransmitter transporter GLYT2 reveals correlation with the distribution of glycine receptor. J. Neurochem., 64, 1026-1033.

Kanatasu-Shinohara, M., Schultz, R. M. and Kopf, G. S. (2000) Acquisition of meiotic competence in mouse oocytes: Absolute amounts of p34(cdc2), cyclin b1, cdc25C, and wee1 in meiotically incompetent and competent oocytes. Biol.

Reprod., 63, 1610-1616.

Kaufman, M. H., Fowler, R. E., Barratt, E. and McDougall, R. D. (1989) Ultrastructural and histochemical changes in the murine zona pellucida during the final stages of oocyte maturation prior to ovulation. Gamete Res., 24, 35-48.

Khatchadourian, C., Guillaud, J. and Menezo, Y. (1994) Interactions in glycine and methionine uptake, conversion and incorporation into proteins in the preimplantation mouse embryo. Zygote, 2, 301-306.

Kidder, G. M. and Mhawi, A. A. (2002) Gap junctions and ovarian folliculogenesis. Reproduction, 123, 613-620.

Kim, J. H., Do, H. J., Wang, W. H., Machaty, Z., Han, Y. M., Day, B. N. and Prather, R. S. (1999) A protein tyrosine phosphatase inhibitor, sosium Orthovanadate, causes parthenogenetic activation of pig oocytes via an increase in protein tyrosine kinase activity. Biol. Reprod., 61, 900-905.

Kim, K. M., Kingsmore, S. F., Han, H., Yang-Feng, T. L., Godinot, N., Seldin, M. F., Caron, M. G. and Giros, B. (1994b) Cloning of the human glycine transporter type 1: molecular and pharmacological characterization of novel isoform variants and chromosomal localization of the gene in the human and mouse genomes. Molec. Pharmacol., 45, 608- 617.

Kleckner, N. W. and Dingledine, R. (1988) Requirement for glycine in activation of NMDA-receptor expressed in Xenopus oocytes. Science, 241, 835-837.

Kolajova, M. and Baltz, J. M. (1999) Volume-regulated anion and organic osmolyte channels in mouse zygotes. Biol. Reprod., 60, 964-972.

Kolajova, M., Hammer, M. A., Collins, J. L. and Baltz, J. M. (2001) Developmentally regulated cell cycle dependence of swelling -activated anion channel activity in the mouse embryo. Development, 128, 3427-3434.

Kouba, A. J., Abeydeera, L. R., Alvarez, I. M., Day, B. N. and Buhi, W. C. (2000) Effects of the porcine oviduct-specific glycoprotein on fertilization, polyspermy, and embryonic development in vitro. Biol. Reprod., 63, 242-250.

Kwon, H. M. and Handler, J. S. (1995) Cell volume regulated transporters of compatible osmolytes. Curr. Opin. Cell Biol., 7, 465-471.

Lane, M. and Gardner, D. K. (2000) Lactate regulates pyruvate uptake and metabolism in the preimplantation mouse embryo. Biol. Reprod., 62, 16-22.

Lang, F., Busch, G. L. and Vokl, H. (1998) The diversity of volume regulatory mechanisms. Cell. Physiol. Biochem., 8, 1-45.

Lang, F., Ritter, M., Gamper, N., Huber, S., Fillon, S., Tanneur, V., Lepple-Wienhues, A., Szabo, I. and Gulbins, E. (2000) Cell volume in regulation of cell proliferation and apoptotic cell death. Cell. Physiol. Biochem., 10, 429-434.

Larsen, W. J., Wert, S. E. and Brunner, G. D. (1987) Differential modulation of rat follicle cell gap junction populations at ovulation. Dev. Biol., 122, 61-71.

Laube, B. (2002) Potentiation of inhibitory glycinergic neurotransmission by Zn2+: a synergistic interplay between presynaptic P2X2 and postsynaptic glycine receptors. Eur. J. Neurosci., 16, 1025-1036.

Law, R. O. (1994) Regulation of mammalian brain cell volume. J. Exp. Zool., 268, 90-96.

Lawitts, J. A. and Biggers, J. D. (1992) Joint effects of sodium chloride, glutamine, and glucose in mouse preimplantation embryo culture media. Mol. Reprod. Dev., 31, 189-194.

Lawitts, J. A. and Biggers, J. D. (1993) Culture of preimplantation embryos. Methods Enzymol., 225, 153-164.

Lawrence, T. S., Dekel, N. and Beers, W. H. (1980) Binding of human chorionic gonadotropin by rat cumuli oophori and granulosa cells: a comparative study. Endocrinology, 106, 1114-1118.

Ledan, E., Polanski, Z., Terret, M. E. and Maro, B. (2001) Meiotic maturation of the mouse oocyte requires an equilibrium between Cyclin B synthesis and degradation. Dev. Biol., 232, 400-413.

Li, T. Y., Colley, D., Barr, K. J., Yee, S. P. and Kidder, G. M. (2007) Rescue of oogenesis in Cx37-null mutant mice by oocyte-specific replacement with Cx43. J. Cell Sci., 120, 4117-4125.

Litscher, E. S., Qi, H. and Wassarman, P. M. (1999) Mouse zona pellucida glycoproteins mZP2, and mZP3 undergo carboxy-terminal proteolytic processing in growing oocytes. Biochemistry, 38, 12280-12287.

Liu, Q. R., Lopez-Corcuera, B., Mandiyan, S., Nelson, H. and Nelson, N. (1993) Cloning and expression of a spinal cord- and brain-specific glycine transporter with novel structural features. J. Biol. Chem., 268, 22802-22808.

Liu, Q. R., Nelson, H., Mandiyan, S., Lopez-Corcuera, B. and Nelson, N. (1992) Cloning and expression of a glycine transporter from mouse brain. FEBS Letters, 305, 110-114.

Lopez-Corcuera, B. and Aragon, C. (1989) Solubilization and reconstitution of the sodium- and-chloride coupled glycine transporter from rat spinal cord. Europ. J.

Biochem., 181, 519-524.

Lopez-Corcuera, B., Geerlings, A. and Aragon, C. (2001) Glycine neurotransmitter transporters: an update. Mol. Membr. Biol., 18, 13-20.

Lopez-Corcuera, B., Kanner, B. I. and Aragon, C. (1989) Reconstitution and partial purification of the sodium-and chloride-coupled glycine transporter from rat spinal cord. Biochim. Biophys. Acta, 983, 247- 252.

Lopez-Corcuera, B., Vazquez, J. and Aragon, C. (1991) Purification of the sodium- and chloride-coupled glycine transporter from central nervous system. J. Biol. Chem., 266, 24809-24814.

Louvet, J. P. and Vaitukaitis, J. L. (1976) Induction of follicle-stimulating hormone (FSH) receptors in rat ovaries by estrogen priming. Endocrinology, 99, 758-766.

Luque, J. M., Nelson, N. and Richards, J. G. (1995) Cellular expression of glycine transporter 2 messenger RNA exclusively in rat hindbrain and spinal cord. Neuroscience, 64, 525-535.

Mamo, S., Gal, A. B., Bodo, S. and Dinnyes, A. (2007) Quantitative evaluation and selection of reference genes in mouse oocytes and embryos cultured *in vivo* and *in vitro*. BMC Dev. Biol., 7:14.

Martina, M., Gorfinkel, Y., Halman, S., Lowe, J. A., Periyalwar, P., Schmidt, C. J. and Bergeron, R. (2004) Glycine transporter type 1 blockade changes NMDA receptor-mediated responses and LTP in hippocampal CA1 pyramidal cells by altering extracellular glycine levels. J. Physiol., 557, 489-500.

Masui, Y. and Market, C. L. (1971) Cytoplasmic control of nuclear behavior during meiotic maturation of frog oocytes. J. Exp. Zool., 177, 129-146.

Matzuk, M. M., Burns, K. H., Viveiros, M. M. and Eppig, J. J. (2002) Intercellular communication in the mammalian ovary: oocytes carry the conversation. Science, 296, 2178-2180.

McKiernan, S. H., Clayton, M. K. and Bavister, B. D. (1995) Analysis of stimulatory and inhibitory amino acids for development of hamster one-cell embryos *in vitro*. Mol. Reprod. Dev., 42, 188-199.

McLaren, A. and Southee, D. (1997) Entry of mouse embryonic germ cells into meiosis. Dev. Biol., 187, 107-113.

McLeskey Kiefer, S. and Saling, P. (2002) Proteolytic processing of human zona pellucida proteins. Biol. Reprod., 66, 407-414.

Mehlmann, L. M., Mikhosiba, K., and Kline, D. (1996) Redistribution and increase in cortical inositol 1,4,5-trisphosphate receptors after meiotic maturation of the mouse oocyte. Dev. Biol., 180, 489-498.

Mehlmann, L. M., Saeki, Y., Tanaka, S., Brennan, T. J., Evsikov, A. V., Pendola, F. L., Knowles, B. B., Eppig, J. J. and Jaffe, L. A. (2004) The Gs-linked receptor Gpr3 maintains meiotic arrest in mammalian oocytes. Science, 3066, 1947-1950.

Mehlmann, L. M., Terasaki, M., Jaffe, L. A. and Kline, D. (1995) Reorganization of the endoplasmic reticulum during meiotic maturation of the mouse oocyte. Dev. Biol., 170, 607-615.

Meijer, L., Borgne, A., Mulner, O., Chong, J. P., Blow, J. J., Inagaki, N., Inagaki, M., Delcros, J. G. and Moulinoux, J. P. (1997) Biochemical and cellular effects of roscovitine, a potent and selective inhibitor of the cyclin-dependent kinase cdc2, cdk2, and cdk5. Eur. J. Biochem., 243, 527-536.

Mitra, J. and Schultz, R. M. (1996) Regulation of the acquisition of meiotic competence in the mouse: Changes in the subcellular localization of cdc2, cyclin b1, cdc25C and wee1, and in the concentration of these proteins and their transcripts. J. Cell. Sci., 109, 2407-2415.

Miyakawa, H., Woo, S. K., Chen, C.-P., Dahl, S. C., Handler, J. H. and H.M., K. (1998) Cis- and trans-acting factors regulating transcription of the BGT1 gene in response to hypertonicity Renal Physiol., 43, F753-F761.

Miyakawa, H., Woo, S. K., Dahl, S. C., Handler, J. S. and Kwon, H. M. (1999) Tonicity-responsive enhancer binding protein, a Rel-like protein that stimulates transcription in response to hypertonicity. Proc. Natl. Acad. Sci. USA, 96, 2538-2542.

Mongin, A. A. and Orlov, S. N. (2001) Mechanisms of cell volume regulation and possible nature of the cell volume sensor. Pathophysiology, 8, 77-88.

Moos, J., Visconti, P. E., Moore, G. D., Schultz, R. M. and Kopf, G. S. (1995) Potential role of mitogen-activated protein kinase in pronuclear envelope assembly and disassembly following fertilization of mouse eggs. Biol. Reprod., 53, 692-699.

Moriyoshi, K., Masu, M., Ishii, T., Shigemoto, R., Mizuno, N. and Nakanishi, S. (1991) Molecular cloning and characterization of the rat NMDA receptor. Nature, 354, 31-37.

Motta, P. M., Makabe, S., Naguro, T. and Correr, S. (1994) Oocyte follicle cells association during development of human ovarian follicle. A study by high resolution scanning and transmission electron microscopy. Arch. Histol. Cytol., 57, 369-394.

Nelson, N. (1998) The family of Na+/Cl- Neurotransmitter Transporters. J. Neurochem., 71, 1785-1803.

Nishiyama, T., Ohsumi, K. and Kishimoto, T. (2007) Phosphorylation of Erp1 by p90rsk is required for cytostatic factor arrest in Xenopus laevis eggs. Nature, 446, 1096-1099.

Norris, R. P., Freudzon, M., Mehlmann, L. M., Cowan, A. E., Simon, A. M., Paul, D. L., Lampe, P. D. and Jaffe, L. A. (2008) Luteinizing hormone causes MAP kinase-dependent phosphorylation and closure of connexin 43 gap junctions in mouse ovarian follicles: one of two paths to meiotic resumption. Development, 135, 3229-3238.

O'Brien, M. J., Pendola, J. K. and Eppig, J. J. (2003) A revised protocol for in vitro development of mouse oocytes from primordial follicles dramatically improves their developmental competence. Biol. Reprod., 68, 1682-1686.

Ochsner, S. A., Day, A. J., Rugg, M. S., Breyer, R. M., Gomer, R. H. and Richards, J. S. (2003) Disrupted function of tumor necrosis factor-alpha-stimulated gene 6 blocks cumulus cell-oocyte complex expansion. Endocrinology, 144, 4376-4384.

Okada, A., Yanagimachi, R. and Yanagimachi, H. (1986) Development of a cortical granule-free area of cortex and the perivitelline space in the hamster oocyte during maturation and following ovulation. J. Submicrosc. Cytol., 18, 233-247.

Okada, Y. (1997) Volume expansion-sensing outward-rectifier Cl^- channel: Fresh start to the molecular identity and volume sensor. Am. J. Physiol., 273, C755-C789.

Okada, Y., Maeno, E., Shimizu, T., Dezaki, K., Wang, J. and Morishima, S. (2001) Receptor-mediated control of regulatory volume decrease (RVD) and apoptotic volume decrease (AVD). J. Physiol. (Lond.), 532, 3-16.

Olivares, L., Aragon, C., Gimenez, C. and Zafra, F. (1995) The role of N-glycosylation in the targeting and activity of the GLYT1 glycine transporter. J. Biol. Chem., 270, 9437-9442.

Otsuka, F., Moore, R. K. and Shimasaki, S. (2001) Biological function and cellular mechanism of bone morphogenetic protein-6 in the ovary. J. Biol. Chem., 276, 32889-32895.

Ozil, J. P., Banrezes, B., Toth, S., Pan, H. and Schultz, R. M. (2006) Ca^{2+} oscillatory pattern in fertilized mouse eggs affects gene expression and development to term. Dev. Biol., 300, 534-544.

Park, J. Y., Su, Y. Q., Ariga, M., Law, E., Jin, S. L. and Conti, M. (2004) EGF-like growth factors as mediators of LH action in the ovulatory follicle. Science, 303,

682-684.

Pastor-Anglada, M., Felipe, A., Casado, F. J., Ferrer-Martinez, A. and Gomez-Angelats, M. (1996) Long-term osmotic regulation of amino acid transport systems in mammalian cells. Amino Acids, 11, 135-151.

Pavlok, A., Lucas-Hahn, A. and Niemann, H. (1992) Fertilization and developmental competence of bovine oocytes derived from different categories of antral follicles. Mol. Reprod. Dev., 31, 63-67.

Peng, X. R., Hsueh, A. J., LaPlot, P. S., Bjersing, L. and Ny, T. (1991) Localization of luteinizing hormone receptor messenger ribonucleic acid expression in ovarian cell types during follicle development and ovulation. Endocrinology, 129, 3200-3207.

Perreault, S. D. (1992) Chromatin remodeling in mammalian zygotes. Mutat. Res., 296, 43-55.

Peters, W. S., Wolfgang, H. and Tomos, A. D. (2000) What makes plants different? Principles of extracellular matrix function in "soft" plant tissues. Comparat. Biochem. Physiol. Part A, 125, 151-167.

Petrunewich, M. A., Trimarchi, J. R., Hanlan, A. K., Hammer, M. A. and Baltz, J. M. (2008) Second Meiotic Spindle Integrity Requires MEK/MAP Kinase Activity in Mouse Eggs. J. Reprod. Dev.

Phillips, D. M. and Shalgi, R. (1980) Surface architecture of the mouse and hamster zona pellucida and oocyte. J. Ultrastruct. Res., 72, 1-12.

Phillips, K. P., Petrunewich, M. A., Collins, J. L. and Baltz, J. M. (2002) The intracellular pH-regulatory HCO3-/Cl- exchanger in the mouse oocyte is inactivated during first meiotic metaphase and reactivated after egg activation via the MAP kinase pathway. Mol. Biol. Cell., 13, 3800-3810.

Pincus, G. and Enzmann, E. V. (1935) The comparative behavior of mammalian eggs in vivo and in vitro. J. Exp. Med., 62, 665-675.

Polanski, Z., Ledan, E., Brunet, S., Louvet, S., Verlhac, M. H., Kubiak, J. Z. and Maro, B. (1998) Cyclin synthesis controls the progression of meiotic maturation in mouse oocytes. Development, 125, 4989-4997.

Qi, H., Williams, Z. and Wassarman, P. M. (2002) Secretion and assembly of zona pellucida glycoproteins by growing mouse oocytes microinjected with epitope-tagged cDNA for mZP2 and mZP3. Mol. Biol. Cell, 13, 530-541.

Quinn, P., Kerin, J. F. and Warnes, G. M. (1985) Improved pregnancy rate in human in

vitro fertilization with the use of a medium based on the composition of human tubal fluid. Fertil. Steril., 44, 493-498.

Richards, J. S. (1980) Maturation of ovarian follicles: Actions and interactions of pituitary and ovarian hormones on follicular cell differentiation. Physiol. Rev., 60, 51-89.

Richards, J. S. (2005) Ovulation: new factors that prepare the oocyte for fertilization. Mol. Cell. Endocrinol., 234, 75-79.

Richards, J. S., Russell, D. L., Ochsner, S. and Espey, L. L. (2002) Ovulation: New dimensions and new regulators of the inflamatory-like response. Annu. Rev. Physiol., 64, 69-92.

Rothman, J. E. (2002) The machinery and principle of vesicle transport in the cell. Nat. Med., 8, 1059-1062.

Rycke, M. D., Liebaers, I. and Van Steirteghem, A. (2002) Epigenetic risks related to assisted reproductive technologies. Hum. Reprod., 17, 2487-2494.

Ryu, O. H., Choi, S. J., Firatli, E., Choi, S. W., Hart, S., Shen, R. F., Wang, G. X., Wu, W. W. and Hart, T. C. (2005) Proteolysis of MIP-1alfa isoforms LD78beta and LD78alfa by neutrophil-derived serine protease. J. Biol. Chem., 280, 17415-17421.

Sadler, S. E. and Jacobs, N. D. (2004) Stimulation of Xenopus laevis oocyte maturation by Methyl-beta-Cyclodextrin. Biol. Reprod., 70, 1685-1692.

Sadler, T. W. (1990) Gametogenesis In Langman's Medical Embryology (Ed, Wilkins, W.) Baltimore, MD, pp. 3-14.

Salustri, A., Fulop, C., Camaioni, A. and Hascall, V. C. (2004) Oocyte-granulosa cell interactions. In The ovary(Eds, Leung, P. C. K. and Y., A. E.), pp. 131-143.

Sato, K., Adams, R., Betz, H. and Schloss, P. (1995) Modulation of a recombinant glycine transporter (GLYT1b) by activation of protein kinase C. J. Neurochem., 65, 1967-1973.

Schatten, H., Simerly, C., Maul, G. and Schatten, G. (1989) Microtubule assembly is required for the formation of pronuclei, nuclear lamin acquisition, and DNA synthesis during mouse, but not sea urchin, fertilization. Gamete Res., 23, 309-322.

Schini, S. A. and Bavister, B. D. (1988) Two-cell block to development of cultured hamster embryos is caused by phosphate and glucose. Biol. Reprod., 39, 1183-1192.

Schultz, G. A., Kaye, P. L., McKay, D. J. and Johnson, M. H. (1981) Endogenous amino acid pool sizes in mouse eggs and preimplantation embryos. J. Rprod. Fertil., 61, 387-393.

Schultz, R. M. (1993) Regulation of zygotic gene activation in the mouse. BioEssays, 15, 531-538.

Sela-Abramovich, S., Chorev, E., Galiani, D. and Dekel, N. (2005) Mitogen-activated Protein Kinase mediates Luteinizing hormone-induced breakdown of communication and oocyte maturation in rat ovarian follicles. Endocrinology, 143, 1236-1244.

Sela-Abramovich, S., Edry, I., Galiani, D., Nevo, N. and Dekel, N. (2006) Disruption of gap junctional communication within the ovarian follicle induces oocyte maturation. Endocrinology, 147, 2280-2286.

Sela-Abramovich, S., Galiani, D., Nevo, N., Dekel, N. (2008) Inhibition of rat oocyte maturation and ovulation by nitric oxide: mechanism of action. Biol. Reprod., 78, 1111-1118.

Senbon, S., Hirao, Y. and Miyano, T. (2003) Interactions between the oocyte and surrounding somatic cells in follicular development: Lessons from in vitro culture. J. Reprod. Dev. , 49, 259-269.

Sheng, Y., Wang, L., Liu, X. S., Montplaisir, V., Tiberi, M., Baltz, J. M. and Liu, X. J. (2005) A serotonin receptor antagonist induces oocyte maturation in both frogs and mice: evidence that the same G protein-coupled receptor is responsible for maintaining meiosis arrest in both species. J. Cell. Physiol., 202, 777-786.

Simon, A. M., Goodenough, D. A., Li, E. and Paul, D. L. (1997) Female infertility in mice lacking connexin 37. Nature, 385, 525-529.

Simons, K. and Ehehalt, R. (2002) Cholesterol. Lipid rafts and disease. J. Clin. Invest, 110, 597-603.

Skinner, M. K. (2005) Regulation of primordial follicle assembly and development. Human Reprod. Update, 11, 461-471.

Smith, K. E., Borden, L. A., Harting, P. R., Branchek, T. and Weinshank, R. L. (1992) Cloning and expression of a glycine transporter reveal colocalization with NMDA receptors. Neuron, 8, 927- 935.

Steeves, C. L. and Baltz, J. M. (2005) Regulation of intracellular glycine as an organic osmolyte in early preimplantation mouse embryos. J. Cell. Physiol., 204, 273-279.

Steeves, C. L., Hammer, M.-A., Walker, G. B., Rae, D., Stewart, N. A. and Baltz, J. M.

(2003) The glycine neurotransmitter transporter GLYT1 is an organic osmolyte transporter regulating cell volume in cleavage-stage embryos. Proc. Nat. Acad. Sci. U.S.A., 100, 13982-13987.

Sternlicht, A. L. and Schultz, R. M. (1981) Biochemical studies of mammalian oogenesis: kinetics of accumulation of total and poly(A)-containing RNA during growth of the mouse oocyte. J. Exp. Zool., 215, 191-200.

Strange, K. (2004) Cellular volume homeostasis. Adv. Physiol. Educ., 28, 155-159.

Strange, K., Emma, F. and Jackson, P. S. (1996) Cellular and molecular physiology of volume-sensitive anion channels. Am. J. Physiol., 270, C711-C730.

Strayer, D. S., Hoek, J. B., Thomas, A. P. and White, M. K. (1999) Cellular activation by Ca^{2+} release from stores in the endoplasmic reticulum but not by increased free Ca^{2+} in the cytosol. Biochem. J., 15, 39-46.

Street, T. O., Bolen, D. W. and Rose, G. D. (2006) A molecular mechanism for osmolyte-induced protein stability. Proc. Natl. Acad. Sci. U.S.A., 103, 13997-14002.

Su, Y. Q., Wigglesworth, K., Pendola, F. L., O'Brien, M. J. and Eppig, J. J. (2002) Mitogen-activated protein kinase (MAPK) activity in cumulus cells is essential for gonadotropin-induced oocyte meiotic resumption and cumulus expansion in the mouse. Endocrinology, 143, 2221-2232.

Summers, M. C. and Biggers, J. D. (2003) Chemically defined media and the culture of mammalian preimplantation embryos: historical perspective and current issues. Hum Reprod Update, 9, 557-582.

Sun, Q. Y. and Fan, H. Y. (2004) Activity of MAPK/p90rsk during fertilization in mice, rats, and pigs. Methods Mol. Biol., 253, 293-304.

Suzuki, O., Toshihiko, A., Yamamoto, Y., Takano, K. and Kouira, M. (1996) Development in vitro of preimplantation embryos from 55 mouse strains. Reprod. Fertil. Dev., 8, 975-980.

Swann, K., Saunders, C. M., Rogers, N. T. and Lai, F. A. (2006) PLCζ(zeta): A sperm protein that triggers Ca2+ oscillations and egg activation in mammals. Semin. Cell Dev. Biol., 17, 264-273.

Takenaka, M., Preston, A. S., Kwon, H. M. and Handler, J. S. (1994) The Tonicity-sensitive Element that mediates increased transcription of the Betaine transporter gene in response to hypertonic stress. J. Biol. Chem., 269, 29379-29381.

Talbot, P. and Dandekar, P. (2003) Perivitelline space: Does it play a role in blocking polyspermy in mammals? Microsc. Res. Tech., 61, 349-357.

Tamakoi, T. (1991) Use and specificity of staurosporine, UCN-01, and calphostin C as protein kinase inhibitors. Methods Enzymol., 201, 340-347.

Thomas, F. H., Ethier, J. F., Shimasaki, S. and Vanderhyden, B. C. (2005) Follicle-stimulating hormone regulates oocyte growth by modulation of expression of oocyte and granulosa cell factors. Endocrinology, 146, 941-949.

Togo, T. and Morisawa, M. (1997) Aminopeptidase-like protease released from oocytes affects oocyte surfaces and suppresses the acrosome reaction in establishment of polyspermy block in oocytes of the Mussel Mytilus edulis. Dev. Biol., 182, 219-227.

Tornell, J., Billig, H. and Hillensjo, T. (1991) Regulation of oocyte maturation by changes in ovarian levels of cyclic nucleotides. Hum. Reprod., 6, 411-422.

Toth, S., Huneau, D., Banrezes, B. and Ozil, J. P. (2006) Egg activation is the result of calcium signal summation in the mouse. Reproduction, 131, 27-34.

Trama, J., Lu, Q., Hawley, R. G. and Ho, S. N. (2000) The NFAT-related NFATL1 (TonEBP/NFAT5) is induced upon T cell activation in a calcineurin-dependent manner. J. Immunol., 165, 4884-4894.

Tsafriri, A., Chun, S. Y., Zhang, R., Hsueh, A. J. W. and Conti, M. (1996) Oocyte maturation involves compartmentalization and opposing changes of cAMP levels in follicular somatic and germ cells: Studies using selective phosphodiesterase inhibitors. Dev. Biol., 178, 393-402.

Tsutsumi, O., Yano, T., Satoh, K., Mizuno, M. and Kato, T. (1990) Studies of hexokinase activity in human and mouse oocyte. Am. J. Obstet. Gynecol., 162, 1301-1304.

Van Winkle, L. J. (2001) Amino acid transport regulation and early embryo development. Biol. Reprod., 64, 1-12.

Van Winkle, L. J., and Campione, A.L. (1996) Amino acid transport regulation in preimplantation mouse embryos: Effects on amino acid content and pre- and peri-implantation development. Theriogeneology, 45, 69-80.

Van Winkle, L. J. and Dickinson, H. R. (1995) Differences in aminoacid content of preimplantation mouse embryos that develop in vitro versus in vivo: in vitro effect of five amino acids that are abundant in oviductal secretions. Biol. Reprod., 52, 96-104.

Van Winkle, L. J., Haghighat, N. and Campione, A. L. (1990) Glycine protects preimplantation mouse conceptuses from a detrimental effect on development of the inorganic ions in oviductal fluid. J. Exp. Zool., 253, 215-219.

Van Winkle, L. J., Haghighat, N., Campione, A. L. and Gorman, J. M. (1988) Glycine transport in mouse eggs and preimplantation conceptuses. Biochem. Biophys. Acta, 941, 241-256.

Van Winkle, L. J., Mann, D. F., Wasserlauf, H. G. and Patel, M. (1992) Mediated Na(+)-independent transport of L-glutamate and L-cystine in 1- and 2-cell mouse conceptuses. Biochim. Biophys. Acta, 1107, 299-304.

Van Winkle, L. J., Patel, M., Wasserlauf, H. G., Dickinson, H. R. and Campione, A. L. (1994) Osmotic regulation of taurine transport system via system β and novel processes in mouse preimplantation conceptuses. Biochem. Biophys. Acta, 1191, 244-255.

Vanderhyden, B. C. and Macdonald, E. A. (1998) Mouse oocytes regulate granulosa cell steroidogenesis throughout follicular development. Biol. Reprod., 59, 1296-1301.

Vidaver, G. A. (1964) Transport of Glycine by Pigeon Red Cells. Biochemistry, 3, 662-667.

Wang, L., Wang, Z. B., Zhang, X., FitzHarris, G., Baltz, J. M., Sun, Q. Y. and Liu, X. J. (2008) Brefeldin A disrupts asymmetric spindle positioning in mouse oocytes. Dev. Biol., 313, 155-166.

Wassarman, P. M. (1988) Zona pellucida glycoproteins. Ann. Rev. Biochem., 57, 415-422.

Wassarman, P. M. (2005) Contribution of mouse egg zona pellucida glycoproteins to gamete recognition during fertilization. J. Cell. Physiol., 204, 388-391.

Wassarman, P. M. (2008) Zona pellucida glycoproteins. J. Biol. Chem., 283, 24285-24289.

Wassarman, P. M., Jovine, L. and Litscher, E. S. (2004) Mouse zona pellucida genes and glycoproteins. Cytogenet. Genome. Res., 105, 228-234.

Wassarman, P. M., Schultz, R. M., Letourneau, G. E., LaMarca, M. J., Josefowicz, W. J. and Bleil, J. D. (1979) Meiotic maturation of the mouse oocytes *in vitro*. Adv. Exp. Med. Biol., 112, 251-268.

Webb, R. J., Marshall, F., Swann, K. and Carroll, J. (2002) Follicle-stimulating hormone induces a gap junction-dependent dynamic change in cAMP and protein kinase A in mammalian oocytes. Dev. Biol., 246, 441-454.

Werman, R., Davidoff, R. A. and Aprison, M. H. (1967) Inhibition of motoneurons by ionophoresis of glycine. Nature, 214, 681-683.

Whitten, W. K. (1956) Culture of tubal ova. Nature, 177, 96.

Williams, Z. and Wassarman, P. M. (2001) Secretion of mouse ZP3 the sperm receptor requires cleavage of its polypeptide at a consensus furin cleavage-site. Biochemistry, 40, 929-937.

Woo, S. K., Dahl, S. C., Handler, J. S. and Kwon, H. M. (2000) Bidirectional regulation of tonicity-responsive enhancer binding protein in response to changes in tonicity. Am. J. Physiol. Renal. Physiol. , 278, F1006-F1012.

Woo, S. K., Lee, S. D. and Kwon, H. M. (2002) TonEBP transcriptional activator in the cellular response to increased osmolarity. Eur. J. Physiol., 444, 579-585.

Wood, J. R. and Strauss, J. F. (2002) Multiple signal transduction pathways regulate ovarian steroidogenesis. Rev. Endocr. Metab. Disord., 3, 33-46.

Yamamoto-Mizuma, S., Wang, G. X., Liu, I. L., Schegg, K., Hatton, W. J., Duan, D., Horowitz, T. L., Lamb, F. S. and Hume, J. R. (2004) Altered properties of volume-sensitive osmolyte and anion channels (VSOACs) and membrane protein expression in cardiac and smooth muscle myocyte from Clcn3-/- mice. J. Physiol., 557, 439-456.

Yamashita, A., Singh, S. K., Toshimitsu, K., Jin, Y. and Gouaux, E. (2005) Crystal structure of a bacterial homologue of Na^+/Cl^--dependent neurotransmitter transporters. Nature, 437, 215-223.

Yanagimachi, R. (1994) Mammalian fertilization. In The physiology of reproduction (Eds, Knobil, E. and Neil, J.) Raven Press, New York, pp. 189-317.

Yancey, P., Clark, M., Hand, S., Bowlus, R. and Somero, G. (1982) Living with water stress: evolution of osmolyte systems. Science, 217, 1214-1222.

Yancey, P. H. (1994) Compatible and counteracting solutes. In Cellular and molecular physiology of cell volume regulation(Ed, Strange, K.) CRC Press, Boca Raton, Florida, pp. 81-109.

Zafra, F., Alcantara, R., Gomeza, J., Aragon, C. and Gimenez, C. (1990) Arachidonic acid inhibits glycine transport in cultured glial cells. Biochem. J., 271, 237-242.

Zafra, F., Aragon., C., Olivares, L., Danbolt, N. C., Gimenez, C. and Strom-Mathisen, J. (1995) Glycine Transporters are differentially expressed among CNS cells. J. Neurosci., 15, 3952-3969.

Zhao, M., Gold, L., Ginsberg, A. M., Liang, L. F. and Dean, J. (2002) Conserved furin cleavage site is not essential for secretion and integration of ZP3 into extracellular egg coat of transgenic mice. Mol. Cell. Biol., 22, 3111-3120.