Pannexin 1 Expression in Bovine Cumulus-Oocyte Complexes and Function During *in vitro* Maturation

by

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Abstract

In this study we investigate the presence of pannexin 1 (PANX1) protein channels in bovine cumulus-oocyte complexes (COCs). Pannexin channels are a recently discovered family of proteins which have been characterized in other tissues and systems and found to be largely ubiquitously expressed. They have been shown to play a role in adenosine triphosphate (ATP) release, the removal of apoptotic cells, neurological functions, and inflammation. However, little is known about their potential roles in reproductive tissues.

Here we investigate the expression of PANX1 in bovine cumulus-granulosa cells. We compare the expression in COCs from early versus late antral follicles and between oocytes with higher developmental competence to those with lower developmental competence.

Furthermore, we characterize the potential role that PANX1 has during oocyte maturation and embryo development, following *in vitro* fertilization (IVF). We investigate oocyte maturation and developmental outcomes *in vitro* with PANX1 channel inhibition during *in vitro* maturation (IVM). We also look at possible functional roles that PANX1 inhibition may have on the maturing oocyte.

Elucidating the role of PANX1 during oocyte maturation stands to further our understanding of the channel. With that knowledge, the foundation for

manipulating the *in vitro* environment to improve bovine embryo production would be improved.

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List of Abbreviations

2N	Diploid
ART	Assisted Reproductive Technologies
ATP	Adenosine Triphosphate
BCB	Brilliant Cresyl Blue
BMP15	Bone Morphogenic Protein 15
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine Monophosphate
CDC25B	Cell Division Cycle 25B
CDK	Cyclin-Dependent Kinase
cGMP	Cyclic Guanosine Monophosphate
CNP	C-type Natriuretic Peptide
COC	Cumulus-Oocyte Complex
CTCF	Corrected Total Cell Fluorescence
CYB	Cyclin B
DCHF-DA	2'-7'-Dichlorodihydrofluorescein Diacetate
DMEM/F-12	Dulbecco Modified Eagle Medium: Nutrient Mixture F-12
DNA	Deoxyribonucleic Acid
ECL	Enhanced Chemiluminescence
EFAF	Essentially Fatty Acid Free

ELISA	Enzyme-Linked Immunosorbent Assay
FSH	Follicle Stimulation Hormone
G6pDH	Glucose-6-Phosphate Dehydrogenase
GPR12	G_sG Protein Receptor 12
GPR3	G_sG Protein Receptor 3
GSH	Glutathione
GV	Germinal Vesicle
GVBD	Germinal Vesicle Breakdown
HIV	Human Immunodeficiency Virus
IBMX	3-Isobutyl-1-Methylxanthine
icAMP	Intracellular Concentration of Cyclic Adenosine Monophosphate
IVD	*In vitro* Development
IVF	*In vitro* Fertilization
IVM	*In vitro* Maturation
IVP	*In vitro* Production
LH	Luteinizing Hormone
MI	Metaphase I
MII	Metaphase II
MPF	Maturation Promoting Factor
mRNA	Messenger Ribonucleic Acid
Myt1	Myelin Transcription Factor 1

N Haploid

NPPC Natriuretic Peptide Precursor C

NPR2 Natriuretic Peptide Receptor 2

ODPF Oocyte-Derived Paracrine Factors

PANX1 Pannexin 1

PANX2 Pannexin 2

PANX3 Pannexin 3

PBS Phosphate Buffered Saline

PDE Phosphodiesterase

PFA Paraformaldehyde

PGCs Primordial Germ Cells

PI Propidium Iodide

PKA Protein Kinase A

PVDF Polyvinylidene Difluoride

qRT-PCR Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction

RIPA Radioimmunoprecipitation Assay

RNA Ribonucleic Acid

ROS Reactive Oxygen Species

SD Standard Deviation

SDS Sodium Dodecyl Sulfate Polyacrylamide

SOF	Synthetic Oviductal Fluid
SPOM	Simulated Physiological Oocyte Maturation
TBS	Tris-Buffered Saline

I. Literature Review

Folliculogenesis

Ovarian folliculogenesis involves complex regulatory mechanisms that incorporate both endocrine and paracrine signaling pathways as well as direct intercellular communication through gap junction channels (Aerts and Bols 2010a;b). Ovarian follicles provide the support required for oocyte growth and maturation and are comprised of germ cells and several types of somatic cells, including granulosa and theca cells. Within the developing follicle, the cumulus-granulosa cells and the oocyte form a functional syncytium called a cumulus-oocyte-complex (COC). Signaling within the COC acts to govern the developmental pathways of the two cell types, coordinating growth of the follicle and maturation of the oocyte.

The formation of primordial follicles begins with the migration of primordial germ cells (PGCs). PGCs initially migrate to gonadal ridges during gestation for the bovine species. During this time, the PGCs multiply by mitotic division up to several thousand cells (Aerts and Bols 2010a). Once the primordial germ cells arrive at the gonads, they are embedded in the proliferating tissue (Lin et al. 2002). Once in the gonad, PGCs continue to multiply, but the population of PGCs drastically decreases as a result of apoptosis with the maximum number being reached during the time of PGC transition from mitosis to meiosis (Gondos

1978). In the cow, the maximum number of PGCs is estimated at 2.1 million and is reduced to an average of 130,000 at birth (Erickson 1966a,b). Clusters of oogonia (PGCs that are mitotically active and in the diploid (2N) state) that are surrounded by somatic cells form in the gonads (Aerts and Bols 2010a). Mitoses of these oogonia occur in syncytium until their deoxyribonucleic acid (DNA) is replicated and the first meiotic division occurs. After this initial meiotic replication, the cells are arrested in the diplotene stage of prophase I, and the oogonia is now considered a primary oocyte (Aerts and Bols 2010a).

At this stage, cytoplasmic processes are sent out from epithelial cells surrounding the oogonia cluster. These processes divide the clusters into individual oocytes that are surrounded by a single layer of flattened (pre)granulosa cells. At the same time, a basal lamina is placed on the surface of the granulosa cells which now isolate the newly formed primordial follicles from the surrounding ovarian stroma (Aerts and Bols 2010a). The primordial follicles are then detached from each other by cytoplasmic processes from the stroma cells between neighboring basal lamina, which happens around day 90 of gestation in cattle (Merchant-Larios and Chimal-Monroy 1989; Yang and Fortune 2008). In cattle, primordial follicles are defined as a quiescent oocyte, arrested in prophase I of meiosis, which is surrounded by a limited number of flattened granulosa cells (Braw-Tal and Yossefi 1997), and they are the ovarian reserve from which all follicles are recruited for the rest of the cow’s reproductive lifespan.

The primary oocytes contained in these follicles will remain meiotically arrested until the female reaches puberty and begins cycling. Primordial follicles are continuously recruited for growth into the follicular pool by a signal that is not completely understood (McGee and Hsueh 2000; Oktem and Oktay 2008). Once this growing stage begins, primordial follicles will go through a series of changes. In the bovine species, follicular growth can be broken in two phases. The first phase is the appearance of cuboidal granulosa and the second stage is continued proliferation of these granulosa cells and volume expansion of the oocyte (Braw-Tawl 2002). After stage one, the follicle is considered a primary follicle, and after phase two it is considered a small pre-antral follicle (Braw-Tal and Yossefi 1997). The next phase of growth is the large pre-antral follicle which is defined by a fully closed zona pellucida and a distinguished theca interna layer (Braw-Tal and Yossefi 1997). Finally, the antral follicle is formed when fluid filled patches within the granulosa cells coalesce into a single cavity (Smitz and Cortvrindt 2002)(Figure 1). Bovine estrous cycles exhibit an average of two to three growth waves, with the last wave resulting in ovulation. During each wave follicle stimulating hormone (FSH) levels increase and a select few (~5-10) antral follicles will escape apoptosis and continue growth but will ultimately regress due to atresia (Driancourt 2001; Aerts and Bols 2010b). However, in the final wave, the largest developing antral follicle will grow and result in a dominate follicle that will ovulate (Aerts and Bols 2010b). The dominant follicle will continue to grow

even though it is the primary suppressor of FSH secretion by way of a negative feedback loop through estrogen and inhibin production (Aerts and Bols 2010b). Low FSH levels prevent a new cohort of growing follicles from being recruited. In concurrence with this drop in FSH, luteinizing hormone (LH) receptors begin to appear on the dominant follicle, suggesting a transition from FSH to LH dependency (Aerts and Bols 2010b). LH pulses begin the production of androgens by thecal cells within the ovary. Basal FSH levels then stimulate the conversion of these androgens into estrogen. As the dominant follicle grows to a pre-ovulatory size, it produces increasing amounts of estrogen (Nuttinck and Peynot 2000). Estrogen will reach a threshold that results in the ovulatory LH surge for the anterior pituitary.

Meiotic Maturation in Mammalian Oocytes

Normal female fertility relies on proper oocyte development. Oocyte development culminates in meiotic maturation which initiates just prior to ovulation. Meiotic maturation describes the transition from prophase I to metaphase II (MII) involving condensation of the chromatin and the acquisition of developmental competence in oocytes (Figure 1). At the time of birth oocytes are arrested at the diplotene stage of the first meiotic prophase (Tripathi et al. 2010). Upon entering the early antral stage of follicular development, the oocytes within these follicles become meiotically competent and if removed from the follicular environment, will

spontaneously resume meiosis (Mehlmann 2005). However, *in vivo*, LH stimulates meiotic resumption indirectly (Jaffe and Egbert 2017; Tripathi et al. 2010). The process of LH stimulation is not well understood. It is known that LH binds to its receptors on the mural granulosa cells that surround the COC (Zhang et al. 2009). This action initiates signaling pathways that involve cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) as second messengers which induces the first meiotic division (Zhang et al. 2009). As the first meiotic division begins, the nuclear envelope or germinal vesicle (GV) holding the nuclear material breaks down into a morphological stage termed germinal vesicle breakdown (GVBD)(Tripathi et al. 2010)(Figure 1). At this stage, cumulus cells begin to expand, and continue to overtime as the oocyte matures (Larsen et al. 1986). This expansion is a response to the changing gonadotrophin profiles during maturation. Meanwhile, the cumulus cells start to produce hyaluronic acid which is deposited into the intercellular spaces and promotes expansion (Tanghe et al. 2002; Sánchez and Smitz 2012). Oocytes are now arrested in metaphase I (MI) until spindle assembly proteins check that all condensed sister chromatids have been properly attached to the bipolar spindle and are aligned at the metaphase plate (Niault et al. 2007; Wassmann et al. 2003)(Figure 1). The first meiotic division occurs, and then the oocyte is rearrested in the MII stage. The arrest at the MII stage is maintained by spindle stability and maturation promoting factor (MPF) stabilization (Brunet and Maro

2005; Tripathi et al. 2010). This stage of oocyte maturation can be visually determined by the presence of the first polar body (Figure 1). This is formed by the unequal division occurring during meiosis forming a structure that is composed of one set of chromosomes and discarded into the perivitelline space of the oocyte (Jaffe and Egbert 2017; Tripathi et al. 2010). At this stage, the oocyte is ovulated and upon fertilization and completion of meiosis II, separation of the chromatids occurs causing the formation of a second polar body. This leaves a haploid (N) genome in the egg which can now combine with the sperm genome to create a new individual (Jaffe and Egbert 2017).

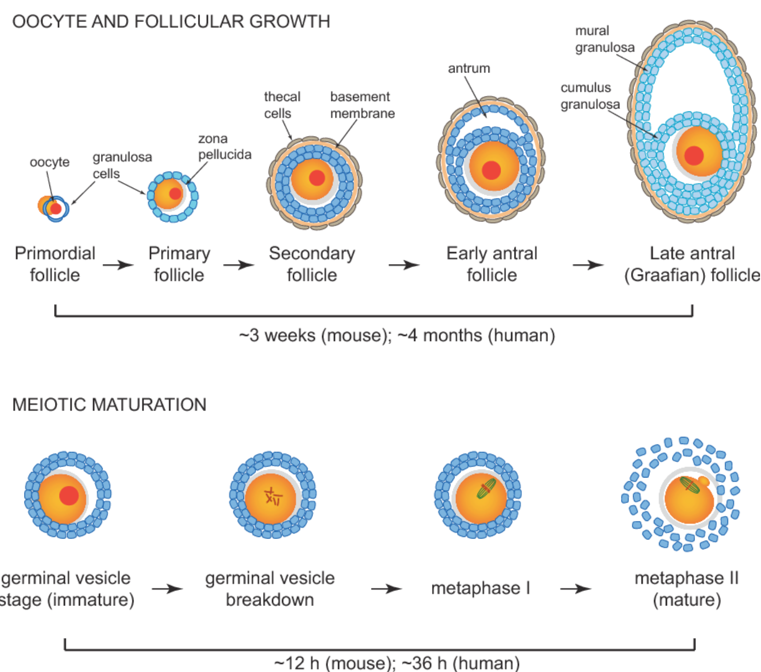

Figure 1: Oocyte and follicular growth and meiotic maturation. Figure from Clarke 2017 and used with publisher's permission.

Cellular Communication, cAMP, and Maturation

Premature oocytes rely on many internal and external signals to progress through maturation in the appropriate timeframe in order to achieve developmental competence. During the primary oocyte stage, meiosis remains paused due to meiosis-inhibitory signals (Figure 2). Meiosis-inhibitory signals preventing maturation are facilitated by gap junctional communication between the granulosa cells and oocyte, as well as, through the follicular fluid (Jaffe and Egbert 2017). If gap junctional communication is blocked or inhibited, meiosis will resume prematurely (Piontkewitz and Dekel 1993; Richard and Baltz 2014; Sela-Abramovich et al. 2006). A similar phenotype is seen when connexin 37 or connexin 43 (two major gap junctional channels in oocyte, cumulus cell, and mural granulosa cell communication) are blocked (Norris et al. 2008; Richard and Baltz 2014). Furthermore, COCs that are incubated in follicular fluid have partially inhibited meiotic resumption after being removed from the follicle, indicating the presence of an extracellular inhibitor (Tsafriri et al. 1976). After the oocyte gains cytoplasmic competence the LH surge occurs causing cumulus expansion to begin and cell to cell communication to breakdown (Eppig 1982; Lorenzo et al. 1994; Sánchez and Smitz 2012; Zhang et al. 1995). Approximately 9 hrs after the induction of meiotic maturation, in mice, communication between the oocyte and its surrounding cumulus cells is greatly reduced, with complete disconnection occurring by full meiotic maturation (Eppig 1982; Larsen et al. 1987; Suzuki et al.

1994). These findings suggest that cell to cell communication and extracellular communication are extremely important to maintain a proper meiotic maturation timeline.

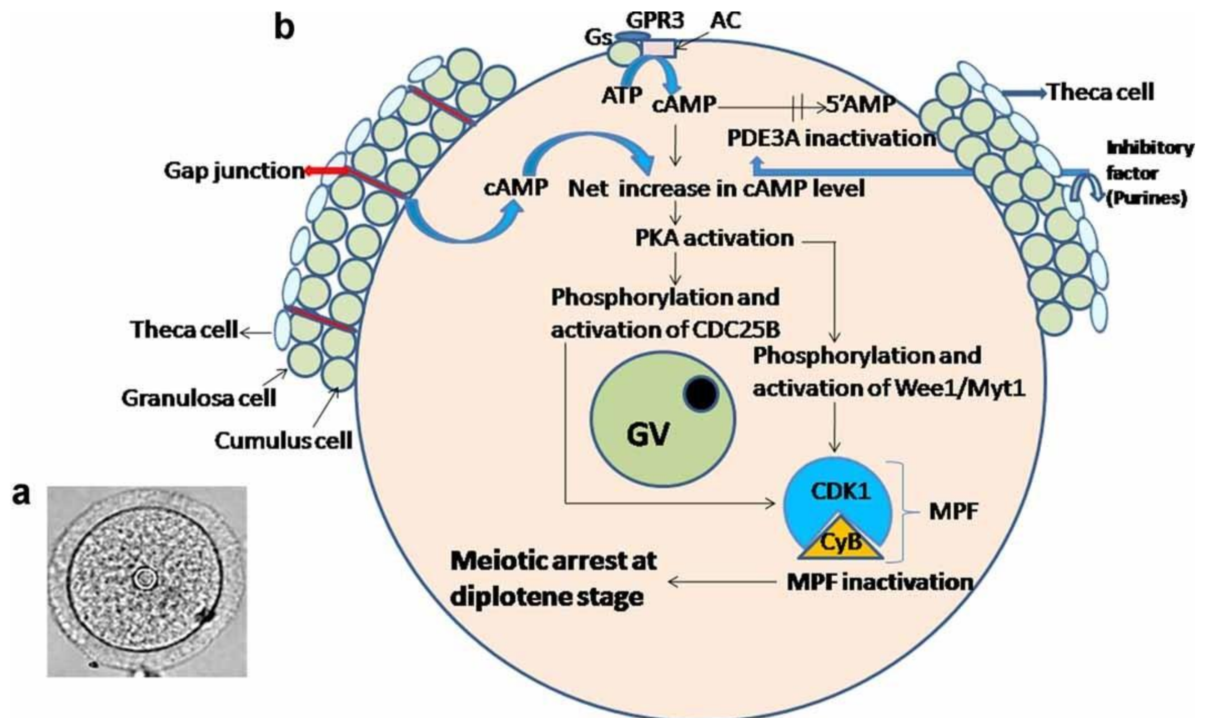

Figure 2: The network of signals that promote high cAMP concentration within the oocyte to maintain meiotic arrest at the GV stage before the LH surge. a) A diplotene-arrested rat oocyte showing the presence of germinal vesicle and nucleolus (magnification 400x). b) A proposed model for the maintenance of meiotic arrest at the GV stage. Figure from Tripathi et al. 2010 and used with publisher's permission.

Meiotic resumption is controlled by many signals. At the center of this signaling pathway is cAMP, which inhibits the resumption of meiosis until the LH surge

(Jaffe and Egbert 2017)(Figure 2). Cyclic AMP is produced in the oocyte from the conversion of ATP to cAMP by adenylyl cyclase which is stimulated by G_s G protein receptors 3 and 12 (GPR3/12) (Jaffe and Egbert 2017; Liu et al. 2013). Cyclic AMP within the oocyte affects the activity of the cyclin-dependent kinase (CDK)/cyclin B (CYB) protein complex (also known as maturation promoting factor (MPF)). High cAMP levels within the oocyte result in phosphorylation of CDK1 rendering it inactive (Duckworth et al. 2002; Mehlmann 2005). The CDK1/CYB complex activates the nuclear envelope breakdown and chromosome condensation of the first meiotic division (Abe et al. 2011; Adhikari et al. 2012; Lénárt et al. 2003). Cyclic AMP acts through protein kinase A (PKA) to maintain meiotic arrest as PKA maintains CDK1 in an inactive form by phosphorylation (Bornslaeger et al 1986; Holt et al. 2013). PKA phosphorylates the phosphatase cell division cycle 25B (CDC25B) which can dephosphorylate CDK1, rendering it active. PKA also promotes the kinases Wee1 and myelin transcription factor 1 (Myt1) which phosphorylate CDK1 (Jaffe and Egbert 2017; Liu et al. 2013). CDC25B is sequestered in the cytoplasm, and prevented from entering the nucleus (Duckworth et al. 2002; Pirino et al. 2009). There is also an influx of cGMP from cumulus cells to the oocyte, through gap junctional channels, which prevents the hydrolysis of cAMP by the phosphodiesterase (PDE) 3A. (Norris et al. 2009; Liu et al. 2013). The propagation of cGMP depends upon natriuretic peptide precursor C (NPPC) and its receptor natriuretic peptide receptor 2

(NPR2). This receptor complex is produced by the mural granulosa cells and stimulates the generation of cGMP (Liu et al. 2013; Zhang et al. 2010). It has been found that oocyte-derived paracrine factors (ODPF) and oestradiol promote NPR2 expression in cumulus cells (Kedem et al. 2011; Liu et al. 2013; Zhang et al. 2010; Zhang and Xia 2012;), thus augmenting the ability of NPPC to produce cGMP for meiotic arrest management (Zhang et al. 2011). The loss of gap junctional coupling between the oocyte and cumulus cells, during maturation, abates the influx of cGMP.

High cAMP levels maintain signaling that keeps CDK1 inactive through a PKA dependent phosphorylation pathway that prevents nuclear envelope/germinal vesicle breakdown and chromosome condensation (Jaffe and Egbert 2017)(Figure 2). Cyclic AMP also inhibits some cytoplasmic changes within the oocyte that promote meiotic progression. These include the transformation of an interphase microtubule network into microtubule asters (Schuh and Ellenberg 2007) as well as the development of endoplasmic reticulum calcium release mechanisms (Mehlmann and Kline 1994). Contributions by the surrounding cumulus cells also maintains high cAMP levels by inhibiting PDE3A by way of cGMP influx (Norris et al. 2009; Sánchez and Smitz 2012).

Maturation Challenges in vitro

Production of embryos *in vitro* has the ability to alleviate many common problems in fertility across different species. However, IVP embryos have developmental challenges that *in vivo* embryos do not (Leal et al. 2018). All developmental molecular and biochemical functions must be controlled until maturation is reached if a viable oocyte is to be produced for fertilization and development (Wrenzycki and Stinshoff 2013). The IVM stage of IVP is stull subpar, in that the common protocols do not have the capacity to efficiently produce many developmentally competent oocytes with the ability to support the development of a viable embryo. This is in part due to the fact that the process of oocyte maturation and its mechanisms are not completely understood (Leal et al. 2018). Immature oocytes that are removed from antral follicles for IVM do have the ability to resume meiosis (Edwards 1965), however, cytoplasmic maturation (required for complete oocyte capacitation) occurs asynchronously with meiotic maturation due to the spontaneous nuclear maturation that occurs after the removal from the follicle (Leal et al. 2018). This has been speculated to be the major mechanistic flaw behind the lower embryo production rates of IVM oocytes, thus the IVM stage of IVP has received attention from researches to improve production rates (Demyda-Peyrás et al. 2013; Leal et al. 2018; Parrish et al. 2014).

Delaying Maturation in vitro

As antral follicles progress toward a preovulatory size, the oocyte continues to grow until it reaches a quiescent state (Gilchrist and Thompson 2007). This latter phase of follicular development may be termed “oocyte capacitation” and describes when ultrastructural modification and full developmental competence are obtained (Hyttel et al. 1997; Sirard et al. 2006). Removal of oocytes from antral follicles for IVM interrupts oocyte capacitation and spontaneous or non-induced oocyte maturation occurs (Gilchrist and Thompson 2007). This spontaneous induction of meiotic maturation without normal *in vivo* signaling, can inhibit an *in vitro* derived oocyte from acquiring full developmental competence and result in the premature breakdown of oocyte-cumulus gap junctional communication (Thomas et al. 2004a). To attenuate these detrimental effects, scientists have been altering standard IVM techniques to delay or temporarily prevent spontaneous nuclear maturation and improve oocyte developmental competence promoting proper development of the ooplasm (Gilchrist and Thompson 2007). In these studies the IVM technique is usually modified with a meiotic inhibiting agent which is then removed and maturation continues until IVF is implemented. The goal of this approach is to prolong gap junctional communication between cumulus cells and the oocyte in an attempt to allow a longer time for messenger ribonucleic acid (mRNA) and protein accumulation in the ooplasm so the oocyte reaches developmental competence (Gilchrist and

Thompson 2007). Cyclic AMP modulation by way of ligand/receptor, PDE inhibitor, and adenylate cyclase activator supplementation in culture media has been implemented during IVM of oocytes to delay spontaneous maturation (Leal et al. 2018; Li et al. 2016; Santiquet et al. 2017; Shu et al. 2008; Thomas et al. 2004a; Vanhoutte et al. 2009; Zeng et al. 2013). Cyclic AMP modulation using these techniques not only delayed maturation of the oocyte *in vitro*, but produced higher embryonic development rates after IVF (Leal et al. 2018; Li at al. 2016; Luciano et al. 1999; Santiquet et al. 2017; Thomas et al. 2004b). Cyclic AMP modulation has also been implemented in a proposed culture system termed simulated physiological oocyte maturation (SPOM) which aims to mimic *in vivo* maturation conditions *in vitro* to generate better developmental competence for clinical applications such as infertility management, though results have varied by lab and species (Albuz et al. 2010; Gilchrist et al. 2015). This method incorporates a "pre-IVM" incubation period of approximately 2 hrs with the COC exposed to cAMP modulators. This incubation period successfully delays meiotic maturation, prolongs gap junctional communication, and attempts to improve cytoplasmic developmental competence. This pre-IVM period is followed by normal IVM and IVF which resulted in higher bovine and murine blastocyst development (Albuz et al. 2010). It is clear from numerous studies that delaying maturation improves the developmental outcomes of oocytes *in vitro*.

Pannexin 1

Pannexins are a family of integral membrane proteins that were discovered relatively recently (Panchin et al. 2000). They were initially thought to have similar functions to that of connexin channels, however, studies show that they have significant functional and structural differences.

Discovery and Structure

Pannexins were first discovered by Panchin and colleagues, as these researchers performed a screen to uncover homologues of the innexin family (a membrane protein family in invertebrates) in the mammalian genome (Panchin et al. 2000). It was found that they had limited sequence homology to innexins (25-33%). The Pannexin family consists of three members, PANX1, PANX2, and PANX3. All three members are predicted to be tetra-spanning membrane proteins with the amino and carboxyl terminal ends present in the cytoplasm, as well as two extracellular loops that contain two cysteine residues each and a N-glycosylation site (Penuela et al. 2013; Sosinsky et al. 2011)(Figure 3). Pannexins also have conserved cysteine residues in putative extracellular loops which form intramolecular disulfide bonds (Foote et al. 1998; Penuela et al. 2007; Penuela et al. 2013). Pannexin proteins were initially thought to have similar functional features to that of connexins, however, pannexin channels do not have the same gap junction forming ability as connexins, except in special cases (Penuela et al. 2013). Pannexins have been shown to be structurally different

from connexins and have the ability to form single membrane channels that provide a pathway for molecular exchanges between the cell's cytoplasm and extracellular environment (Dahl 2018; Locovei et al. 2006a; Sosinsky et al. 2011). PANX1 and PANX3 share a greater structural similarity to each other, while PANX2 exhibits a larger carboxy terminal domain (Penuela et al. 2007; Yen and Saier 2007). PANX1 requires six subunits to form a functional channel from oligomerization called a pannexon (Boassa et al. 2007). It is important to note that pannexons do not refer to hemichannels but single membrane channels since they are not destined to form gap junctional or intercellular channels (Sosinsky et al. 2011).

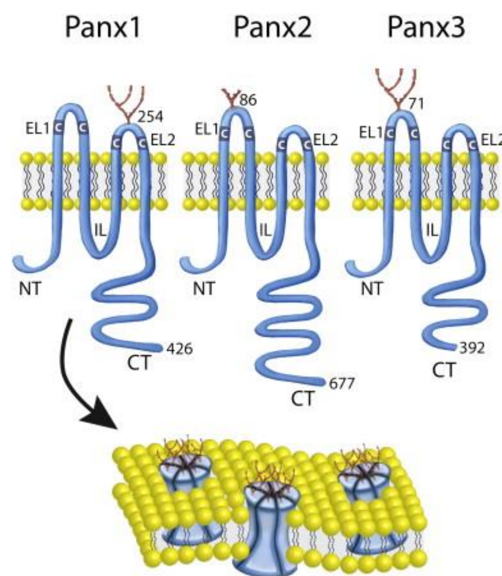

Figure 3: Structural formation of the pannexin family of proteins. Figure from Penuela et al. 2013 and used with publisher's permission. EL (extracellular loop), IL (intracellular loop), CT (carboxyl-terminus), NT (amino-terminus), C (cysteine), 254, 86, 71 (predicted glycosylation sites), 426, 677, 392 (amino acid length).

Expression and Function

PANX1 is the most extensively studied of the pannexin family and has been found to be ubiquitously expressed in many tissue types. Using northern blot analysis for ribonucleic acid (RNA), PANX1 was found to be expressed in the human brain, heart, skeletal muscle, skin, testis, ovary, placenta, thymus, prostate, lung, liver, small intestine, pancreas, spleen, colon, blood endothelium and erythrocytes (Baranova et al. 2004). PANX1 has also been found to be extensively expressed in the central nervous system, with transcripts being detected in the cerebellum, cortex, lens, retina, pyramidal cells, interneurons of the neocortex and hippocampus, amygdala, substantia, nigra, olfactory bulb, neurons and glial cells (Penuela et al. 2013). At the protein level, Panx1 was found expressed in the brain, lung, kidney, spleen, heart ventricle, skin, and ear cartilage of young mice (Penuela et al. 2007). Panx1 protein expression was also found in rodent cochlea (Wang et al. 2009b). In reproductive tissues, Panx1 has been reported in the basal compartments of the seminiferous epithelium and epididymis, as well as the apical region of efferent ducts of adult rats (Turmel et al. 2011). Recently PANX1 has been confirmed to be expressed in human cumulus-granulosa cells (Sang et al. 2019). Overall, information on the expression of PANX1 in female reproductive tissues and cells is lacking, however, PANX1 has been recently shown to play a role in oocyte health in humans (Sang et al. 2019).

PANX1 has three distinct species that are different based on glycosylation that occurs at asparagine 254 in the second extracellular loop. The three species include a non-glycosylated core species (Gly0), a endoplasmic reticulum resident high mannose species (Gly1), and the complex glycosylated species (Gly2)(Penuela et al. 2007; Penuela et al. 2013). Glycosylation is important for proper trafficking of PANX1 to the cell surface, with glycosylation-deficient mutants having reduced or absent cell surface localization (Boassa et al. 2007; Penuela et al. 2007).

Functionally, Panx1 has been implicated in many physiological processes including the removal of apoptotic cells (Chekeni et al. 2010; Poon et al. 2014), inflammation (Lohman et al. 2015), viral infection (Séror et al. 2011), ischemia (Thompson et al. 2006), and neurological functions (Gulbransen et al. 2012; Karatas et al. 2013). One major role involves facilitating the release of ATP into the extracellular environment (Bao et al. 2004; Dahl 2015; Dahl 2018). ATP release is achieved after pannexon channel activation by way of purinergic receptors or cytoplasmic calcium (Locovei et al 2006b; Locovei et al. 2007; Pelegrin et al. 2006). PANX1 has also been linked to functional roles in vasodilation and constriction, taste sensation, and human immunodeficiency virus (HIV) infection (Billaud et al. 2011; Huang et al 2007; Séror et al. 2011). Panx1 appears to have different functional roles based upon stimulation type

such as voltage activation or truncation; leading to more potential roles for the channel (Wang and Dahl 2018).

While PANX1 expression has been found in the gonads of both males and females, its biological role is not clearly defined as Panx1 knockout mice are fertile (Qu et al. 2011). However, recently a study by Sang et al. found mutations in the PANX1 sequence of humans that had unexplained infertility. These individuals attempted ART, however oocytes produced from standard IVP degraded either before or after fertilization (Sang et al. 2019). Sang termed this phenotype “oocyte death”. A look into real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis revealed higher levels of *PANX1* expression in human oocytes, 8-cell embryos, and brain compared to other tissues (Sang et al. 2019). Immunofluorescence found the localization of PANX1 mainly on the cell membrane of oocytes and zygotes, or cell to cell interfaces of early embryos (Sang et al. 2019). Unfortunately they did not investigate the expression of PANX1 in cumulus-granulosa cells. They then investigated if the glycosylation state of PANX1 was compromised in the “oocyte death” mutants (p.Q392*, p.K346E, p.C347S, and p.21_23delTEP). It was found that the “oocyte death” mutations caused PANX1 to remain in the immature Gly1 state (Sang et al. 2019). GV stage oocytes were collected from mutant mice that had a knock in mutation of the p.Q392* strain mutant. These oocytes were matured without cumulus cells. After 3 hrs of culture 80% of the oocytes degenerated, and after

10 hrs 90% had degenerated before reaching the MII stage (Sang et al. 2019). The mutant PANX1 channels had higher channel activity and thus higher extracellular ATP concentrations with PANX1 mediating ATP release (Sang et al. 2019). A PANX1 specific inhibitor (probenecid) was used in rescue experiments to save *in vitro* maturing mutant oocytes. Probenecid was successful in preventing the oocyte death phenotype, however, none of these treated oocytes extruded the first polar body at the MII stage. This suggests PANX1 has multiple roles in oocyte maturation (Sang et al. 2019). Overall, this study showed the presence of PANX1 in the human oocyte, along with potential roles it facilitates in oocyte development.

Reactive Oxygen Species

The reactive oxygen species (ROS), superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH), are ubiquitous, highly reactive, diffusible molecules that are generated within the cell as by-products of aerobic respiration and metabolism (Al-Gubory et al. 2010). The generation of O_2^- radical is the precursor of most ROS, and dismutation of O_2^- produces H_2O_2 (Al-Gubory et al. 2010; McCord and Fridovich 1969). H_2O_2 is a stable molecule that can cross cell membranes (Al-Gubory et al. 2010). With the presence of iron, H_2O_2 and O_2^- react to form OH (Halliwell 1978; Kehrer 2000). OH is thought to be the molecule responsible for oxidative damage (Halliwell and Gutteridge 1989). ROS

however represent a wide class of molecules including: free radicals, non-radicals, and oxygen derivatives (Agarwal and Prabakaran 2005).

Oxidative stress/damage is caused by an imbalance of pro-oxidants and antioxidants (Al-Gubory et al. 2010; Khazaei and Aghaz 2017). This ratio can be changed by the addition of pro-oxidants to a system or cell (such as ROS), or a reduction in antioxidant defense (Burton and Jauniaux 2011; Cindrova-Davies et al. 2007; Ruder et al. 2009). ROS can react with nucleic acids, lipids, proteins, and carbohydrates to become stable (Khazaei and Aghaz 2017). However, this addition of a stabilizing electron causes downstream reactions that result in cell damage (Attaran et al. 2000, Pierce et al. 2004). Controlled regulation of ROS generation is important to many processes including: cell signaling (Finkel 1998), gene expression (Allen and Tresini 2000), and maintenance of redox homeostasis and signal transduction pathways involved in cell function, growth, differentiation, and death (Al-Gubory et al. 2010; Valko et al. 2007). Intracellular ROS production is controlled by complex and integrated antioxidant systems (Al-Gubory et al. 2010). The seemingly critical antioxidant glutathione (GSH), is a thiol tripeptide that protects cells from ROS-induced oxidative damage (Schafer and Buettner 2001).

Reactive Oxygen Species and Oocyte Development

It has been well established that ROS buildup in oocytes is detrimental to embryo development. Increased levels of ROS beyond physiological range may lead to

deterioration in oocyte quality (Chaube et al. 2014). ROS have been implicated in causing negative developmental outcomes in embryos *in vitro* (Guerin et al. 2001; Takahashi 2012). ROS cause many different types of damage to developing embryos including a rise in lipid peroxides, increase in protein oxidation, and DNA strand breaks (Guerin et al. 2001; Nasr-Esfahani and Johnson 1992; Orsi and Leese 2001). It has been shown that high levels of ROS in the *in vitro* culture environment are detrimental to bovine embryo development (Favetta et al. 2007).

The role of ROS during IVM remains controversial. High levels of ROS due to excessive glucose in the maturation medium have a negative effect on bovine embryos until the blastocyst stage (Hashimoto et al. 2000). However, there is supportive evidence that ROS can regulate cellular functions by activating key downstream signaling pathways and controlling biologically active substances (Buhimschi et al. 2000; Marshall et al. 2000; Schreck et al. 1991).

Project Rationale

Currently, very little is known about the presence and function of the membrane protein PANX1 in reproductive tissues. PANX1 is ubiquitously expressed in many tissues across species and has been found to have various functions, primarily as an exchange point between the cell and its extracellular environment. It was recently found that PANX1 is expressed in human oocytes and embryos, and

that mutations in the PANX1 protein sequence can have detrimental effects on oocyte maturation and development *in vitro*, though mechanisms for the "oocyte death" phenotype observed from these mutations is still unknown.

In this study, we sought to investigate the expression of PANX1 in *Bos taurus* COCs, as well as, possible functional roles that the channel could facilitate during oocyte maturation and development. With oocyte IVM improvement having the attention of researchers, our focus was primarily on PANX1 function during IVM culture and the developmental outcomes from PANX1 inhibition. With that in mind, we investigated the effects of inhibiting PANX1 on IVM processes including cumulus expansion and meiotic maturation, as well as, functional roles such as cAMP concentration overtime and ROS levels during maturation. This was followed by IVF with developmental outcomes observed.

Identifying the expression of PANX1 in reproductive tissues, as well as, functional roles that the channel has in reproductive processes, could not only help us better understand the channel itself, but also improve ARTs and embryo production *in vitro*.

II. Pannexin 1 Inhibition Delays Maturation and Improves the Developmental Competence of *Bos Taurus* Oocytes, *in vitro*

HYPOTHESIS

PANX1 is expressed in bovine COCs and plays a communicational role between the COCs and their extracellular environment during IVM and subsequent *in vitro* development (IVD).

AIMS

AIM 1

To investigate the expression of PANX1 in bovine COCs.

AIM 2

To assess the consequences to maturation of inhibiting PANX1 function.

AIM 3

To investigate the effects on embryo development, following IVF, of inhibiting PANX1 function during IVM.

SPECIFIC OBJECTIVES

1. Determine if PANX1 is expressed in bovine COCs.

2. Investigate if PANX1 inhibition has an effect on bovine oocyte maturation by way of cumulus expansion, meiotic maturation, and intracellular cAMP concentration.
3. Determine if PANX1 inhibition alters early developmental outcomes at the cleavage and blastocyst stages.
4. Investigate the role PANX1 may play in ROS accumulation of maturing oocytes.

ABSTRACT

Intracellular exchange between the oocyte and its surrounding cells in the follicular environment is critical for oocyte maturation and subsequent development. In vertebrates this exchange is facilitated through gap junctions formed by connexin membrane proteins. Another family of membrane proteins, named pannexins, are able to form single membrane channels that allow cellular exchanges with the extracellular environment. The most ubiquitously expressed and studied member, PANX1, has yet to be described thoroughly in female reproductive tissues or functionally studied during oocyte maturation. Here, we look into the expression of PANX1 in bovine COCs, as well as, its potential role in oocyte maturation and development. We show that PANX1 is expressed in bovine COCs, and that the expression of PANX1 was significantly lower in COCs isolated from large antral follicles (≥5mm) compared to those isolated from small

antral follicles (≤2mm). Supporting this we also found lower expression of PANX1 in more developmentally competent oocytes (brilliant cresyl blue (BCB) positive) when compared to less developmentally competent oocytes (BCB negative). We further found that PANX1 channel inhibition during IVM resulted in a delay in meiotic maturation and improved *in vitro* developmental outcomes, while decreasing oocyte intracellular ROS levels. We found that PANX1 is differentially expressed during the antral stages of folliculogenesis, during the critical stage when oocytes are acquiring developmental competence. We also found that PANX1 plays a functional role during oocyte maturation, by way of maintaining a high cAMP concentration.

INTRODUCTION

During folliculogenesis, communication between the oocyte and its surrounding cells is critical for proper maturation of the oocyte following ovulation. Intracellular exchange between the oocyte and follicular cells is facilitated by gap junctional communication (Mitchell and Burghardt 1986). Gap junctions, in mammals, are formed by the family of membrane proteins called connexins (Bruzzone et al. 1996). Connexins facilitate the exchange of nutrients, metabolites, and secondary messengers between neighboring cells (Evans and Martin 2002). Connexins have also been shown to play an important role in oocyte maturation and development, with the loss or reduction of connexin 43

having negative implications on fertility in multiple species (Ackert et al. 2001; Tong et al. 2006; Wang et al. 2009a; Winterhager and Kidder 2015).

A more recently identified family of membrane proteins named pannexins, with limited sequence homology to the invertebrate gap junction family of innexin proteins, was discovered by Panchin et al. (Panchin et al. 2000). The pannexin family of glycoproteins consists of three members: PANX1, PANX2, and PANX3. All three are predicted to be tetra-spanning membrane proteins with the amino and carboxy terminal ends present in the cytoplasm, as well as two extracellular loops that contain two cysteine residues each and a N-glycosylation site (Penuela et al. 2013; Sosinsky et al. 2011). Though initially thought to form gap junctions like connexins, pannexins have also been shown to be structurally different and form single membrane channels that provide a pathway for molecular exchanges between the cell's cytoplasm and the extracellular environment (Dahl 2018; Locovei et al. 2006a; Sosinsky et al. 2011).

The most extensively studied, PANX1, has been found to be ubiquitously expressed in many tissue types. In human tissues, PANX1 is expressed in the heart, skeletal muscle, testis, ovary, brain, placenta, thymus, prostate, and small intestine (Baranova et al. 2004). Panx1 is also highly expressed in central nervous tissues and neuronal cells in mice (Baranova et al. 2004; Zappala et al. 2006). Though shown to be expressed in both female and male gonads the role of PANX1 in reproductive cells remains very limited. However, PANX1 has been

recently shown to play a role in oocyte health in humans (Sang et al. 2019). A mutation in PANX1 in humans has been described that results in a loss of control of cellular functioning (Sang et al. 2019). Oocytes having the mutation, following retrieval, released more ATP to the extracellular space and degenerated. The mutation appeared to affect maturation potential in the oocytes as they were able to collect very few mature oocytes with the majority being immature and all degenerating at or very shortly after fertilization (Sang et al. 2019).

Functionally, Panx1 has been implicated in many physiological processes including the removal of apoptotic cells (Chekeni et al. 2010; Poon et al. 2014), inflammation (Lohman et al. 2015), viral infection (Séror et al. 2011), ischemia (Thompson et al. 2006), and neurological functions (Gulbransen et al. 2012; Karatas et al. 2013). One major role involves facilitating the release of ATP into the extracellular environment (Bao et al. 2004; Dahl 2015; Dahl 2018). ATP release is achieved after pannexon channel activation by way of purinergic receptors or cytoplasmic calcium (Locovei et al. 2006b; Locovei et al. 2007; Pelegrin and Surprenant 2006). PANX1 has also been linked to functional roles in vasodilation and constriction, taste sensation, and HIV infection (Billaud et al. 2011; Huang et al. 2007; Séror et al. 2011). Panx1 appears to have different functional roles based upon stimulation type such as voltage activation or truncation; leading to more potential roles for the channel (Wang and Dahl 2018). While PANX1 expression has been found in the gonads of both males and

females, its biological role is not clearly defined as Panx1 knockout mice are fertile (Qu et al. 2011). However, recently a mutation in PANX1, found in humans, has been shown to result in female infertility suggesting a potentially important role in fertility (Sang et al. 2019). The mutation altered the glycosylation pattern of PANX1 and influenced the subcellular localization resulting in more ATP released into the extracellular environment. This led to a loss of oocytes through degeneration (Sang et al. 2019).

While the specific role of pannexins in oocyte maturation remains undefined, connexins and innexins have been clearly implicated. It has been shown that the resumption of meiotic maturation out of prophase-arrest is inhibited in cattle and mice if connexin channels are inhibited or knocked-out (Simon et al. 1997; Vozzi et al. 2001). Furthermore, innexin gap junction channels are required for proper meiotic maturation to occur in *C. elegans* (Whitten and Miller 2007). Mechanistically, gap junctional communication in bovine COCs facilitates the decline in cAMP which is one proposed mechanism of oocyte meiotic resumption (Luciano et al. 2004; Thomas et al. 2004a).

To date there is little information regarding the expression and functioning of pannexin channels within ovarian tissues. Here we investigated the expression of PANX1 in bovine COCs and its potential role during oocyte maturation and early embryo development, following IVF.

MATERIALS AND METHODS

All reagents were purchased from VWR (Radnor, USA) unless otherwise noted.

IVM and IVF System

COCs were collected from abattoir-sourced, mixed breed *Bos taurus* ovaries. COCs underwent maturation and IVF followed by culturing to the blastocyst stage as previously described (Read et al. 2018), with modifications. Briefly, follicles ranging from 2-5mm were aspirated using a 10ml syringe and 18-gauge, short-beveled needle. COCs, granulosa cells, and follicular fluid were collected into 50ml conical tubes at room temperature. COCs with a uniform cytoplasm and at least three layers of cumulus cells were collected into TCM-199 supplemented with 8.3mM sodium bicarbonate, 20mM Hepes, 10% fetal bovine serum, 50µg/ml gentamicine, and 22µg/ml pyruvate. COCs were matured in TCM-199 (Gibco, Thermo Fisher Scientific, Waltham, MA) supplemented with 8.3mM sodium bicarbonate, 10% FBS, 50µg/ml gentamicin, 22µg/ml pyruvate, 1.1mM glutamine, and 10ng/ml EGF. Selected COCs were placed in groups of 20-25 per 90µl microdrops consisting of oocyte maturation media either supplemented with 100µM 10Panx (a PANX1 mimetic inhibitory peptide, Tocris, Bristol, United Kingdom) or the vehicle only, under mineral oil (Sigma Life Science, Darmstadt, Germany) and cultured for 22 hrs. COCs were then used for experiments or fertilized in groups of 200 in 1.9ml of fertilization media containing

10^6 spermatozoa/ml for 18-22 hrs. To eliminate variation due to the male component, semen from a single ejaculation from a bull with proven *in vitro* fertility was used. Fertilization media consists of synthetic oviductal fluid (SOF (Caisson Labs, Smithfield, UT), 1.17mM $CaCl_2 2H_2O$, 0.49mM $MgCl_2 6H_2O$, 1.19mM KH_2PO_4, 7.16mM KCl, 107.7mM NaCl, 25.07mM $NaHCO_3$, and 5.3mM Na-lactate) supplemented with 50µg/ml gentamicine, 22µg/ml pyruvate, 10µg/ml heparin, 194.2µg/ml caffeine, and 6mg/ml bovine serum albumin (BSA) fraction V essentially fatty acid free (EFAF). Following fertilization, the presumptive zygotes were denuded by gently pipetting them up and down with a glass pipette. The presumptive zygotes were then cultured in groups of 25 in 50µl culture media under mineral oil. Culture media consisted of 5ml SOF supplemented with 6mg/ml BSA fraction V EFAF, 1.1mM glutamine, 2.8mM myo-inositol, 0.57mM sodium citrate, 22µg/ml pyruvate, 50µg/ml gentamicin, 1X essential amino acids, and 1X nonessential amino acids. Presumptive zygotes were then cultured in 5% CO_2, 5% O_2, and 90% N_2 at 38.5ºC. Forty-eight hours after fertilization, cleavage rates were recorded based on the initial total number of oocytes (number cleaved (divided into two cells)/ number of oocytes fertilized). On day 7 after fertilization, morula/blastocyst rates were recorded based on the initial total number of oocytes (number in morula/blastocyst stage (transitioning to a blastocyst with fluid cavity or in the blastocyst stage and has a fluid cavity)/ number of oocytes

fertilized). COCs in all experiments in this study were cultured at 38.5^{o}C and 5.0% CO_2. All experiments were repeated at least 3 times.

Immunocytochemistry of COCs

To determine if PANX1 is expressed in bovine COCs, COCs were aspirated from bovine ovarian follicles using a syringe and 18 gauge needle. This was followed by their collection under a stereoscope and washing in TCM199. Selected COCs were washed in phosphate buffered saline (PBS) then fixed for 15 min in 4% paraformaldehyde (PFA), blocked 30 min with PBS containing 3% BSA and 0.5% Triton-X, incubated overnight with a primary antibody (1:500 Rabbit anti-PANX1 CT-412; Western University, London, Ontario (Penuela et al. 2007; Shao et al. 2016)) (Table 1), washed with blocking solution, and incubated 1 hr with a secondary antibody (1:500, Goat anti-Rabbit FITC; ImmunoReagents Inc., Raleigh, NC) (Table 1). Cells were counterstained with Hoechst 33342. A Nikon A1 Confocal Scanning Laser Microscope (Nikon Instruments, Melville, NY) was used to randomly image a single plane of the cumulus cells with or without the oocyte present. The anti-PANX1 CT-412 antibody was produced using a human epitope that has 86.67% homology with the bovine protein (unpublished results)(Penuela et al. 2007).

BCB Staining of Oocytes

To determine if PANX1 protein was differentially expressed based upon oocyte developmental competence, oocytes were aspirated from bovine ovarian follicles ranging from 2-5 mm in size using a syringe and 18-gauge needle. This was followed by their collection under a stereoscope and washing in TCM199. Oocytes with at least three continuous layers of surrounding cumulus cells and a homogeneous cytoplasm were then selected for BCB staining. Selected COCs were then washed three times in TCM199 and cultured 90 mins in TCM199 supplemented with 26μM BCB. After staining COCs were washed in PBS and examined under a stereoscope. They were classified into different groups of high or low developmental competence, according to the level of BCB coloration in the ooplasm. The categorized COCs were then pelleted and resuspended in radioimmunoprecipitation assay (RIPA) Lysis and Extraction buffer containing proteinase inhibitors.

Western Blotting

To determine if PANX1 protein was differentially expressed at different stages of folliculogenesis, as well as, at different levels of developmental competence, COCs or follicular fluid content were washed in PBS, pelleted, and frozen at -80° Celsius. When selecting for different antral follicle stages of development, follicles were aspirated separately from small antral (≤2mm) and large antral

(≥5mm) follicles. Protein from the frozen samples was isolated using RIPA Lysis and Extraction buffer supplemented with protease inhibitors (1.2mM AEBSF, 0.46μM Aprotonin, 14μM Bestatin, 12.3μM E-64, 112μM Leupeptin, 1.16μM Pepstatin). Protein samples were quantified using the Pierce BCA Protein Assay Kit (ThermoFisher, Waltham, MA). Samples were combined with loading dye, heated at 95°C for 5 min, and ran through a 12% sodium dodecyl sulfate polyacrylamide (SDS) page gel, followed by transfer to a polyvinylidene difluoride (PVDF) membrane (Invitrogen, Carlsbad, CA). The membrane was blocked with tris-buffered saline (TBS) containing 5% BSA fraction V, washed with TBS containing 0.5% Tween-20, incubated overnight with primary antibody, washed with TBS-Tween, incubated 1 hr with a secondary antibody, washed with TBS-Tween, and exposed with enhanced chemiluminescence (ECL) for 4 min followed by chemiluminescent detection on an Azure c400 (Azure Biosystems, Dublin, CA). For PANX1 staining, a rabbit anti-PANX1 CT-412 (1:1K, Western University, London, Ontario (Penuela et al. 2007; Shao et al. 2016)) (Table 1) primary and goat anti-rabbit horseradish peroxidase (HRP) (1:2K, Columbia Biosciences, Frederick, MD) (Table 1) secondary were used. GAPDH staining used a mouse anti-GAPDH (1:1K, Sigma Life Science) (Table 1) primary and a goat anti-mouse HRP (1:10K, Novagen, Darmstadt, Germany) (Table 1) secondary. The blots were densitometry analyzed and normalized to GAPDH

using ImageJ analysis software (Java, NIH, v1.52a). Tissues were derived from bovine samples.

Name	Dilution	Company/ Supplier	Research Resource Identification (RRID)
Rabbit anti-PANX1 CT-412	1:500	Western University	See Materials and Methods
Mouse anti-GAPDH	1:1000	Sigma	AB_1078991
Goat anti-rabbit FITC	1:500 (IF)	Immuno Reagents, Inc.	
Goat anti-rabbit HRP	1:2000 (WB)	Columbia Biosciences	
Goat anti-mouse HRP	1:10,000 (WB)	Novagen	

Table 1. *Primary and secondary antibodies used for Western blotting (WB) and Immunofluorescence (IF).*

Propidium Iodide (PI) Dye Uptake

To determine Dye uptake studies were performed on bovine granulosa cells derived from follicular fluid collections (described above). Granulosa cells were collected from the follicular fluid by pelleting the cell content and washing with Dulbecco Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) media (D-MEM/F12 base powder (Gibco, ThermoFisher Scientific, Waltham, MA), 0.24%

sodium bicarbonate, 1X Antibiotic-Antimycotic). Isolated granulosa were plated in 6-well culture plates and incubated at 38.5°C with 5% CO_2 in DMEM/F12 supplemented with 10% fetal bovine serum (FBS). The media was changed every 24 hrs. 10Panx (100μM, a PANX1 mimetic inhibitory peptide, Tocris, Bristol, United Kingdom) or vehicle control was added to the appropriate wells following 48hr of culture and incubated at 38.5°C and 5% CO_2 for 30 min. A propidium iodide (PI) dye uptake protocol was utilized using methods previously described (Ortiz-Escribano et al. 2017), with modifications. Basal dye uptake was measured by adding PI (1mM) to all wells and placing the plate back into the incubator for 30 min. Cells were then washed in PBS and fixed for 25 min in 4% PFA protected from light. Cells were washed with 3% PBS-BSA and nuclei were stained with NucBlue Live Cell Stain Hoechst33342 (Life Technologies, ThermoFisher Scientific, Waltham, MA) for 10 min protected from light. Images were captured using an Evos FL Cell Imaging System (ThermoFisher Scientific, Waltham, MA) with the RFP fluorescent filter, and PI positive cells were counted. Cells were considered positive if the nuclear material was stained with a concentrated red fluorescence. Three repetitions were conducted, and counting was performed by the same researcher for each repetition.

Cumulus Expansion Measurement

To determine the expansion distance of the cumulus around the maturing oocyte, COCs were matured in groups of ~20-25 with or without 10Panx treatment (100μM) for 22 hrs using methods described above. Culture plates with droplets were removed from incubation and bright light images were taken using an Evos FL Cell Imaging System (ThermoFisher Scientific, Waltham, MA). Only completely visible COCs, where the distance from the center of the oocyte to the edge of the expanded cumulus could be measured, were included. Five repetitions were conducted with a total of n=134 and n=132 COCs for control and 10Panx groups respectively. ImageJ analysis software was used for measurements.

Meiotic Maturation of Oocytes

To determine the stage of nuclear maturation, COCs were matured with 10Panx treatment (100μM) or vehicle control for 6 or 22 hrs using methods described above. Each treatment group and time point was removed from incubation when appropriate, washed in PBS, and placed in 0.25% trypsin for 10 min at 38.5°C. The trypsinized COCs were then vortexed and denuded mechanically using a glass pipet. Denuded oocytes were selected and washed in PBS, then fixed in 4% PFA for 15 min. Fixed oocytes were permeabilized in 0.5% PBS-Triton for 15 min, washed in 3% PBS-BSA, and the nuclei were stained with NucBlue Live Cell

Stain Hoechst 33342 (Life Technologies, ThermoFisher Scientific, Waltham, MA) for 10 min protected from light. Stained oocytes were mounted with coverslips on Superfrost microscope slides (Fisher Scientific, ThermoFisher Scientific, Waltham, MA) using VectaShield antifade mounting medium (Vector Laboratories, Burlingame, CA). Oocyte nuclear maturation state was recorded using the Evos FL Cell Imaging System (ThermoFisher Scientific, Waltham, MA) set to the DAPI fluorescent filter. Oocytes were classified as being in the GV (nucleus intact), GVBD (nucleus broken and nuclear material condensing), or MII (presence of two polar bodies) stage of meiotic maturation. Four repetitions were conducted with n=73, n=93, n=120, and n=114 oocytes for 6hr control, 6hr 10Panx, 22hr control, and 22hr 10Panx groups respectively.

Cyclic AMP ELISA

To determine the intracellular concentration of 3’,5’-cyclic adenosine monophosphate (icAMP) in COCs, COCs were matured with or without 10Panx treatment (100μM) for 0, 3, 6, or 22 hrs using methods described above. Each treatment group and time point was removed from incubation when appropriate, and placed into maturation media supplemented with 0.5mM 3-isobutyl-1-methylxanthine (IBMX). COCs were then washed again in maturation media supplemented with 0.5mM IBMX, followed by a wash in maturation media without IBMX. The COCs were moved to PBS and then snap frozen in a 1.7ml conical

tube. Samples were stored at -80°C until the assay. The icAMP concentration of the COCs was determined using the Enzo Direct cAMP enzyme-linked immunosorbent assay (ELISA) kit (Enzo Life Sciences, Farmingdale, NY, Cat# ADI-900-066) using the acetylation protocol according to the manufacturer's instructions. Optical density was then measured at 405nm using an EMax Plus Microplate Reader (Molecular Devices, San Jose, CA) and icAMP concentration was calculated. Three experimental replicates were repeated with samples collected for each time point and treatment with n=10 COCs per sample.

2'-7'-Dichlorodihydrofluorescein diacetate (DCHF-DA) Staining of Oocytes for ROS levels

To determine if intracellular levels of ROS were different in PANX1 inhibited oocytes, oocytes were matured for 22 hrs and were mechanically denuded using a glass pipette. The denuded oocytes were then collected under a stereoscope and cultured for 30 min in PBS supplemented with 3% BSA, 10μg/ml Hoechst 33342, and 10μM 2’-7’-dichlorodihydrofluorescein diacetate (DCHF-DA). The stained oocytes were then washed briefly three times in PBS and immediately imaged for fluorescence under an Evos FL Cell Imaging System (ThermoFisher Scientific, Waltham, MA) using the DAPI and GFP fluorescent filters. Staining intensity was quantified using ImageJ analysis software by calculating the corrected total cell fluorescence (CTCF, CTCF = Integrated Density–(Area of

selected cell * Mean fluorescence of background readings)) of five areas of an individual oocyte and averaging them. The individual averaged CTCFs between 10Panx treated and vehicle only controls were then used to compare the DCFH-DA staining. 7 repetitions were performed with averages and imaging done by the same researcher.

Statistics

Statistical analysis was conducted using GraphPad Prism software (v6.01). Unpaired two-tailed Student t-tests were performed for all data sets besides meiotic maturation data, in which a one-way ANOVA was implemented per time point with Tukey's *post hoc* testing for multiple comparisons. Data for PI dye uptake studies were log transformed for statistical analysis, however graphical representation is shown using non-transformed data. Data for developmental outcomes were arcsine transformed for statistical analysis, however graphical representation is shown with non-transformed data. Results are reported using standard deviation (SD) from the mean. Comparisons were considered statistically significant if $p < 0.05$ and contained a significant trend if $p < 0.055$.

RESULTS

PANX1 expression in bovine COCs

We initially wanted to determine if PANX1 was expressed in bovine COCs. Bovine COCs were isolated and stained for PANX1 expression using an anti-PANX1 antibody followed by a FITC-conjugated secondary antibody, and counterstained with Hoechst33342 nuclear stain. PANX1 protein was expressed in bovine COCs as seen by immunofluorescence. PANX1 is localized in cumulus cells and surrounds the oocyte itself with a ubiquitous expression pattern (Figure 4).

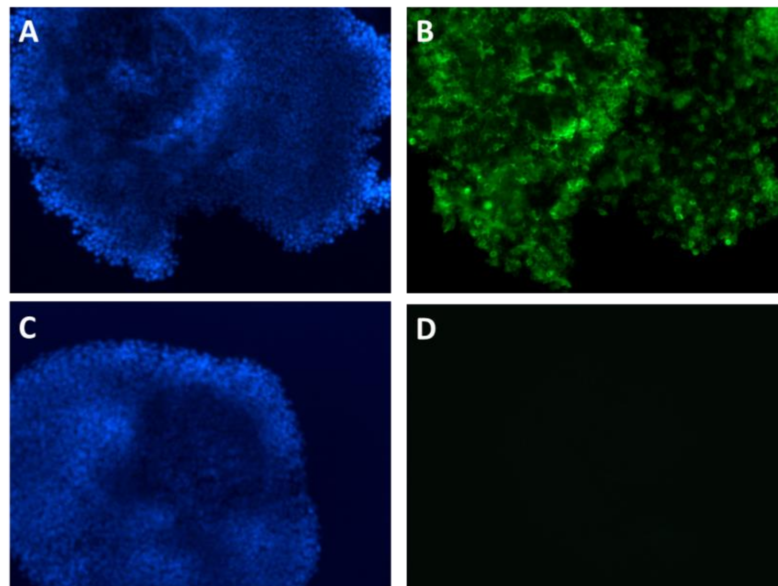

Figure 4. *PANX1 expression in Bovine COCs.* A) Representative immunofluorescent images of bovine COCs stained with Hoechst33342 (blue, A) and labeled with PANX1 antibody (green, B). C) A representative image depicting a bovine COC stained with Hoechst33342 and stained with secondary antibody only (D). Magnification 10x. A&B are the same COC. C&D are the same COC.

PANX1 protein expression is greater in early follicular stages

In order to determine if there was a difference in PANX1 expression levels during different phases of folliculogenesis, the level of protein expression was quantified using western blotting. The expression of PANX1 in granulosa cells isolated from small antral follicles (≤2mm) was compared to those from large antral follicles (≥5mm). Granulosa isolated from small follicles had a significantly higher expression of PANX1 (0.827±0.217) compared to granulosa isolated from large follicles (0.394±0.176, p=0.0211)(Figure 5).

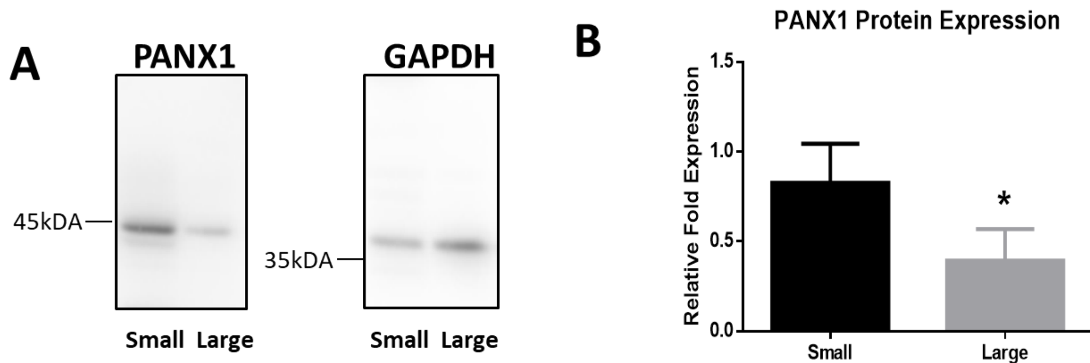

Figure 5. *PANX1 Expression during Different Follicular Stages.* A) Representative blot images depicting the levels of PANX1 expression in small (≤2mm) and large (≥5mm) antral follicles. GAPDH was used as a loading control. B) Densitometry results showing the expression level of PANX1 in small and large antral follicles. *Denotes a significant difference, p < 0.05. Error bars are ± SD from mean. 4 replicates were analyzed.

PANX1 protein expression is greater in less developmentally competent COCs

Next we wanted to see if there was a difference in PANX1 expression levels at varying oocyte developmental potentials. COCs were isolated and BCB stained to determine their developmental competence level. COCs containing oocytes that were BCB negative (lower competence) had significantly higher expression of PANX1 when compared to BCB positive (higher competence) oocyte containing COCs (2.043±0.314 and 1.000±0.0 respectively, p=0.0045) (Figure 6).

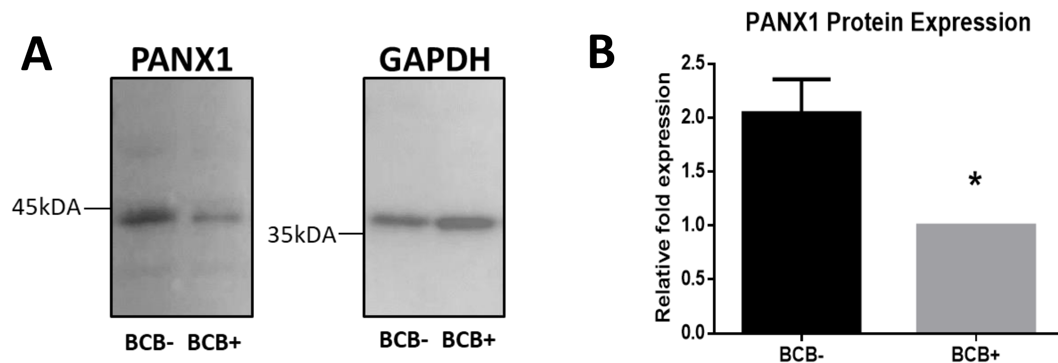

Figure 6. *PANX1 Expression at Varying Stages of Oocyte Developmental Competence.* A) Representative blot images depicting the levels of PANX1 in BCB- and BCB+ COCs. GAPDH was used as a loading control. B) Densitometry results showing the expression of PANX1 in BCB- and BCB+ groups of COCs. *Denotes a significant difference, $p < 0.05$. Error bars are ± SD from mean. 3 replicates were analyzed.

PANX1 channels are inhibited by 10Panx mimetic inhibitory peptide

Before conducting inhibitory studies, we wanted to ensure that the 10Panx mimetic peptide inhibited PANX1 channels. To ensure this, a dye uptake study

was performed using the hemichannel permeable dye PI. Cultured granulosa cells that had been incubated for 30 mins with or without 10Panx were measured for PI uptake. A significant decrease in dye uptake was found in 10Panx treated cells compared to the control cells (p=0.0143). The relative fold proportion of PI positive cells was greater in the control with this group having 1.846±0.353 fold more positive cells than the 10Panx group (Figure 7, Figure shows data that was non-transformed ratios, log transformed data was used for statistics).

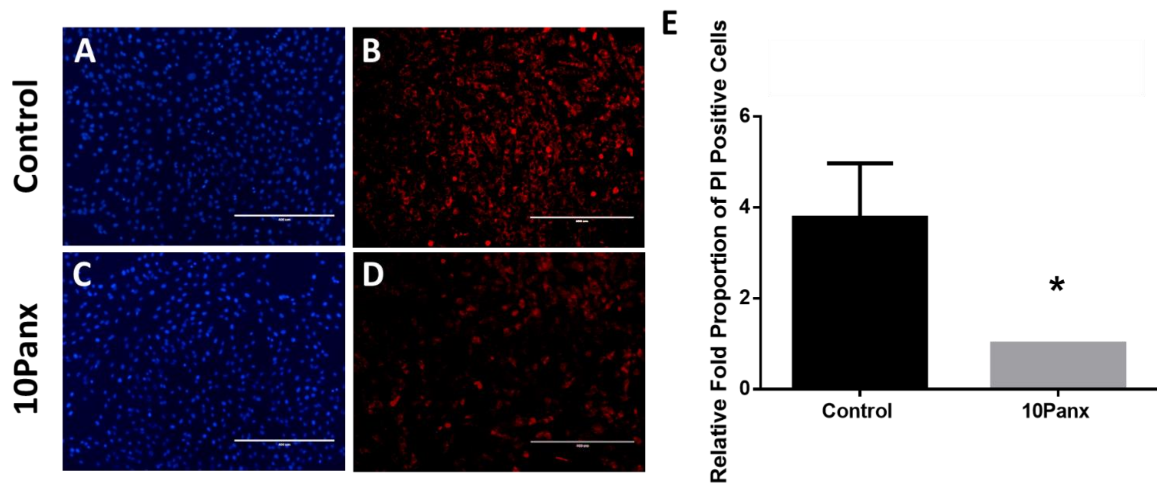

Figure 7. *Inhibiting the PANX1 Channel.* Representative images depicting PI dye uptake (red) by untreated granulosa cells (B) and granulosa cells treated with 10Panx (D). Cells were nuclear stained with Hoechst33342 (blue, A&C). Bars = 400µm. E) The relative number of PI positive cells in control or 10Panx treated granulosa cells. *Denotes a significant difference, $p < 0.05$. Error bars are ± SD from mean. 3 replicates were analyzed.

Maturing COCs with inhibited PANX1 function causes less cumulus cell expansion

To study the initial effects of PANX1 inhibition on the IVM of COCs, we measured the expansion of cumulus cells in COCs after a maturation time of 22 hrs with or without 10Panx supplementation. The 10Panx treated COCs were found to have less cumulus expansion compared to the vehicle only control group (152.60±30.46μm and 193.20±13.89μm respectively, $p=0.0264$)(Figure 8).

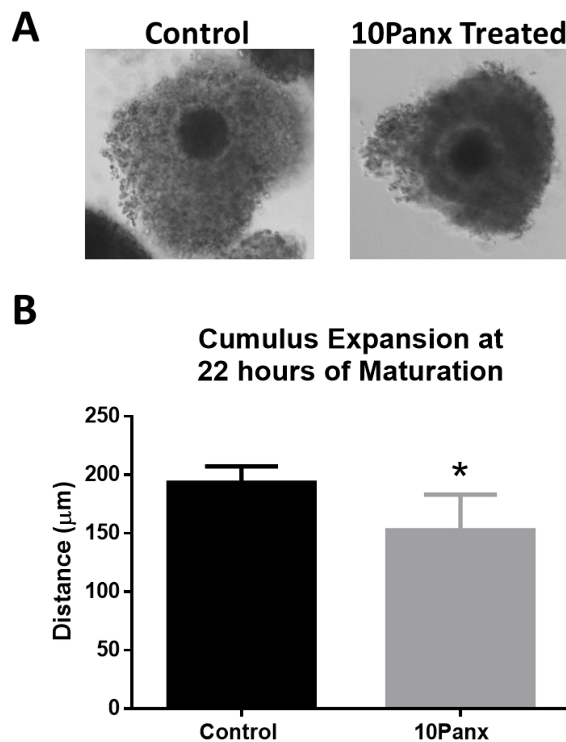

Figure 8. *Cumulus Expansion after PANX1 Inhibition.* A) Representative images of cumulus expansion after 22 hrs of IVM in control COCs and PANX1 inhibited COCs. Magnification was 20x. B) Cumulus expansion in the control and 10Panx treated groups. Denotes a significant difference, $p < 0.05$. Error bars are ± SD from mean. 5 replicates were analyzed.

Meiotic maturation of COCs is delayed with inhibited PANX1 function

We next looked into the effects that the inhibition of PANX1 had on the stage of DNA maturation after 6 and 22 hrs of maturation. COCs were matured *in vitro*, stripped of cumulus cells, stained with Hoechst33342, and DNA maturation stage was observed under fluorescence. DNA maturation stages were categorized into GV, GVBD, and MII stages (Figure 9B). Following 6 hrs of IVM, COCs treated with 10Panx had a significantly higher proportion (0.600±0.094) of DNA in the GV stage compared to the control group (0.340±0.073, $p<0.0001$). There was a significantly higher proportion of oocytes in the GVBD stage after 6 hrs in the control group compared to the 10Panx treated group (0.576±0.097 and 0.385±0.083 respectively, $p<0.0001$). The proportion of COCs at the MII stage after 6 hrs was not significantly different between the 10Panx and control groups (0.015±0.030 and 0.084±0.107 respectively). After 22 hrs of maturation, the proportion of COCs was no longer significantly different between the 10Panx treated and the control groups in the GV stage (0.027± 0.034 and 0.023± 0.046 respectively). There was also no significant difference between the proportions of 10Panx treated and control oocytes in the GVBD (0.140±0.075 and 0.237±0.102 respectively) or MII (0.833±0.061 and 0.741±0.080 respectively) stages after 22 hrs of maturation (Figure 9A).

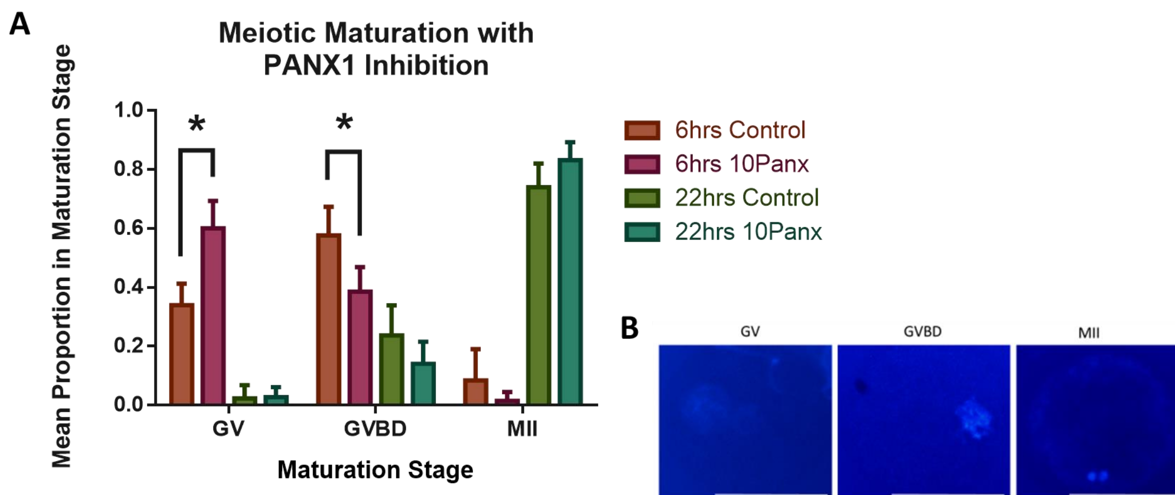

Figure 9. *Meiotic Maturation During PANX1 Inhibition*. A) Meiotic maturation proportions of oocytes at the GV, GVBD, and MII stages after 6 hrs and 22 hrs of IVM with or without PANX1 inhibition. B) Representative images of GV, GVBD, and MII DNA categorizations. Bars = 100µm. *Denotes a significant difference, $p < 0.05$. Error bars are ± SD from mean. 4 replicates were analyzed.

Intracellular cAMP concentration in maturing COCs with inhibited PANX1 function corresponds to a delay in meiotic maturation

To further understand the functional role PANX1 has in the bovine COC during maturation, we studied the icAMP levels of intact COCs at various time points during maturation. COCs were matured *in vitro* with or without 10Panx for 3, 6, or 22 hrs and were removed, washed, and snap frozen. This was followed by a direct cAMP ELISA to measure icAMP. After 3 hrs of maturation, the COC icAMP concentration was not significantly different between the 10Panx treated and control groups (14.330±3.434 pmol/ml and 14.910±1.625 pmol/ml respectively, p=0.8034). After 6 hrs of maturation, the COC icAMP concentration was

significantly higher in the 10Panx treated group (18.880±2.052 pmol/ml) versus the vehicle only control (13.350±1.345 pmol/ml, p=0.0175). After 22 hrs of maturation, the COC icAMP was no longer significantly different between the 10Panx treated and control groups (12.690±0.646 pmol/ml and 12.770±2.928 pmol/ml respectively, p=0.9639)(Figure 10).

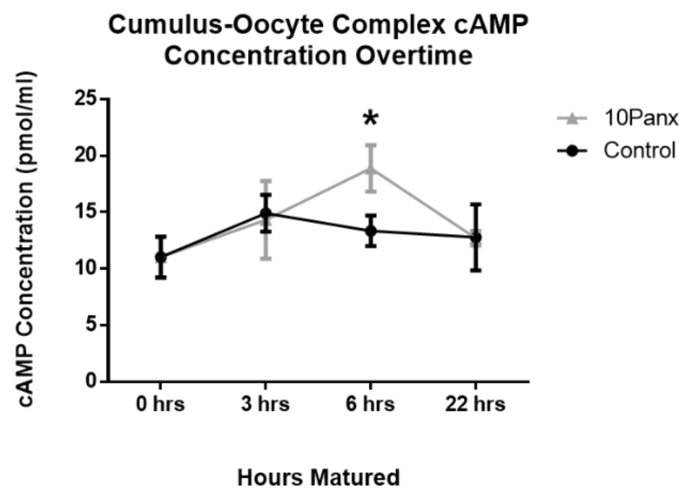

Figure 10. *Intracellular cAMP Concentration of COCs throughout IVM with PANX1 Inhibition.* The average icAMP concentration of COCs treated with or without 10Panx at 0, 3, 6, and 22hrs of IVM. *Denotes a significant difference, $p < 0.05$. Error bars are ± SD from mean. 3 replicates were analyzed.

Early embryo development is improved with PANX1 inhibition during IVM

To study the effects of PANX1 inhibition during maturation on future preimplantation developmental potential, COCs were isolated from abattoir sourced ovaries, washed, matured *in vitro* with or without 10Panx supplementation. Matured COCs were then fertilized and cultured *in vitro* to the

blastocyst stage. The embryonic cleavage rate contained a significant trend with the 10Panx treated COCs having higher cleavage (1.141±0.071%) compared to the untreated control COCs (1.006±0.049%, p=0.0529). This was followed by a significantly higher blastocyst rate in 10Panx treated fertilized COCs (0.696±0.016%) compared to untreated fertilized COCs (0.466±0.036%, p=0.0005)(Figure 11, Figure shows data that was non-transformed percentages, arcsine transformed data was used for statistics).

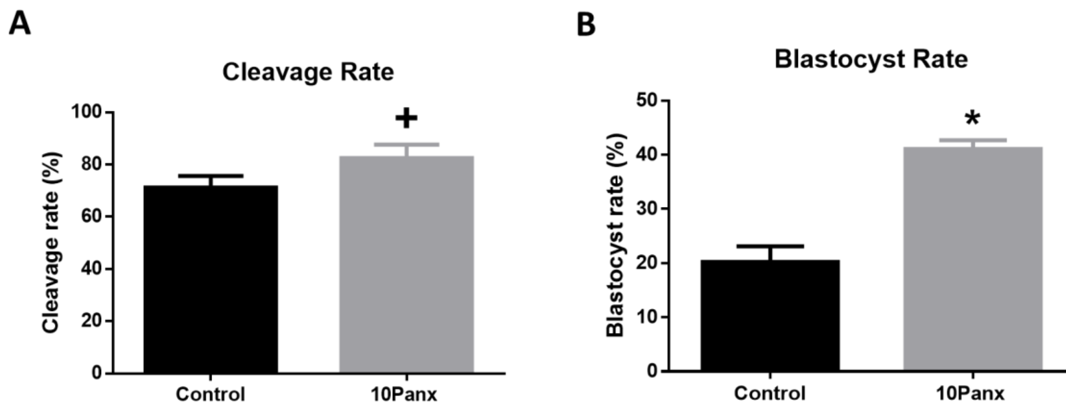

Figure 11. *Embryo Development Post PANX1 Inhibition.* A) Cleavage rate at day 2 post IVF with or without 10Panx treatment during IVM. B) Blastocyst rate at day 7 post IVF with or without 10Panx treatment during IVM. *Denotes a significant difference, $p < 0.05$. +Denotes a significant trend, $p < 0.055$. Error bars are ± SD from mean. 3 replicates were analyzed.

ROS levels are decreased in oocytes that are matured with PANX1 inhibition

In order to measure the levels of free radicals in the oocytes following maturation in the presence of 10Panx we compared the DCFH-DA staining level in oocytes

following treatment to untreated controls. The level of ROS in the oocytes cultured in the presence of 10Panx was found to be significantly lower (144418±23134) when compared to the untreated vehicle only controls (177665±32725, p=0.0486)(Figure 12).

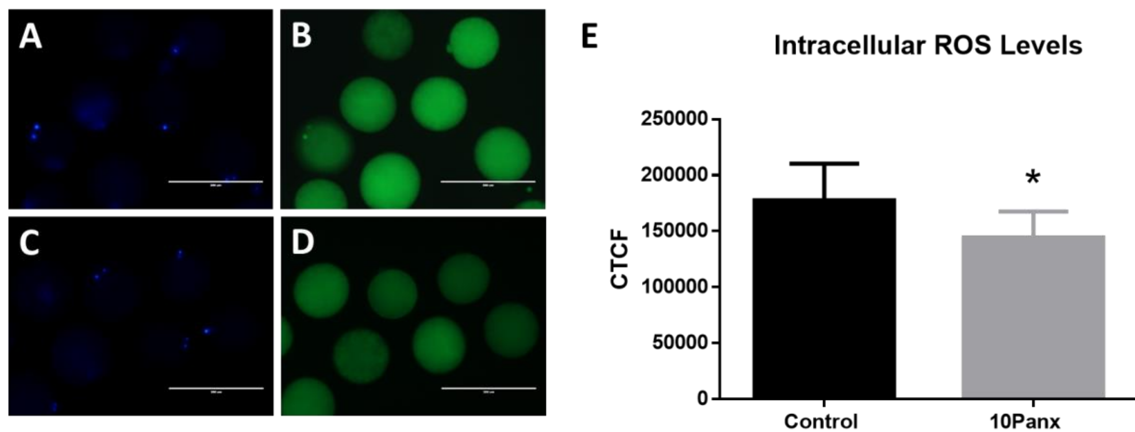

Figure 12. *PANX1 Inhibiton and Oocyte Intracellular ROS Levels.* Representative images depicting the staining intensity of DCFH-DA showing levels of free radicals within the oocyte after 22 hrs of IVM without (B) or with (D) PANX1 inhibition. Cells were nuclear stained with Hoechst33342 (blue, A&C). Bars = 200μm E) Quantitation of the staining intensity of DCFH-DA using CTCF. *Denotes a significant difference, $p < 0.05$. Error bars are ± SD from mean. 7 replicates were analyzed.

DISCUSSION

In this study, we describe the expression of PANX1 in bovine oocyte cumulus cells. Interestingly, PANX1 is differentially expressed by follicle size with COCs isolated from large antral follicles having less PANX1 compared to COCs isolated from smaller follicles. This is consistent with our findings that cumulus cells from

BCB+ oocytes have a lower expression level of PANX1 when compared to less developmentally competent BCB- oocytes. BCB staining is used to determine glucose-6-phosphate dehydrogenase (G6pDH) activity, with more developmentally advanced and competent bovine oocytes staining positive (Pujol et al. 2004). This suggests that during folliculogenesis, and the development of competence in oocytes, the expression of PANX1 decreases *in vivo*. This is consistent with the findings of Sang et al. who described a mutation in humans that resulted in a loss of control of PANX1 cellular functioning (Sang et al. 2019). In that case oocytes having the mutation released more ATP to the extracellular space and following retrieval degenerated. One described mutation appeared to affect maturation potential in the recovered oocytes as very few mature oocytes were obtained with the majority being immature and all degenerating at or very shortly after fertilization (Sang et al. 2019). Ours and their findings suggest an important role for PANX1 during oocyte maturation.

In order to explore this further we studied the maturation rate of oocytes exposed to the PANX1 inhibitor 10Panx (Manohar et al. 2012). We initially found that treatment of oocytes with 10Panx following maturation for 22 hrs did not change the number of oocytes reaching the MII stage. However, when we looked at the stage of maturation at 6 hrs we found a significantly higher number of treated oocytes were at the GV stage. These results suggest that the inhibition of the PANX1 single membrane channel effectively delays oocyte maturation.

Moreover, following exposure to 10Panx the oocytes had higher cleavage and blastocyst rates when compared to untreated controls. This is consistent with others showing that delaying maturation effectively improves the developmental competence of aspirated oocytes.

Delaying meiotic maturation has been shown to improve developmental competence using different techniques. Ligand/receptor supplementation has been recently studied as a method to delay spontaneous maturation *in vitro* to better mimic *in vivo* conditions. It was found that supplementing culture media with the physiologic ligand/receptor reagents C-type natriuretic peptide (CNP), estradiol, FSH, and bone morphogenic protein 15 (BMP15) before standard IVM delayed maturation and improved oocyte developmental competence (Santiquet et al. 2017). Cyclic AMP modulation has been the major focus for improving the IVM system. Cyclic AMP modulation has been implemented in different studies to either prevent the spontaneous drop in cAMP (PDE inhibitors) or stimulate the production of cAMP (adenylate cyclase activators) (Leal et al. 2018). When used in culture before standard IVM, adenylate cyclase activators, and/or PDE inhibitors have been shown to delay maturation and improve embryo development (Li et al. 2016; Shu et al. 2008; Thomas et al. 2004b; Vanhoutte et al. 2009; Zeng et al. 2013). Cyclic AMP modulation in culture has also been implemented in a proposed culture system termed SPOM, which aims to mimic *in vivo* maturation conditions *in vitro* to generate better developmental competence

for clinical applications such as infertility management, though results have varied by lab and species (Albuz et al. 2010; Gilchrist et al. 2015). We found the inhibition on PANX1 channels during maturation resulted in significantly higher cAMP concentrations following 6 hrs of maturation. Consistent with this finding we found, at 6 hrs of maturation, a significantly higher number of treated oocytes remained at the GV stage. This suggests that inhibiting the PANX1 channels delayed maturation though it appears temporary as by 22 hrs a similar level of cAMP and number of mature oocytes were seen compared to differences found at 6 hrs of maturation. Though, this delay appears to benefit the developmental competence of the oocytes as a higher proportion of treated oocytes successfully cleaved and developed to the blastocyst stage following maturation in the presence of 10Panx. Further evidence of delayed maturation can be seen following treatment with cAMP modulators.

Treatment of COCs with cAMP modulators during IVM lengthens the time of cumulus-oocyte gap junctional communication (Franciosi et al. 2014; Luciano et al. 2011). Our findings that cumulus expansion is decreased with 10Panx treatment suggests prolonged gap junctional communication similar to that during cAMP modification since cumulus expansion is a signal for gap junction communication loss (Larsen et al. 1986). These studies help support that PANX1 channels play a role in oocyte maturation; in that inhibition of these channels

causes a delay in maturation and improved developmental competence by way of cAMP modulation.

Finally, to determine a potential functional result to impairing PANX1 channel function we compared the level of ROS in the oocytes following maturation with exposure to 10Panx. We found that oocytes that were matured while inhibiting PANX1 channels had significantly less ROS when compared to our untreated controls. It has been well established that ROS buildup in oocytes is detrimental to continued embryo development. ROS have been implicated in causing negative developmental outcomes in embryos *in vitro* (Guerin et al. 2001; Takahashi 2012). ROS cause many different types of damage to developing embryos including a rise in lipid peroxides, increase in protein oxidation, and DNA strand breaks (Guerin et al. 2001; Nasr-Esfahani and Johnson 1992; Orsi and Leese 2001). It has been shown that high levels of ROS in the *in vitro* culture environment are detrimental to bovine embryo development (Favetta et al. 2007). A more recent study by Li et al. showed the effects of cAMP modulation during IVM on ROS defense. They found that implementing a pre-IVM culture period with forskolin (an adenylate cyclase activator) and IBMX (PDE inhibitor) not only delayed maturation and improved embryo developmental outcomes, but also increased the levels of antioxidant GSH and decreased the intra-oocyte concentration of the ROS hydrogen peroxide (Li et al. 2016). This study could lead to experiments that connect inhibited PANX1 channels during IVM and

cAMP modulation, with improved *in vitro* oocyte developmental competence and lower ROSs.

In conclusion, we have found that PANX1 is expressed in bovine cumulus cells. It is also expressed at higher levels in less developmentally competent oocytes. PANX1 channel inhibition during IVM leads to a delay in meiotic maturation and greater developmental competence following IVF. Functionally, PANX1 inhibition during IVM potentially delays maturation by maintaining elevated cAMP levels, keeping spontaneous maturation from occurring as quickly, which in turn can lead to improved developmental outcomes. ROS levels were also decreased in the oocyte with PANX1 inhibition. These findings support that PANX1 channels are important in oocyte maturation and development and can be manipulated to provide better developmental outcomes during *in vitro* settings.

III. Results, Conclusions, and Future Studies

Embryo production by way of IVM and IVF needs to be improved if maximum reproductive outcomes are to be realized. Efforts have been put into place to alter IVM by modulation of culture media to delay spontaneous maturation and improve developmental outcomes. Pannexins, being a relatively newly discovered family of protein channels, have not been extensively studied in female reproductive tissues. Here we show that not only are they expressed in bovine COCs, but the channels play a functional role in oocyte maturation. As implemented by cAMP or ligand/receptor modulation, inhibition of the PANX1 channel during maturation delays spontaneous maturation and improves cleavage and blastocyst rates of fertilized oocytes. These findings could support PANX1 inhibition as a culture modification to improve bovine IVF outcomes if implemented appropriately. Our studies show that PANX1 plays a role in the maturation of bovine COCs. This is shown in that PANX1 inhibition delays maturation, slows cumulus expansion, and maintains higher cAMP concentrations. Further investigation should take place to determine if PANX1 is expressed throughout the ovary, follicles, and other reproductive tissues. While we have shown that inhibition of PANX1 communication in COCs improves maturation and development of bovine oocytes, the mechanisms involved remain to be studied. The temporal expression and behavior of PANX1 during

folliculogenesis, ovulation, maturation, and fertilization will all be critical to our understanding. Here we see that PANX1 inhibition causes a drop in intracellular ROS levels. With the varying functions that PANX1 has been connected to in other tissues, the possibilities are numerous for experimentation into PANX1 reproductive functions.

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