

Investigating the role of connexin 43 in oocyte developmental competence

by

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A thesis submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Auburn, Alabama
August 4, 2018

Keywords: Connexin 43, Oocyte, Cumulus, *In Vitro* Fertilization (IVF)

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Abstract

In this study we are committed to understanding the role of connexin 43 (CX43)-based, cumulus cell cellular coupling and its relationship to bovine embryo development. Several studies have provided clear evidence that CX43-based coupling between the cumulus cells is critical for producing a healthy oocyte. Previously, the expression and localization of CX43 has been linked to resulting embryo outcomes in several studies. However, the relationship has largely remained undefined due to limitations inherent in group culture *in vitro* fertilization (IVF). To better associate the expression and localization of CX43 with *in vitro* embryo outcomes we first developed a bovine single cumulus-oocyte-complex (COC) IVF and embryo culture system.

Furthermore, all-*trans* retinoic acid (ATRA) signaling has been shown to enhance Cx43 expression and cellular coupling in a variety of cell types. ATRA has been shown to have beneficial effects on oocyte maturation, fertilization, and embryo development in a number of species. Therefore, we investigated the ability of ATRA to enhance cumulus cell coupling and influence bovine embryo production.

Clarifying the relationship between cumulus CX43 expression and embryo outcomes has the potential to allow for the selection of COCs with higher developmental capacities, limiting the current inefficiency of bovine IVF systems.

Furthermore, the enhancement of CX43 expression may allow for improvements in the number of COCs available for use in advanced reproductive technologies.

Acknowledgements

First, and most importantly, I would like to take this opportunity to thank my supervisor, Dr. Paul Dyce. When I first joined your lab I was excited to be working with someone as excited and passionate about research as I was. Since then, I have valued your guidance, encouragement, and confidence in my scientific abilities throughout the course of my Masters. I have especially appreciated you helping me learn how to focus my “hail mary” experiments into ones that better and more efficiently answer the questions being asked. I truly learned a lot, enjoyed every minute of it, and couldn’t have asked for a better mentor. I’d next like to thank my committee members. Dr. Fernando Biase for your patience while teaching me RNA sequencing and your kind words throughout my project. Dr. Chad Foradori for allowing me to work in your lab during my undergraduate degree and introducing me to research. Finally, Dr. Terry Brandebourg, many thanks for the support, advice, and friendship throughout my time at Auburn. You introduced me to both my undergraduate and masters supervisors- I truly wouldn’t be where I am today without your help. I would also like to thank my family and friends. Notably Velislov Stamenov, Taylor Papstein-Novak, Nate Fairbank, Tessa Dimick, and John Perkins for making the hours not spent in the lab fun and memorable. I would also like to thank my many undergraduate research assistants, my labmates and the members of Dr. Biase’s

lab for the support and assistance with my project. Additionally, I would like to thank Mama Mocha's coffee shop for being the perfect place to write, Brown Packing Co. for the assistance with sample collection, and Novus International for honoring me with their graduate research award.

Last, but not least, I would like to thank the Alabama Cattlemen's Association, the Alabama Agricultural Experiment Station, and the Hatch program of the National Institute of Food and Agriculture, US Department of Agriculture for providing funding for these studies.

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List of Abbreviations

ADH	alcohol dehydrogenase
AI	artificial insemination
ALDH	acetaldehyde dehydrogenase
ATRA	all- <i>trans</i> retinoic acid
BMP15	bone morphogenetic protein-15
BSA	bovine serum albumin
cDNA	complementary DNA
COC	cumulus-oocyte-complex
CRBP-II	cellular retinol binding protein type II
CTCF	corrected total cell fluorescence
CX32	connexin 32
CX37	connexin 37
CX43	connexin 43
CYP19A1	cytochrome P450 19A1
CYP26A1	cytochrome P450 26A1
CYP26B1	cytochrome P450 26B1
DAPI	4', 6'-diamidino-2-phenylindole
DMC1	dosage suppressor of mck1 homolog
DNA	deoxyribonucleic acid

ECL	enhanced chemiluminescence
EFAF	essentially fatty acid free
EGFr	epidermal growth factor receptor
ET	embryo transfer
FSH	follicle stimulating hormone
GDF9	growth differentiation factor 9
GJA1	gap junctional protein alpha 1
GJIC	gap junctional intercellular communication
HDC	high developmental competence
IVF	<i>in vitro</i> fertilization
IVP	<i>in vitro</i> production
KGN	immortalized granulosa cell line
KL	kit ligand
LDC	low developmental competence
LH	luteinizing hormone
LHR	luteinizing hormone receptor
LRAT	lecithin:retinol acyl transferase
MAPK	mitogen-activated protein kinase
mRNA	messenger RNA
PGC	primordial germ cell
PI3K	phosphoinositide-3 kinase

PPAR	peroxisome-proliferator activated receptors
QPCR	quantitative polymerase chain reaction
RAR	retinoic acid receptor
RARE	retinoic acid response element
RXR	retinoid X receptor
RBP	retinol binding protein
RNA	Ribonucleic Acid
RT	reverse transcribed
SOF	synthetic oviductal fluid
STRA6	stimulated by retinoic acid 6
STRA8	stimulated by retinoic acid 8
TUNEL	terminal deoxynucleotidyl transferase-dUTP nick end labeling
TZP	transzonal projection
μl	Microliter
μM	Micromolar
μm	Micron
VAD	vitamin A deficient

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. 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.

In domesticated animal species, including cattle, the primordial follicle pool is established during fetal development. Primordial follicles consist of an immature oocyte surrounded by a single layer of flattened granulosa cells (Aerts 2010a). In cattle, primordial germ cells (PGCs) originate in the epithelium of the yolk sac and migrate to the gonadal ridges (Rüsse 1991). During this migration, the PGCs multiply via mitotic division (Aerts 2010a). Once they arrive in the gonads, the germ cells form mitotically active clusters that consist of multiple oogonia surrounded by somatic cells (Aerts 2010a). Following mitotic expansion they enter the first meiotic division and arrest in the diplotene stage of prophase I,

forming primary oocytes (Aerts 2010a). Once they enter meiosis, the cluster of oogonia is divided by the surrounding somatic cells resulting in the formation of a primordial follicle. In cattle, the number of PGCs in the fetal gonad is estimated at around 2,100,000, but through apoptosis is reduced to ~130,000 at birth (Erickson 1966a and 1966b). These remaining germ cells form the follicular reserve available to the adult animal and contribute to their reproductive lifespan. Primary oocytes formed during fetal development remain meiotically arrested until the female undergoes puberty and begins cycling. A subset of the primordial follicles begin to grow in response to signals, eventually becoming atretic or forming antral follicles. The activation of primordial follicles is continuous, while the selection of the resulting antral follicles during the estrous cycle occurs in waves (Aerts 2010a). The bovine estrous cycle consists of two to three of these growth waves, the last of which results in ovulation (Aerts 2010b). These growth phases are further subdivided into recruitment, selection, and dominance phases. In the recruitment phase of cattle, an increase in circulating follicle stimulating hormone (FSH) causes a cohort of around 5-10 antral follicles to escape apoptosis (Driancourt et al. 2000). The selection phase consists of the selection of a single follicle, usually the largest, to become the dominant follicle and progress towards ovulation. During the dominance phase, this follicle grows in size and begins secreting estradiol and inhibin, decreasing the production of FSH. The remaining follicles still rely on FSH for growth and, therefore, undergo atresia and regress (Jiang et al. 2017). Around the time of FSH inhibition, the dominant follicle begins to express luteinizing hormone receptors (LHRs),

suggesting a transition from FSH to luteinizing hormone (LH) dependency (Aerts 2010b). LH pulses stimulate the production of androgens by thecal cells within the ovary. Stimulated by basal FSH levels, granulosa cells subsequently convert these androgens to estrogen. As the dominant follicle grows to pre-ovulatory size it produces increasing amounts of estrogen (Nuttinck and Peynot 2000). Estrogen reaches a threshold resulting in an ovulatory surge of LH from the anterior pituitary.

Oocyte health and fertility

Oocyte maturation is coordinated with the growth of the surrounding follicle. Kit ligand (KL), produced by the granulosa cells, is one factor involved in the early growth of the oocyte. Upon binding of KL to the KIT receptor on the oocyte, several signaling pathways are activated that regulate cell survival/apoptosis. These include RAS, RAF, MAP kinase, and Akt (Kidder and Vanderhyden 2010). Two oocyte secreted factors, growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15), have also been identified. The oocyte secretes BMP15 and GDF9 which induce the expression of epidermal growth factor receptor (EGFr) by cumulus cells and stimulates cumulus cell growth, decreases cumulus cell apoptosis, and stimulates energy metabolism and expansion (Coticchio 2015). The importance of oocyte signaling to granulosa cell function is shown in a study by el-Fouly *et al.* (1970). *In vivo* removal of the oocyte from the pre-ovulatory follicle caused the granulosa cells to spontaneously luteinize and form a corpus luteum. This study suggested that

either the oocyte or oocyte-secreted factors suppress LH-induced differentiation of its corresponding granulosa cells. This concept is supported by studies showing that oocytes secrete paracrine factors that inhibit LH receptor expression and progesterone production by granulosa cells in mice, pigs, and cattle (Eppig and Wigglesworth 1997, Vanderhyden 1995, Coskun et al. 1995, Li et al. 2000). The bidirectional signaling between the oocyte and cumulus cells is imperative for both the maturation of the oocyte and proper functioning of the cumulus cells. Oocytes and cumulus cells are also coupled via gap junctions which allow for the passage of small molecules (<1kDa) like nutrients, ions, cAMP, and cGMP between the oocyte and granulosa cells (Winterhager and Kidder 2015, Norris et al., 2009, Mao et al., 2013). Connexin 43 (CX43), one of the most prevalent gap junction proteins in the gonads (Kidder 2002, Winterhager 2015), is essential for proper oocyte maturation and will be discussed in further detail later in this chapter.

Oocyte developmental competence is broadly defined as the ability of the oocyte to undergo meiosis, be fertilized, and develop into a healthy embryo. While paternal influences are of importance after fertilization, they typically exert their influence after the embryonic genome is activated, around 3-4 cellular divisions in cattle (Graf and Krebs 2014). Because of this, oocyte growth and acquisition of developmental competence during folliculogenesis is imperative. Oocyte maturation occurs in two stages: cytoplasmic maturation and nuclear maturation. Cytoplasmic maturation involves the passage of molecular messages between the granulosa cells and oocyte via paracrine signaling and gap junctional

communication (Winterhager and Kidder 2015, Ikeda et al. 2005). Cytoplasmic maturation involves an increase in oocyte size, increased transcripts, and increased nutrient and energy stores in order to support cleavage post fertilization until the embryonic genome is activated (Graf and Krebs 2014, Ikeda et al. 2005). Nuclear maturation involves the resumption of meiosis and occurs after gap junctions have been decoupled and the meiosis-inhibiting cGMP/cAMP is no longer transported to the oocyte (Winterhager and Kidder 2015). Nuclear and cytoplasmic maturation of the oocyte are critical steps in forming a developmentally competent oocyte.

Bovine *in vitro* embryo production

In cattle production systems, *in vitro* embryo production has the potential to improve both crossbreeding- and genetic selection-based programs. Under artificial insemination (AI) and natural cover production systems, the female's contribution to genetic improvement is limited. Genetically valuable females are, at best, only able to produce one or two offspring per year. This results in an underutilization of the female's genetic potential. *In vitro* maturation and fertilization and subsequent embryo transfer (ET) systems have the ability to increase progeny from these genetically superior females, increasing their reproductive potential. Incorporating *in vitro* produced (IVP) embryos into a production system brings with it many benefits. A decreased generation interval combined with the ability to select embryos with the desired allelic variant of a specific gene allow for an increase in the selection rate of given phenotypic traits

(Hansen and Block 2004). Additionally, sexed semen can be more efficiently utilized in these systems. Sexed semen is able to fertilize more oocytes *in vitro* than in AI based systems (Hansen 2006). IVP embryos can also be used to improve production in herds with low fertility. This is especially important in the dairy industry, which has experienced declines in fertility over time (Royal et al. 2000, Lucy 2001). This decline in fertility is largely attributed to early embryo loss (Berg et al. 2010). While fertilization remains high, accepted to be close to 90%, the pregnancy rate per service is around 40-55% and is declining (Leroy 2008). The greatest loss of pregnancy occurs in the developmental stages between fertilization and days 5-7, the morula/blastocyst stage (Hansen et al. 2011). This stage of embryo development is largely the responsibility of the oocyte and relies on nutrients and genetic material stores acquired during maturation. While paternal influences are of importance after fertilization, they typically exert their influence after the embryonic genome is activated, around 3-4 divisions in cattle (Graf and Krebs 2014). Because of this, proper maturation of the oocyte is imperative for the acquisition of developmental competence in the oocyte.

As mentioned, oocyte developmental competence is largely defined by the ability of the oocyte to undergo meiosis, be fertilized, and develop into a healthy embryo. In intensive beef and dairy production systems, cows are rebred following a period of negative energy balance that is a result of lactation (Butler, 2003). This causes them to be in a state of negative energy balance during oocyte production and conception. Negative energy balance has been associated with early embryonic mortality and linked to the follicular fluid

environment in cattle (Leroy et al. 2008a). This results in the production of low quality oocytes that possess poor developmental competence due to deficiencies in maturation (Leroy et al. 2008a, Leroy et al. 2008b, and Sartori et al. 2002). The use of IVP embryos circumvents these issues as shown in a study by Demetrio et al. in 2007. They compared the conception rates of artificially inseminated lactating cattle (n=227) to lactating cattle that received embryos from non-lactating cows (n=160). Pregnancy checks on day 42 showed that the embryo transfer group had an increase in pregnancy rate compared to the AI group (47% vs 34%, respectively).

While the embryo transfer group had an increased pregnancy rate, their numbers were still low. Despite the promise embryo transfer holds for improving genetic selection and fertility in the cattle industry, the field has room for improvement. A big limitation in IVP systems includes embryo and fetal survival rates that are less than desirable. While IVP techniques have improved over the years, the percentage of embryos that develop to the blastocyst stage has remained stagnant at around 40% (Guimarães et al. 2015). This poor development is attributed to selection of poor quality oocytes that lack developmental competence. Currently, selection of oocytes for use in IVP systems is based on morphologic criteria (Kussano et al. 2016). As evidenced by the low blastocyst and pregnancy rates, this has proven to be a poor indicator of developmental potential.

Due to the importance of proper oocyte maturation on developmental competence, the ability to pick the best quality oocytes would be advantageous.

Several oocyte characteristics have been used to differentiate between competent and incompetent oocytes including gene expression, organelle distribution, and oocyte diameter (Kussano et al. 2016). While useful tools, the majority of these methods are invasive to the oocyte and prevent its further use in an embryo production system. Due to the intimate relationship between development of the oocyte and the surrounding cumulus cells, it is thought that these cells can be used as noninvasive indicators of oocyte quality. Messenger RNA expression in cumulus cells has been linked to oocyte quality (Saini et al. 2015) and transcript analysis has identified potential markers in multiple mammalian species (Uyar et al. 2013), including cattle (Golini et al. 2014, Girard et al. 2015, Bunel et al. 2015, Read et al. 2018). Additionally, novel, single COC culture systems have been developed that allow researchers to link gene expression in cumulus cells to specific oocyte developmental outcomes (Read et al. 2018, Bunel et al. 2015). In these systems, COCs are placed in individual media droplets and small cumulus cell biopsies are removed and stored until developmental data is gathered after fertilization of the corresponding oocyte. Oocytes are assigned to high or low developmental potential groups depending on their embryo developmental outcomes. The corresponding cumulus cell biopsies are then analyzed for transcript and/or protein expression. Expression differences between the cumulus cells can then be determined and used as potential biomarkers of oocyte health and developmental potential in *in vitro* embryo production systems. While this single COC culture system holds promise for the identification of oocyte health biomarkers, current research

identifying the expression profiles of specific mRNA and protein biomarkers in cumulus cells is lacking. There is a need for research in this area as the selection of oocytes destined to fail leads to a substantial loss of efficiency in the cattle industry (Berg et al. 2010).

Connexin 43

A hallmark of multicellularity includes the ability of groups of cells to coordinate a reaction to a stimulus (Bruzzone et al. 1996). This allows for a more rapid response and is important for cells undergoing differentiation during development and for homeostasis during adult life. Cells primarily communicate through the exchange of small molecules, second messengers, and small metabolites (Bruzzone and White 1996). Coordination of cells in this manner requires a system that allows for the exchange of signaling molecules between neighboring cells and must be able to be rapidly modulated to continuously adapt to the needs of the group (Bruzzone et al. 1996).

Connexins are a group of channel-forming proteins (Evans and Vuyst 2006) that meet these needs. Connexins form specialized structures, called intercellular channels, which allow for the coupling of neighboring cells and the exchange of small molecules between them. They are found in a variety of vertebrate species and within a majority of organ systems including the brain, skin, gonads, liver, and heart (Bruzzone and White 1996, Willecke et al. 2002). Their rapid turnover rate, 1-5 hours (Falk et al. 2014), allows for a dynamic regulation of intercellular communication.

The connexin family of proteins have molecular masses that range from 25 to 62kDa and have ~20 members (Bruzzone and White 1996, Evans and Vuyst 2006, Winterhager and Kidder 2015). Connexin paralogues may differ in molecular mass between species. Because of this, both the species and molecular mass are used to identify the different proteins (Bruzzone and White 1996). Connexin 43 (CX43), however, is homologous between species and is the most prevalent within an organism (Bruzzone and White 1996, Solan and Lampe 2014). It is one of the primary connexins expressed in the gonads (Kidder 2002, Winterhager and Kidder 2015) and has been linked to oocyte health and embryo developmental outcome in humans (Wang et al. 2009, Best et al. 2015), rodents (Gittens 2005), and cattle (Read et al. 2018). For a list of connexins expressed in bovine reproductive tissues, see Table 1.

Table 1: Connexin expression in bovine tissues

Connexin	Tissue	Citation
Cx26	Placentomes	Pfarrer CD et al. 2006
Cx32	Placentomes, atretic follicles	Pfarrer CD et al. 2006, Johnson et al. 1999
Cx37	Oocyte, cumulus cells	Nuttinck et al. 2000
Cx43	Placentomes, cumulus cells	Pfarrer CD et al. 2006

Assembly and function

Connexins are transmembrane proteins located in the plasma membrane of cells. They are cotranslationally inserted into the rough endoplasmic reticulum and subsequently transported to the Golgi apparatus (Bruzzone and White 1996). Within the Golgi apparatus, individual connexins oligomerize to form a connexon made up of six connexin subunits (Evans and Vuyst 2006). These connexons are inserted into the plasma membrane and result in the formation of hemichannels. Hemichannels span the cell's plasma membrane and connect its cytoplasm to the extracellular environment. Intercellular channels form when connexin hemichannels of neighboring cells dock together, joining the cytoplasm of the two cells. These intercellular channels travel through the membrane and cluster together, forming gap junctional plaques (Evans and Vuyst 2006). Once these intercellular plaques are formed, transport of signaling molecules through these channels is neither passive nor promiscuous. Cells can regulate whether the channels are open or closed and can internalize entire gap junctional plaques (Evans and Vuyst 2006). Connexin channels themselves also limit intercellular signaling depending on the connexin composition of the channel (Bruzzone and White 1996). The various connexins form channels with differing molecular size permeability (typically up to 1 kDa) and ionic selectivity. Additionally, only hemichannels composed of compatible connexins can dock with one another.

The structure of connexins also plays an important role in its regulation by the cell. Connexins consist of four transmembrane domains, two extracellular loops, one cytoplasmic loop, and cytoplasmic N- and C-termini (Kidder 2002). The C-

terminal tail of connexins contain several phosphorylation sites recognized by various kinases (Solan and Lampe 2014). CX43, in particular, has 21 serines and 6 tyrosine residues that are able to be phosphorylated. Examples of kinases that interact with these sites include protein kinase A, protein kinase C, mitogen-activated protein kinase (MAPK), and Akt (Lampe 2004, Dun 2014). Variations in the phosphorylation of the C-terminal tail affect several stages in the lifecycle of CX43 including trafficking, assembly/disassembly, degradation, and the gating of the channels themselves (Lampe 2004). Phosphorylation of CX43 channels by different kinases changes their permeability (Bruzzone and White 1996). For example, open CX43 channels are permeable to both Lucifer Yellow fluorescent dye and current carrying ions. However, when they are phosphorylated by protein kinase C, they are no longer permeable to Lucifer Yellow dye (Kwak and van Veen 1995). Additionally, when they are phosphorylated by viral SRC tyrosine kinase they are no longer permeable to either Lucifer Yellow or current carrying ions (Swenson and Piwinca-Worms 1990). In addition to phosphorylation of the C-terminal tail, connexins can be stimulated to open under other stimuli including low extracellular Ca^{2+} , membrane depolarization, and metabolic inhibition (Bruzzone and White 1996). This dynamic control of connexin expression is integral to its function in intercellular communication. A diagram of CX43 assembly and disassembly can be seen in figure 1.

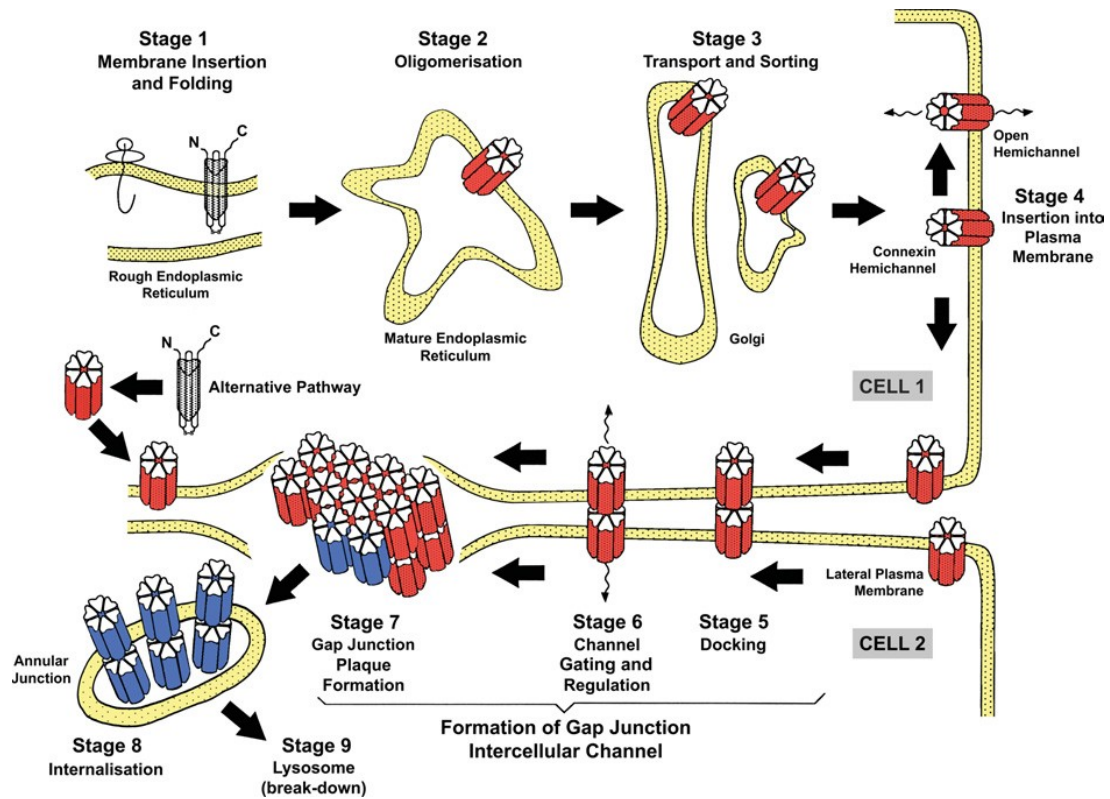


Figure 1: Assembly and function of CX43 gap junctional plaques. Figure from Evans et al. 2006 and used with publisher's permission.

Connexin 43 in the Ovary

While multiple connexins have been identified in ovarian follicles in several species, targeted gene ablation studies revealed that CX43 and connexin 37 (CX37) are essential for follicular growth in mice (Wang et al. 2009). Other connexin knockouts resulted in females that were fertile or died *in utero* (Winterhager and Kidder 2015). Multiple other studies have gone on to show the importance of CX43 to follicular development in other species including mice, cattle, and humans (Wang et al. 2009, Calder and Caveney 2003, Dyce et al. 2012).

Connexin 43 in folliculogenesis and oocyte developmental competence

Mammalian follicles consist of an oocyte surrounded by layers of granulosa and theca cells. Cell to cell interactions between these somatic cells and the oocyte are essential for oocyte development and folliculogenesis. Gap junctional intercellular communication (GJIC) has been shown to be involved in the control of cell differentiation (Bruzzone and White 1996, Kidder 2001, Dyce et al. 2014), cell proliferation, and cell migration (Kidder 2002). These actions are all important in folliculogenesis, from the migration of primordial germs cells to the proliferation and differentiation of cumulus granulosa cells.

As mentioned previously, both CX37 and CX43 have repeatedly been shown to be expressed in both growing and mature follicles. These two connexins are primarily responsible for the passage of molecular molecules between the oocyte and surrounding cumulus cells. CX37 is primarily expressed by the oocyte while CX43 is expressed in granulosa cells (Gittens 2005). These connexins allow for the passage of small molecules between the oocyte and granulosa cells through the formation of transzonal projections (TZPs) (Kidder 2002). TZPs are formed when granulosa cell membranes travel through the zona pellucida and contact the oocyte's surface. CX37 is largely responsible for coupling the oocyte with the surrounding cumulus cells, and CX43 with coupling cumulus cells to each other. Figure 2 depicts the location of CX37 and CX43 in the COC as well as the TZPs linking the two connexins. In mice, the expression of CX43 can be seen as early as primordial follicle formation (Juneja and Barr 1999) and increases in response

to rising FSH levels (Kidder and Vanderhyden 2010) while CX37 is present from the primary follicle stage onwards (Simon et al. 1997). This early presence of CX43 and CX37 shows that the oocyte and granulosa cells are coupled with each other at the onset of folliculogenesis.

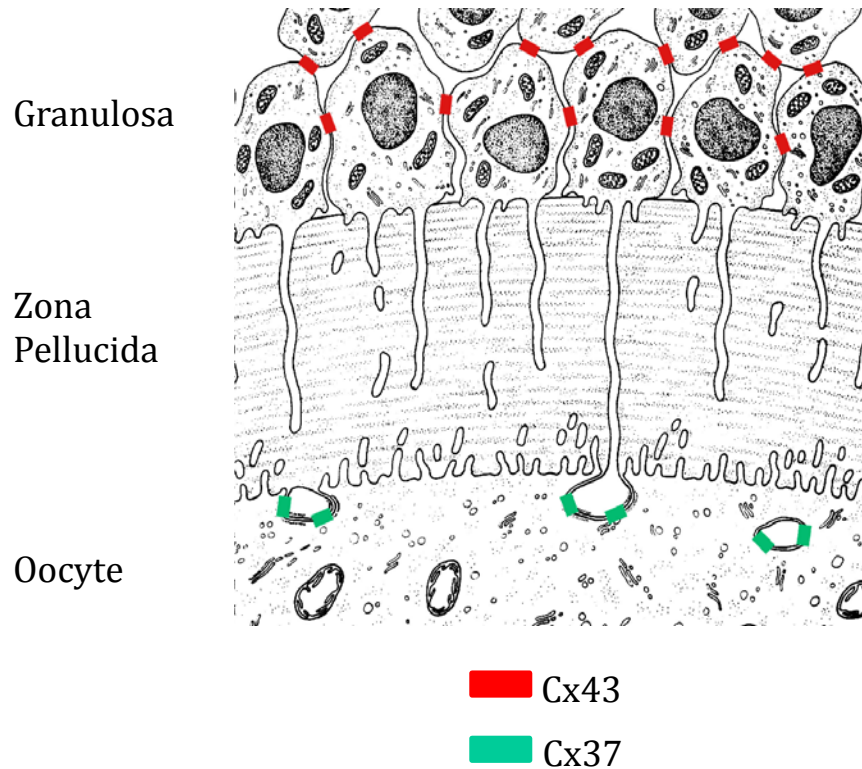


Figure 2: Localization of CX43 in cumulus-oocyte-complexes. CX43 is primarily expressed by the granulosa cells while CX37 is primarily expressed by the oocyte. Transzonal projections can be seen connecting the two gap junctions.

Adapted from Baker 1982.

These connexins are imperative for oocyte and follicular development as shown in a study by Gittens and Kidder (Gittens and Kidder 2005). They used chimeric,

reaggregated mouse ovaries to show the importance of CX43 and CX37 expression to oocyte development. The reaggregated ovary technique is an elegant way to study the cell-specific knock-out of gene products, whose knockout would render the organism unviable, on folliculogenesis. Using this method, genetic knock-out cells can be combined with wildtype cells to study cell-specific gene deficiencies. They showed that CX43-deficient granulosa cells paired with a wild-type oocyte and CX37-deficient oocytes paired with wild-type granulosa cells caused both follicle development and oocyte maturation to be impaired. Further support for the interactions of cumulus cells and oocytes via connexins 37 and 43 are shown in studies by Simon *et al.* (1997), Carabatsos *et al.* (2000), Juneja *et al.* (1999), and Ackert *et al.* (2001). Additionally, studies in CX37 knockout mice showed that folliculogenesis stalled in late preantral stages and oocyte growth at that stage was 74% of normal size (Simon *et al.* 1997, Carabatsos and Sellitto 2000). These studies also demonstrated the importance of oocyte secreted factors on the differentiation state of granulosa cells. For example, granulosa cells of CX37 knockout mice underwent premature luteinization (Simon 1997). CX43 knockout mice had very few germ line cells and folliculogenesis was retarded, arresting development prior to becoming multilaminar follicles (Juneja and Barr 1999, Ackert *et al.* 2001).

Cellular coupling, via gap junctions, within COCs creates a functional syncytium that allows for the passage of molecular messages. In mice, these messages have been shown to include nutrients like amino acids and glucose to support the oocyte's metabolism, ions to regulate the oocyte's pH, and cGMP to maintain

meiotic arrest in the oocyte (Winterhager and Kidder 2015). As follicle growth approaches the pre-ovulatory stages, the LH surge results in decoupling of the granulosa cells (Granot 2002). LH is thought to activate MAP kinase, resulting in the phosphorylation of CX43, disrupting the gap junctions and, subsequently, the flow of cGMP through to the oocyte (Norris et al. 2008). This allows the oocyte to resume meiosis and progress towards nuclear maturation.

The findings in these papers show the importance of gap junctional coupling between granulosa cells and the oocyte and granulosa cells and each other. This coupling is essential for growth and differentiation of the oocyte and differentiation of the granulosa cells. Passage of molecules and ions from the granulosa cells to the oocyte are required for growth, maturation, and survival. Because of its role in the acquisition of oocyte developmental competence, CX43 expression levels have been suggested as a potential biomarker for oocyte health (Wang et al., 2009). Not only do they pass important molecules to the oocyte, but they are located in the cumulus cells which are easily biopsied.

Retinoic Acid

Vitamin A is a fat-soluble vitamin that functions in many physiological processes. In cattle, a vitamin A deficiency has been shown to increase marbling (Oka et al. 1998) and aid in adaptive immunity (Yano et al. 2009). Vitamin A also functions in eye development and the production of visual pigments (Clagett-Dame and Knutson 2011). In addition to these functions, Vitamin A is especially important in reproductive processes. Metabolites of Vitamin A induce the expression of genes

that are important for germ cell entry into meiosis and oocyte development and maturation (Clagett-Dame and Knutson 2011, Li and Clagett-Dame 2009).

Vitamin A cannot be produced by the body and, therefore, must be obtained from the diet-primarily in the form of retinoids. In the case of grazing animals, vitamin A is obtained through β -carotene present in forages. β -carotene is cleaved into retinoids in the small intestine of these animals (Parker et al. 2017). Vitamin A is then stored in the liver until it is released into circulation to act on its target tissues. In keeping with a review by Blomhoff and Blomhoff (2006), the term vitamin A will be used to refer to any compound possessing the biological activity of retinol.

Once ingested, vitamin A is converted into multiple metabolites. These include retinol, retinal, and retinoic acid (Kawai et al. 2016). All-*trans* retinoic acid (ATRA) is one of the most abundant forms of retinoic acid and is considered the active form of vitamin A in the body (Blomhoff and Blomhoff 2006). Additionally, while many retinoids are fat soluble and require binding proteins for transport through hydrophilic phases, ATRA is also soluble to some extent in water.

Retinoic Acid Metabolism

In cattle, carotenoids primarily obtained from forages are cleaved into retinal by enterocytes in the small intestine. The retinal product is subsequently reduced to retinol. Cellular retinol binding protein type II (CRBP-II) binds this retinol and facilitates its esterification by lecithin:retinol acyl transferase (LRAT) into retinyl esters (Blomhoff and Blomhoff 2006). The majority of absorbed vitamin A is

released into intestinal lymph as chylomicrons composed of triacylglycerol, phospholipids, retinyl esters, and other vitamin A metabolites. These chylomicrons are subsequently released into portal circulation where they are degraded into chylomicron remnants that primarily consist of retinyl esters (Blomhoff and Blomhoff 2006).

Once in portal circulation, retinyl esters are filtered out by hepatocytes and hydrolyzed back into retinol. The retinol then either associates with retinol binding protein (RBP) and is secreted into circulation or is stored in perisinusoidal stellate cells. Once in the stellate cells, CRBP-I and LRAT convert the retinol back into retinyl esters. The ability of these cells to both store and control the release of retinol allows for a steady blood concentration of retinol despite dietary fluctuations (Blomhoff and Blomhoff 2006).

Once RBP-retinol is released into circulation, retinol is taken in by target tissues via CRBPI and CRBP II, the transporter stimulated by retinoic acid 6 (STRA6), or by passive transport into cells that interact with blood vessels (Kawai et al. 2016). Once in the cell, retinol is converted to retinal via alcohol dehydrogenase (ADHs), primarily ADH1, ADH3, and ADH4 (Kawai et al. 2016, Blomhoff and Blomhoff 2006). It is further converted to ATRA by acetaldehyde dehydrogenases (ALDHs), primarily ALDH1A1 and ALDH1A2 (Kawai et al. 2016, Blomhoff and Blomhoff 2006). ATRA can be degraded by cytochrome P450 enzymes (CYP26A1 and CYP26B1). CYP26A1 and CYP26B1 gene expression are induced by the genomic effects of ATRA. Increased ATRA concentrations in the cell cause increased expression of these enzymes. This mechanism acts as a

way to regulate the amount of ATRA present in the cell (Blomhoff and Blomhoff 2006, Jiang et al. 2017).

Signaling pathways: genomic and nongenomic effects

Intracellular ATRA is transported to retinoic acid receptors (RARs) by cytosolic proteins (Reichrath et al. 2007). These RARs come in three forms: RAR α , RAR β , and RAR γ . Nuclear RARs form heterodimers where one RAR is complexed with one retinoid X receptor (RXR). RARs and RXRs are nuclear receptors that act as ligand-dependent transcription factors (Reichrath 2007). RXRs solely bind 9-*cis* retinoic acid, a stereoisomer of ATRA, while both ATRA and 9-*cis* retinoic acid can bind RARs. As the effects of 9-*cis* retinoic acid on cellular function are controversial and ATRA is widely accepted to be the active form, ATRA will be the focus of this lit review. ATRA binds to the RAR present in the heterodimer and the complex enters the nucleus where it binds to retinoic acid response elements (RAREs) on the DNA. These RAREs are present in the promoter region of the affected gene. The consensus sequence of these RAREs consists of direct repeats of “(a/g)g(g/t)tca” separated most commonly by five or one nucleotide (Blomhoff and Blomhoff 2006). Once bound to the consensus sequence, the RAR complex regulates transcription of target genes by recruiting corepressors and coactivators (Parker et al. 2017). A diagram of ATRA metabolism and genomic actions can be seen in figure 3.

Nongenomic actions of ATRA include modulating the actions of proteins. ATRA has been shown to exert its effects on the MAPK, peroxisome-proliferator

activated receptors (PPAR), and the Janus kinase/STAT5 signaling pathways (Tanoury and Piskunov 2013). ATRA has also been shown to activate the Akt pathway in multiple cell types (García-Regalado et al. 2013, Bastien et al. 2006). The activation of these pathways has been shown to occur within minutes after exposure to ATRA (Tanoury and Piskunov 2013, García-Regalado et al. 2013). ATRA activation of these pathways varies by cell type, but involves the activation of upstream cascades involving Rho-GTPases, phosphoinositide-3 kinase (PI3K), and/or Akt (Tanoury and Piskunov 2013). These pathways play a key role in cellular proliferation, survival, and differentiation.

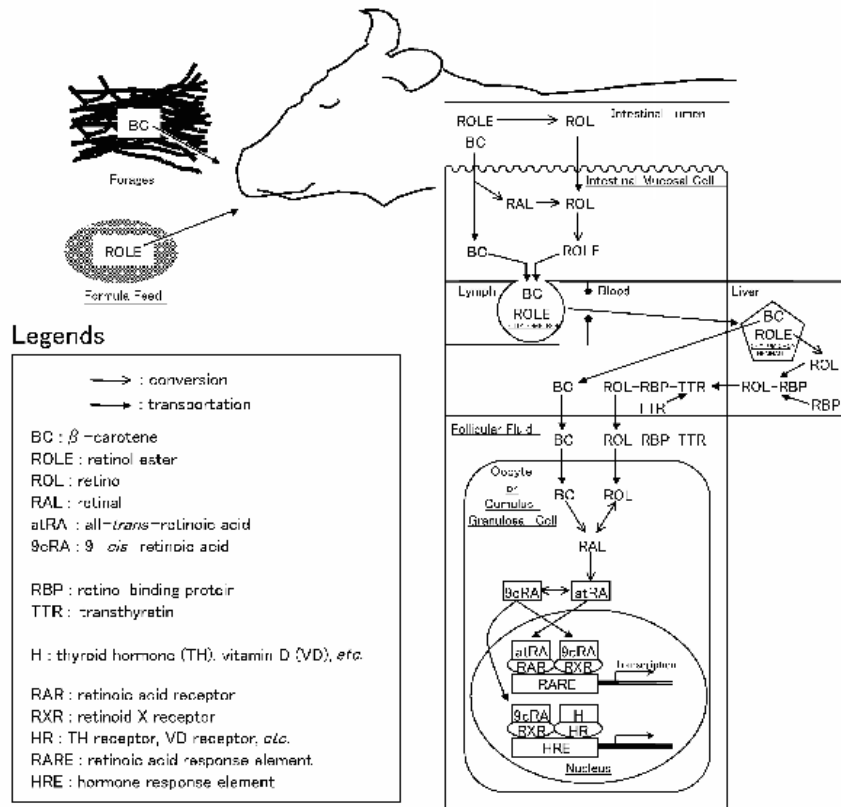


Figure 3: Metabolism and signaling pathways of ATRA in bovine. Figure from Ikeda et al. 2005 and used with publisher's permission.

Retinoic Acid in the Ovary

Several studies have shown that ATRA signaling is necessary for the development of both the male and female gonad and that severe vitamin A deficiency leads to reproductive failure in both sexes (Li and Clagett-Dame 2009). The genomic functions of ATRA are important for signaling processes involved in germ cell entry into meiosis, oocyte development and maturation, embryonic development, and epithelial cell differentiation and maintenance

(Clagett-Dame and Knutson 2011, Li and Clagett-Dame 2009, and Kawai et al. 2016).

Retinoic acid and folliculogenesis

In mammalian females, germ cells enter meiosis during embryogenesis, forming oocytes. This differentiation is signaled by the gonadal environment and is thought to be influenced by retinoic acid. Retinoic acid is present in the mesonephros in close proximity to the gonadal ridges (Bowles and Koopman 2007). During female embryogenesis, germ cells are exposed to ATRA, initiating their entry into meiosis while in the male, CYP26B1 enzymes produced by Sertoli cells degrades ATRA and prevents germ cell entry into meiosis (Jiang et al. 2017). In the female, primordial germ cells exposed to ATRA have an increased expression of the gene stimulated by retinoic acid 8 (*Stra8*). *Stra8* expression is followed by the initiation of meiosis in these cells. A study by Li and Clagett-Dame in mice (2011) showed significantly reduced expression of *Stra8* as well as dosage suppressor of mck1 homolog (*Dmc1*), a marker of cells undergoing meiosis, in the gonads of female embryos of vitamin A deficient (VAD) dams compared to vitamin A sufficient dams. This shows that ATRA is required for primordial germ cell entry into meiosis. Additional studies *in vitro* showed that ATRA treatment causes cultured murine ovaries to enter meiosis (Livera et al. 2000) and stimulates *Stra8* expression via RAR signaling pathways (Koubova et al. 2006). While ATRA deficiencies can block meiotic entry of primordial germ cells, so can high ATRA levels. Complementary expression of CYP26B1 and

ALDH1A1 are needed to produce optimal levels of ATRA in the fetal ovary and result in proper formation of the primordial follicle pool (Jiang et al. 2017). The germ cells formed during embryonic development make up the follicular reserve of the adult female. Errors in formation of the follicular reserve can lead to reduced fertility in the adult animal.

Retinoic Acid and Oocyte developmental competence

As the female animal undergoes embryonic development, the oocytes formed are arrested at the prophase stage of meiosis I. Once the female reaches puberty, these oocytes resume meiosis in response to LH and pause again at the metaphase stage of meiosis II. Resumption of meiosis is known as nuclear maturation. In the developing oocyte, this nuclear maturation is accompanied by cytoplasmic maturation, or acquisition of developmental competence (Ikeda et al. 2005). By eliciting genomic and nongenomic effects on the surrounding cumulus-granulosa cells, ATRA influences cytoplasmic maturation in the oocyte.

Initial evidence in support of ATRA's effects on oocyte development is the presence of retinol in follicular fluid. Retinol is filtered from the blood and transported to the follicular fluid. It has been shown that serum levels of retinol (Haliloglu et al. 2002) and RBP4 (Makimura et al. 2009) increase around the time of estrus. In addition, the expression of enzymes involved in the internalization of serum retinol by target cells and the conversion of retinol to ATRA also increases in antral and pre-ovulatory follicles (Kawai et al. 2016). Additionally, a study by Schweigert and Zucker (1988) showed that, in bovine, retinol concentrations

present in the follicular fluid of nonatretic follicles was significantly greater than retinol concentrations of atretic follicles. It has also been shown, in cattle, that oocytes and early embryos express the genes for RARs, RXRs, RBP, and ALDH2 (Mohan 2003). In addition to containing the enzymes needed to produce ATRA, cumulus-granulosa cells also contain biologically active RARs (Zhuang et al. 1994), showing they are capable of responding to ATRA.

ATRA treatment of cumulus-granulosa cells *in vivo* has been shown to change the expression of genes that influence cellular differentiation and growth. These genes include decreased expression of gonadotrophin receptors, increased midkine expression, and decreased expression of cyclo-oxygenase-2 and nitric oxide synthase. These changes are caused by the genomic actions of ATRA and prevent premature differentiation and apoptosis of the cumulus granulosa cells. This prevents premature and abnormal maturation of the oocyte (Ikeda and Kitigawa 2005). Nongenomic actions of ATRA vary by cell type and involve the modification of translation, post-translational processing, induction of protein synthesis and modification of cellular proteins (Livingston 2003). Currently, there is no research showing the nongenomic effects of ATRA in female ovarian cells. However, in the male, ATRA has been shown to phosphorylate Kit, activating it. This results in the activation of Kit's downstream effectors PI3K and MAPK, initiating meiosis (Pellegrini et al. 2008).

Improved maturation of the oocyte caused by ATRA's effects on the cumulus-granulosa cells is further shown by looking at post-fertilization development. A study by Kawai et al. (2016) showed the importance of vitamin A to oocyte

health. Initially, they showed that mice fed a VAD diet had decreased retinol in their follicular fluid compared to a vitamin A sufficient control. They then went on to show these VAD mice ovulated fewer oocytes. These VAD oocytes also had a lower maturation rate and, once they were fertilized, had a lower blastocyst rate. They then went on to treat mice with an ADH inhibitor, preventing the synthesis of ATRA. The inhibited group ovulated fewer oocytes and, once they were fertilized, had a lower fertilization and blastocyst rate compared to control mice. Additionally, they treated a group of ADH inhibited mice with RA and saw ovulated oocyte numbers and developmental rates return back to control levels. These results suggest that vitamin A deficiency affects cytoplasmic maturation of the oocytes- the acquisition of developmental competence for embryonic development after fertilization. Another study in humans showed that oocytes obtained from follicles with higher ATRA concentrations yielded a significantly higher percentage of embryos with a higher quality grade (Pauli et al. 2013). A study by Pu et al. (2014) shows that goat oocytes treated with ATRA have an increased nuclear maturation rate compared to control. Retinol treatment of superovulated cattle resulted in embryos with increased embryo quality grades. It also increased the number of transferable embryos and the blastocyst rate (Shaw et al. 1995). *In vitro*, retinol treatment of bovine COCs improved the blastocyst rate compared to control.

These studies show that cumulus cells, the oocyte, and the early embryo express key components of retinoid metabolism and signaling. They also suggest that the

reproductive tract delivers vitamin A to the COC and early embryo, eliciting changes in gene expression, differentiation, and development.

Retinoic Acid and Connexin 43

ATRA treatment of bovine cumulus cells *in vivo* has been shown to change the expression of genes that influence cellular differentiation and growth (Makimura et al. 2009). This change in expression is thought to prevent premature apoptosis and differentiation of the cumulus cells, preventing premature and abnormal maturation of the oocyte. Proper maturation of the oocyte is critical as it has been clearly demonstrated that oocyte health plays a major role in the resulting embryo's success following fertilization (Breukman et al. 2012). GJIC has also been shown to be involved in the control of cell differentiation and proliferation (Willecke et al. 2002, Winterhager and Kidder 2015, and Kidder and Vanderhyden 2010). Connexin 43 (CX43), a gap junctional protein, has been shown to increase throughout the progression of folliculogenesis (Nuttinck et al. 2000), concurrent with increases in ATRA concentrations.

ATRA treatment has been shown to increase CX43, and subsequently GJIC, in multiple cell types including human leukemic bone marrow stromal cells, human oral cancer cells, and canine primary lens epithelial cells (Wang et al. 2013, Liu et al. 2015, and Long et al. 2010). ATRA has also been shown to increase CX43 expression and GJIC in reproductive tissues, as shown in studies by Tanmahasamut and Sidell (2005) and Wu et al. (2013) looking at human endometrial stromal cells. Additionally, primary human granulosa cell cultures and

an immortalized granulosa cell line (KGN) treated with ATRA had changes in CX43 phosphorylation while the KGN cells also had increases in GJIC (Best et al. 2015).

CX43's structure plays an important role in its regulation by the cell. CX43 has a long, cytoplasmic C-terminal tail that contains several phosphorylation sites (Solan and Lampe 2014). These sites are recognized by various kinases including protein kinase A, protein kinase C, MAPK and Akt (Lampe 2004, Dunn 2014). Variations in the phosphorylation of the C-terminal tail affect several stages in the lifecycle of CX43 including trafficking, assembly/disassembly, degradation, and gating of the channels themselves (Lampe 2004). Phosphorylation of CX43 at S373 of its C-terminal tail by Akt results in enlarged gap junctions and increased gap junctional communication (Solan and Lampe 2014, Dunn 2014).

Project Rationale

Currently, COCs are morphologically assessed to determine the quality of the oocyte. This leads to inefficiencies in embryo production systems that result in losses of both time and money. In an effort to noninvasively test for oocyte developmental competence, single COC culture protocols have been developed to allow researchers to link oocyte developmental competence to transcript and protein expression in cumulus cells. In this study, our lab sought to investigate the relationship of CX43-based cumulus cell coupling to embryo developmental outcome. Previous studies have shown that the expression and localization of

CX43 is linked to embryo outcomes. Our lab developed a bovine single COC IVF and embryo culture system to establish CX43 expression as a potential biomarker for oocyte health.

Additionally, multiple studies have shown ATRA signaling to enhance CX43 expression and cellular coupling. Furthermore, oocyte maturation, fertilization, and embryo development have been shown to benefit from ATRA signaling. Because of this, we decided to investigate the role of ATRA in CX43 expression and its effects on bovine oocyte developmental potential.

Identifying CX43 as a potential biomarker for oocyte developmental competence has the potential to allow improved selection of COCs for use in embryo production systems. Additionally, the ability to increase CX43 expression in COCs may improve the number of developmentally competent COCs available for use in embryo production systems.

II. Connexin 43 coupling in bovine cumulus cells during the follicular growth phase, and its relationship to *in vitro* embryo outcomes

DOI: 10.1002/mrd.22993

OBJECTIVE

Investigate the relationship between CX43 and embryo developmental outcome.

SPECIFIC AIMS

1. Develop a single COC IVF assay.
2. Determine the effect biopsies have on oocyte development and resulting blastocyst health.
3. Compare the expression of CX43 and GJIC in cumulus cells corresponding to embryos with high and low developmental potential.

ABSTRACT

Gap junctional coupling between cumulus cells is required for oocytes to reach developmental competence. Multiple connexins, which form these gap junctions, have been found within the ovarian follicles of several species including bovine. The aim of this study is to determine the role of connexin 43 (CX43) and its relationship to embryo development, following *in vitro* fertilization (IVF). Cumulus-oocyte-complexes (COCs) were obtained from abattoir-sourced, mixed breed, bovine ovaries. COCs were isolated from follicles ranging from 2-5mm in size, representing the preselected follicle pool. Immediately following isolation, two cumulus cell biopsies were collected and stored for analysis pending determination of developmental outcomes. Using *in vitro* procedures, COCs were individually matured, fertilized, and cultured to the blastocyst stage. Biopsies were grouped as originating from COCs that arrested at the two cell stage (low developmental competence, LDC) or having developed to the late morula/blastocyst stage (high developmental competence, HDC), following IVF and embryo culture. The expression level of CX43 was found to be significantly higher in cumulus cells from COCs that had a HDC when compared to those that had a LDC. Moreover, the gap junctional intercellular coupling (GJIC) rate was significantly higher in cumulus from COCs deemed to have a HDC. Significantly higher expression of the cumulus health markers luteinizing hormone receptor (LHR) and cytochrome p450 19A1 (Cyp19A1) was found in the cumulus originating from oocytes with high developmental competence, suggesting this

system may provide a mechanism for non-invasively testing for oocyte health in preselected bovine follicles.

INTRODUCTION

It has been clearly demonstrated that oocyte health plays a major role in the resulting embryo's success, following fertilization (Breukelman et al. 2012). Important critical aspects of oocyte health include the ability to resume meiosis, cleave, and continue development to the blastocyst stage following fertilization. These attributes are largely believed to be the responsibility of the oocyte. During follicular development, cellular interactions between the oocyte and its surrounding cumulus cells encourage oocyte maturation and development. The symbiotic relationship between cumulus-granulosa cells and the oocyte is critical for both oocyte growth and acquisition of competence and granulosa cell functioning. In mice, oocyte secreted factors including GDF9 and BMP15 have been shown to be potent regulators of cumulus cell differentiation and function (Gilchrist et al. 2008; Diaz et al. 2007). It has been shown, in mice, that the ability of oocytes to undergo maturation and support post-fertilization development is compromised in denuded oocytes when compared to oocytes enclosed by cumulus cells (Chang et al. 2005). Cumulus cells have been demonstrated to have positive effects on oocyte growth and maturation through their response to follicle stimulating hormone (FSH) and epidermal growth factor (EGF)(Downs et al. 1988). Gonadotropins produce these beneficial effects on the oocyte by binding to receptors that are expressed exclusively by the cumulus cells (Chang

et al. 2005). This bidirectional relationship is supported through the cellular coupling of cumulus cells to the oocyte that allows for small molecule communication between these two cell types.

Cellular coupling, via gap junctions, within COCs creates a functional syncytium that allows for the passage of molecular messages. Gap junctions are groups of intercellular membrane channels formed by members of the connexin family of proteins. These channels allow for the coupling of cells in order to exchange small molecules, including nutrients, metabolites, and secondary messengers (for review see Evans and Martin, 2002). Among these small molecules are signals regulating oocyte maturation including Ca^{2+} and cAMP. The coupling of the oocyte to surrounding cumulus has been shown to effectively arrest meiosis with a drop in connexin 43 (CX43) being associated with meiotic progression (Calder et al. 2003; Edry et al. 2006). Recent findings have implicated gap junctional proteins in many aspects of fertility and suggested a role in female infertility (Winterhager and Kidder, 2015). Multiple proteins make up the connexin family with different family members forming channels with differing permeability to specific molecules. This supports the different physiological roles played by connexins in different cell types. Multiple connexins have been identified in ovarian follicles in several species (Winterhager and Kidder, 2015). The specific roles of connexins within the ovarian system have been clearly determined using targeted gene ablation in mice. Granulosa cells from CX43 knockout mice do not show evidence of gap junctional coupling and their oocytes fail to achieve meiotic competence (Ackert et al. 2001; Gittens et al.

2005). Conversely, connexin 37 knockout mice show lack of gap junctional coupling between the oocyte and surrounding cumulus cells and results in oocyte loss and premature luteinization of the follicles (Simon et al. 1997). This supports the important role of connexins in developing competent oocytes.

Evidence has shown that granulosa cells in mouse and human are largely coupled through the expression of CX43 and the formation of gap junctions (Ackert et al. 2001; Gittens and Kidder 2005; Wang et al. 2009; Kidder and Mhawi 2002; Gittens et al. 2005). In the bovine ovary, CX43 has been shown to be expressed in the granulosa cell compartment and to increase concurrent with antrum formation and growth, with maximum expression at the onset of ovulation (12-20mm in size)(Nuttinck et al. 2000). In cows there are two or three waves of follicles entering the growing pool each estrous cycle (Fortune et al. 1991; Ginther et al. 1996). Selection of the dominant follicle and the onset of atresia in subordinate follicles occurs when one follicle becomes larger (≥ 8 mm) than the remaining cohort (≥ 5 mm)(for review see Rodgers and Irving-Rodgers, 2010). While it is well characterized that ovulatory follicles (≥ 8 mm) express CX43 at a higher level than small follicles (≤ 5 mm), the differential expression as it relates to pre-selection follicles remains uncharacterized (Nuttinck et al. 2000). It is well understood that bovine follicles ≥ 8 mm have a better developmental potential *in vitro* than smaller follicles (Fortune et al. 1991; Ginther et al. 1996). The ability to discriminate between follicles based on developmental potential, within the larger heterogeneous small follicle population, would benefit *in vitro* embryo production efficiencies.

Currently, there is limited knowledge on the relationship between CX43 cellular coupling and oocyte health. However, studies in mice have shown that nutrients to support the growing oocyte's metabolism, ions to regulate the oocyte's pH, and cGMP to support meiotic arrest pass from the cumulus cells to the oocyte via the gap junctions of this syncytium (for review see Winterhager and Kidder, 2015). However, the relationship between CX43 expression levels in cumulus cells and oocyte quality remains controversial. Recently, a study in humans looking at oocytes aspirated from dominant follicles, provided evidence that increased CX43 expression and cellular coupling had a positive relationship with human *in vitro* fertilization (IVF) pregnancy outcomes (Wang et al. 2009). Conversely, a study looking at the mRNA levels of the CX43 transcript gap junctional protein alpha 1 (*GJA1*) in human cumulus cells found a lower level of *GJA1* transcripts in cumulus cells from human oocytes that developed to the blastocyst stage than those that did not (Feuerstein et al. 2007). These results are likely explained by the stage at which the oocytes were isolated and the significant post-translational modifications that affect the location and functioning of CX43 (Solan and Lampe 2014). For example, in bovine it has been demonstrated that COCs with a higher quality grade prior to maturation had cumulus cells that expressed more *GJA1* transcripts than those with a lower grade, but found that the overall level of CX43 mRNA decreased as oocytes matured (Calder et al. 2003). Therefore, studies limited to comparing mRNA expression in cumulus cells often fail to identify *GJA1* as differentially expressed (Bunel et al. 2015; Melo et al. 2017).

In order to provide clarity to this controversy we have utilized an *in vitro* single COC fertilization and embryo development system to retroactively compare the level and strength of cumulus cell coupling to eventual embryo outcomes. We utilized COCs isolated from pre-selection follicles (2-5mm) in order to establish the use of CX43 as a marker for oocyte developmental potential. The ability to reliably select oocytes with a high developmental potential from the pre-selection follicle pool will allow improvements in the efficiency of using IVF for *in vitro* embryo production. Therefore, the objectives of this study are to compare CX43 expression and gap junctional coupling rate to eventual embryo outcomes in individual COCs isolated from pre-selection follicles.

MATERIALS AND METHODS

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

Single COC IVF System

COCs were collected from abattoir-sourced, mixed breed, *Bos taurus* ovaries. For comparing the expression of CX43 in large and small follicles, follicles less than or equal to 2mm were considered small and follicles 8mm or larger were considered large. In all other experiments, follicles ranging from 2-5mm (pre-selection follicles) 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 3 layers of cumulus cells were collected into oocyte collection media. The oocyte collection media consists of TCM-199, 0.42M sodium bicarbonate, 0.02M

Hepes, 1X glutamine, 10% FBS, 22µg/ml pyruvate, and 50µg/ml gentamicine. Each IVF repetition consisted of 20-40 singly isolated COCs. Utilizing small numbers of COCs per repetition allowed us to minimize processing time and avoid influencing oocyte health. Selected COCs were placed in individual 20ul microdrops consisting of oocyte collection media under mineral oil (Sigma Life Science, Darmstadt, Germany). COCs in all experiments in this study were cultured at 38.5°C and 5.0% CO₂. Two small samples (~50-100 cells) of cumulus cells were carefully dissected under a stereoscope using 18 gauge needles. One sample was snap frozen in liquid nitrogen for real-time quantitative polymerase chain reaction (qPCR) analysis and Western blot assay and the other was cultured overnight for immunofluorescence and Lucifer Yellow scratch assays. Cumulus cell samples were cultured in granulosa cell media consisting of DMEM/F12 supplemented with 10% FBS and 1X Penicillin/Streptomycin. Each COC and cumulus cell sample was correlated to allow for data analysis. All experiments were repeated at least 3 times.

COCs underwent maturation and *in vitro* fertilization (IVF) followed by culturing to the blastocyst stage as previously described with modifications (Bunel, et al. 2015, Loureiro et al. 2009). Following cumulus cell biopsy, COCs were matured in 20ul oocyte maturation media for 24 hours. The oocyte maturation media consists of TCM-199 and 0.03M sodium bicarbonate supplemented with 10% FBS, 50µg/ml gentamicine, 22 µg/ml pyruvate, 1.1mM glutamine, and 10ng/ml EGF prior to use. COCs were then fertilized in their individual droplets using fertilization media containing 10⁶ spermatozoa/ml for 18-22 hours. 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 a synthetic oviductal fluid (SOF, 1.17mM CaCl₂·2H₂O, 0.49mM MgCl₂·6H₂O, 1.19mM KH₂PO₄, 7.16mM KCl, 107.7mM NaCl, 25.07mM NaHCO₃, 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 20ul culture media under mineral oil. During denuding, presumptive zygotes were not completely stripped of their cumulus cells. A small group of approximately 30 were allowed to remain attached. Those presumptive zygotes that were completely denuded had a small group of their cumulus cells (approximately 30) placed into the microdrop with them. 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. Initially, the cleavage and blastocyst rates were compared between individually cultured presumptive zygotes, in three separate IVFs, in normal culture conditions (5% CO₂, 95% air, n=79) and hypoxic conditions (5% CO₂, 5% O₂, and 90% N₂, n=78). After determining normal culture conditions produced higher cleavage and blastocyst rates, the remaining experiments were conducted in normal conditions. Forty-eight hours after fertilization, cleavage rates were recorded based on the initial total number of

oocytes. Oocytes failing to fertilize were included in cleavage and blastocyst rate determination but excluded from all other analysis while those reaching the cleavage stage were included. On day 8 after fertilization, morula/blastocyst rates were recorded based on the initial total number of oocytes. Those reaching the morula/blastocyst stage possessed high developmental competence and were labeled as such. Those that stalled at the two cell stage had poor developmental competence and formed the low developmental competence group. This classification applies to all experiments in this study.

Embryo quality assessment

To determine the total cell number and the percentage of cells that were apoptotic, blastocysts were collected on day 7 following IVF and stained with terminal deoxynucleotidyl transferase-dUTP nick end labeling (TUNEL) and 4',6'-diamidino-2-phenylindole (DAPI, NucBlue Fixed Cell Stain Ready Probes Reagent, Life Technologies, Carlsbad, CA). Blastocysts from the individual oocyte culture (n=25) and the group oocyte cultures (n=25) were collected on day 7 and washed in PBS prior to fixation in 4% paraformaldehyde for 20 minutes. After washing in PBS supplemented with 3% BSA the blastocysts were incubated in 0.5% Triton-X in PBS for 80 minutes. The blastocysts were then washed and positive and negative controls were selected and incubated with 1 unit of DNase I diluted in 1X DNase I reaction buffer for 30 minutes. Following incubation, the control blastocysts were washed in PBS with 3% BSA and then separated into positive and negative groups. Not including the negative control group, the blastocysts were then stained with the TUNEL enzyme labeling

mixture (Invitrogen, Carlsbad, CA) for 60 minutes at 37°C, washed 3 times in PBS with 3% BSA and then incubated for 30 minutes at 37°C in the TUNEL reaction cocktail. Blastocysts were then washed 3 times in PBS with 3% BSA and DAPI (Invitrogen, Carlsbad, CA) stained for 10 minutes prior to mounting on glass slides and observing under a fluorescent microscope. For each blastocyst, the total cell number (DAPI, blue nuclei) and the total number of apoptotic cells (TUNEL, green nuclei) were determined. The percentage of apoptotic cells were determined by dividing the number of TUNEL positive cells by the number of DAPI positive cells.

RNA isolation, cDNA synthesis and Real-Time qPCR

The snap frozen cumulus biopsy samples were stored at -80°C for up to 9 months until RNA was isolated. Two to four cumulus cell biopsies corresponding to oocytes with high- and low developmental competence were combined and underwent RNA isolation using the MicroElute Total RNA kit (Omega Bio-tek Inc., Norcross, GA). Concentrations of isolated RNA were measured using a NanoDrop (ThermoFisher, Waltham, MA) and ranged from 0.1 to 2.1 ng/μl. To account for the variations in RNA concentrations, C_q values of the samples were normalized to the the C_q values of the reference gene GAPDH. For each developmental outcome, three samples total were used: one from three different experimental repetitions. The isolated RNA was then reverse transcribed (RT) to cDNA using qScript cDNA supermix (Quanta BioSciences Inc., Beverly, MA) according to the manufacturer's recommended protocol. Primers for *GAPDH*, *GJA1*, luteinizing hormone receptor (*LHR*), and Cytochrome P450 19A1

(*CYP19A1*) were validated for product specificity and efficiency tested prior to use (Table 2). A Roche LightCycler 480 Real-time qPCR machine was utilized to compare the expression levels of the target transcripts using the delta-delta C_q method (Schmittgen and Livak 2008) and *GAPDH* as an internal loading control. 10 μ l reactions were run in a clear, 96-tube plate sealed with a clear, adhesive cover. The qPCR reactions were run using PerfeCTa SYBR Green Supermix (Quanta Biosciences Inc., Beverly, MA) according to the manufacturer's protocol.

Table 2: Primer sequences and expected product information.

Name	Primers	Annealing T _m (°C)	Product (bp)
<i>Gapdh</i>	F: CGTAACTTCTGTGCTGTGCC R: ATTGATGGCGACGATGTCCA	59.49 59.82	136
<i>Gja1</i>	F: AGTACCAAACAGCAGCAGACT R: CCCATGTTGCCTGGGTACTG	59.58 60.68	155
<i>Lhr</i>	F: TGCATTCAATGGGACAACGC R: GCCCGCAGTTTAGTGGAAGA	59.76 60.32	132
<i>Cyp19a1</i>	F: CACCCATCTTTGCCAGGTAGTC R: ACCCACAGGAGGTAAGCCTATAAA	62.10 61.00	78
<i>Adh4</i>	F: AGCCACTGACTGCCTCAATC R: ACTGTTACACTGTCCAGGGC	60.04 59.60	144

<i>Adh5</i>	F: AGTTTGCCTTCTGGGTTGTG	58.60	144
	R: GCACCAGCCATCTTACAGCC	61.38	
<i>Aldh1a1</i>	F: TCACCTGTGTTCCAGGAGCG	62.10	134
	R: ACCACTCACTGAACTATGCCATT	59.99	
<i>Rara</i>	F: CTCAGGGTCAGGGCATA CG	59.56	140
	R: CCTCAGGATGCCGAAAAGGA	59.75	
<i>Rarβ</i>	F: GCAAGCCTCACATGTTCCCA	60.9	159
	R: GCTCGTGCCCTTCAGAGTTC	61.02	
<i>Rary</i>	F: CCTTTCGAGATGCTGAGCCC	60.81	119
	R: GAGCTTGGCACCATCTCCTC	60.46	

Western Blotting

The snap frozen samples corresponding to oocytes with high- and low developmental competence were analyzed using a western blot. Protein was isolated from frozen samples using RIPA Lysis Buffer with Protease inhibitors. Within each repetition, four samples per embryo developmental outcome were combined at concentrations of 10µl per sample with the exception of one repetition that only yielded two 2 cell-arrested cumulus samples. Samples were combined with loading dye, heated at 95°C for 5 min. and ran through a 10% SDS Page gel, followed by transfer to a PVDF membrane (Invitrogen, Carlsbad, CA). The membrane was blocked with TBS containing 5% BSA fraction V, washed with TBS containing 0.1% Tween-20 incubated overnight with primary,

washed with TBS-Tween, incubated 1 hour with secondary antibody, washed with TBS-Tween, and exposed with enhanced chemiluminescence (ECL) for 5 minutes. For CX43 staining, a rabbit anti CX43 (1:8K, Sigma Life Science, Darmstadt, Germany) primary and goat anti rabbit HRP (1:2K, Columbia Biosciences, Frederick, MD) secondary were used. GAPDH staining utilized a mouse anti GAPDH (1:1K, Sigma Life Science, Darmstadt, Germany) primary and a goat anti mouse HRP (1:5K, Novagen, Darmstadt, Germany) secondary. The blots were densitometry analyzed, normalized to GAPDH, using ImageJ image analysis software. Antibody use and descriptions are listed in Table 3.

Table 3: Primary and secondary antibodies used for western blotting and immunofluorescence.

Primary Antibody	Company	Dilution	Secondary Antibody	Company	Dilution
Rabbit anti-CX43	Sigma	1:8000	Goat anti-rabbit HRP	Columbia Biosciences	1:2000
Mouse anti-GAPDH	Sigma	1:1000	Goat anti-mouse HRP	Novagen	1:5000
Rabbit anti-CX43	Sigma	1:8000	FITC 488 Goat anti-rabbit	ImmunoReagents Inc.	1:500

Immunocytochemistry

20-40 cumulus cell biopsies were cultured each repetition, mirroring the number of COCs cultured per repetition. Following culture for 24 hours, cumulus cells were fixed for 15 min. in 4% paraformaldehyde, blocked 30 minutes with PBS containing 3% BSA and 0.5% Triton-X, incubated overnight with primary antibody (1:8K Rabbit anti CX43, Sigma Life Science, Darmstadt, Germany), washed with

blocking solution and incubated 1 hour with secondary antibody (1:500, Goat anti Rabbit FITC, ImmunoReagents Inc., Raleigh, NC). Cells were counterstained with DAPI. Confocal microscopy was used to randomly image a single plane of the biopsy and, once developmental data was collected, images were processed using ImageJ image analysis software. Antibody use and descriptions are listed in Table 3.

Scratch Assay

Cumulus cell biopsies were placed into 24 well plates with 400 μ l granulosa cell media (1X Pen/Strep and 12.5% FBS in DMEM:F12). They were cultured for 72 hours prior to testing for cellular coupling. To test for passive diffusion, some samples were washed three times with PBS and treated with 100 μ M carbenoxolone (Sigma Life Science, Darmstadt, Germany) in serum free medium, or vehicle only serum free medium, for 30 min prior to scratch testing. Samples were then washed 3 times with PBS, and Lucifer Yellow (0.05%) was added. Biopsies were then scratched with a scalpel and incubated for 90 seconds. Biopsies were washed 3 more times with PBS, fixed for 15 minutes in 4% paraformaldehyde, and imaged immediately. A scratch assay was done on three separate single COC IVFs with 24 COCs per experiment (El-fouly et al. 1987). Dye transfer distance was measured and compared between cumulus samples corresponding to oocytes with high- and low developmental competence using ImageJ software.

Image Analysis

All western blot and fluorescence images were analyzed using ImageJ software.

For corrected total cell fluorescence (CTCF), the total fluorescence (integrated density) of five randomized subsets of equal area of the cumulus cell image was obtained. Background fluorescence was then obtained. CTCF of each subset was then calculated as $CTCF = \text{integrated density} - (\text{selected area} \times \text{background fluorescence})$. These values were then averaged to find the average CTCF of the entire cumulus cell biopsy (Burgess et al. 2010). All immunofluorescence (CX43) in the analyzed images was included in the quantification. Gap junctional plaque counts were also performed using ImageJ software. The background of all images were normalized. Briefly, brightness and contrast were altered to remove background staining and make the plaques more prominent. Then, images were converted to binary using ImageJ's thresholding algorithms. Randomized sections of the images were chosen and the plaque and nuclei number counted. Three subsets of larger area were taken and ImageJ's particle counting algorithms were used to count the gap junctions (Le et al. 2014). The same subsets were taken on the nuclear image and nuclear counts were performed by hand. Number of plaques per 100 nuclei were then calculated for each subset and then averaged to obtain the average number of plaques per 100 cells for the cumulus cell biopsy. Plaque areas were also evaluated using ImageJ particle analysis algorithms (Le et al. 2014). For scratch testing, images were collected and unaltered images were measured for the distance travelled by the Lucifer Yellow dye. This distance was converted into a cell layer measurement by normalizing the data to an average cell size. The average cell size was calculated by measuring 50 random cells per image and averaging.

Statistics

Statistical analysis was conducted using Prism software. Unpaired two-tailed Student's t-tests or ANOVA with post-hoc Tukey HSD were performed and results were considered statistically significant if $p < 0.05$. Where appropriate, data was arcsine square root transformed.

RESULTS

Single COC culture development is not significantly different from group culture development

We initially needed to determine if the removal of cumulus cell biopsies and the individual IVF system had negative effects on the development of bovine embryos. COCs were isolated from abattoir-sourced ovaries, washed, and two cumulus cell biopsies were removed under a stereoscope prior to performing *in vitro* fertilization and embryo culture. The single COC culture was compared in 5% CO₂, 95% air (normal) and in 5% CO₂, 5% O₂, and 90% N₂ (hypoxic). We found the cleavage rate to be significantly better in the normal conditions when compared to the hypoxic, $63.5 \pm 7.3\%$ vs $45.0 \pm 8.4\%$ respectively ($p = 0.002$). Moreover, we found the blastocyst rate to be significantly better in the normal conditions when compared to the hypoxic, $28.4 \pm 7.9\%$ vs $15.4 \pm 4.2\%$ respectively ($p < 0.028$). Therefore, we compared the group and single COC IVF protocols under the 5% CO₂, 95% air conditions. In addition, the cleavage rate of the individually cultured COCs ($70.8 \pm 2.5\%$) was not significantly different from the cleavage rate seen in group cultured COCs ($68.4 \pm 3.8\%$, $p > 0.05$). Blastocyst

rates, determined at day 8, were also not significantly different between the group and individually cultured bovine embryos, $24.8 \pm 3.4\%$ and $22.5 \pm 3.2\%$, respectively ($p > 0.05$). In addition, there were no significant differences in the quality of the blastocysts generated using the individual or group culture methods as determined by the mean total cells of the day 7 blastocysts, 84.4 ± 5.4 and 82.4 ± 4.9 respectively, or in the percentage of apoptotic cells, 3.8 ± 0.6 and 3.9 ± 0.6 (Figure 4). Complete developmental data can be found in Table 4.

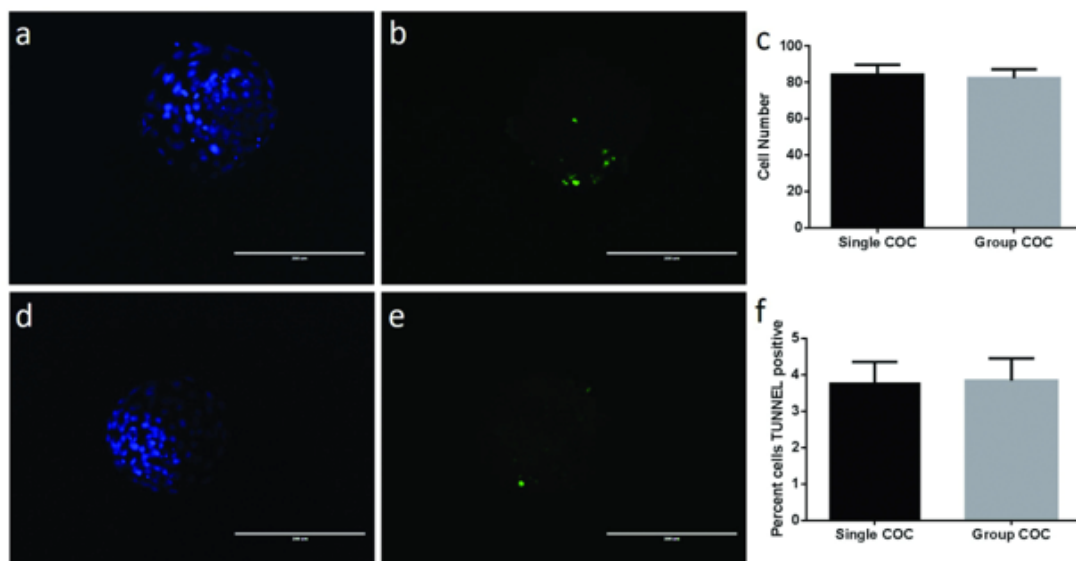


Figure 4: Comparing the total cell number and percent TUNEL positive (apoptotic) cells in bovine COCs cultured individually or in groups. Panels (a) and (d) depicts DAPI nuclear staining. Panels (b) and (e) depict TUNEL staining of apoptotic cells. Panels (a) and (b) are representative images corresponding to the (singly cultured/group cultured) COCs. Panels (d) and (e) correspond to (singly cultured/group cultured) COCs. Bar=100 μ m in panels a, b, d, and e. Panel (c) depicts the analysis of total blastocyst cell number. Panel (f) depicts the

analysis of percent TUNEL positive (apoptotic) cells in blastocysts resulting from singly cultured COCs and group cultured COCs.

Table 4: Comparison between culture methods and atmosphere conditions. Superscripts different from each other denote $p < 0.05$.

	Cleavage Rate (%)	Blastocyst Rate (%)	Blastocyst cell number	Blastocyst apoptosis (%)
Hypoxic Conditions	45.0±8.4% ^a	15.4±4.2% ^c	--	--
Normal Conditions	63.5±7.3% ^b	28.4±7.9% ^d	--	--
Group Culture	68.4±3.8%	24.8±3.4%	82.4±4.9	3.9±0.6
Single COC Culture	70.8±2.5%	22.5±3.2%	84.4±5.4	3.8±0.6

COCs isolated from large follicles have a higher expression of CX43 than those isolated from small follicles

We next aimed to determine if the expression of CX43 changed as follicles developed from early to late antral stages. The cumulus cells from COCs isolated from large follicles ($\geq 8\text{mm}$) had a higher overall expression level of CX43 when compared to COCs isolated from small follicles ($\leq 2\text{mm}$, Figures 5-8).

Furthermore, cumulus cells originating from large follicles expressed CX43 at a CTCF of $204,464 \pm 38,142$ vs $142,900 \pm 33,542$ in the cumulus cells originating from small follicles ($p=0.01$, Figure 6). Comparing the expression of CX43 using western blot, the large follicle-originating cumulus cells had 2.26 ± 0.35 fold higher expression level when compared to those originating from small follicles (0.78 ± 0.21 fold expression)(Figure 7).

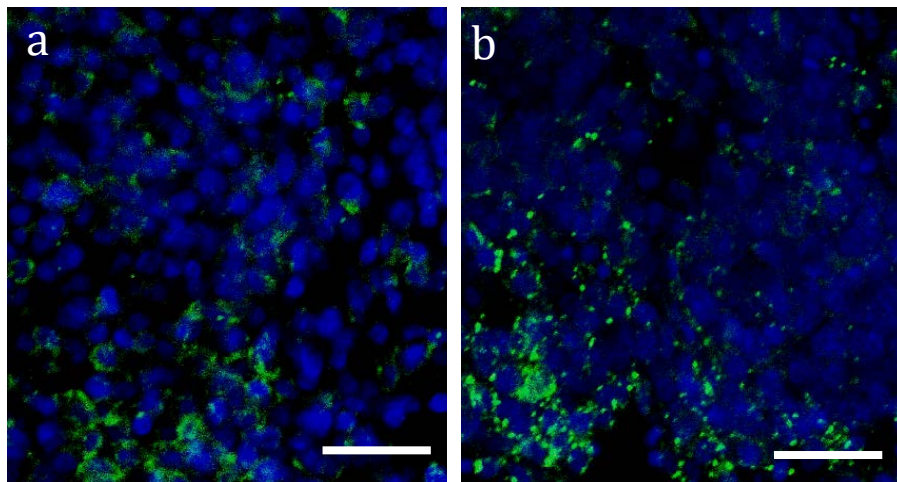


Figure 5: Comparing the expression of CX43 in bovine COCs isolated from small and large antral follicles. Representative images depicting immunofluorescent localization of CX43 (FITC, green) in cumulus cells from small follicles (≤ 2 mm; a) and large follicles (≥ 8 mm; b). Cells were nuclear stained with DAPI (blue). Bar=100 μ m.

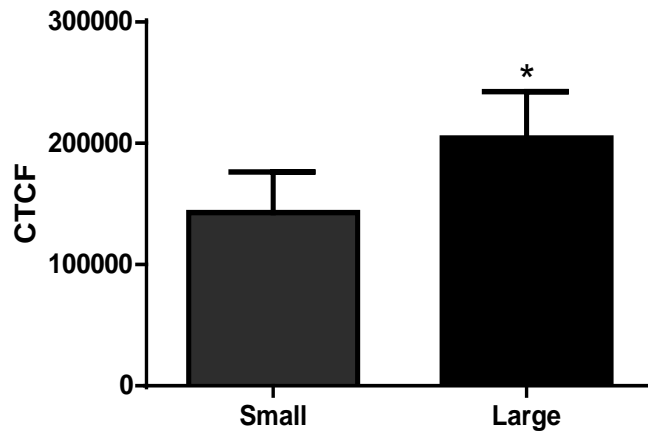


Figure 6: Comparing the expression of CX43 in bovine COCs isolated from small and large antral follicles. Analysis of total CX43 expression, corrected total cell fluorescence (CTCF), from small and large antral follicles. *Denotes a significant difference, $p < 0.05$.

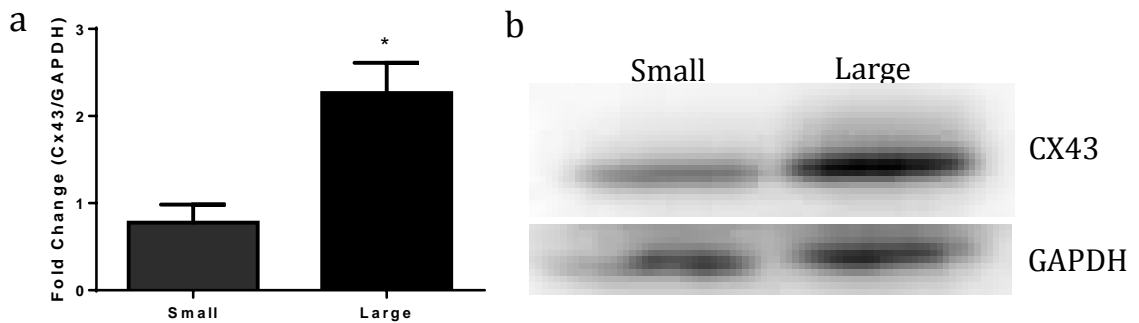


Figure 7: Comparing the expression of CX43 in bovine COCs isolated from small and large antral follicles. Panel (a) depicts the densitometry quantification of total CX43 as detected using western blotting for COCs isolated from small and large follicles. Panel (b) depicts representative immunoblots showing total CX43 expression. GAPDH was used as a loading control *Denotes a significant difference, $p < 0.05$.

COCs originating from 2mm follicles cannot be discriminated from COCs isolated from 5mm follicles based on CX43 expression

In order to determine if the expression of CX43 could be differentiated during the preselection timeframe we compared the expression level of CX43 at the low end of our collected size range (2mm) to the expression level at the high end of our collected size range (5mm). Comparing the expression level of CX43 using western blot, the COCs isolated from 2mm follicles had 1.14 ± 0.15 fold expression compared to 1.17 ± 0.05 fold expression in the COCs isolated from 5mm follicles showing no statistically significant difference ($p > 0.05$, Figure 8).

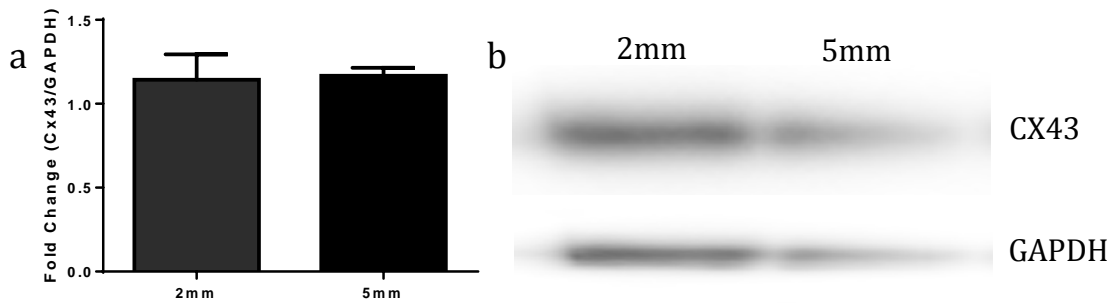


Figure 8: Comparing the expression of CX43 in bovine COCs isolated from 2mm and 5mm antral follicles. Panel (a) depicts the densitometry analysis of CX43 expression in COCs isolated from follicles 2mm in size and follicles 5mm in size. Panel (b) depicts representative immunoblots showing total CX43 expression. GAPDH was used as a loading control.

Cumulus cells originating from COCs with high developmental competence have a higher expression of CX43 when compared to cumulus cells from those with a low developmental competence

Comparing the expression level of CX43 in our cumulus cell biopsies showed that cell samples from COCs with a high developmental competence (HDC) had a 2.29 ± 0.78 fold higher level than those with a low developmental competence (LDC) (Figure 9). Immunofluorescent staining of CX43 in the cumulus biopsies showed an increased level of CX43 positive gap junctional plaques in the cumulus of COCs with a HDC (Figure 10). The number of CX43 gap junctional plaques was found to be significantly higher in the cumulus of COCs with a HDC when compared to those with a LDC, 77.1 ± 37.4 plaques/100 cells vs. 34.3 ± 19.5 plaques/100 cells, respectively ($p=0.01$, Figure 11). There was no significant difference in the number of large plaques ($1.21 \mu\text{m}^2$ or greater) per 100 cells between the high and low developmental competence groups, 10.3 ± 3.7 and 14.3 ± 3.4 , respectively (Figure 12).

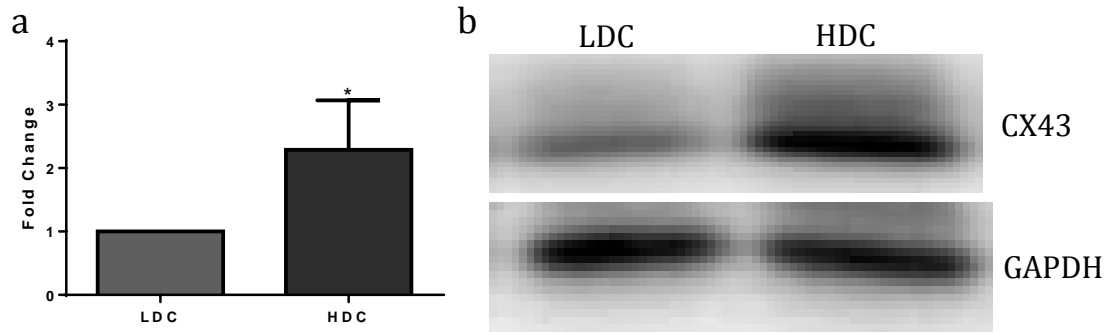


Figure 9: Comparing the expression of CX43 in bovine COCs with a low developmental competence (LDC) with that of COCs with a high developmental competence (HDC) after IVF. Panel (a) depicts the densitometry quantification of total CX43 as detected using western blotting. Panel (b) depicts representative immunoblots showing total CX43 expression. GAPDH was used as a loading control. *Denotes a significant difference, $p < 0.05$.

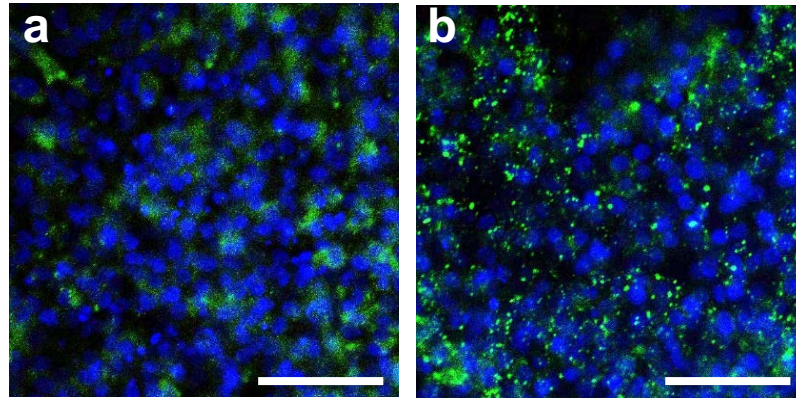


Figure 10: Comparing the expression of CX43 in bovine COCs with a low developmental competence (LDC) with that of COCs with a high developmental competence (HDC) after IVF. Representative images depicting immunofluorescent localization of CX43 (FITC, green) in cumulus cells from COCs with an LDC (a) and COCs with an HDC (b). Cells were nuclear stained with DAPI (blue). Bar=100 μ m.

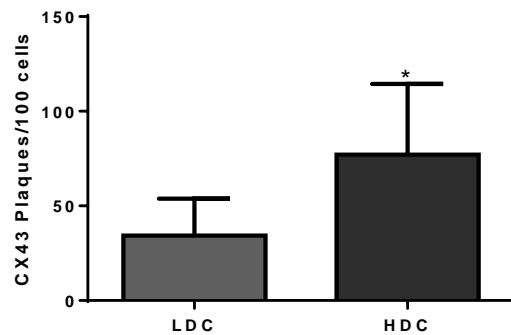


Figure 11: Analysis of CX43 localized to plaques from COCs with an LDC and those with an HDC. *Denotes a significant difference, $p < 0.05$.

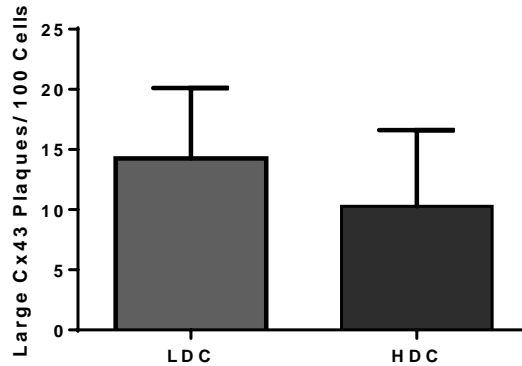


Figure 12: Analysis of the number of large plaques per 100 cells in COCs with an LDC and an HDC. $p > 0.05$

Cumulus cells have enhanced GJIC in COCs that are more developmentally competent

Scrape loading with Lucifer Yellow was used to test the GJIC between the cumulus cells in our biopsies. As shown in the representative images in Figure 13, cumulus cells from COCs with a HDC had increased dye transfer when compared to those with LDC. This suggests enhanced GJIC between the cumulus cells of the HDC COCs. On average, the small-molecule dye Lucifer Yellow was able to spread through 2.82 ± 1.06 layers of cells in cumulus from COCs with a LDC following IVF and embryo culturing (Figure 13beg). In comparison, cumulus from COCs with a HDC had dye travel through 5.56 ± 2.10 layers of cells (Figure 13cfg). When dye coupling was blocked using the gap junction inhibitor carbenoxolone, dye transfer was limited to 1.95 ± 0.33 layers of cells (Figure 13adg).

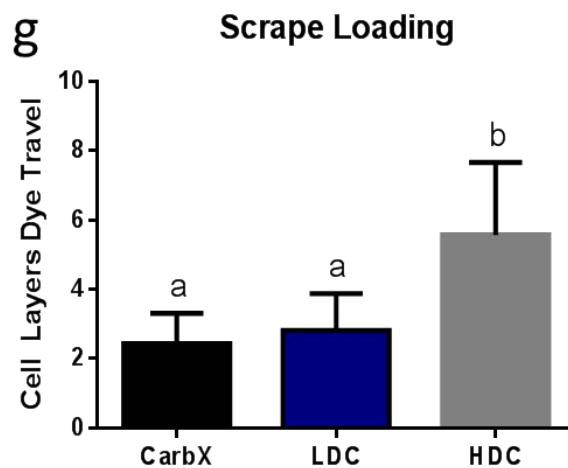
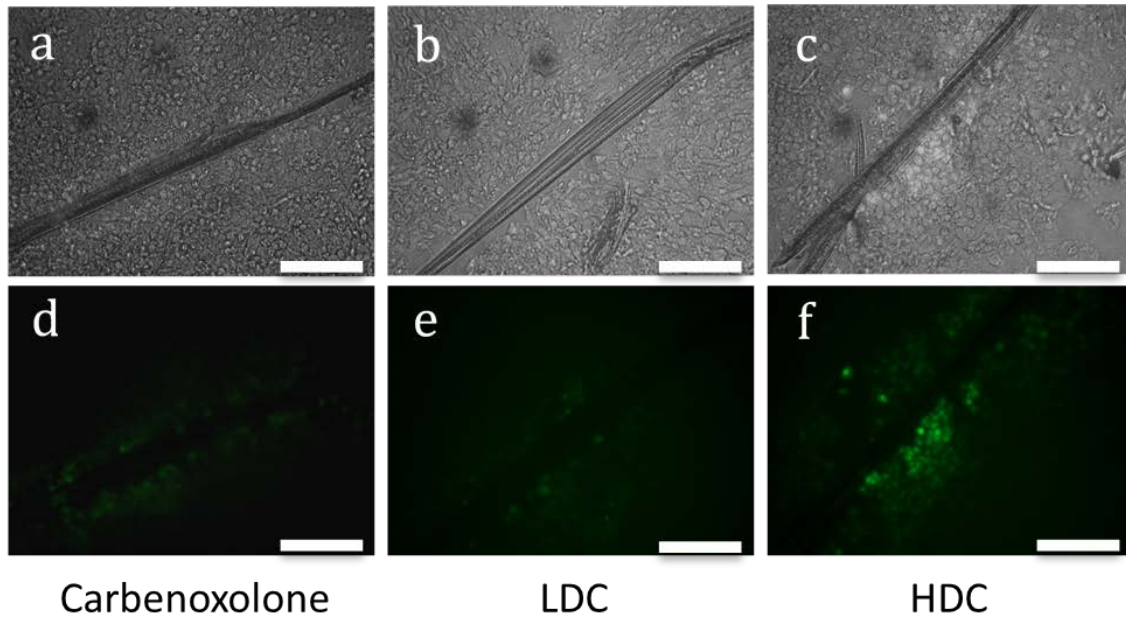


Figure 13: Scrape loading results. Panels (a), (b), and (c) depict white light micrographs of confluent cumulus cell biopsies from COCs treated with carbenoxolone, those with a low developmental competence (LDC), and those with a high developmental competence (HDC), respectively. Panels (d), (e), and (f) depict dye transfer in the cumulus biopsies from COCs treated with 100 μ M carbenoxolone (CarbX), those with an LDC, and those with an HDC, respectively. The green staining depicts cells that have taken up and transferred the Lucifer

yellow dye. Bar=100µm in panels a-f. Panel (g) depicts the cell layers that transferred the dye through biopsies after the scrape loading. Different letters denote a significant difference, $p < 0.05$.

Cumulus cell biopsies can be used to test biomarkers of developmental competence

We initially tested the expression of the CX43 transcript *GJA1* to determine if effects were solely at the protein level. Not surprisingly, we found *GJA1* expression to be significantly higher, 7.04 ± 2.49 fold, in the cumulus originating from oocytes found to have a HDC when compared to those with a LDC ($p=0.01$, Figure 14a). We found the expression of *LHR* to be 2.50 ± 0.95 fold higher in the cumulus from oocytes with a HDC when compared to those with a LDC ($p=0.04$, Figure 14b). Similarly, expression of *CYP19A1* was found to be 2.29 ± 0.45 fold higher in the cumulus from oocytes with a HDC when compared to those with a LDC ($p=0.01$, Figure 14c).

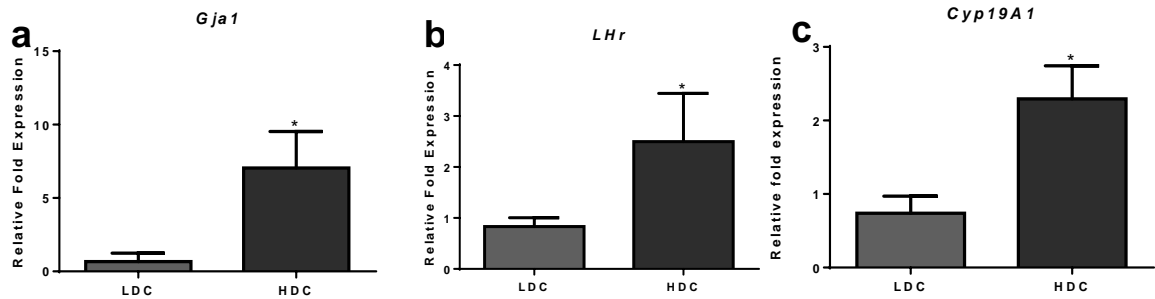


Figure 14: Analysis of gene expression differences detected in the biopsies from COCs with a low development competence (LDC) after IVF and those with a high developmental competence (HDC). Quantitative real-time PCR analysis of *Gja1*, *Lhr*, and *Cyp19a1* expression in cumulus cell biopsies. *Denotes a significant difference, $p < 0.05$.

DISCUSSION

Gap junctions have been shown to contribute to a variety of functions through the coupling of the oocyte and surrounding somatic cells. Several studies have shown that gap junctions are crucial for coordinating cumulus cell functions and permitting oocyte-somatic cell communication (Cecconi et al. 2004; Eppig et al. 1997). The coupling between cumulus cells is a requirement to maximize their proliferative response to oocyte-derived GDF9 (Gittens et al. 2005). Moreover, gap junctional coupling between granulosa cells plays an important role in the oocyte's development of competence including maintaining pH, oxidative metabolism and regulating the progression through meiosis (Edry et al. 2006; FitzHarris and Baltz, 2006; Fitzharris et al. 2007; Johnson et al. 2007).

Ovarian follicles have been shown to express several connexin family

members, depending on the species, including CX26, CX30.3, CX32, CX37, CX40, CX43, CX45 and CX60 (Gittens and Kidder, 2005). While most of the research into connexins has been garnered from mouse gene knockout studies, their expression in livestock species has been observed (Knapczyk-Stwora et al. 2013; Feranil et al. 2005). For example, the protein for CX43 has also been localized to pre-antral stages in the bovine follicle (Knapczyk-Stwora et al. 2013). Moreover, CX43 has been shown to increase in healthy antral follicles while decreasing in follicles undergoing atresia (Johnson et al. 1999). Along with this, connexin 32 (CX32) was absent in healthy follicles but present in bovine granulosa cells of antral follicles undergoing atresia (Johnson et al. 1999). This data suggests that connexin proteins may be useful non-invasive indicators of follicle, and thereby oocyte, health in the bovine. Recently, the function and expression of CX43 has been implicated in human embryo quality. The strength and expression level of CX43 in human cumulus cells was positively correlated with embryo quality using single COC assessments (Wang et al. 2009). One major difference of the study by Wang et al. is that analysis was restricted to after human chorionic gonadotropin treatment and, presumably, many of the oocytes would be mature or maturing. It has been previously demonstrated that higher grade immature COCs have cumulus cells that expressed more *GJA1* transcripts than those grading lower, but that the overall level of CX43 mRNA decreased as oocytes matured, in the bovine (Calder et al. 2003). This supports a positive relationship, prior to maturation, between Cx43 and oocyte quality. Unfortunately, the size of follicles from which the COCs were isolated was not reported. In the

present study, cumulus cell CX43 expression was analyzed specifically during the follicular growth phase. There is little information looking at the effects that the differential expression of CX43 has on oocyte quality during the early antral stages of development. This led us to investigate the relationship between cumulus cell coupling and oocyte developmental potential following IVF in bovine preselected follicles.

Initially, in order to carry out our experiments, we required a system allowing us to obtain developmental outcomes based on a single isolated COC. We adapted previously described protocols, but had additional manipulations and therefore wanted to ensure we were not effecting embryo outcomes (Bunel et al. 2015, Loureiro et al. 2009). We determined the success of the system by comparing cleavage rate at 48hr and blastocyst rate on day 8 following IVF between the individually fertilized COCs and traditional group cultures isolated from follicles ranging from 2-5mm in size. A major difference from the study by Bunel et al. was the culturing of our single COC putative zygotes in 5% CO₂ in air as opposed to culturing in 5% CO₂, 5% O₂, and 90% N₂. When we were setting up the IVF system, we found that hypoxic conditions were detrimental to the cleavage and blastocyst rates in our single COC culturing system. It is of note that several laboratories have found enhanced cleavage and blastocyst rates using hypoxic embryo culture conditions with bovine IVF when compared to normal oxygen tension (Takahashi et al. 1996; Van Soom et al. 2002; Takahashi and Kanagawa 1998). These studies utilized group culture dynamics and had differences in the media utilized which may explain our differing results.

Moreover, many studies have seen no or detrimental effects when comparing the culture of presumptive bovine embryos in 5% O₂ as opposed to in air (Rocha-Frigoni et al. 2015; Somfai et al. 2010; Mingoti et al. 2011). It has been suggested that when presumptive zygotes are cultured in the presence of cumulus cells they show improved blastocyst rates at a higher O₂ levels than in the hypoxic 5% O₂ conditions (Correa et al. 2008). We found no significant difference between the cleavage rate or blastocyst rate when fertilizing and culturing the COCs individually, showing the feasibility of using this system under our conditions.

We next aimed to confirm that CX43 cellular coupling increased in cumulus cells as antral follicles progress towards dominance. In order to do this, we isolated COCs into two groups based on the size of the antral follicles. COCs isolated from antral follicles ≤ 2 mm in diameter were grouped as small and COCs isolated from antral follicles ≥ 8 mm were grouped as large. Previous studies, in bovine, have provided evidence that follicular selection occurs after follicles reach >5 mm in size (Fortune et al. 1991; Ginther et al. 1996). Our goal was to determine if CX43 expression was discernably different between follicles prior to selection of dominance and follicles undergoing/achieving follicle dominance. We compared the expression of CX43 using western blot and compared the number of CX43 gap junctional plaques using immunofluorescent staining. We found that there is an increase in CX43 expression and CX43 positive gap junctional plaques as follicles progress from early to later antral stages (Figure 1; Nuttinck et al. 2000). This supports our hypothesis that increased gap junctional coupling,

as oocytes approach ovulation during the antral stage, is a characteristic during the timeframe when oocyte's are undergoing development of competence.

We isolated COCs from abattoir-sourced bovine ovaries by aspirating from follicles 2-5mm in size. This stage should precede follicle selection and removal by atresia (Rodgers and Irving-Rogers, 2010). Initially, we wanted to determine if there is an increase in CX43 expression when follicles transition to larger sizes during the early antral growth stages. We collected COCs from 2mm follicles and COCs from 5mm follicles and compared the expression using western blot. Using densitometry analysis there was no change in CX43 expression levels between the two follicle sizes (Figures 9 and 10). It has been well demonstrated that, in bovine, follicles less than 6mm in size have a lower developmental competence when compared to larger follicles, 34.3% blastocyst rate vs 65.9% respectively (Lonergan et al. 1994). We were interested in comparing the developmental potential of pre-selected follicles and to determine if there is a relationship between CX43 expression, gap junctional intercellular communication (GJIC) rate and their developmental outcomes. Therefore, we isolated COCs from follicles ranging from 2mm to 5mm in size for our studies. Using the single COC IVF system we are able to retrospectively study cumulus cells knowing the resulting embryo outcomes of the COCs from which they are isolated. Following single COC IVF, and embryo culture, we identified cumulus biopsies from COCs that had low developmental competence and those that had high developmental competence. Comparing the expression level of CX43 using western blot, we initially found that cumulus cells from COCs that had a low developmental

competence had an over 2 fold decrease in CX43 protein when compared to those with high developmental competence (Figures 11 and 12). Due to the fact that CX43 only functions for cellular coupling when present in the membrane in the form of gap junctional plaques, we next aimed to compare the number of CX43 positive gap junctional plaques (Winterhager and Kidder, 2015). Overall, we found that cumulus cells from COCs with HDC, following IVF and embryo culture, had a higher number of CX43 positive gap junctional plaques (Figure 11). This supported our hypothesis that increased CX43-based gap junctional coupling is an important step in the oocyte's acquisition of competence. We also determined that plaque size did not differ between the high and low developmentally competent COCs. This shows that the decrease in plaque number was not compensated for by an increase in plaque size in the COCs with low developmental competence.

While we were able to show an increased expression of CX43 positive gap junctional plaques, we also wanted to link this to increased cumulus cell coupling. We utilized a scrape loading assay to relatively compare the rate of GJIC between cumulus cells from COCs with LDC and those with HDC. Scrape loading has been shown to accurately measure the rate of GJIC between cells (El-Fouly et al. 1987; Opsahl and Rivedal 2000). We found that cumulus cells from COCs with LDC have a significantly lower rate of GJIC when compared to cumulus from COCs with HDC (Figure 13). To control for passive diffusion we utilized carbenoxolone to block gap junctional coupling (Sagar and Larson, 2006). Taken together with the localization data, this supports a role for CX43 in

bovine cumulus cell coupling. Moreover, the expression level of CX43 and the rate of GJIC may be useful in discriminating between oocytes isolated from pre-selection follicles based on predictive embryo outcomes following IVF.

The requirement for the expression of CX43 in cumulus cells in order for the progression of folliculogenesis to proceed unperturbed has been well characterized (Winterhager and Kidder, 2015; Gittens and Kidder, 2005; Dyce et al. 2012). There is also convincing evidence that meiosis inhibition is removed during oocyte maturation through the decoupling of the cumulus cells via breakdown of CX43 gap junctions. However, the breakdown of CX43 gap junctions too early or a decrease in the expression of CX43 during the acquisition of developmental competence has an inhibitory effect on bovine oocyte maturation (Vozzi et al. 2001). Moreover, inhibition of CX43 in bovine COCs results in a large decrease in the rate of GJIC supporting the critical role of CX43 in bovine cumulus cell coupling (Vozzi et al. 2001). The role of CX43 during antral follicle development and growth, before the antral follicle transitions to dominance, is less well defined. On a per cumulus cell level, the expression of CX43 has been shown to increase as antral follicles transition from the early growing phase to the dominant phase (Nuttinck et al. 2000). This suggests that stronger cumulus cell coupling may play an important role during the critical time period when the oocyte is transitioning to a competent gamete. Utilizing a single COC IVF system, we were able to show the relationship between cumulus cell CX43 expression, rate of GJIC, and developmental outcome of the oocyte during the growing phase.

Finally, we aimed to use our categorized cumulus cell samples to compare the expression of markers linked to oocyte health. Not surprisingly, we found the expression of *GJA1* was significantly lower in cumulus from COCs with LDC (Figure 14a), consistent with our protein findings (Figures 12 and 13). The expression of *LHR* and *CYP19A1*, markers linked to oocyte health, were both found to have an increased expression in cumulus cells from oocytes with HDC (Figure 14bc)(Hamel et al. 2008). LHRs are acquired in cumulus cells only during the later stages of follicle development in rats appearing in the antral stages of ovulatory females (Bukovsky et al. 1993). In porcine cumulus, LHR has been reported to play an important role in oocyte resumption of meiosis and the acquisition of oocyte competence (Shimada et al. 2003). Moreover, LH supplementation has been shown to positively affect bovine oocyte maturation, fertilization, and embryo development, *in vitro* (Zuelke and Brackett 1990). *Cyp19A1* has been identified as a marker of oocyte health in human cumulus cells (Hamel et al. 2008). This provided confirmation that the CX43 expression levels and the rate of GJIC are correctly identifying cumulus from oocytes with a higher developmental competence during the growing phase.

In conclusion, our data indicates that CX43 forms gap junctions in bovine cumulus cells and is higher in the cumulus cells from COCs with high developmental competence in the pre-selected bovine follicle pool. We have also shown that CX43 levels and the rate of GJIC, in bovine cumulus cells, can serve as an indicator of oocyte potential prior to the determination of follicle dominance. The ability to distinguish high developmentally competent COCs from low

developmentally competent COCs, prior to IVF and *in vitro* embryo production, provides the ability to improve their efficiency.

III. The effects of ATRA treatment on connexin 43 expression in bovine cumulus cells and *in vitro* embryo production

OBJECTIVE:

Investigate whether ATRA can induce increased CX43 expression in COCs and determine its effects on embryo development.

SPECIFIC AIMS:

1. Determine whether bovine cumulus cells express the transcripts needed to synthesize and respond to ATRA
2. Determine the optimal concentration of ATRA that is able to induce CX43 expression
3. Determine ATRA's effects on CX43-based gap junctions and GJIC
4. Determine the effect of ATRA on embryo developmental outcomes

ABSTRACT

Previously, our lab has shown CX43's relationship to embryo development. We next sought to investigate the effect of all-*trans* retinoic acid (ATRA) treatment on CX43 expression and subsequent embryo developmental outcome. ATRA has been shown to affect CX43 expression in many different cell types. When present in cells, ATRA elicits responses through both genomic and nongenomic pathways. Intracellular ATRA is transported to and binds retinoic acid receptors (RARs) where they travel to the nucleus and bind retinoic acid response elements (RAREs) on the DNA, influencing transcription. CX43 does not possess RAREs in its sequence therefore, our lab investigated potential nongenomic mechanisms behind ATRA's effects on CX43 expression. ATRA's nongenomic actions vary by cell type and include modification of cellular proteins. Our study utilized a group *in vitro* maturation and fertilization system to compare the expression of CX43 in, and the developmental potential of, COCs treated with 0, 0.1, 1, 10, and 20 μ M ATRA. In addition to expression, localization of CX43 in gap junctional plaques was also compared in the cumulus cells of control and ATRA-treated groups. Treatment of COCs with 10 μ M ATRA was shown to have the most significant increase in both CX43 mRNA (*Gja1*) expression and CX43 protein expression. This was the concentration used for further experiments. COCs treated during maturation with 10 μ M ATRA had significantly higher expression of CX43 and more gap junctional plaques. In addition, there was a significant increase in the maturation rate, cleavage rate, and blastocyst rate in COCs treated with ATRA. Data from these studies suggest

that not only can CX43 can be used as a biomarker for oocyte health, it can also be manipulated in *in vitro* embryo production systems using ATRA to increase the number of oocytes that achieve developmental competence.

INTRODUCTION

ATRA is one of the most abundant metabolites of vitamin A and is considered the active form in the body (Blomhoff and Blomhoff 2006). Retinol, an ATRA precursor that circulates in the blood, is internalized by cells and converted to retinal by alcohol dehydrogenases (ADHs) 1, 3, and 4 and subsequently converted to ATRA by acetaldehyde dehydrogenases (ALDHs) 1A1 and 1A2 (Kawai et al. 2016, Blomhoff and Blomhoff 2006). When present in cells, ATRA elicits responses through both genomic and nongenomic pathways. Intracellular ATRA is transported to and binds retinoic acid receptors (RARs). RARs form heterodimers with retinoic X receptors (RXRs) and, once bound to ATRA, they travel to the nucleus and bind retinoic acid response elements (RAREs) on the DNA, influencing transcription (Blomhoff and Blomhoff 2006). ATRA's nongenomic actions vary by cell type and include the modification of cellular proteins (Tanoury et al. 2013, García-Regalado et al. 2013). It has been shown that ATRA signaling is necessary for the development of the male and female gonads and that severe vitamin A deficiency results in reproductive failure in both sexes (Li and Clagett-Dame 2009). Initial evidence in support of ATRA's effects on oocyte development is the presence of retinol in follicular fluid. In cattle, growing, nonatretic follicles have been shown to have significantly increased

concentrations of retinol in their follicular fluid compared to those follicles undergoing atresia.(Schweigert and Zucker 1988). In addition to an increase in retinol present in the follicular fluid, as follicles approach antral and pre-ovulatory stages, the expression of ADHs and ALDHs, needed to convert that retinol into ATRA, also increase (Kawai et al. 2016). The presence of ATRA in follicular fluid has been linked to oocyte health in multiple studies. Cumulus cells of mice contain biologically active RARs, showing they are capable of responding to ATRA (Zhuang et al. 1994). In humans, Pauli et al. (2013) showed that oocytes originating from follicles with higher follicular fluid concentrations of ATRA yielded embryos of a higher quality grade. Additionally, mice treated with an inhibitor of ATRA synthesis not only produced fewer oocytes, but those oocytes had significantly decreased fertilization and blastocyst rates (Kawai et al. 2016). Treatment of these mice with RA saw oocyte numbers and developmental rates return to normal. The effects of ATRA on ruminant oocytes has also been shown. A study by Pu et al. (2014) showed that goat oocytes treated with ATRA have an increased nuclear maturation rate compared to control. Additionally, retinol treatment of superovulated cattle resulted in embryos with increased embryo quality grades as well as the number of transferable embryos and the blastocyst rate (Shaw et al. 1995). To our knowledge, the effects of ATRA on oocyte health and subsequent embryo development in the bovine have not been studied.

ATRA treatment of bovine cumulus cells *in vivo* has been shown to change the expression of genes that influence cellular differentiation and growth (Ikeda et al. 2005). This change in expression is thought to prevent premature apoptosis

and differentiation of the cumulus cells, preventing premature and abnormal maturation of the oocyte. Proper maturation of the oocyte is critical as it has been clearly demonstrated that oocyte health plays a major role in the resulting embryo's success following fertilization (Breukman et al. 2012). Gap junctional intercellular communication (GJIC) has also been shown to be involved in the control of cell differentiation and proliferation (Bruzzone and White 1996, Kidder 2001, and Kidder 2002). Connexin 43 (CX43), a gap junctional protein, has been shown to increase throughout the progression of folliculogenesis (Nuttinck et al. 2000), concurrent with increases in ATRA concentrations. Within the COC, CX43 is located in the plasma membrane of cumulus cells. This allows for the coupling of neighboring cells and the passage of small molecules between them. Among these small molecules are Ca^{2+} and cAMP, important signals for oocyte maturation. A previous study by our lab has linked increased expression of CX43 in cumulus cells to improved embryo developmental outcomes (Read et al. 2018).

ATRA treatment has been shown to increase CX43, and subsequently GJIC, in multiple cell types including human leukemic bone marrow stromal cells, human oral cancer cells, and canine primary lens epithelial cells (Wang et al. 2013, Liu et al. 2015, and Long et al. 2010). ATRA has also been shown to increase CX43 expression and GJIC in reproductive tissues, as shown in studies by Tanmahasamut and Sidell (2005) and Wu et al. (2013) looking at human endometrial stromal cells. Additionally, primary human granulosa cell cultures and an immortalized granulosa cell line (KGN) treated with ATRA had changes in

CX43 phosphorylation while the KGN cells also had increases in GJIC (Best et al. 2015).

With an apparent critical role on reproductive success *in vivo* we set out to determine the effect of ATRA on bovine oocyte *in vitro* developmental potential. We cultured bovine oocytes in the presence of ATRA to determine its effects on oocyte developmental potential. The ability to influence oocyte developmental potential will allow improvements in the efficiency of using IVF for *in vitro* embryo production.

MATERIALS AND METHODS

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

Group IVF System

COCs were collected from abattoir-sourced mixed breed, *Bos Taurus* ovaries. COCs underwent maturation and *in vitro* fertilization (IVF) followed by culturing to the blastocyst stage as previously described (Read et al. 2018) with modifications. Follicles ranging from 2-5mm (pre-selection follicles) 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 oocyte collection media. Selected COCs were placed in groups of 20-25 per 90µl microdrops consisting of oocyte maturation media under mineral oil (Sigma Life Science, Darmstadt, Germany) and cultured for 24 hours. Maturation media was supplemented with vehicle control (0.1%

DMSO) and 0, 1, 5, 10, or 20 μ M ATRA. Each IVF repetition consisted of 40 COCs per treatment group. COCs were collected after 22 hours of maturation for qPCR analysis (10 COCs per treatment) and western blotting (30 COCs per treatment) for the dosage testing. In subsequent experiments, 10 μ M of ATRA was used. COCs in all experiments in this study were cultured at 38.5°C and 5.0% CO₂. All experiments were repeated at least 3 times.

COCs were also collected at 22 hours to determine maturation rate. Briefly, COCs were denuded of their cumulus cells and the presence of a polar body was determined. Forty-eight hours after fertilization, cleavage rates were recorded based on the initial total number of oocytes. Oocytes failing to fertilize were included in cleavage and blastocyst rate determination. On day seven, eight, and nine after fertilization, blastocyst rates were recorded based on the initial total number of oocytes.

RNA Isolation, cDNA synthesis, PCR, and Real-time qPCR

COCs matured in the presence of vehicle control (0.0005% DMSO) or ATRA were collected and stored (25 COCs per repetition) at -80°C until RNA was isolated using the MicroElute Total RNA kit (Omega Bio-tek Inc., Norcross, GA). Concentrations of RNA were measured using Nanodrop or Qubit. To account for the variation in RNA concentrations, C_q values were normalized to the C_q values of the reference gene GAPDH. For each treatment group, three samples were used: one from three different experimental repetitions. The isolated RNA was then reverse transcribed to cDNA using qScript cDNA supermix (Quanta Biosciences Inc., Beverly, MA) according to the manufacturer's recommended

protocol. Primers for *GAPDH* and *GJA1* were validated for product specificity and efficiency prior to use (Table 2). A Roche LightCycler 480 Real-time qPCR machine was utilized to compare the expression levels of the target transcripts using the delta-delta C_q method and *GAPDH* as an internal loading control. 10µl reactions were run using PerfeCTa SYBR Green Supermix (Quanta Biosciences Inc., Beverly, MA) according to the manufacturer's protocol.

For gel electrophoresis assays, RNA was isolated as described above. *Adh4*, *Adh5*, *Aldh1a1*, *Rara*, *Rarβ*, and *Rary* primers were combined with Accustart II PCR Supermix (Quanta Biosciences Inc, Beverly, MA) and bovine cumulus cells cDNA. Samples were run on a PCR machine on a gradient (55 °C-65 °C) according to the manufacturer's protocol. After PCR, samples were combined with 1X loading dye and run on a 1% agarose gel. Gels were imaged and assessed for the presence of bands.

Western Blotting

COCs from each treatment were analyzed using a western blot. Protein was isolated from samples using RIPA lysis buffer with protease inhibitors. The RIPA mixture was added at a concentration of 13µl per 10 COCs collected. Samples were combined with loading dye, heated at 95°C for 5 minutes and run through a 10% SDS Page gel, followed by transfer to a PVDF membrane (Invitrogen, Carlsbad, CA). The membrane was blocked with TBS containing 5% BSA fraction V, washed with TBS containing 0.5% Tween-20, incubated overnight with primary, washed with TBS-Tween, incubated 1 hour with secondary antibody, washed with TBS-Tween, and exposed with ECL for 5 minutes. For Cx43

staining, a rabbit anti CX43 (1:8K, Sigma Life Science, Darmstadt, Germany) primary and goat anti rabbit HRP (1:2K, Columbia Biosciences, Frederick, MD) secondary were used. GAPDH staining utilized a mouse anti GAPDH (1:1K, Sigma Life Science, Darmstadt, Germany) primary and a goat anti mouse HRP (1:10K, Azure Biosystems, Dublin, CA) secondary. The blots were densitometry analyzed, normalized to GAPDH, using ImageJ image analysis software. Antibody use and descriptions are listed in Table 4.

Immunocytochemistry

Approximately 150 COCs per treatment per experiment were collected to look at localization of CX43. Collected COCs were fixed for 15 minutes in 4% paraformaldehyde, blocked 30 minutes with PBS containing 3% BSA and 0.5% Triton-X, incubated overnight with primary antibody (1:8K rabbit anti CX43, Sigma Life Science, Darmstadt, Germany), washed with PBS containing 3% BSA, and incubated 1 hour with secondary (1:500 goat anti rabbit FITC, ImmunoReagents Inc., Raleigh, NC). Cells were counterstained with DAPI (NucBlue Fixed Cell Stain Ready Probes Reagent, Life Technologies, Carlsbad, CA). An inverted fluorescence scope was used to image the COCs using the 20X objective. Images were processed using ImageJ image analysis software. Antibody use and descriptions are listed in Table 4.

Granulosa Cell Isolation and Culture

Once COCs were isolated, the remaining follicular fluid was centrifuged at 500xg for 5 min. The supernatant was removed and the remaining pellet was resuspended in 15 ml DMEM/F12 media containing 2X penicillin/streptomycin.

Suspension was recentrifuged at 500 x g for 5 min. The pellet was resuspended and centrifuged two more times. DMEM/F12 was added to the final cell pellet to bring the final volume up to 15 ml and cells were resuspended. Cell concentration was determined using a haemocytometer. Cells were recentrifuged and suspended in 1ml DMEM/F12. Cells were added to a 6 well plate at a concentration of 1.75×10^7 cells/well. Each well contained 2ml of DMEM/F12 supplemented with 1X FBS and 1X penicillin/streptomycin. Cells were cultured at 38.5°C and 5.0% CO₂. Cells were cultured for 12 hours, washed 2 times with PBS, and given fresh culture media. Media was refreshed again at 36 hours and 60 hours after initial placement in the incubator. Cells were confluent after 60 hours and utilized for scratch assay experiments prior to 72 hours.

Scratch Assay

Granulosa cells were isolated and cultured in 6 well plates with 2 ml of granulosa cell media (1X Pen/Strep and 12.5% FBS in DMEM:F12). They were cultured for 72 hours prior to testing for cellular coupling. Samples were washed three times with PBS and treated with 100µM carbenoxolone (Sigma Life Science, Darmstadt, Germany) in serum free medium, vehicle only serum free medium, or ATRA (10 µM), for 30 min prior to scratch testing. Samples were then washed 3 times with PBS, and Lucifer Yellow (0.05%) was added. Cultured cells were then scratched with a scalpel in six different places and incubated for 10 minutes at 37 degrees Celsius. Cells were washed 3 more times with PBS and imaged immediately. A scratch assay was done on three separate granulosa isolations measuring dye transfer in each scratch (6 per sample). Dye transfer distance

was measured using ImageJ software.

Image Analysis

All western blot and fluorescence images were analyzed using ImageJ image analysis software. Gap junctional plaque counts were also performed using ImageJ software. The background of all images was normalized. Briefly, brightness and contrast were altered to remove background staining and make the plaques more prominent. Then, the images were converted to binary using ImageJ's thresholding algorithms. Randomized sections of the images were chosen and the plaque and nuclei number counted. Five subsets of larger area were taken and ImageJ's particle counting algorithms were used to count the gap junctions. The same subsets were taken on the nuclear image and nuclear counts were performed by hand. Number of plaques per 100 nuclei were then calculated for each subset and then averaged to obtain the average number of plaques per 100 cells for each COC. For scratch testing, images were collected and unaltered images were measured for the distance travelled by the Lucifer Yellow dye by measuring from the scratch front to the dye termination site.

Statistics

Statistical analysis was conducted using Prism software. Unpaired Student's t-tests were performed and results were considered statistically significant if $p < 0.05$. Where appropriate, data was arcsine square root transformed.

RESULTS

Adh5, Aldh1a1, Rar α and Rar β are expressed by bovine cumulus cells

We initially needed to determine whether bovine cumulus cells possessed the enzymes necessary to convert retinol into retinoic acid. Cumulus cell cDNA was amplified using primers for *Adh4*, *Adh5*, or *Aldh1a1*. The amplified cDNA was then run on a gel electrophoresis assay. Bands were seen at 144 bp (*Adh5*) and 134 bp (*Aldh1a1*) (Figure 15bc), but not at 129 bp (*Adh4*) (Figure 15a). Next, we wanted to see if bovine cumulus cells possessed the receptors for retinoic acid and, therefore, able to respond to it. Cumulus cell cDNA was amplified using primers for *Rar α* , *Rar β* , or *Rar γ* primers. The cDNA was then run on a gel electrophoresis assay. Bands were seen at 140 bp (*Rar α*) and 159 bp (*Rar β*) (Figure 16ab), but not 119 bp (*Rar γ*) (Figure 16c).

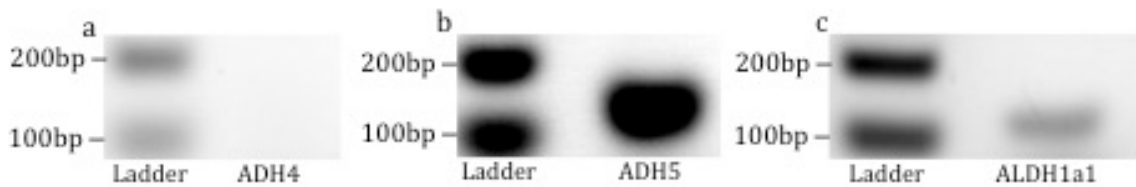


Figure 15: Representative gels depicting the absence of (a) ADH4 and the presence of (b) ADH5 and (c) ALDH1a1 as detected by gel electrophoresis in bovine cumulus cells.

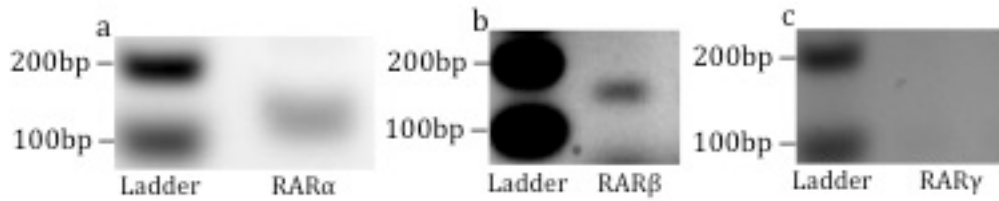


Figure 16: Representative gels depicting the presence of (a) RAR α and (b) RAR β and the absence of (c) RAR γ as detected by gel electrophoresis in bovine cumulus cells.

Connexin 43 mRNA expression was significantly increased after treatment with 10 μ M ATRA

We next needed to determine the optimal concentration of ATRA to include in the maturation media. 0, 0.1, 1, 10, and 20 μ M ATRA were included in maturation media. COCs matured in 10 μ M ATRA showed the greatest fold increase in *Gja1* (4.12 \pm 0.93 fold over control) (Figure 17). The 0.1, 1, and 20 μ M treatments were 1.12 \pm 0.11, 1.28 \pm 0.77, and 2.23 \pm 1.82 fold over control, respectively (Figure 17).

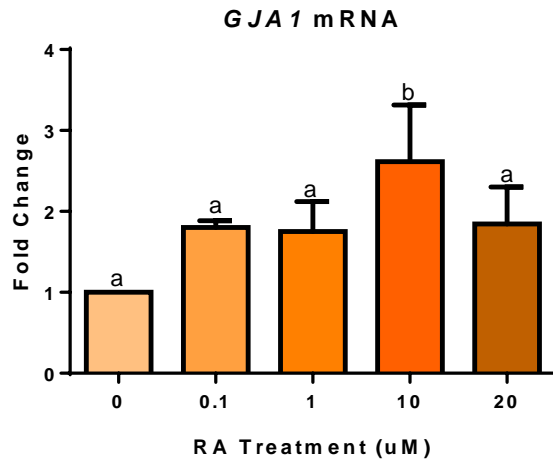


Figure 17: Effect of ATRA treatment on CX43 expression in COCs. Quantitative real-time PCR analysis of *Gja1* expression in cumulus cells following ATRA treatment. GAPDH was used as a loading control. Different letters denote a significant difference, $p < 0.05$.

Connexin 43 protein expression was significantly increased after treatment with 10 μ M ATRA

We also utilized CX43 protein expression to determine the optimal concentration of ATRA to use when supplementing the maturation media. Again, we saw the most significant fold increase in CX43 expression after treatment with 10 μ M ATRA treatment (2.61 ± 1.21) (Figure 18). The 0.1, 1, and 20 μ M treatments were 1.80 ± 0.14 , 1.75 ± 0.64 , and 1.85 ± 0.79 fold over control, respectively (Figure 18).

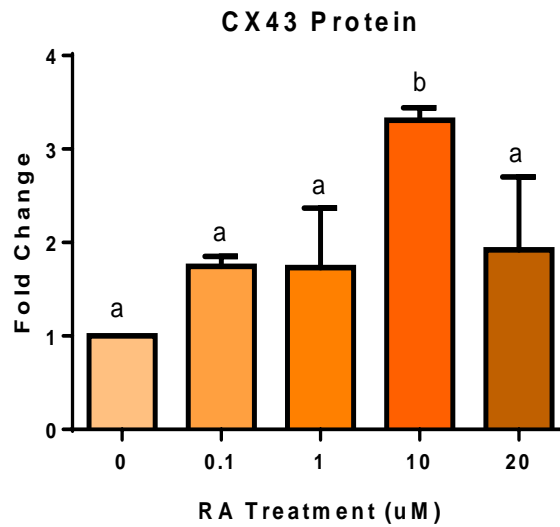


Figure 18: Densitometry analysis of CX43 expression following treatment with ATRA. Figure depicts the densitometry quantification of total CX43. GAPDH was used as a loading control. Different letters denote a significant difference, $p < 0.05$.

Cumulus cells in COCs treated with 10 μ M ATRA had significantly increased CX43-based gap junctional plaques compared to cumulus cells from control COCs

Immunofluorescence staining of CX43 in COCs matured with or without 10 μ M ATRA showed a significant increase CX43-gap junctional plaques 2.30 ± 0.81 vs 1.33 ± 0.59 gap junctions/cell, respectively (Figures 19 and 20).

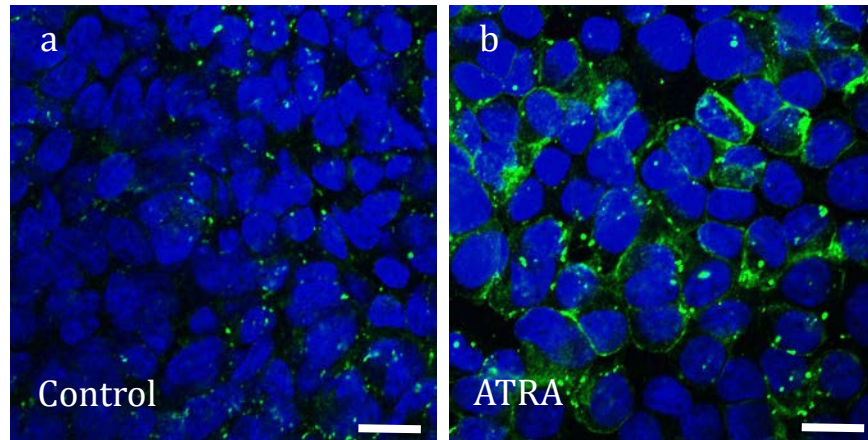


Figure 19: Comparing the expression of CX43 in bovine COCs treated with 0 μ M ATRA (Control) with that of COCs treated with 10 μ M ATRA. Representative images depicting immunofluorescent localization of CX43 (FITC, green) in cumulus cells from control COCs (a) and ATRA-treated COCs (b). Cells were nuclear stained with DAPI (blue). Bar=100 μ m.

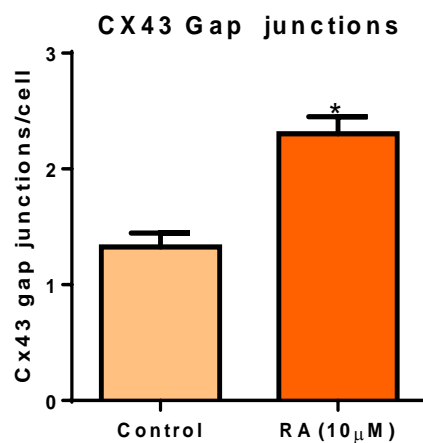


Figure 20: Analysis of CX43 localized to plaques from control COCs and ATRA-treated COCs. *Denotes a significant difference, $p < 0.05$.

Granulosa cells treated with 10 μ M ATRA had enhanced gap junctional intercellular communication

A scrape loading/dye transfer assay was used to test the GJIC between primary culture granulosa cells treated with 0 and 10 μ M ATRA. As seen in the representative images (Figure 21), granulosa cells treated with 10 μ M ATRA had significantly increased dye transfer when compared to control. This suggests enhanced GJIC between the granulosa cells treated with 10 μ M ATRA. On average, the small-molecule dye Lucifer yellow was able to spread 23.67 ± 3.46 μ m (Figure 21) of granulosa cells treated with control media while those treated with ATRA had the dye spread 55.61 ± 9.56 μ m (Figure 21). When dye coupling was blocked using carbenoxolone, dye transfer was limited to 20.05 ± 2.52 μ m. When both carbenoxolone and ATRA (10 μ M) were included, the dye travelled 19.62 ± 3.22 μ m (Figure 21).

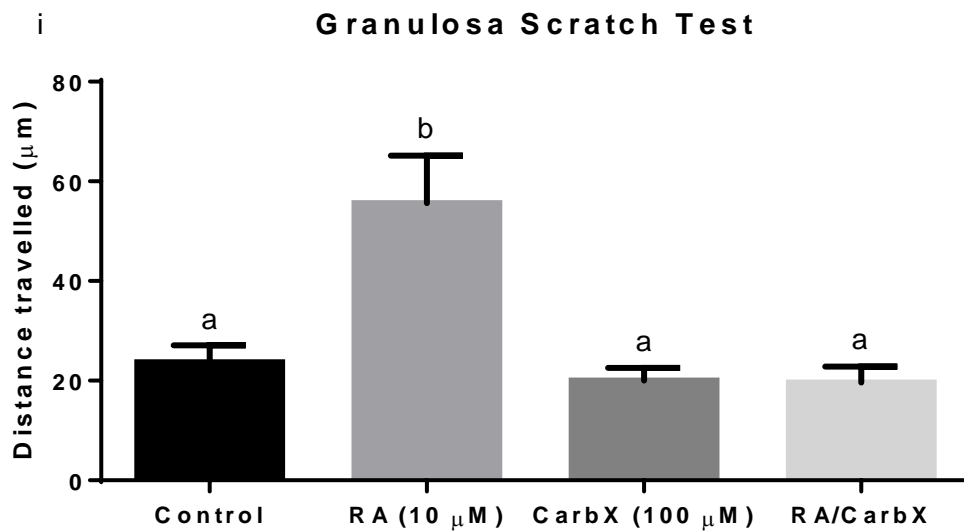
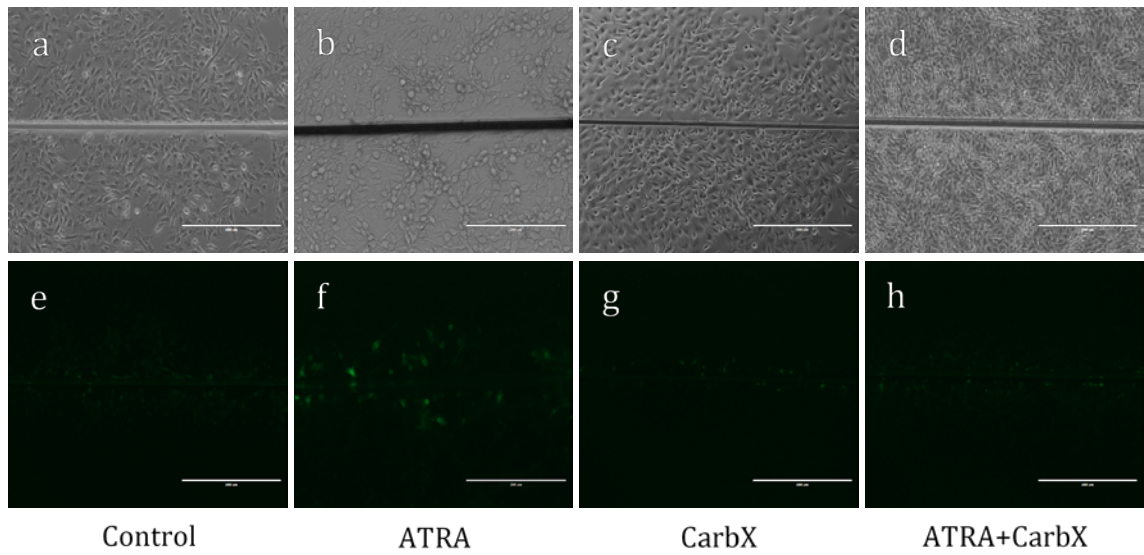


Figure 21: Representative images depicting dye transfer in cumulus cell biopsies. Panels (a), (b), (c), and (d) depict white light micrographs of confluent primary culture granulosa cells treated with control, 10 μM ATRA, 100 μM carbenoxolone, and 10 μM ATRA + 100 μM Carbenoxolone, respectively. Panels (e), (f), (g), and (h) depict dye transfer in the primary culture granulosa cells

treated with control, 10 μ M ATRA, 100 μ M carbenoxolone, and 10 μ M ATRA + 100 μ M Carbenoxolone, respectively. The green staining depicts cells that have taken up and transferred the Lucifer yellow dye. Bar=100 μ m in panels a-h. (i) Quantification of scrape loading/dye transfer assay in primary culture granulosa cells. Different letters denotes significant difference (ANOVA, $p < 0.05$).

Treatment of COCs with 10 μ M ATRA significantly improved the developmental competence of the oocytes

COCs treated with either 0 or 10 μ M ATRA were collected after maturation, stripped and evaluated for the presence of a polar body as evidence of maturation within the oocyte. COCs treated with 10 μ M ATRA showed significantly increased maturation when compared to control ($87.47 \pm 2.71\%$ vs $79.58 \pm 1.42\%$, respectively) (Figure 22). In subsequent experiments, COCs were matured in the presence of either control or 10 μ M ATRA, fertilized, and allowed to develop. Cleavage rates were collected 48 hours after fertilization and found to be significantly higher in the 10 μ M ATRA group when compared to the untreated controls ($80.27 \pm 4.12\%$ vs $66.67 \pm 6.12\%$, respectively) (Figure 23). On day 7 after fertilization, blastocyst data was collected. Blastocyst rate was found to be significantly higher in the 10 μ M ATRA treatment group when compared to control (27.50 ± 3.70 vs $19.25 \pm 4.57\%$, respectively) (Figure 24).

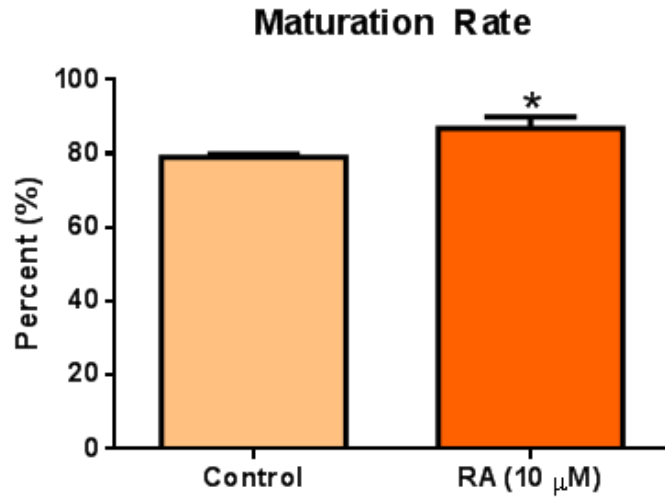


Figure 22: Effect of ATRA treatment on maturation of COCs after 24 hours.

*Denotes a significant difference, $p < 0.05$. $n=219$ (control), 225 (ATRA).

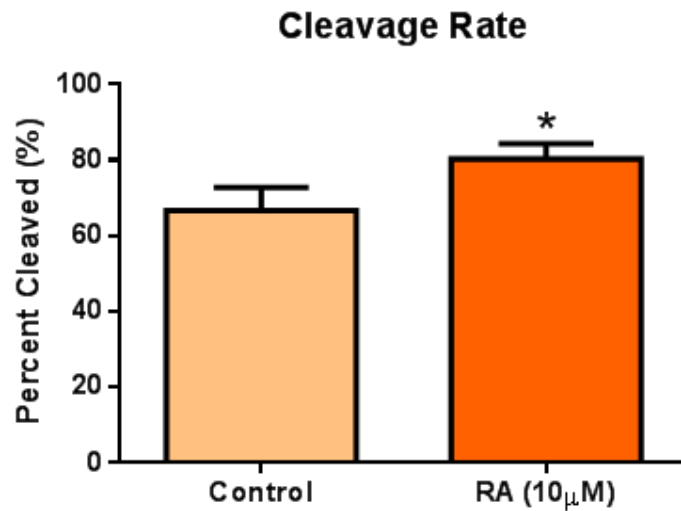


Figure 23: Cleavage rate of embryos 48 hours after IVF following ATRA treatment during maturation. *Denotes a significant difference, $p < 0.05$. $n=219$

(control), 225 (ATRA).

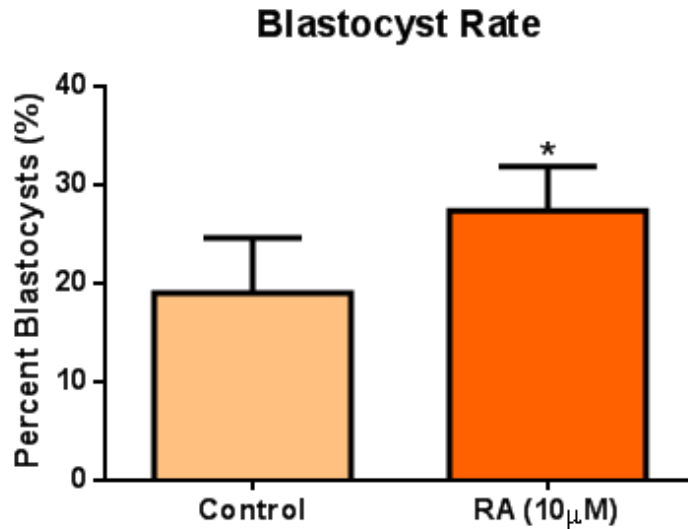


Figure 24: Comparison of the blastocyst rate following ATRA treatment during maturation. *Denotes a significant difference, $p < 0.05$. $n=219$ (control), 225 (ATRA).

DISCUSSION

The ovary is a highly vascularized organ and, therefore, exposed to large amounts of retinol via the blood circulation. This retinol is present in follicular fluid and can be used as an indicator of follicular health in developing follicles. In cattle, dominant follicles possess higher concentrations of retinol than small follicles. Additionally, these developing, nonatretic follicles had significantly greater retinol concentrations than atretic follicles (Schweigert and Zucker 1988). The increase in follicular fluid concentrations of retinol as follicles develop suggests that retinol plays a role in oocyte maturation. Additionally, a study in mice showed that the enzymes needed to convert retinol to ATRA increase in expression as follicular development progresses (Kawai et al. 2016). This

suggests that ATRA plays a role in oocyte development. We showed that the cumulus cells of cattle possess these same transcripts (Figure 15). They also express the receptors for ATRA, suggesting that cattle can both synthesize and respond to ATRA within the follicle (Figure 16).

ATRA has been shown to influence CX43 expression in multiple cell types including reproductive tissues like human endometrial stromal cells (Tamahasamut and Sidell 2005, Wu et al. 2013). This increase in CX43 expression has been accompanied by an increase in GJIC. ATRA's effects have also been shown in *in vitro* studies of human COCs (Best et al. 2015, Pauli et al. 2013). Best et al. showed that ATRA treatment of human granulosa cells treated had increased CX43 expression as well as increased GJIC while Pauli et al. showed that COCs originating from follicles with increased ATRA concentrations yielded higher quality embryos. Our lab has previously shown that increased CX43 expression by cumulus cells is linked to improved developmental competence in the oocyte (Read et al. 2018). In this study we sought to influence CX43 expression and subsequently enhance developmental competence of the oocytes. We saw increases in CX43 mRNA and protein expression in COCs treated with 10 μ M ATRA (Figures 11, 12, and 18a). Because CX43 is only functional when located in gap junctional plaques, we looked at its localization via immunofluorescence. We saw increased CX43-based gap junctional plaques in the COCs treated with ATRA (Figure 11). We then performed a scrape loading/dye transfer assay to link the increased number of gap junctional plaques to cumulus cell coupling. The increase in CX43-based gap junctional plaques

was accompanied by an increase in GJIC, as evidenced by the increased dye travel distance in granulosa cells treated with ATRA (Figure 13). Scrape loading has been shown to accurately measure the rate of GJIC between cells (El-Fouly et al. 1987, Opsahl and Rivedal 2000). To control for passive diffusion we used carbenoxolone, an inhibitor gap junctional intercellular communication (Sagar and Larson 2006). Combined with the immunofluorescence data, this supports a role for ATRA in CX43 expression and CX43-based GJIC. Because increased CX43 expression is linked to oocyte developmental outcome, we next sought to investigate whether ATRA treatment of COCs improved post-fertilization development.

Multiple studies have shown a relationship between ATRA and developmental competence of oocytes. ATRA treatment of cumulus cells has been shown to change the expression of genes involved in cellular differentiation and growth. These changes in the cumulus cells prevent abnormal maturation of the corresponding oocyte (Ikeda and Kitigawa 2005). Additional evidence for ATRA's role in oocyte maturation is shown when looking at post-fertilization development. Oocytes obtained from VAD mice had both a decreased maturation and blastocyst rate when compared to vitamin A sufficient controls (Kawai et al. 2016). In this same study, mice treated with an inhibitor of ATRA synthesis also had decreased development when compared to vitamin A sufficient controls. Retinoic acid supplementation of these mice showed developmental rates return to control levels. Another study, by Pauli et al. (2013) showed that oocytes obtained from follicles with higher ATRA concentrations

yielded a higher percentage of embryos with higher quality grades. Further, goat oocytes treated with ATRA had increased nuclear maturation compared to control oocytes (Pu et al. 2014). While it has been shown that retinol treatment improves the blastocyst rate of bovine COCs (Shaw et al. 1995), currently there are no studies that look at ATRA's effects on development. Our study showed that supplementation of maturation media with 10 μ M ATRA significantly improved the development of the corresponding oocytes (Figures 28-30). Oocytes had a significant increase in nuclear maturation as evidenced by the extrusion of polar bodies (Figure 22). The ATRA-treated group also had significantly improved post-fertilization development, shown by the increased cleavage and blastocyst rates (Figure 23 and 24). This further supports our hypothesis that ATRA treatment improves developmental competence in bovine COCs.

In conclusion, our data indicates that ATRA treatment of bovine COCs not only increases CX43 expression, but it increases the number of CX43-based gap junctions. This increase in gap junction plaques allowed for increases in GJIC communication. We also showed that ATRA treatment significantly improved developmental competence in bovine COCs. The ability to improve the developmental competence of COCs has the potential to improve the efficiencies of bovine *in vitro* embryo production.

IV. Results, Conclusions and Future Studies

As mentioned previously, there is room for improvement in *in vitro* embryo production systems. Currently, COCs are morphologically assessed for quality which is often inaccurate and leads to the fertilization of oocytes that are destined to fail. This leads to inefficiencies in embryo production that contribute to losses of time and money. In an effort to noninvasively test for oocyte developmental competence, researchers have begun to investigate cumulus cell biomarkers. Through utilization of a single COC culture system, our lab linked increased CX43 expression in cumulus cells to an increased developmental potential. The ability to select oocytes that are more developmentally competent could lead to improvements in cattle embryo production technologies. We then went on to show that the addition of ATRA to the maturation media increased the expression of CX43 in bovine cumulus cells. This increase in CX43 expression was accompanied by increases in embryo developmental outcome. This data could improve embryo production efficiency in two ways: the first is to use CX43 as an indicator of oocyte developmental potential. Being able to test the oocytes for competence prior to entry into an embryo production system would allow technicians to transfer embryos that are more likely to result in a calf. Additionally, ATRA could be used to increase the developmental potential of oocytes used in an embryo transfer system. This increase in developmentally competent oocytes could result in the transfer of more embryos that are likely to result in a calf, increasing the number of offspring gained with each embryo production session.

The mechanism behind ATRA's effects on CX43 expression remains to be elucidated. Future studies by this lab will involve investigating the mechanisms by which ATRA effects CX43 expression. Additionally, the production of more blastocysts through ATRA treatment does not necessarily mean better blastocysts. ET will have to be performed to fully determine the functioning of these embryos.

V. Literature Cited

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