THE ROLE AND MECHANISM OF NODAL IN REGULATION OF OOCYTE MATURATION IN ZEBRAFISH

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ABSTRACT

Zebrafish is beneficial as a model organism to study biological processes that are conserved among vertebrates, such as the endocrine and paracrine pathways involved in follicle development and oocyte maturation. Luteinizing hormone (LH) induces the production of maturation inducing hormone (MIH) from the ovary to initiate oocyte maturation in fish. Late vitellogenic (stage IIIb) follicles possess the capacity to respond to these hormone signals to undergo oocyte maturation. Nodal, a member of the transforming growth factor-β (TGF-β) superfamily, plays an important role in embryo development. However, little is known about its functions in the ovary. To determine whether Nodal regulates ovarian function in zebrafish, stage IIIb follicles were subjected to Nodal treatment. Our findings indicate Nodal induces oocyte maturation in a dose- and time-dependent manner. However, Nodal does not enhance hCG- or MIH-induced oocyte maturation. *In vivo*, *ndr2*$^{+/-}$ knockouts had higher progesterone levels but lower 17α-hydroxyprogesterone (17α-HP), suggesting ndr2 promotes conversion of progesterone to 17α-HP. The cAMP pathway is known to be activated in granulosa cells by LH but inactivated by MIH in oocytes. To determine if Nodal regulates the cAMP pathway, I examined the expression and regulation of several cAMP responsive element binding proteins (CREB). CREB-1a levels were increased in stage IIIb oocytes when compared with stage IIIa. Treatment of follicles with hCG or Nodal resulted in a significant increase in CREB-1a levels in oocytes, suggesting its potential role in regulating oocyte maturation. Taken together, this study demonstrates that Nodal promotes zebrafish oocyte maturation, probably by upregulating 17α-HP production in follicular cells and CREB-1a and downregulating CREB-5a expression in oocytes.

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cAMP	cyclic adenosine monophosphate
E2	estradiol
FSH	follicle stimulating hormone
Fshr	FSH receptors
GDFs	growth and differentiation factors
GnRH	gonadotropin-releasing hormone
GV	germinal vesicle
GVBD	germinal vesicle break down
hCG	human chorionic gonadotropin
Hsd3b	3β-hydroxysteroid-dehydrogenase
LH	luteinizing hormone
Lhcgr	LH receptors
MIH	maturation inducing hormone
MPF	maturation promoting factor
mPRs	membrane progestin receptors
ndr	nodal-related
qPCR	real-time PCR
spaw	southpaw
sqt	squint
StAR	steroidogenic acute regulatory protein
TGF-β	transforming growth factor-β
WT	wildtype
rhNodal	recombinant human Nodal
CREB	cAMP responsive element binding protein

I. INTRODUCTION

Zebrafish as a model organism

In recent years, zebrafish (*Danio rerio*) have been growing into a popular model organism for scientific research (Link and Megason, 2008). Out of all teleost fish, zebrafish is the most studied (Nelson, 2006). Zebrafish are popular in research because their genome is highly conserved among higher vertebrates with a 70% homology to human genes (Howe et al., 2013). Additionally, zebrafish have a short generation time; their eggs develop into larvae in three days, and they reach sexual maturity within four months (Aluru, 2017). Reproductive biology processes involving folliculogenesis and oogenesis in zebrafish are conserved generally among vertebrates. Zebrafish ovaries are asynchronous, with follicles at different developmental stages simultaneously within the same ovary. Therefore, zebrafish have been used to study endocrine and paracrine pathways and are a valuable model to study follicle development and oocyte maturation (Clelland and Peng, 2009).

Regulation of oocyte growth

In zebrafish, oocytes develop and mature asynchronously over a period of approximately 10 days. Oogenesis in zebrafish has been classified into five stages based on morphology and biochemical events (Selman et al., 1993; Figure I.1; Table 1). During the primary growth stage (stage I), oocytes are arrested in prophase I. Follicles initially exist in groups, then grow in size and form the follicular layer. In the second stage (stage II or cortical alveolus stage), the vitelline envelope forms around the oocyte. Additionally, oocytes accumulate yolk bodies containing zona pellucida proteins. During the third stage (stage III or vitellogenesis), follicles increase significantly in size due to the accumulation of vitellogenin leading to yolk formation within the oocyte.

Follicle development in zebrafish

The zebrafish ovary, surrounded by a thin epithelium, contains hundreds of follicles and oogonia. The follicles consist of an oocyte surrounded by a vitelline envelope (zona radiata), an inner layer of granulosa cells, and an outer layer of theca cells; the two somatic cell layers make up the follicular layer (Clelland and Peng, 2009). Folliculogenesis occurs in two phases: growth and maturation. This process is regulated by various hormones and growth factors. Gonadotropins - follicle stimulating hormone (FSH) and luteinizing hormone (LH) - secreted from the pituitary, in response to the gonadotropin releasing hormone (GnRH) neuropeptide, act directly on the ovary to control the growth and maturation of ovarian follicles (Nagahama 1994; Nagahama et al., 1995; Patino et al., 2001; Figure I.2).

Follicle development and oocyte maturation in vertebrates are complex events that require the coordination of hormones originating from the hypothalamus-pituitary-gonadal axis. The hypothalamus produces gonadotropin-releasing hormone (GnRH), which stimulates the secretion of gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH), from the pituitary gland. In fish, FSH plays a major role in promoting follicle development by inducing estradiol production from ovarian follicular cells while LH acts on follicular cells to induce the production of 17α, 20β-dihydroxy progesterone, known as the maturation-inducing hormone (MIH) (Ge, 2005; Clelland and Peng, 2009). MIH then binds to membrane progestin receptors (mPRs), particularly mPRα, expressed on the surface of oocytes (Hanna and Zhu, 2011; Thomas, 2017). This, in turn, activates the maturation promoting factor (MPF), leading to the release of the oocyte from its meiotic arrest and the maturation of the oocyte (Nagahama et al., 1995; Patino et al., 2001). In addition, many signaling molecules produced within the follicular cells and/or oocytes also act locally to regulate follicle development and oocyte maturation (Ge, 2005; Clelland and Peng, 2009).

Prior to engaging in the maturation process, zebrafish follicles develop through three stages. In stage I or the primary growth phase, oocytes begin to grow, and follicles start to form. Stage II is known as the cortical alveolus or previtellogenic stage in which cortical alveoli accumulate within the oocytes. Stage III is characterized by vitellogenesis (Selman et al., 1993). During this stage, follicles not only increase in size but also develop maturational competency. It has been reported that small early vitellogenic follicles are unable to undergo maturation when treated with human chorionic gonadotropin (hCG, used as an analog of LH), or MIH. In contrast, larger follicles in mid to late vitellogenesis can be induced by these hormones and enter the maturation phase (Selman et al., 1993; Wu et al., 2000). Therefore, the small and mid-late vitellogenic follicles are described as stages III-1 (or IIIa) and III-2 (or IIIb), respectively (Wu et al., 2000; Gioacchini et al., 2012). Signaling molecules produced within the follicles, such as members of the transforming growth factor-β (TGF-β) superfamily, have been shown to regulate maturational competency (Pang and Ge, 2002; Clelland et al., 2007; Peng et al., 2009).

Oocyte maturation occurs during stage IV as meiosis resumes. Oocytes change in appearance from opaque to translucent due to yolk protein proteolysis. Nuclear membrane breakdown is followed by the germinal vesicle (GV) migration to the animal pole and undergoes breakdown (GVBD) (Selman et al., 1993). Oocytes are arrested again at metaphase I. In the final stage, stage V, proteolytic enzymes digest the follicular layer resulting in a fully mature egg with a transparent ooplasm. The egg is released into the ovarian lumen during ovulation. Upon fertilization of the egg, the metaphase II block is released, and the second meiotic division is completed (Selman et al., 1993; Clelland and Peng, 2009).

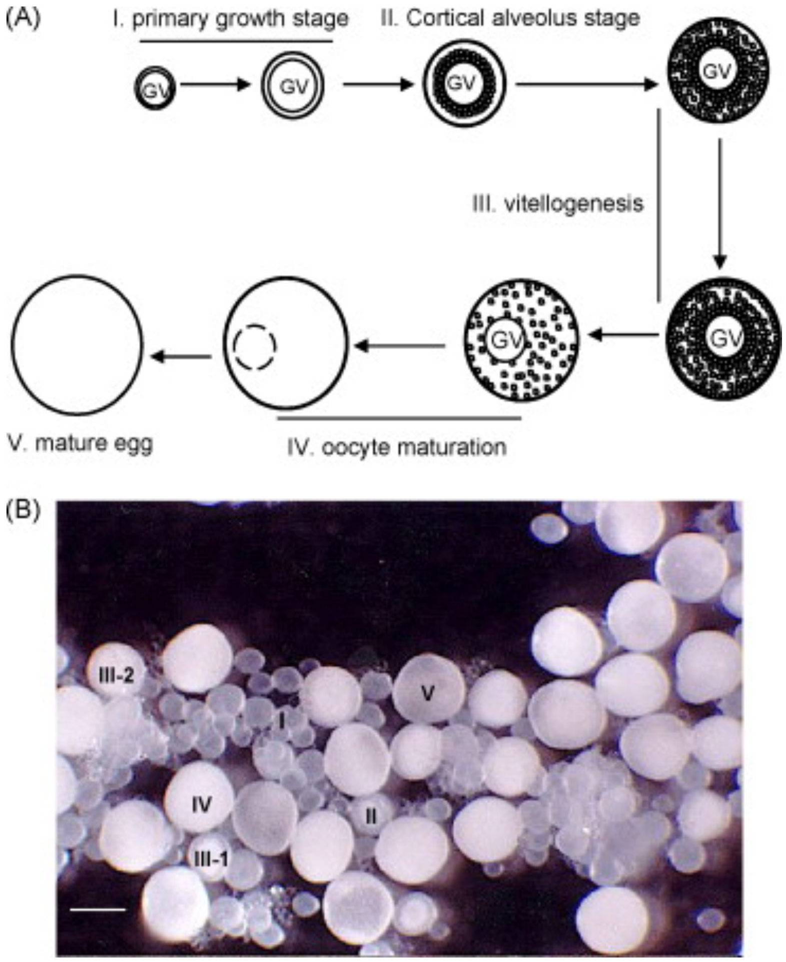

Figure I.1. Stages of follicle development in zebrafish. (A) A schematic illustration representing the five stages of follicle development. The primary growth stage (stage I) marks the beginning of follicle formation, as oocytes begin to increase in size. Cortical alveoli accumulate within the oocytes in stage II. Follicles undergo vitellogenesis during stage III, which significantly increases their size. Oocyte maturation occurs during stage IV, when the germinal vesicle (GV) migrates to the periphery of the oocyte and the nuclear membrane breaks down. In stage V, the mature eggs are ovulated and ready for spawning. (B) A micrograph displaying zebrafish follicles at the five different development stages. Scale bar: 0.70mm. Reproduced with permission from Clelland and Peng (2009).

Table I.1: Zebrafish follicles are classified into five stages (adapted from Selman, et al., 1993; Selman et al., 1994, Wu et al., 2000).

Stage	Size (mm)	Characteristics
I	0.07 to 0.14	*Primary Growth Stage* • Oocytes are arrested in prophase I • Stage I-1: follicles exist in groups • Stage I-2: follicles grow in size and form the follicular layer
II	0.14 to 0.34	*Cortical Alveolus Stage* • Vitelline envelope forms around oocyte • Accumulation of yolk bodies within oocyte
III	0.34 to 0.69	*Vitellogenesis* • Vitellogenin accumulates to form yolk within oocyte • Stage IIIa: early vitellogenic follicles do not respond to in vitro exogenous hormone treatment due to maturational incompetency • Stage IIIb: late vitellogenic follicles possess the ability to mature in vitro in response to stimulation from exogenous hormones
IV	0.69 to 0.74	*Oocyte Maturation* • Oocytes change in appearance from opaque to translucent due to yolk protein proteolysis • Germinal vesicle (GV) migrates to the animal pole and undergoes breakdown (GVBD) • Oocyte meiosis arrest at prophase I is released • Oocytes are arrested again at metaphase I
V	> 0.75	*Mature Egg* • Proteolytic enzymes digest the follicular layer • The egg is ovulated into the ovarian lumen • Upon fertilization of the egg, the metaphase II block is released and the second meiotic division is completed

Regulation of oocyte maturation

The progression of ovarian follicles between the different development stages outlined above is a process regulated by several hormones and transcription factors. Oocyte maturation occurs in three steps: 1) the pituitary releases LH to stimulate MIH production in follicular cells (Nagahama and Yamashita, 2008), 2) formation of maturation promoting factor (MPF) complex is initiated by MIH through binding of receptors on the oocyte surface, and 3) GVBD occurs leading to the release of the first polar body and oocyte maturation.

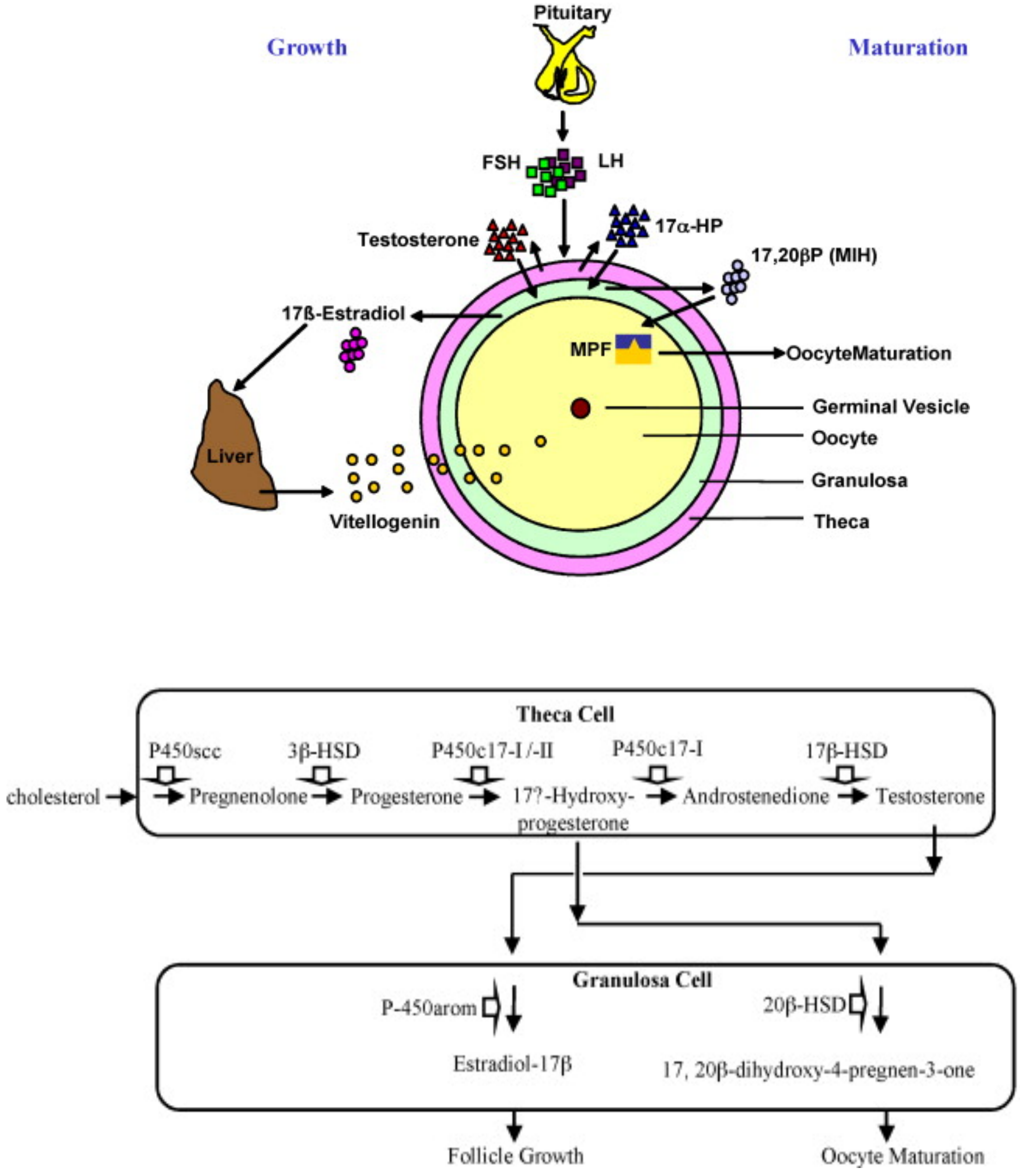

Figure I.2. Control of follicle growth and maturation in teleosts. FSH stimulates the conversion of testosterone to estradiol in the granulosa cells; this causes the production of vitellogenin in the liver, leading to follicle growth. LH produces 17 α-HP in the granulosa cell, which is converted to 17,20βP, a common form of MIH. Reproduced with permission from Clelland and Peng (2009).

Membrane progestin receptors

Membrane progestin receptors (mPRs) are integral membrane proteins with a distinct role in mediating the signaling of progestins, including maturation inducing steroids (MIS) (Hanna and Zhu, 2009). Progestin hormones bind to mPRs to induce oocyte maturation (Tan et al., 2009). In zebrafish, the oocyte membrane contains a progestin receptor that binds MIH, triggering a series of events in the oocyte, including germinal vesicle breakdown and meiosis resumption (Hanna and Zhu, 2009). The MIS in zebrafish (4-pregenen-17,20b-diol-3-one or 17,20b-DHP) can bind to, and activate, both of the zebrafish mPR isoforms: mPRa and mPRb (Hanna and Zhu, 2009).

Consistent with results from other species, including seatrout and goldfish, it has been shown through mRNA and protein expression levels that both mPRa and mPRb are present in abundance in zebrafish oocytes (Hanna et al, 2006). As zebrafish ovarian follicles develop from stage I to stage IV, the amount of mPRa increases; in contrast, mPRb expression levels remain relatively the same across all stages of intact follicles (Hanna et al, 2006). Through immunoreaction studies, it was found that mPRa and mPRb are localized near the membrane of late-stage IV oocytes (Hanna et al, 2006). Although mPR proteins are also observed in ovarian follicular cells, they are at lower levels than in denuded oocytes (Hanna et al., 2006). One study in zebrafish follicles explored the regulation of mPRs by hormones and growth factors, including hCG, bone morphogenetic protein-15 (BMP-15), and TGF-β1 (Tan et al., 2009). It was found that BMP-15 overexpression reduced mPRb levels but did not affect mPRa levels, hCG treatment increased mPRb levels but did not affect mPRa levels, and TGF-beta1 treatment suppressed mPRb expression but did not affect mPRa expression (Tan et al., 2009). Recent knockout studies show oocyte maturation cannot take place without mPRa.

Steroidogenesis

An important step occurring during oocyte maturation in teleost species is the shift in steroidogenesis (Nagahama & Yamashita, 2008; Senthilkumaran et al., 2004). For follicle growth to take place, the thecal cells convert cholesterol to testosterone in order to produce estradiol in the granulosa cells. Cholesterol is first transferred into the mitochondria with the help of the steroidogenic acute regulatory protein (StAR), where it is converted into pregnenolone by Cyp11a1 (P450scc) (Ings and Van Der Kraak, 2006; Nagahama and Yamashita, 2008; Nagahama et al., 1995; Fig. I.2). Pregnenolone is converted to progesterone by 3β-hydroxysteroid-dehydrogenase (Hsd3b). Progesterone is then converted to 17α-hydroxyprogesterone through the 17α-hydroxylase activity of Cyp17a (P450c17); this is followed by the conversion of 17α-hydroxyprogesterone to androstenedione by the 17,20-lyase activity of Cyp17a (P450c17). Androstenedione is then converted to testosterone by 17β-hydroxysteroid-dehydrogenase (Hsd17b). Testosterone is secreted from the thecal cells and diffuses into the granulosa cells to be converted to estradiol 17β estradiol (E_2) by the enzyme ovarian cytochrome P450 aromatase (Cyp19a1a) (Nagahama & Yamashita, 2008; Clelland and Peng, 2009). E2 promotes vitellogenin synthesis in the liver, which accumulates within oocytes during the growth phase.

The levels of E_2 gradually increase during oocyte growth and decrease at the start of oocyte maturation in what is known as the steroidogenic shift (Senthilkumaran & Joy, 2001). 17α-HP is converted to MIH instead of E2 by Hsd20b (Nagahama and Yamashiita, 2008). MIH is then released from the granulosa cells to induce oocyte maturation through its action on membrane progestin receptors (mPRs) (Zhu et al., 2003). The enzyme 20β-hydroxysteroid dehydrogenase (20β-HSD*)* produces the maturation inducing steroid 17α, 20β dihydroxyprogesterone (17α, 20β-DP) in ovarian granulosa cells (Nagahama & Yamashita, 2008). 17α, 20β-DP remains at a consistent basal level during oocyte growth and increases sharply with maturation (Senthilkumaran & Joy, 2001).

The synthesis of steroids is catalyzed by steroidogenic enzymes within the ovary and is regulated both spatially and temporally within the follicular granulosa and theca cells (Summers et al., 2014). The expression and activity of steroidogenic enzymes is regulated mainly by gonadotropins LH and FSH (Summers et al., 2014). Among the proteins that appear to be upregulated in follicles at the vitellogenic stage are StAr and hsd3b (Lubzens et al., 2017).

The steroidogenic acute regulatory protein (StAR) participates in the first committed step of steroid hormone synthesis by delivering cholesterol to the mitochondria (Ings and Van Der Kraak, 2006; Nagahama and Yamashita, 2008). In Nile tilapia, StAR is expressed in ovarian interstitial cells from as early as 5 days after hatching (Yu et al., 2014). Hsd3b is an enzyme that catalyzes steroid and sex hormone synthesis, including progesterone, in the adrenals and gonads (Lin et al., 2015). Certain fish species, such as medaka and trout, contain a single hsd3b gene; however, other species, including Japanese eel and zebrafish, contain multiple hsd3b genes (Lin et al., 2015). Zebrafish contain two hsd3b paralogs: hsd3b1 and hsd3b2, both of which are expressed in zebrafish gonads and head kidney (Lin et al., 2015). Zebrafish hsd3b is present in abundance as a maternal transcript and later in the embryo (Hsu et al., 2009). A study in coho salmon found expression levels of StAR and hsd3b increased during oocyte growth, with highest levels being detected at the post-vitellogenic or pre-ovulatory stage (Guzman et al., 2014). In salmon ovarian follicles, it was found that FSH increases transcript levels of StAR and hsd3b; similar expression levels were found in trout, European sea bass, and Japanese eel (Guzman et al., 2014). However, a study conducted on different ovarian follicle stages in zebrafish found expression levels of StAr and hsd3b decreased with maturation (Ings and Van der Kraak, 2006).

The 20β-HSD and Cyp19a1a expression levels are regulated by gonadotropins via cyclic adenosine monophosphate (cAMP). Serving an important role as a second messenger in various biological processes of different organisms, cAMP is responsible for intracellular signal transduction. Additionally, gonadotropins use cAMP to mediate actions on steroidogenic enzyme genes (Senthilkumaran et al., 2015). cAMP employs the corresponding cAMP response element-binding protein (CREB), which regulates the expression of genes. It does this through binding to cAMP responsive elements (CRE) on gene promoters, such as the teleost cyp19a1a gene. Spatial and temporal CREB expression could be responsible for the transcriptional regulation of such genes (Senthilkumaran et al., 2015). In a study conducted by Wang and colleagues (2019), 4 forms of CREB were identified in zebrafish: CREB1a, CREB1b, CREB5a, and CREB5b.

Transforming Growth Factor-β superfamily

The TGF-β superfamily is a large group of growth and differentiation factors that have important functions in many physiological processes, including reproduction. Different peptidyl factors from the TGF-β family have different effects on oocyte maturation. For example, activin induces oocyte maturation (Wu et al., 2000; Pang and Ge, 2002) while TGF-β1 inhibits oocyte maturation and its mRNA expression is decreased in mature follicles (Kohli et al. 2005). Additionally, Bmp-15 in the zebrafish ovary shows suppression of oocyte maturation (Clelland et al. 2006; Clelland et al. 2007). TGF-β peptides are mostly expressed near their targets and act as paracrine or autocrine factors in order to modify cellular activity in the local environment (de Kretser et al. 2002).

Nodal, a secretory protein belonging to the TGF-β superfamily, is functionally conserved across species (Mohapatra et al., 2009). Nodal was first discovered from a cDNA library of a 7.5-day post-coitum mouse embryo (Zhou et al., 1993). Based on studies with zebrafish, mice, and *Xenopus*, Nodal has been determined to play an important role in the regulation of early embryogenesis. It is involved in cell differentiation, specifically the formation of the mesoderm and endoderm, and axial organization (left-right axes and anterior-posterior patterning) (Feldman et al., 1998; Brennan et al., 2002; Bennett et al., 2007). The importance of Nodal can be seen from knockout models, which result in severe defects in embryogenesis, causing lethality (Gu et al. 1998). Studies in rat ovarian granulosa cells, human trophoblast cells, and ovarian epithelial cancer cells also indicate that Nodal possesses growth-inhibitory and pro-apoptotic effects (Wang et al., 2006; Munir et al. 2004; Xu et al. 2004).

The mature form of Nodal is obtained through proteolytic cleavage of the larger Nodal precursor to produce a biologically active disulfide-linked homodimer (Zhou et al., 1993). The mature Nodal homodimer contains seven cysteine residues, which are essential in forming the cysteine knot that is characteristic of molecules in the TGF-β family (Zhou et al., 1993). It signals via receptor complexes on the membrane: activin type I (ALK4 and ALK7) and type II (ActRII and ActRIIB) serine/threonine kinase receptors. Upon activation by type II receptors, ALK4/7 phosphorylate cytoplasmic signal transducers, Smad2 and Smad3. The phosphorylated Smad2 and/or Smad3 form a ternary complex with Smad4, which translocates to the nucleus to associate with tissue-specific transcription factors to regulate the expression of target genes. Nodal also interacts with extracellular proteins, including Cripto and Lefty. Cripto is a coreceptor on the plasma membrane that is required for the ALK4 Cripto-dependent receptor to promote a stable high-affinity complex. Nodal expression is tightly regulated by the antagonist Lefty, which blocks Nodal by competing for the binding site of Cripto (Hill, 2018; Kelber et al., 2008; Gray et al., 2003; Cheng et al., 2004; Schier and Shen, 2000; Reissmann et al., 2001).

Nodal spatial distribution is time- and function-dependent. In the embryo, after implantation, Nodal is symmetrically distributed throughout the ectoderm (Brennan et al., 2001; Schier and Shen, 2000). However, the expression is downregulated after the formation of the primitive streak until Nodal can only be detected on the edges of distinct nodes. Although Nodal was once thought to be expressed only in embryonic tissues, recent studies have shown it is also expressed in tissues that undergo remodeling, such as the islet cells of the pancreas and lactating mammary glands (Kenney et al., 2004). Nodal expression is dynamic throughout pregnancy; it plays roles in the maternal reproductive tract, embryo implantation, and placenta development (Park and Dufort, 2011; Bianco et al., 2002; Zhang et al., 2008).

Zebrafish contain three paralogs of the Nodal gene: *squint* (sqt or *ndr1*), *cyclops* (cyc or *ndr2*), and *southpaw* (spaw or *ndr3*). Through the cloning and characterization of the first Nodal proteins expressed specifically in zebrafish embryo dorsal structures, it was found that sequence patterns were different from nodal-related factors in vertebrates. Although they cannot be considered orthologs, the expression patterns and functions of Nodal in zebrafish and vertebrates do show similarity (Rebagliati et al. 1998, Feldman et al. 1998). *Ndr1* mRNA is expressed uniformly in zebrafish oocytes of all stages and can be detected in the egg (Gore and Sampath, 2002; Sampath and Robertson, 2016; Rebagliati et al., 1998). In the embryo, it is suggested to be involved in the formation of mesoderm (Rebagliati et al., 1998). One study showed that Nodal functions as a signaling molecule in the regulation of embryonic endodermal sorting through an autocrine positive feedback loop: that is, cells receiving Nodal release more Nodal (Liu et al., 2018). *Ndr2* mRNA is expressed at low levels maternally and during early and late embryonic development (Erter et al., 1998; Rebagliati et al., 1998). It is also involved in mesoderm formation, although to a lesser extent than *ndr1*, and mainly functions in nervous system patterning (Rebagliati et al., 1998).

During folliculogenesis, both Nodal and its receptor ALK7 are expressed in a cell-type-specific and follicular stage-dependent manner. A study conducted on rats suggests Nodal and ALK7 are involved in regulating follicular cell apoptosis and, consequently, follicle atresia; an overexpression of Nodal or activation of ALK7 induced apoptosis in follicular cells (Wang et al., 2006).

II. RATIONALE, HYPOTHESES, AND OBJECTIVES

There are many advantages for conducting research with zebrafish animal models, one of which is the 70% homology to human genes (Howe et al., 2013). Due to this conserved reproductive endocrinology, studying zebrafish allows us to understand the reproductive health in humans better. While Nodal is known for its critical role in embryo development, little is known about its role within the ovary. Using real-time PCR, our lab has identified zebrafish Nodal orthologs, *ndr1* and *ndr2*, expressed within the zebrafish ovary across all stages of follicle development. Additionally, mRNA levels of *ndr1*, *ndr2*, and Nodal type 1 receptors (acvr1b and acvr1c) were significantly upregulated in stage IIIb follicular cells compared to stage IIIa. Preliminary results from a previous PhD student (Yara Zayed) suggest that recombinant human Nodal (rhNodal) induces the phosphorylation of Smad3, CREB, and transient activation of ERK, indicating possibly the pathways through which Nodal induces oocyte maturation. Furthermore, Nodal was shown to inhibit follicular cell proliferation while stimulating steroidogenic mRNA levels of hsd3b2, cyp17a1, and mprb (Zayed et al., 2020). Investigating the role of Nodal is significant due to its involvement in the human reproductive pathway, such as its role in placenta and preeclampsia (Nadeem et al., 2011)

Based on these findings, we hypothesized that Nodal induces oocyte maturation in stage IIIb follicles. The objectives of my research were to determine 1) the effect of Nodal on oocyte maturation and 2) the mechanisms by which Nodal induces oocyte maturation.

III. METHODOLOGY

III.1 Animals

Wild-type TL zebrafish (*Danio rerio*) were maintained in acrylic tanks in a high-density rack system designed especially for zebrafish (Aquaneering Inc., San Diego, USA) at 28 °C, under a 14-h light, 10-h dark cycle. Fish were fed twice a day, once with brine shrimp and then with a commercial pelleted diet (Zeigler adult zebrafish diet, Zeigler Bros Inc). The use of zebrafish in this study was approved by York University's Animal Care Committee. All experiments were performed according to the *Guide to the Care and Use of Experimental Animals* published by the Canadian Council on Animal Care.

Additionally, Nodal knockouts (ndr2$^{+/-}$) were generated by Yara Zayed in our lab using CRISPR/Cas9. The line used in this project has been confirmed to have a 4bp deletion (Zayed Y, PhD thesis).

III.2 Isolation of Ovarian Follicles

Female adult gravid zebrafish were euthanized in tricaine methanesulfonate (MS222; 250mg/L) followed by decapitation, approximately 1 hour after lights on. The ovaries were extracted and maintained in a 100-mm culture dish containing 60% Leibovitz L-15 medium without phenol red (Life Technologies, ThemoFisher Scientific, Burlington, ON). Stage IIIb follicles (0.52-0.65mm) were manually isolated by gentle pipetting and separated according to size and morphological characteristics (opaque appearance and centrally located germinal vesicle). Some experiments required the isolation of the follicular layer, which was performed manually using fine forceps.

III.3 Oocyte Maturation Assays

Maturation assays were performed as previously described (Kohli et al. 2003; Clelland et al. 2007). Briefly, gravid female zebrafish were anesthetized, decapitated, the ovaries were removed and healthy stage IIIb follicles were selected. Triplicate groups of 20-25 follicles or denuded oocytes per well were distributed randomly into 24-well culture plates, washed with 60% L-15, and then incubated at 28°C with treatment hCG (20IU/ml) and/or MIH (500ng/ml, all purchased from Sigma) as positive controls, EtOH (0.25g/mL) as a negative control, or rhNodal (50ng/mL, 100ng/mL, 250ng/ml, R&D Systems, Inc., Minneapolis) in 60% Leibovitz L-15 medium without phenol red (Life Technologies Canada Inc., Burlington). Percentage oocyte maturation based on GVBD (number of mature oocytes/total number of oocytes), or a translucent appearance, were scored after 6 and 18h of treatment.

III.5 RNA Extraction, Reverse Transcriptions (RT), PCR, and qPCR

Total RNA was extracted using TRIzol Reagent (Life Technologies Canada Inc., Burlington) according to the manufacturer's instructions. Briefly, intact follicles, follicular cells, or oocytes were homogenized in TRIzol reagent using a syringe, chloroform was added after incubation to allow for dissociation of nucleoproteins, centrifuged for 15 mins at 13,500 rpm at 4°C, and the aqueous top layer was removed without disrupting the other layers. After incubation and centrifugation with isopropanol, the RNA pellet was washed once with 70% EtOH, once with 100% EtOH, and then air-dried. The pellet was dissolved in 20 to 30 uL of DEPC H_2O and stored at 4°C for 15 mins to allow the dissolving of RNA completely. RNA quantification was done using a NanoVue Plus spectrophotometer (GE Healthcare Life Sciences, Baie d'Urfe, Canada) and only samples with A260/280 > 1.9 were chosen for further experimentation.

1 mg of total RNA was used for cDNA synthesis through RT. The reaction set-up consisted of 5mM of oligo dT primer, 1mM of dNTP mix, M-MLV 10X buffer, RNAse inhibitor, M-MLV enzyme, and 1ug of RNA; the final reaction volume was made up to 20uL with DEPC H_2O. The oligo dT primer, dNTP mix, and RNA were first heated for 5 min at 70°C and placed on ice before the addition of the other components. The running conditions were 42°C for 1 hour followed by 90°C for 10 mins. cDNA was stored at -20°C for later use.

q-PCR was performed using Rotor-Gene Q (QIAGEN) to analyze the expression of genes using elongation factor-1-alpha *(ef1a)* as an internal housekeeping gene control. The reaction set-up consisted of 10uL EvaGreen qRT-PCR Master Mix (ABM), 0.5uL of forward and reverse primers (10uM), and 4uL of cDNA (1:4 dilution or 1:40 dilution for internal control); the final reaction volume was made up to 20uL with DEPC H_2O. The running conditions were initial denaturation at 95°C for 10 mins, followed by 40 cycles of 95°C for 15 secs, 60°C for 60 secs, and a 10-min extension at 72°C. All samples were run in triplicates; CT values were aimed to be between 20 to 30 and data were normalized against *ef1a* internal control. All mRNA quantification data were presented as fold changes to relative control groups using the delta-delta CT method.

Table III.1. List of primers used in real-time PCR

Gene	**Orientation**	**Sequence**	**Accession No.**
ef1α	Forward	AGGACATCCGTCGTGGTAA	NM_131263.1
	Reverse	AGCCTTGGGGTTGTCTTCA	
creb-1a	Forward	AGGAAAAACTCAGTCACATTTGTT	NM_200909.1
	Reverse	AGATGGCACCAGAATGACCG	
creb-1b	Forward	GCAAAAGTCTGCCAGATCACC	NM_001017818.1
	Reverse	TGCCAGTTATATGTTGTGAGCCT	
creb-5a	Forward	TCGCTCAGAACAGAAGTCACTC	XM_009294189.3
	Reverse	TGGGCTGTAATATGATGTGCAAG	

III.6 ELISA

Steroid measurement was performed on zebrafish ovaries according to the manufacturer's protocol. Wild-type (n=8) and *ndr2* knockout (n=8) female zebrafish were anesthetized with MS-222 and ovaries dissected. The weight of each ovary was measured to ensure an equal amount before placing in PBS in Eppendorf tubes. Ovaries were homogenized by sonication (10% amplitude for 1 min on ice), centrifuged at 15,000g for 5 min at 4°C, and the supernatant collected. This procedure was repeated twice to increase the efficiency of extraction. 17α-HP, progesterone, estradiol, and testosterone steroid levels were determined using their respective ELISA kits (Cayman Chemical Company or Accubind). The assays were conducted following the manufacturer's instructions. The standard series and all samples were conducted in duplicates and the concentrations were normalized by taking the sample dilution into account. Some experiments measured steroid levels in stage IIIb follicles or condition media collected after treatment with rhNodal for 18h.

III.7 Statistical Analysis

Each experiment was done 3 times. Data presented are mean ± SEM for the number of experiments or replicates in one experiment, as indicated in figure legends. Data were analyzed by student's t-test to determine statistical significance between two groups or ANOVA, followed by a Tukey test, using GraphPad Prism to determine statistical significance within multiple groups. Equal variance and normal distribution were ensured. All experiments were performed with at least 3 biological replicates.

RESULTS

Nodal induces oocyte maturation in follicles in a dose- and time-dependent manner

To determine the effect of Nodal on oocyte maturation, intact stage IIIb follicles were treated with different concentrations of Nodal (50ng/mL, 100ng/mL, and 250ng/mL). Oocyte maturation was scored after 18 hours based on GVBD. The results showed significant oocyte maturation that was Nodal dosage-dependent (Fig. IV.1). Nodal-induced oocyte maturation was also compared at two different time-points, 6h and 18h, by treating stage IIIb follicles with Nodal (250ng/mL). The results showed significant oocyte maturation at 18h, indicating that Nodal-induced oocyte maturation was also time-dependent (Fig. IV.2).

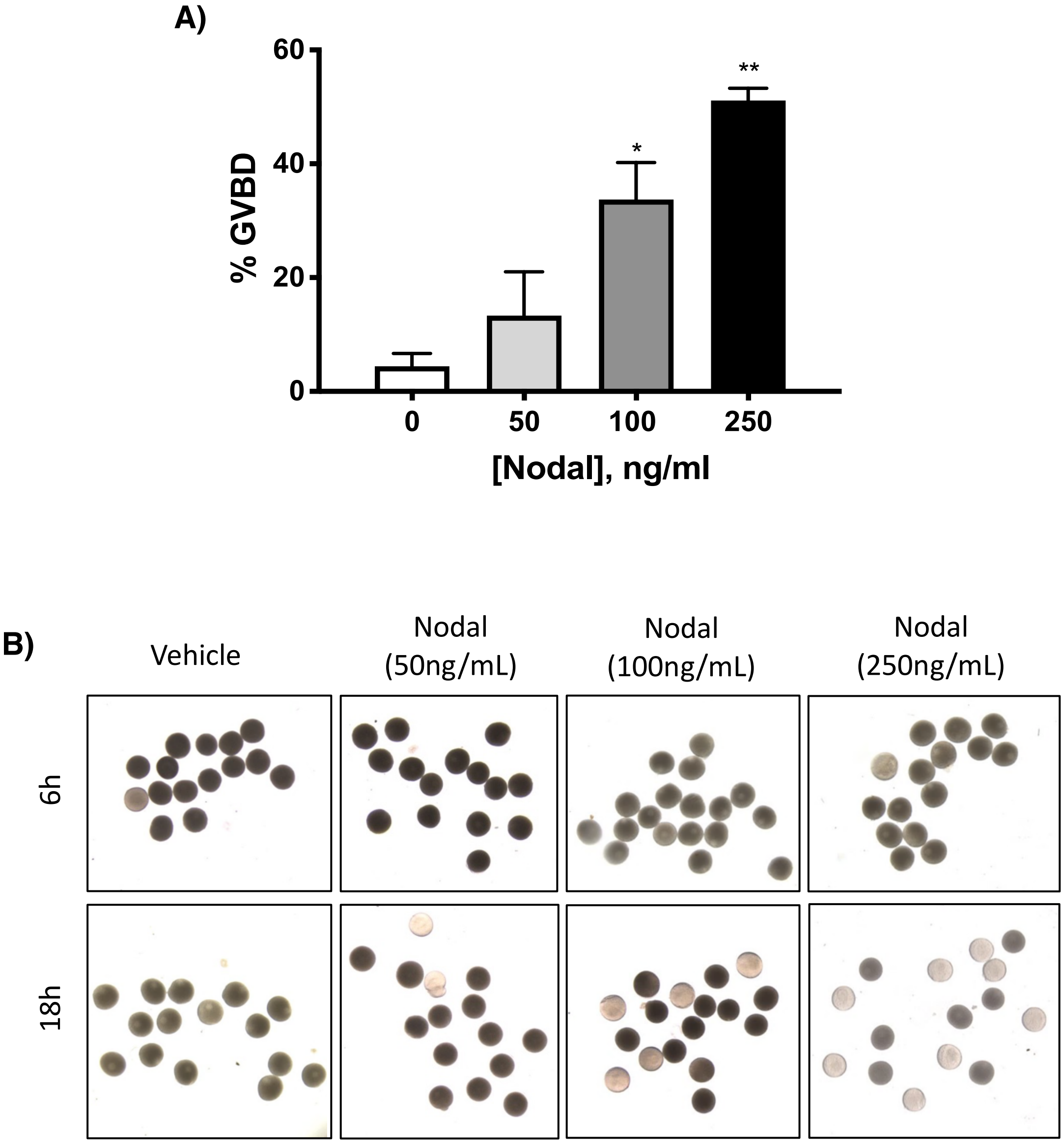

Figure IV.1: Nodal induces oocyte maturation in a dose-dependent manner. Stage IIIb follicles were treated with different concentrations of Nodal for 18h and oocyte maturation was scored as a percentage of germinal vesicle breakdown (GVBD). Representative graphs and pictures are shown. Mature oocytes were identified by their translucent appearance. Data represent mean ± SEM (n = 3 wells). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs control as analyzed by one-way ANOVA, followed by the Tukey's test. All experiments were performed three times with similar results.

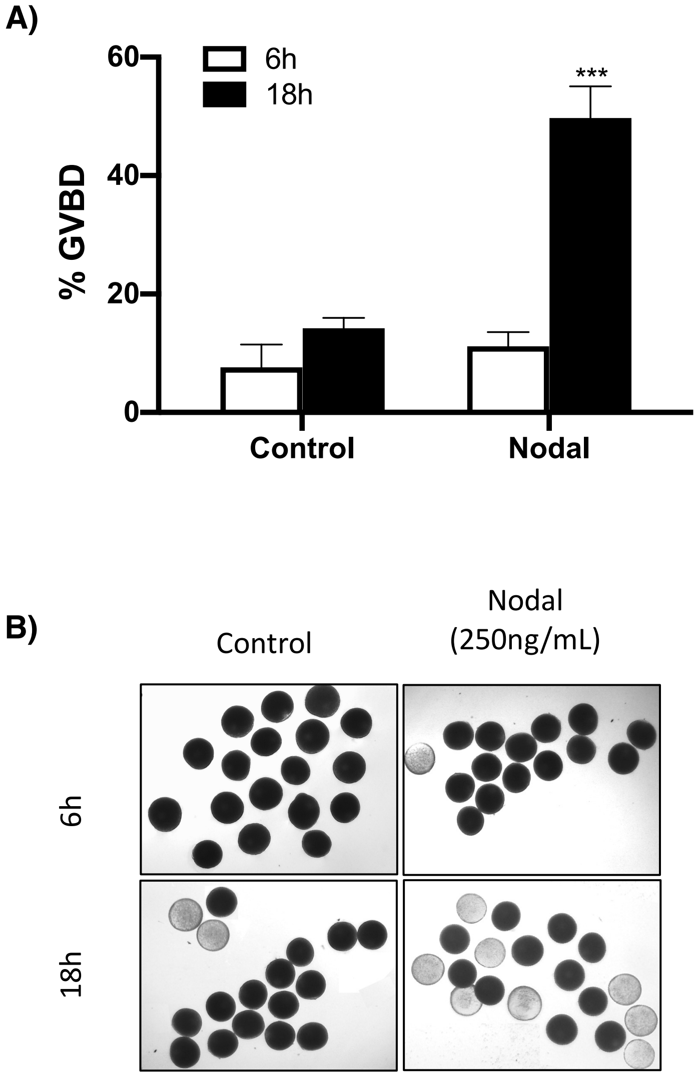

Figure IV.2: Nodal induces oocyte maturation in a time-dependent manner. Stage IIIb follicles were treated with Nodal (250ng/mL) for 6h and 18h and oocyte maturation was scored as a percentage of germinal vesicle breakdown (GVBD). Representative A) graphs and B) pictures are shown. Mature oocytes were identified by their translucent appearance. Data represent mean ± SEM (n = 3 wells). *P<0.05; **P < 0.01; ***P < 0.001 vs control. All experiments were performed three times with similar results.

Nodal induces oocyte maturation in denuded oocytes.

Components of Nodal signaling are present on oocytes. To determine if Nodal can act directly on the oocytes, the follicular layer was manually removed, and the resulting denuded oocytes were treated with different concentrations of Nodal. At the doses of 100 and 250 ng/ml, Nodal significantly induced oocyte maturation (Fig. IV.3).

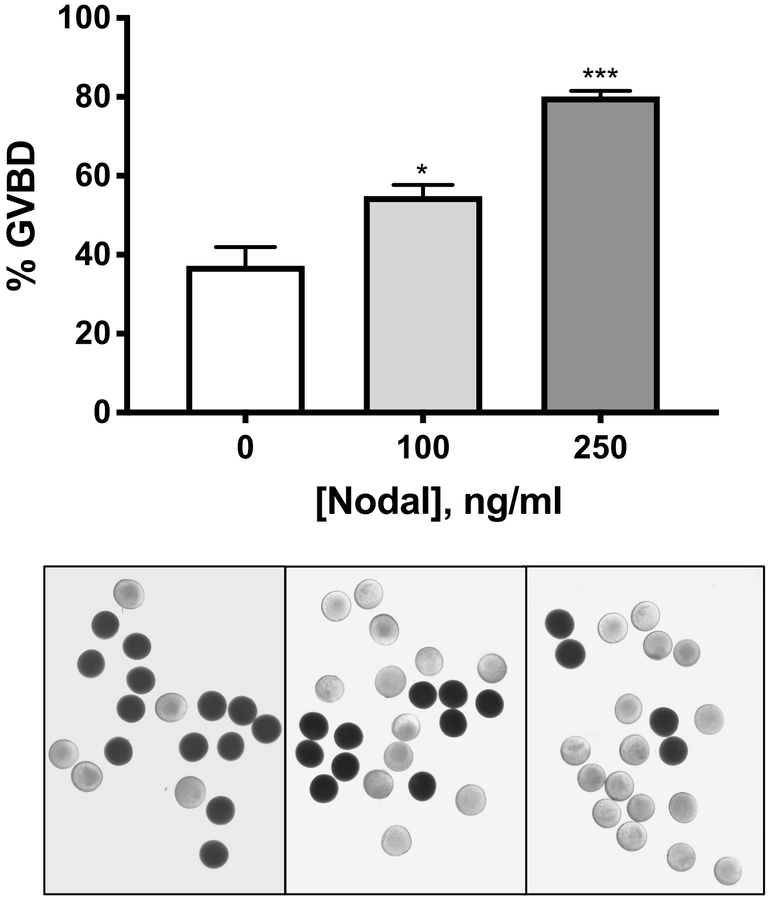

Figure IV.3: Nodal induces oocyte maturation in denuded oocytes. Stage IIIb follicles were denuded by removal of the follicular layer manually using fine-tip forceps. Denuded oocytes were treated with Nodal (100ng/mL or 250ng/mL) for 18h and oocyte maturation was scored as a percentage of germinal vesicle breakdown (GVBD). Data was arcsine transformed before analyzed by ANOVA, followed by Tukey’s test. Representative pictures and graphs are shown. Mature oocytes were identified by their translucent appearance. Data represent mean ± SEM (n = 3 wells). *P < 0.05; ***P < 0.001 vs control. All experiments were performed three times with similar results.

Nodal does not enhance hCG- or MIH-induced oocyte maturation.

Intact stage IIIb follicles were treated with either MIH or hCG alone or in combination with Nodal to determine if Nodal could further enhance the induction of oocyte maturation. The results showed that Nodal does not enhance hCG- or MIH-induced oocyte maturation (Fig. IV.4).

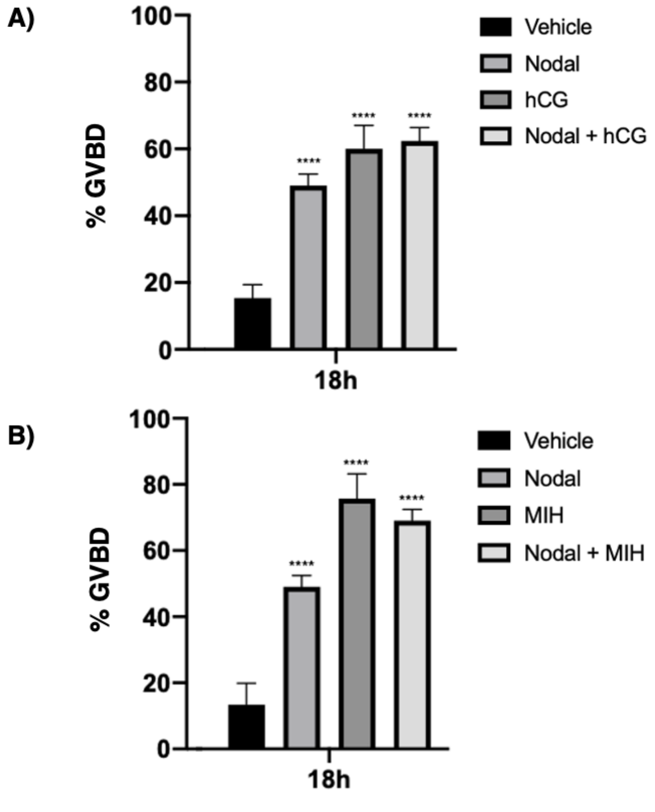

Figure IV.4: Nodal does not enhance hCG- or MIH-induced oocyte maturation. Stage IIIb follicles were treated with either A) hCG (20 IU/mL) or B) MIH (500 ng/mL), alone or in combination with Nodal (250 ng/mL), for 18h, and oocyte maturation was scored as a percentage of germinal vesicle breakdown (GVBD). Data was arcsine transformed before statistical significance was determined using ANOVA followed by Tukey's test. Data represent mean ± SEM (n = 3 wells). ****P<0.00001 vs control.

Nodal induces 17α-HP production

A previous study in our lab has found that Nodal upregulates the mRNA levels of hsd3b2 and cyp17a1, which encode for two steroidogenic enzymes, 3β-HSD and 17α-hydroxylase, respectively (Zayed et al., 2020). This finding suggests that Nodal may regulate the production of progesterone and 17α-HP. Since 17α-HP is the precursor of MIH and can induce oocyte maturation in fish (Nagahama and Yamashita, 2008), stage IIIb follicles were treated with either control or different concentrations of Nodal. The conditioned media was collected and processed to measure 17α-HP concentration using ELISA. The results show that 17α-HP levels increased in a dose-dependent manner after 18h (Fig. IV.5A). In another experiment, hCG was also included as a positive control. Both hCG and Nodal increased 17α-HP levels (Fig. IV.5B).

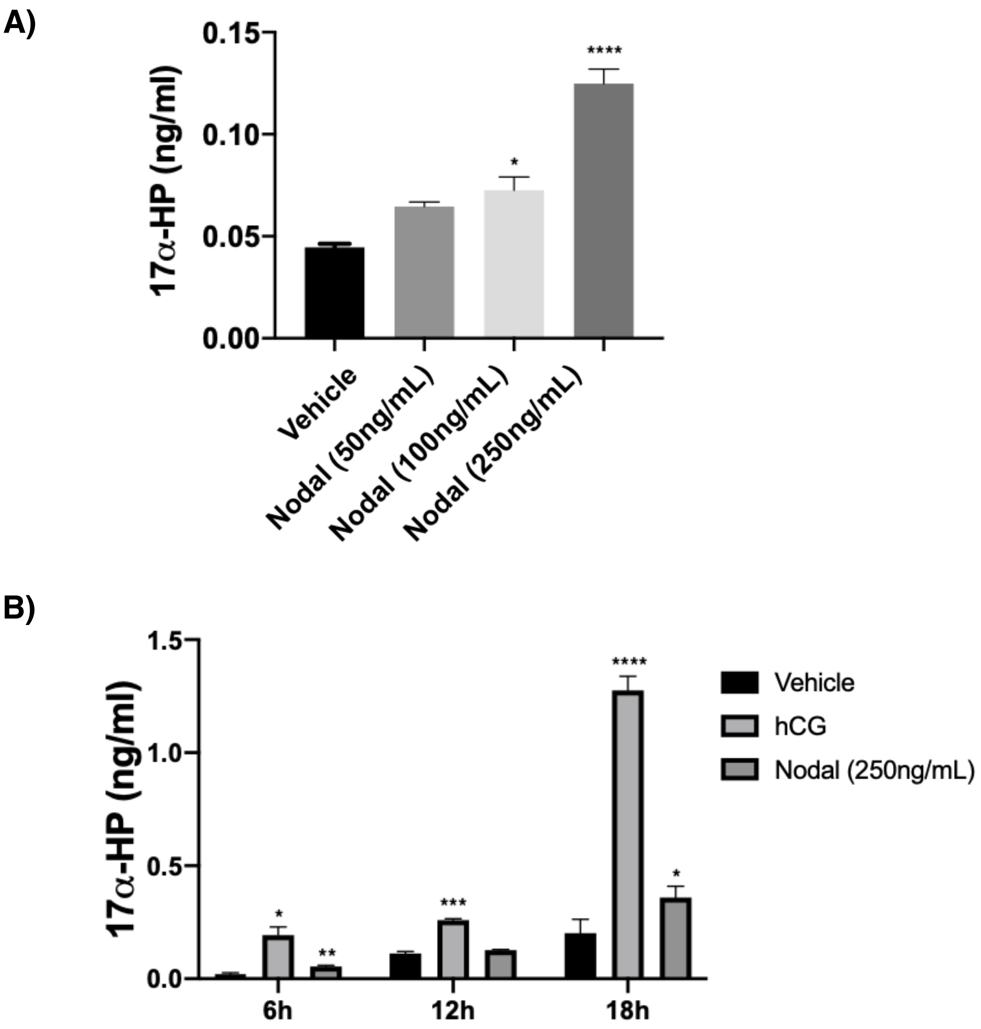

Figure IV.5: Nodal induces 17α-HP production. A) Stage IIIb follicles were treated with control and different doses of Nodal for 18h. The conditioned media was collected and processed to measure 17α-HP concentration. B) Follicles were also treated with control, hCG (20IU/mL), or Nodal (250ng/mL) over a time-course, and condition media was collected after 6h, 12h, and 18h to measure 17α-HP concentration. Statistical significance was determined using ANOVA followed by Tukey's test. Data represent mean ± SEM (n = 3 wells). *P < 0.05; **P<0.01; ***P<0.001; ****P<0.0001 vs control within the same exposure group. All experiments were performed three times with similar results.

Steroid hormone production in *ndr2*$^{+/-}$ zebrafish

To study the *in vivo* functions of zebrafish, our lab has generated *ndr2* knockout fish. Homozygous *ndr2* knockout resulted in embryonic lethality; however, heterozygous *ndr2* knockout (*ndr2*$^{+/-}$) was able to develop into adults (Zayed et al., unpublished). To determine if steroid levels were changed in Nodal knockout models, whole ovaries were collected from WT and *ndr2*$^{+/-}$ fish 1h after lights on and processed to measure 17α-HP, progesterone, estradiol, and testosterone concentrations using ELISA. The results (Fig. IV.6) show 17α-HP levels were significantly lower in *ndr2*$^{+/-}$ fish compared to WT. In contrast, progesterone levels were significantly higher in the knockouts. On the other hand, testosterone levels were significantly lower in *ndr2* mutants while estradiol levels were unchanged.

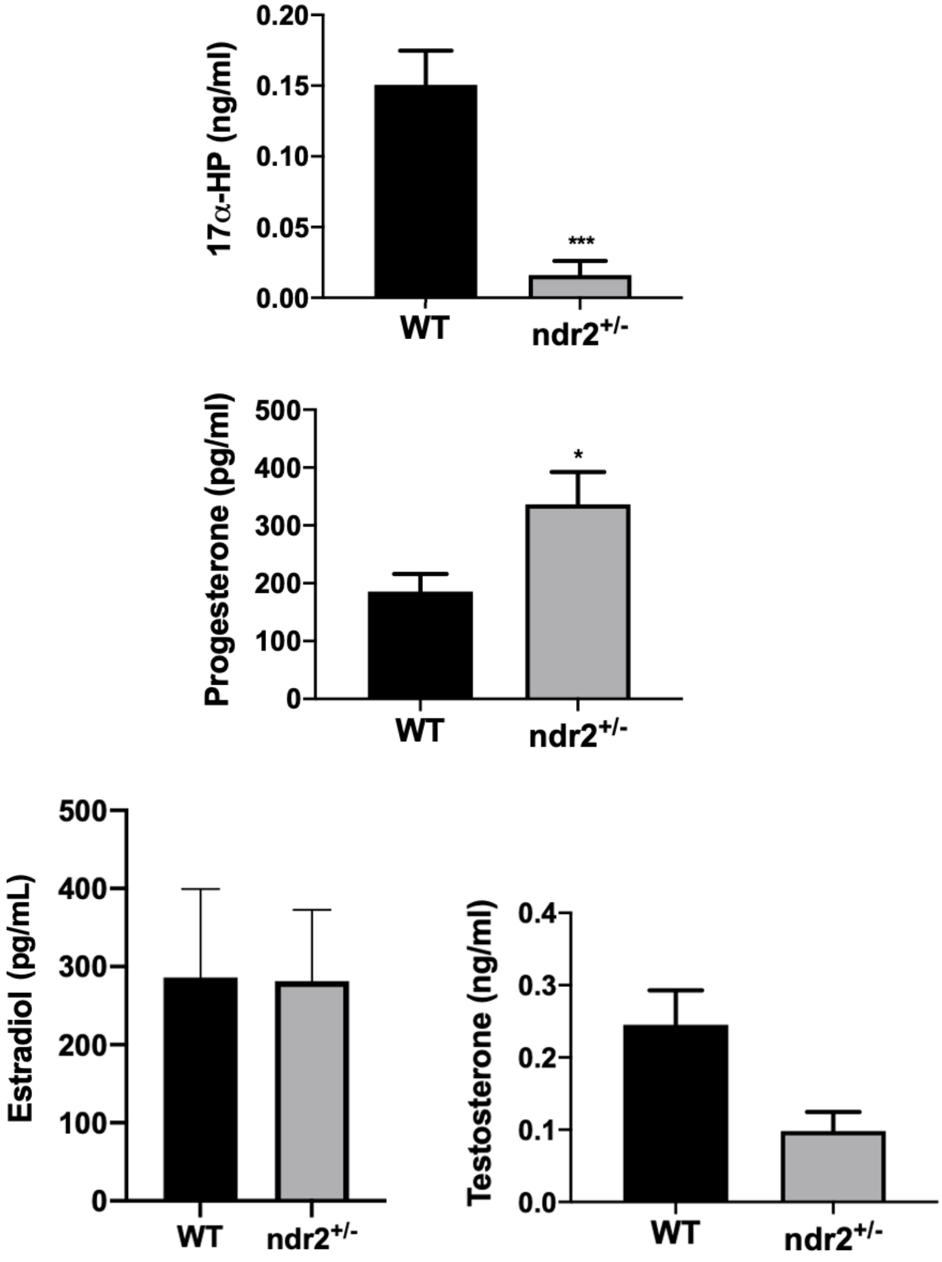

Figure IV.6: Expression profile of steroids in *ndr2*$^{+/-}$ knockouts. Whole ovaries were collected from wild-type (n = 8) and *ndr2* knockouts (n = 8) 1h after lights on and processed to measure the concentration of 17α-HP, progesterone, estradiol, and testosterone. Statistical significance was determined using a two-tailed t-test. Data represent mean ± SEM (n = 8). *P < 0.05; ***P<0.001 vs control.

Expression profile of CREB in follicle development

To determine the expression levels of CREB in follicles, stage IIIa and IIIb follicles were collected 1h after lights on. After RNA extraction, CREB mRNA levels were determined by qPCR. As shown in Fig. IV.7, CREB-1a mRNA levels were significantly higher in stage IIIb follicles when compared to stage IIIa. In contrast, both CREB-1b and CREB-5a mRNA levels were significantly lower in stage IIIb than in stage IIIa follicles.

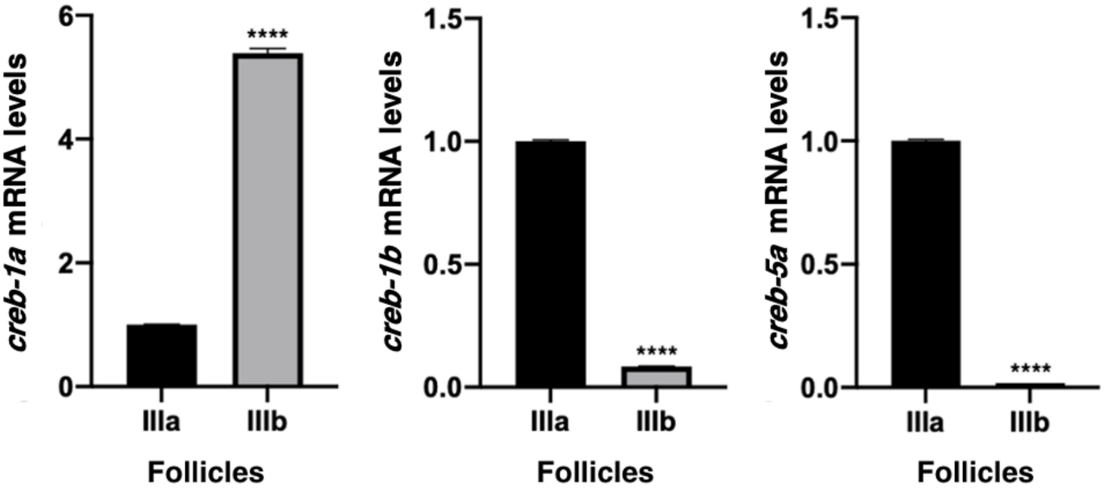

Figure IV.7. CREB mRNA levels in stage III follicles. Stage IIIa and IIIb follicles were collected 1h after lights on and processed to measure *creb-1a*, *creb-1b*, and *creb-5a* mRNA levels. Relative mRNA levels are represented as fold-change to stage IIIa after normalized to the internal control, elongation factor-1-alpha (*ef1a*). Statistical significance was determined using a two-tailed t-test. Data represent mean ± SEM (n = 3). ****P < 0.0001 vs control.

Expression profile of CREB in follicular cells

To determine the mRNA levels of CREB in follicular cells, stage IIIa and IIIb follicles were collected 1h after lights on and the follicular layer removed manually using fine-tip forceps. After RNA extraction, CREB mRNA levels were determined by qPCR. The results (Fig. IV.8) show relative CREB-1a mRNA levels are significantly lower in stage IIIb follicular cells compared to stage IIIa. In contrast, relative mRNA levels of CREB-1b and CREB-5a are significantly higher in stage IIIb follicular cells compared to stage IIIa.

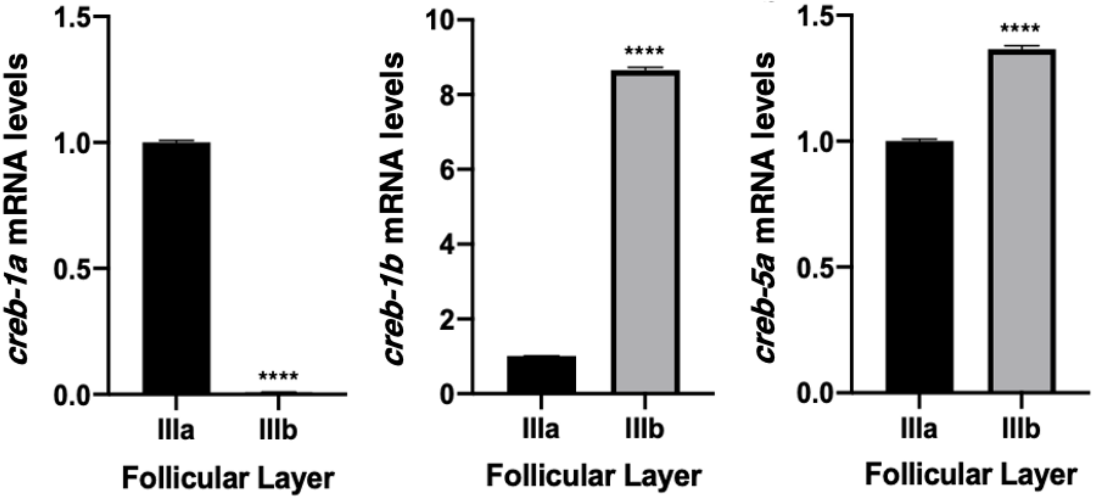

Figure IV.8. CREB mRNA levels in follicular cells. Stage IIIa and IIIb follicles were collected 1h after lights on and the follicular layers were manually separated from the oocytes. Total RNA was extracted from the follicular layers and processed to measure *creb-1a*, *creb-1b*, and *creb-5a* mRNA levels. Relative mRNA levels are represented as fold-change to stage IIIa after normalization to elongation factor-1-alpha (*ef1a*). Statistical significance was determined using a two-tailed t-test. Data represent mean ± SEM (n = 3). ****P < 0.0001 vs control.

Expression profile of CREB in Nodal-treated denuded oocytes

To determine if Nodal affects CREB mRNA levels, stage IIIb follicles were collected and treated with either control, hCG (20IU/mL), or Nodal (250ng/mL). Follicles were collected after 18h and their follicular layer were manually removed. Denuded oocytes were processed through RNA extraction and CREB mRNA levels were determined by qPCR. The results (Fig. IV.9) show relative *creb-1a* mRNA levels were significantly higher in hCG- and Nodal-treated follicles compared to the control. While there was no significant difference in *creb-1b* mRNA levels across treatments, *creb-5a* levels were significantly lower in hCG- and Nodal-treated samples.

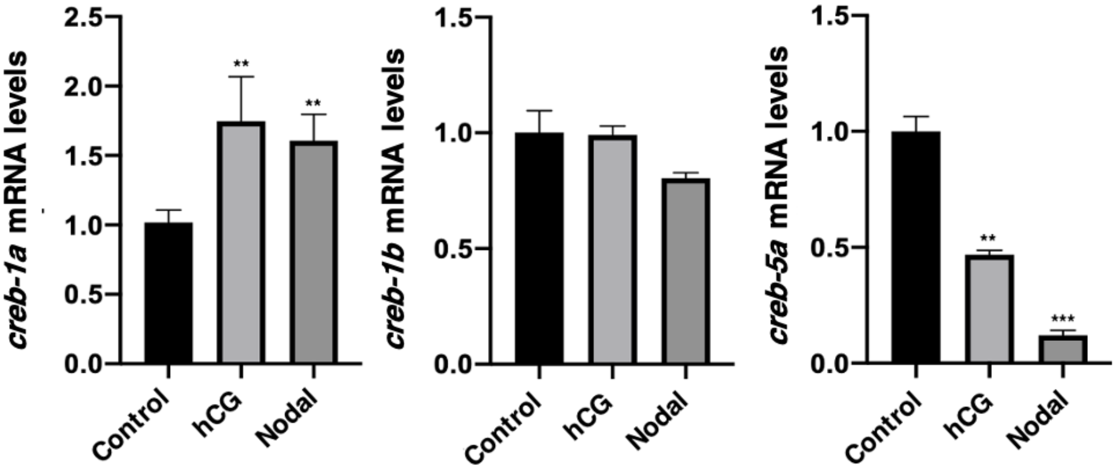

Figure IV.9: Nodal regulates CREB mRNA levels in oocytes. Stage IIIb follicles were collected and treated with control, hCG (20IU/mL), or Nodal (250ng/mL). After 18h, the denuded oocytes were processed to measure *creb-1a*, *creb-1b*, and *creb-5a* mRNA levels. Relative mRNA levels are represented as fold-change to the control after normalized to zebrafish elongation factor-1-alpha (*ef1a*). Statistical significance was determined using one-way ANOVA. Data represent mean ± SEM (n = 3 wells). **P<0.01; ***P<0.001 vs control.

DISCUSSION

The TGF-β superfamily has important functions in many physiological processes, including follicle development, growth, and oocyte maturation. While activin induces oocyte maturation (Wu et al., 2000; Pang and Ge, 2002), TGF-β1 and BMP-15 inhibit oocyte maturation (Clelland et al. 2006; Clelland et al. 2007). Nodal, a member of the TGF-β superfamily, is known for its critical role during embryo development. However, little is known about its involvement in ovarian function regulation. Nodal signals through two types of serine/threonine kinase receptors, type I (ALK4 and ALK7) and type II (ActR11A and ActRIIB) (Hill, 2018; Wang and Ge, 2003).

A previous study in our lab showed the presence of Nodal signaling components within zebrafish oocytes and follicular cells. The study found endogenous mRNA levels of Nodal homologs, *ndr1* and *ndr2*, showed different expression patterns during follicle development; *ndr1* decreased from stages I-IV and increased at stage V while *ndr2* levels increased at stage II and decreased thereafter. When comparing stage IIIa and IIIb follicles, *ndr1* and *ndr2* mRNA levels increase in stage IIIb follicular cells. Similarly, acvr1b and acvr1c, which encode ALK4 and ALK7, respectively, also have higher mRNA levels in stage IIIb follicular cells. These findings suggest that Nodal signaling may play a role in follicle development, specifically in transitioning from stage IIIa to IIIb.

In this study, I demonstrate that Nodal induces oocyte maturation. Treatment of rhNodal resulted in dose- and time-dependent increases in GVBD. This is observed in both follicles and denuded oocytes, indicating that Nodal can act both on follicular cells and oocytes. While the role of Nodal in embryonic development is well established, to the best of our knowledge, this is the first study that shows Nodal regulates oocyte maturation. The finding suggests that Nodal signaling plays an important role in female reproduction. This notion is further supported by our recent observations that *ndr*$^{+/-}$ female fish produce fewer eggs than wild-type fish (Alvi et al., unpublished observations).

A recent study from our lab has demonstrated that Nodal induces expression levels of hsd3b2 and cyp17a1 (Zayed et al., 2020). Hsd3b2 encodes 3b-HSD, an enzyme involved in the production of progesterone (Lin et al., 2015), while cyp17a1 encodes 17a hydroxylase, an enzyme involved in the conversion of progesterone to 17α-HP and production of androgen precursors (Wang and Ge, 2004). The upregulation of these enzymes by Nodal may stimulate production of progesterone, 17α-HP, or androgens, which could be further converted to MIH or estradiol (Fig. IV.10). In this study, I observed 17α-HP levels measured by ELISA were increased in Nodal-treated stage IIIb follicles in a dose-dependent manner.

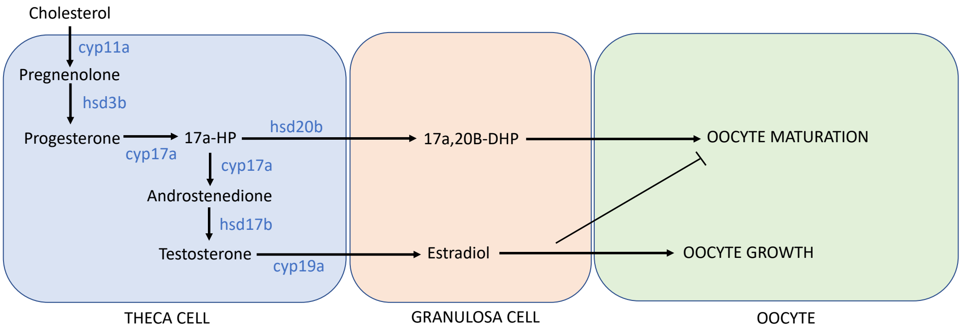

Figure IV.10: Follicle growth and maturation in zebrafish. The process of follicles growth and oocyte maturation in zebrafish involves several hormones and enzymes within the different cell layers. Cholesterol is first converted to pregnenolone by cyp11a within the theca cell, which, in turn, is converted to progesterone by hsd3b. The enzyme cyp17a then facilitates the conversion of progesterone to 17a-HP. When undergoing oocyte maturation, 17a-HP is converted into 17a,20B-DHP. During oocyte growth, 17a-HP undergoes conversion by cyp17a and hsd17b to testosterone, which then converts to estradiol in the granulosa cell by cyp19a. While estradiol promotes oocyte growth, it inhibits oocyte maturation. (Adapted from Clelland and Peng, 2009.)

I also measured changes in steroid hormone levels in heterozygous *ndr2* knockout ovaries. Although estradiol levels were unchanged, levels of progesterone were higher in *ndr2*$^{+/-}$ fish compared to wild-type while 17α-HP were significantly lower. Although testosterone levels appear to be lower in the *ndr2*$^{+/-}$ ovaries, the difference was not statistically significant. These results demonstrate that *ndr2* plays a critical role in promoting the conversion of progesterone to 17α-HP. High levels of progesterone observed in *ndr2*$^{+/-}$ could be caused by the suppression of 17α-hydroxylase, which facilitates conversion from pregnenolone to progesterone. It is possible that *ndr2* exerts a more prominent role in regulating cyp17a1 than hsd3b2, leading to more progesterone accumulation but lower 17α-HP levels in the *ndr2*$^{+/-}$ovary. Interestingly, estradiol levels remained unchanged, which suggests the pathway of conversion of testosterone to estradiol was undisturbed. This is consistent with our observations that Nodal did not alter the mRNA level of cyp19a1a, which encodes aromatase responsible for the conversion of testosterone to estradiol, in follicular cell culture (Zayed et al., 2020).

MIH is the most well-documented maturation steroid in zebrafish and many other teleosts. 17α-HP, which can be directly converted into MIH, and progesterone have also been shown to induce maturation *in vivo* (Tokumoto et al., 2011). In addition, previous studies have proposed the importance of androgens in follicle development and oocyte maturation in several species, including fish (Li et al., 2019). A study showed hCG-induced oocyte maturation transiently increased testosterone concentration in serum and was also transiently stimulated in ovarian follicles, suggesting testosterone levels could be elevated in the ovary by LH in zebrafish (Li et al., 2019). Follicles treated *in vitro* with testosterone-induced oocyte maturation and activated the MPF component cyclin B, suggesting that testosterone induces the activity of MPF and oocyte maturation (Li et al., 2019). Since treatment with Nodal induced, while *ndr2* knockout reduced, 17α-HP levels, it is possible that Nodal induces oocyte maturation by increasing 17α-HP production. It remains to be determined if Nodal also induces MIH production. In addition, more studies are needed to confirm whether Nodal plays a role in regulating testosterone production and thereby promoting oocyte maturation.

One pathway that regulates steroid production, follicle development, and oocyte maturation is the cAMP pathway (Qi et al., 2016; Duarte et al., 2014; Manna et al., 2002). CREB is a transcription factor known to be activated by a cAMP-dependent protein kinase, PKA, and several signaling pathways, such as the Ca^{2+}-CAMKII signaling pathway, and the phosphoinositide 3-kinase (PI3K)/AKT signaling pathway, have also been reported to activate CREB (Wang et al., 2019). Interestingly, the cAMP pathway has distinct roles in follicular cells and oocytes. While it mediates the role of gonadotropins in steroid hormone production (Senthilkumaran et al., 2015), cAMP is also known to maintain meiosis arrest and inhibit oocyte maturation (Das et al., 2018). Preliminary observation from our lab suggests that Nodal may activate CREB in follicular cells. Therefore, I attempted to explore the CREB expression in zebrafish follicles and determine if Nodal induces oocyte maturation by regulating CREB.

Multiple forms of CREB have been identified in Nile tilapia and found to be differentially expressed during folliculogenesis and oocyte maturation (Senthilkumaran et al., 2015; Wang et al., 2019). Based on their expression profile, the authors suggested CREB1 may be involved in oocyte growth while CREB2 and CREB3 may be important for oocyte maturation (Senthilkumaran et al., 2015). In zebrafish, I found CREB-1a had higher mRNA levels in stage IIIb follicles than stage IIIa follicles, while CREB-1b and CREB-5a had lower mRNA levels. Interestingly, the opposite pattern was observed in follicular cells, with lower CREB-1a but higher CREB-1b and CREB-5a mRNA levels found in stage IIIb. This could be due to the fact that most mRNA in follicles come from oocytes. Therefore, it is possible that the CREB expression patterns in oocytes are opposite to those in follicular cells. This suggests the expression of CREB is spatially dependent within the different components of the follicle.

To explore the potential role of Nodal in regulating CREB expression, I treated follicles with hCG and Nodal and analyzed CREB mRNA levels in follicular cells and oocytes separately. The effect of Nodal and hCG on follicular cell CREB mRNA levels was inconsistent and requires further investigation; however, I observed significant changes in CREB-1a and CREB-5a mRNA levels in oocytes after both Nodal and hCG treatments. Specifically, oocyte CREB-1a mRNA levels were elevated, while CREB-5a mRNA levels were down-regulated, by hCG and Nodal, suggesting that these two CREBs may be involved in the regulation of oocyte maturation. Additionally, CREB-1b mRNA levels did not change upon treatment with either hCG or Nodal, suggesting it does not play a role in oocyte maturation. It remains to be determined which signaling pathway(s) activates CREB-1a and CREB-5a and what roles these transcription factors play during oocyte maturation. The down-regulation of CREB-5a mRNA levels after hCG and Nodal treatment suggests that it may be downstream of the cAMP pathway, as cAMP in oocytes is blocked before oocyte maturation occurs. On the other hand, CREB-1a is likely acting downstream of a signaling pathway that promotes oocyte maturation. While these findings are novel, they are preliminary in nature and more studies are required to further investigate the regulation of different CREB isoforms in follicular cells and oocytes and their functional significance in oocyte maturation.

CONCLUSION AND FUTURE DIRECTIONS

While little is known about the role of Nodal in the ovary, this study provides evidence of its involvement in oocyte maturation in zebrafish. We demonstrated that Nodal induces oocyte maturation in a time- and dose-dependent manner in both intact follicles and denuded oocytes. We also showed that Nodal significantly increased 17α-HP levels in the conditioned media of cultured follicles while *ndr2* knockout reduced 17α-HP contents in the ovary. These findings strongly suggest that Nodal induces oocyte maturation by enhancing the production of 17α-HP, which could then be converted into MIH. To further investigate the mechanisms by which Nodal induces oocyte maturation, I also explored CREB expression and regulation in zebrafish follicles. Three forms of CREB (1a, 1b, and 5a) were found to be expressed differentially within oocytes and follicular cells. Preliminary data suggest that Nodal and hCG regulate CREB-1a and CREB-5a mRNA levels in oocytes.

For future study, it would be useful to characterize the expression of CREB isoforms within all follicle development stages (stage I-V) and to further determine their regulations and functions during follicle development and oocyte maturation. Additionally, the CREB expression levels could be measured in *ndr* knockouts to further explore the relationship between Nodal and CREB. To further investigate the mechanism through which Nodal exerts its effects on oocyte maturation, inhibitors could be used to block different pathways, such as CREB or ERK. Additional *ndr* knockout lines (such as $ndr1^{+/-}$ or $ndr3^{+/-}$) could also be used to examine the functions of different *ndr* genes within zebrafish ovaries and oocyte maturation.

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