Temporal mRNA expression of transforming growth factor-beta superfamily members and inhibitors in the developing rainbow trout ovary 

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During mammalian ovarian development transforming growth factor-beta (TGFβ) superfamily members and their inhibitors are critical paracrine regulators, yet the intraovarian functions of these proteins have received less attention in fish. Using quantitative real-time RT-PCR, changes in ovarian mRNA expression of six TGFβ members and two inhibitors were investigated in rainbow trout across a wide range of fish ovarian stages (i.e., early perinucleous stage through acquisition of maturational competence). Transcript changes for insulin-like growth factor 1 and 2, and five enzymes associated with steroidogenesis, as well as plasma levels of three sex steroids were also measured to provide a framework of established intraovarian regulators in trout. Expression of bone morphogenetic protein 4 (bmp4), bone morphogenetic protein 7 (bmp7), and growth differentiation factor 9 (gdf9) peaked during pre-vitellogenic stages and steadily decreased through advancing stages implicating these genes in early ovarian development. A dramatic increase in inhibin betaA and decrease in follistatin expression occurred during early to mid-vitellogenic stages, which corresponded with increased 17α-estradiol plasma levels suggesting a vitellogenic role for ovarian activin A. Follicles that were competent to respond to the maturation-inducing hormone had decreased levels of inhibin betaA and increased expression ofambi(bmp and activin membrane-bound inhibitor) suggesting their roles in maturation processes. Furthermore, bmp4, bmp7 and gdf9 are primarily expressed in the oocyte whereas the inhibin subunits, follistatin, and ambis are primarily expressed in the somatic follicle cells. These results support TGFβ superfamily members and their inhibitors have wide-ranging and disparate roles in regulating ovarian development in fish.

1. Introduction

Ovarian development is well described in fishes, but the majority of our knowledge on regulatory mechanisms centers on the functions of the hypothalamic–pituitary–gonadal axis endocrine products. The relative importance of autocrine and paracrine growth factor regulators of germ cell and somatic cell interactions, as well as inter-somatic cell interactions (i.e., theca to granulosa cell) has largely been overlooked. Described intraovarian actions of TGFβ superfamily members and their innate inhibitors in mammals and birds include stimulating granulosa cell proliferation, altering GTH receptor densities in follicle cells, and altering steroidogenesis, which appears to be species specific (Knight and Glister, 2006; Shimasaki et al., 2004; Al-Musawi et al., 2007; Elis et al., 2007). Investigations into domesticated mammals with natural mutations in GDF9 and BMP15 (i.e., Inverdale and Hanna ewes) have even led investigators to suggest that these oocyte derived proteins are primary determinates of ovulation quota in these species (Moore et al., 2004). In spite of these findings, little information on the reproductive function and ovarian expression of TGFβ superfamily members and their innate inhibitors is available in fishes.

The most extensive knowledge of TGFβ superfamily systems in fish supports an intact ovarian inhibin–activin system (Ge, 2005). Inhibins and activins are closely related dimeric proteins that share the same β subunits; inhibins consist of an inhibin α subunit and an inhibin β subunit, while activins are composed of two inhibin β subunits (Vale et al., 1990). The few studies that have investigated the functions of these peptides in the fish ovary indicate that activins are involved in the process of oocyte maturation (Pang and Ge, 1999, 2002; Wu et al., 2000; Petrino et al., 2007), while the classic stimulatory actions of activin on pituitary follicle-stimulating hormone (Fsh) synthesis and release appear to remain intact (Davies and Swanson, 1997; Ge, 2000). A few recent studies suggest that TGFβ, BMP and GDF members are also operating in the fish ovary including studies describing changes in transcript expression (Bobé et al., 2004, 2008; Baron et al., 2005; von
Schalburg et al., 2006; Liu and Ge, 2007; Sawatari et al., 2007; Lunkenbach et al., 2008), and actions on oocyte maturation (Kohli et al., 2003, 2005; Clelland et al., 2006, 2007; Peng et al., 2009; Tan et al., 2009), but the number of species and reproductive stages that have been investigated are limited.

Changes in BMP4, 7, and GDF9 transcript expression have been characterized in the rainbow trout ovary during gonadal differentiation and the onset of gametogenesis (Baron et al., 2005), as well as in somatic cells during follicle maturation (Bobe et al., 2003, 2004). However, the temporal expression patterns during the intervening stages of ovarian development, as well as the expression of oocyte-derived transcripts during follicle maturation have been largely ignored. Therefore, the objective of the present study was to provide temporal mRNA expression profiles of TGβfI superfamily members and their inhibitors in follicle-enclosed oocytes across multiple ovarian development stages, including those not previously investigated, as well as the differential expression between somatic cells and the oocyte in competent follicles, which are those with the ability to respond to the maturation-inducing hormone (MIH). The expression patterns of bone morphogenetic protein 4 (bmp4), bone morphogenetic protein 7 (bmp7), and growth differentiation factor 9 (gdf9), inhibin α-subunit (inha), inhibin βα subunit (inha), inhibin ββ subunit (inhbb), follistatin (fst), and bmp and activin membrane-bound inhibitor (bambi) were quantified in six histologically verified ovarian developmental stages in rainbow trout. In addition, due to their well established roles in both mammalian and fish reproduction, the transcripts encoding insulin-like growth factor 1 (igf1) and 2 (igf2), and five enzymes related to steroidogenesis (see Section 3.1 for transcript list) were measured, as well as the circulating levels of 17b-estradiol (E2), testosterone (T) and the progesterone MIH, 17α,20β-dihydroxy-4-pregnene-3-one (17,20βP) to ascertaining the biological relevance of intraovarian TGβfI-related transcript expression patterns.

2. Materials and methods

2.1. Fish care and tissue sampling

The rainbow trout used in temporal analysis of mRNA and the steroidogenic portion of this study were obtained from Troutlodge Inc. (Sumner, Washington, USA) as eyed eggs and reared at the National Center for Cool and Cold Water Aquaculture (NCCWA/USDA) in Kearneysville, West Virginia, USA, while the rainbow trout use in the spatial analysis were obtained as adults from the White Sulphur Springs National Fish Hatchery (USFWS), White Sulphur Springs, West Virginia, USA. All fish were reared and maintained in continuous-flow ground water with an ambient temperature of 13 ± 1 °C and dissolved oxygen content near air saturation while at NCCWA. Photoperiod was maintained with artificial lighting that was adjusted weekly to simulate the ambient photoperiod. Fish were fed Zeigler Gold food (Zeigler Bros. Inc., Gardners, Pennsylvania) at 1% body weight daily. Fish care and experimentation followed the guidelines outlined by the USDA and the NCCWA Animal Care and Use Committee, which are in line with the National Research Council publication Guide for Care and Use of Laboratory Animals.

Ovarian fragments were dissected from maturing virgin rainbow trout at monthly intervals beginning on March 17 (1+ year fish) and continuing through March 3 of the following year (reproductively mature fish; 2+ year fish). At the time of collection fish were anesthetized by immersion in MS-222 (150 mg L−1), the length and weight were recorded, a blood sample was collected for hormonal analysis and fish were euthanized by decapitation. Both ovarian lobes were dissected out and weighed for determination of the gonadosomatic index (GSI: [gonad weight/body weight – gonad weight] × 100]) and sub-samples were either flash frozen in liquid nitrogen and stored at −80 °C for RNA isolation or fixed in Prefer<sup>TM</sup> (Anatech Ltd., Battle Creek, MI) for histological verification of reproductive stage. Ovarian fragments collected for assessing maturational competence or for mechanical separation of the somatic follicular cells from the oocyte for spatial analysis of transcripts were placed in ice-cold trout mineral medium (TMM; Bobe et al., 2003).

2.2. Histological verification of maturational stage

Ovarian fragments were initially assigned to one of six developmental stages by the average diameter of ~20 follicle-enclosed oocytes. The stages were designated early pre-vitellogenesis (E pre-Vg: ≤0.5 mm), late pre-vitellogenesis (L pre-Vg: 0.51–0.65 mm), early vitellogenesis (E-Vg: 0.66–1.1 mm), mid-vitellogenic (M-Vg: 1.11–2.1 mm), late vitellogenic (L-Vg: 2.11–4 mm) and competent, capable of responding to the MIH to induce resumption of meiosis as indicated by germinal vesicle breakdown (GVBD) as determined by bioassay. The diameter requirements are according to Tyler and coworkers (Tyler et al., 1994). Histological verification of maturational stage was performed in paraffin embedded, 4 μm, hematoxylin and eosin stained sections (See Table 1 for Summary). All oocytes contained within three randomly selected objective fields (20×) were staged for the E pre-Vg, L pre-Vg and E-Vg samples, while all oocytes contained within the section were staged for the M-Vg samples. Histological verification was not necessary for the L-Vg and competent samples since all were well beyond the minimum size for reaching the yolk-granule stage. A combination of size, appearance of nucleus and nucleolus, and the ooplasmic inclusions were considered when assigning individual oocytes to one of four maturational stages (i.e., early perinucleous, late perinucleous, cortical alveoli/oil drop and yolk globule). These stages were based upon the description summarized by Nagahama (1983).

2.3. In vitro bioassay for verification of maturational competence

To verify that oocytes were competent to respond to rainbow trout MIH, triplicate samples of 15 follicle-enclosed oocytes from each fish (n = 12) were incubated with 1 μg mL−1 of 17,20βP for 96 h in 12-well Falcon culture plates (Becton Dickson, Franklin Lakes, NJ) filled with 8 mL of ice cold TMM at 10 °C on a orbital oscillator at 80 RPM. The tissue was then fixed and cleared in Davidson’s solution (2 parts 37% formaldehyde:3 parts 95% ETOH:1 part acetic acid:3 parts H<sub>2</sub>O) for verification of GVBD. Only the oocytes (n = 5) that displayed 100% GVBD after 96 h were considered competent.

2.4. Separation of somatic follicular tissue from oocyte

A total of 25 sets of somatic follicular layers (i.e., theca and granulose layers) and 25 intact follicle-enclosed oocytes were collected in triplicate from three individual fish that were between the L-Vg and competent stages. The follicular layers were isolated by mechanically rupturing the follicular layers with fine forceps followed by a series of four rinsing steps, which each involved gently vortexing the follicular components in ice cold TMM, centrifugation at 615 rcf for 10 min, discarding the supernatant, and resuspension of the follicular components in fresh TMM. Tri Reagent (Sigma, St. Louis, MO) was used to resuspend the components after the final wash and RNA was isolated as described below.

2.5. Total RNA isolation and quantitative real-time RT-PCR techniques

Approximately 100 mg fragment of ovarian tissue was homogenized in Tri Reagent using a MM300 multi-tube homogenizer
(Retsch Inc., Haan, Germany) and total RNA was isolated according to the manufacturer’s protocol with the inclusion of a high salt solution (Molecular Research Center, Cincinnati, OH) modified pre-precipitation step to remove the excess glycosylated proteins from the eggs. The RNA was then treated with DNase (Promega, Madison, WI) according to the manufacturer’s instructions and re-isolated. The RNA was then quantified using a NanoDrop ND-1000 (Wilmington, DE USA) and integrity was verified by analysis of the 260/280 ratio and electrophoresis (1.5% agarose). First-strand cDNA was synthesized by allowing 1 μL of RNA for 5 min at 70 °C in a final volume of 10 μL and then adding 15 μL reverse transcription reaction mix (1 × MMLV buffer, 12.5 mM each dNTP, 25 U RNasin, 200 U MMLV-RT) and incubating at 37 °C for 1 h, followed by incubation at 95 °C for 5 min. Negative controls (no RT) were also included for verification that no genomic DNA contamination was present.

Quantitative real-time RT-PCR reactions were carried out in triplicate and each consisted of 0.3 μL of the first-strand cDNA reaction, 0.1–0.4 μL each primer and 1 × SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) in a 15 μL volume. The thermal cycling profile was 50 °C for 2 min, 95 °C for 10 min and 40 cycles of 95 °C for 30 s, 60 °C for 20 s, and 72 °C for 30 s. A final dissociation step was performed to assess the specificity of the reaction. Relative quantification of the unknown gene expression was determined by the standard curve method described in Applied Biosystems, User Bulletin #2. All samples were normalized to the expression of elongation factor-1-alpha (ef1α). Products were sequenced to confirm identity. Details of the quantitative real-time RT-PCR reactions are provided in Table 2.

### Table 2

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<th>Gene</th>
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<th>Forward sequence</th>
<th>Reverse sequence</th>
<th>Size</th>
<th>Mean Ct</th>
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- a Bobe et al. (2004).
- b Number of amplicon base pairs.
- c Mean Ct across all six ovarian stages.

### 2.6. Measurement of plasma hormone levels

Plasma levels of E2, T and 17,20βP were measured by specific radioimmunoassay according to Woods and Sullivan (Woods and Sullivan, 1993). Antisera were validated for use in salmonid species (Young et al., 1983; Fitzpatrick et al., 1986).

### 2.7. Statistical analysis

Statistical analyses were performed using SigmaStat 3.0 (Jandel Scientific, San Rafael, CA) software. The relative expressions of all genes measured were individually compared across all six developmental stages by one-way analysis of variance (ANOVA) model followed by Holm-Sidak multiple comparisons test. Data were log10 transformed when necessary to meet normality and equal variance assumptions and reported as means ± SE. Plasma hormone levels were compared across all six developmental stages by a one-way ANOVA model, followed by either a Holm-Sidak or ANOVA on Ranks multiple comparison test and reported as means ± SE. All data were considered significant at the α = 0.05 level.

### 3. Results

#### 3.1. Plasma hormone concentrations and mRNA expression of steroidogenic enzymes and star during ovarian development

Plasma levels of E2, T and 17,20βP remained low and unchanged through the E-Vg stage (Fig. 1). Plasma levels of E2 were significantly elevated at M-Vg and further increased to a peak concentration of 31.4 ± 2.7 ng mL⁻¹ in the L-Vg stage. Plasma levels of
T remained unchanged until the L-Vg stage and peaked at the competent stage with a concentration of 59.0 ± 5.8 ng mL⁻¹/C₀. Plasma levels of 17,20βP remained unchanged and low until the competent stage where they rose slightly to 1.6 ± 0.2 ng mL⁻¹/C₀.

The transcripts encoding steroidogenic acute regulatory protein (star) and side-chain cleavage cytochrome p450 (cyp11a1), general indicators of steroidogenic capacity, significantly increased in the E-Vg stage and then increased dramatically in the competent stage (Fig. 2a). Transcripts for 17-alpha-hydroxylase cytochrome p450 (p450c17), aromatase cytochrome p450 (cyp19a1), and 3-beta-hydroxysteroid dehydrogenase/D5-4-isomerase (3bhsd), which are more specific indicators of androgen, estrogen and progestogen pathways, showed their first significant increases after star and cyp11a1 (Fig. 2b). Transcript abundances for p450c17 and cyp19a1 first increased by the M-Vg stage whereas 3bhsd abundance did not significantly increase until competence. All of the transcripts for these enzymes appear to be exclusively expressed in the somatic follicular tissue as evidenced by the similar expression levels between the follicular tissue and the follicle-enclosed oocyte samples (Table 3). Since there was the same number of follicular layers in both samples, one would expect similar relative expression values if the follicular cells account for nearly all of the transcript expression. This method of comparison is clearly not perfect due to the possibility of incomplete separations and the potential loss of follicular cells during processing. For those reasons the authors interpret transcripts with ratios of less than two as being predominately expressed in the follicular cells, whereas if the ratio is greater than two, it is likely that the additional transcripts are expressed in the oocyte itself.

3.2. Expression of igf1 and igf2

The igf1 and igf2 transcripts were at their lowest in the pre-Vg through E-Vg stage (Fig. 2c). Expression of igf1 mRNA displayed a significant increase by the L-Vg stage and a further increase in the competent stage. The expression profile of igf2 was similar to igf1, but the maximum increase was attenuated in comparison. Interestingly, the mean Cₘ for igf2 was much lower than that of igf1 suggesting much higher igf2 than igf1 expression (Table 2; 22.7 versus 27.9 mean Cₘ, respectively). Transcripts for igf1 and igf2 appear to be ubiquitously expressed in the follicle-enclosed oocyte due to a ~3-fold greater abundance in the follicle-enclosed oocyte samples compared to the somatic follicular tissue isolate (Table 3). This suggests an approximately equal level of expression in both the somatic follicular tissue and oocyte.

3.3. Expression of bmp4, bmp7, and gdf9

Transcripts of bmp4, bmp7, and gdf9 were most highly expressed in the earlier stages of ovarian development (Fig. 3a). Expression of bmp4 mRNA was significantly reduced by E-Vg and displayed further reduction by L-Vg, which was maintained through the compe-
tent stage resulting in an 8.3-fold reduction compared to E pre-Vg levels. The expression of bmp7 mRNA paralleled bmp4 and was reduced by 6.3-fold in the competent stage. Transcripts of gdf9 displayed a similar pattern, but were not significantly reduced until the L-Vg stage and were further reduced by 11.1-fold in the competent stage when compared to E pre-Vg. The majority of the mRNA coding for bmp4, bmp7, and gdf9 appears to be in the oocyte compared to the somatic follicular tissues, as evidenced by the respective 12-, 27- and 20-fold higher expression in follicle-enclosed oocyte when compared to the somatic follicular tissue isolate (Table 3). However, all three transcripts were detectable in somatic follicular tissues.

3.4. Expression of inha, inhba, and inhbb

Transcript abundance of inha and inhba were at their lowest in the E pre-Vg stage (Fig. 3b). Expression of inha mRNA was relatively stable throughout ovarian development, with the exception of a significant elevation at the M-Vg stage. Transcripts of inhba were significantly elevated by the E-Vg stage and expressed a dramatic up regulation by the M-Vg stage. Elevated transcript levels were statistically maintained through the competent stage, where they reached a peak 34.4-fold increase compared to the E pre-Vg stage. Alternatively, inhbb transcripts remained unchanged through the M-Vg stage (Fig. 3b), which appeared to be peak expression, and reached the lowest expression (18.5-fold reduction compared to E pre-Vg) during the competent stage. The mRNA coding for all three subunits appears to be predominately located in the somatic follicular tissue due to the similar level of expression between the somatic follicular tissue isolate and the follicle-enclosed oocyte samples (Table 3).

3.5. Expression of fst and bambi

The highest fst mRNA expression was in the pre-Vg ovary and drastically decreased (~5-fold) upon the initiation of exogenous Vg (Fig. 3c). The expression of bambi mRNA remained statistically stable through the L-Vg stage and was significantly elevated (~2-fold) in the competent stage when compared to the E pre-Vg stage (Fig. 3c). The location of fst and bambi mRNA appears to be in the somatic follicular tissues due to similar level of expression between the somatic follicular tissue isolate and the follicle-enclosed oocyte samples (Table 3).

4. Discussion

The temporal expression patterns of the intraovarian transcripts related to the TGFβ superfamily investigated show they are differentially regulated through ovarian development suggesting involvement in wide range of reproductive processes in the rainbow trout. Changes in expression in both the oocyte and somatic follicle cells are consistent with their playing roles in intercellular communication between the germ and somatic cells. To our knowledge in addition to gdf9, the inhibin β subunits, and fst, in
zebrafish (Wang and Ge, 2004; Liu and Ge, 2007) these data are among the first to provide a normalized comparison of these transcripts across these ovarian events in fish.

Changes in levels of plasma steroids and mRNAs for steroidogenic enzymes and star measured in our study are consistent with what has been reported previously in rainbow trout and other salmonid species (Van Der Kraak and Donaldson, 1986;Estay et al., 1998; Nakamura et al., 2005). In general, all of these components remained low and relatively unchanged through the E-Vg stage, reflecting the gonadotropin-independent and steroid-independent nature of primary oocyte growth in fishes (Tokarz, 1978). Expression of the earliest acting enzymes in the steroidogenic pathway, star and cypl1a1, increased by E-Vg (Fig. 2a), indicating the onset of increasing steroidogenic capacity. Transcript levels of p450c17 and cypl9a1 increased by M-Vg (Fig. 2b), together with increasing plasma E2 concentrations (Fig. 1). The increase in E2 and T production at L-Vg without a further increase in the normalized expression of cypl9a1 and p450c17 mRNA, can be explained by the increase in follicle growth during this period. The significant increase in 3βhsd transcripts in the competent stage (Fig. 2b) would also suggest a decreased P450c17:3βhsd ratio, which would direct steroidogenesis towards the synthesis of M1H via enhanced 17α-hydroxyprogesterone (17αOHP) synthesis (Nakamura et al., 2005; Sakai et al., 1994). Keep in mind that the fish sampled for the competent stage follicles were not spawning when sampled, and the reported plasma steroid concentrations, particularly the relatively low M1H concentrations, are also not indicative of spawning rainbow trout.

The increases in igf1 and igf2 expression beginning at M-Vg and peaking in competent follicles (Fig. 2c), suggest involvement in the final stages of ovarian growth and development. Expression of igf1 and 2 have been shown to increase in concert with the acquisition of competence and oocyte maturation in rainbow trout (Bobe et al., 2003, 2004;Aegerter et al., 2004) and Igfs were shown to stimulate steroid synthesis in the granulosa cell layers and inhibit steroid synthesis in the theca cell layers of preovulatory coho salmon suggesting a role for Igfs during oocyte maturation (Mae stro et al., 1997). We did not see an increase in igf1 or igf2 transcript abundance in E-Vg stages as observed in coho salmon (Campbell et al., 2006) suggesting possible differences in Igf roles in the onset of secondary growth among salmonids. Furthermore, based on mean Cv values, igf2 mRNA was found to be approximately 30-fold greater in abundance when compared to igfs (Table 2), suggesting igf2 is the prevalent ligand for activating igf1 receptors in the trout ovary.

Although no significant changes in TGFβ-related transcript expression were observed between E pre-Vg, L pre-Vg, and M pre-Vg, many transcripts were at their highest concentrations, including bmp4, bmp7, the closely related gdf9, and fst. The majority of the oocytes grouped into these stages were in the early perinucleolus (i.e., approaching diplotene stage of meiosis I) and late perinucleolus (i.e., arrested in diplotene stage of meiosis I) stages (Table 1) of primary growth. However, a small portion of the oocytes grouped into the E pre-Vg and L pre-Vg stages had already initiated the secondary growth phase, as indicated by the presence of cortical alveoli (Table 1). High levels of bmp4, bmp7, gdf9, fst, and to a lesser extent inha and inhbb, during these stages suggest potential roles in oogenesis, primary oocyte growth, and the transition into secondary growth. Expression of inha was at its lowest levels and bambi did not change until an increase at the competent stage. We have little data from fish with which to compare our results from these stages, but the expression pattern of gdf9 in trout corroborates the pattern reported for zebrafish over a similar range of stages, primary growth through maturation (Liu and Ge, 2007). Although we observed no changes in inha and fst between the E-pre-Vg and L-pre-Vg stages, both transcripts increased in zebrafish con-
In zebrafish, **inhba** expression also increases throughout the transition from pre-vitellogenesis through mid-vitellogenesis, but in contrast to trout, **fst** expression parallels **inhba** expression, with both transcripts appearing to decrease in full-grown follicles (Wang and Ge, 2004). Differences in expression are not surprising considering the zebrafish is a multiple-clutch spawner, completing primary growth through ovulation within a day, whereas this process takes over a year in the single-clutch spawning rainbow trout.

The continual decline in **bmp4**, **bmp7**, and **gdf9** transcripts during vitellogenesis also supports a potential direct involvement in steroidogenesis. In the mammal, steroidogenesis is influenced by BMPs and GDF9, resulting in the general pattern of enhanced synthesis of E2 and inhibition of P4 synthesis (Shimasaki et al., 2004). Also in the hen, BMP4 and 7 stimulated basal, IGF-I, and GTH-stimulated P4 production by granulosa cells in vitro (Onagbesan et al., 2003). Given that the lowest levels of **bmp4**, **bmp7**, and **gdf9** transcripts are observed in competent stage follicles (Fig. 3a) when steroidogenesis is switching from E2 to the progestogen MIH (Fig. 1), it is tempting to speculate that their steroidogenic functions are conserved among vertebrates. Nevertheless, Bobe and colleagues (2004) observed greater **bmp4** and **bmp7** mRNA levels in follicle layers of rainbow trout undergoing maturation as compared to incompetent and competent fish, which would argue against this idea. However, the current data suggest that **bmp4** and **bmp7** transcripts are primarily located in the oocytes (Table 3) and not in the follicle layers, suggesting differences in oocyte compared with somatic follicle cell regulation of these transcripts in the trout follicle. Few changes were observed in the abundance of TGFβ member transcripts as the follicles progressed from the L-Vg to the competent stage. This is in contrast to the increased expression in both **igf1** and **igf2** (Fig. 2c), and all of the steroidogenic enzyme transcripts except **cyp19a1** (Fig. 2a and b), which remained unchanged. However, transcript levels of **inhba** were at their highest levels in the competent stage fish, while **inhbb** transcripts were at their lowest (Fig. 3b), suggesting the production of activin A. Although 17.20βP is accepted as the MIH in trout, as in many teleost fishes, activins have been found to promote the acquisition of competence and GVBD in several species of fish (Ge, 2000; Wu et al., 2000). Both activin A and B induced GVBD in competent zebrafish oocytes, and induced the acquisition of competence in mid-vitellogenic oocytes (Ge, 2000; Pang and Ge, 2002). However, activin A only augmented the steroid-induced GVBD in killifish (Petirno et al., 2007), suggesting some level of species differences. The current expression pattern for **inhba** agrees in general with the expression pattern found in zebrafish (Wang and Ge, 2004), however, **inhbb** expression in zebrafish remains high from E-Vg through the remainder of follicle growth. Curiously, **bambi** mRNA was only increased in competent follicles (Fig. 3c), which suggests a regulatory role in the acquisition of competence or oocyte maturation. Since BAMBI functions as a type-I pseudoreceptor lacking a serine/threonine kinase domain, it inhibits signaling of most TGFβi superfamily members (Onichtchouk et al., 1999). Expression of **bambi** was primarily in the follicle cell layers (Table 3) and since it functions as a pseudoreceptor its direct actions are limited to the somatic cells. This would suggest an increase in its expression would not interfere with activin actions at the oocyte but would decrease activin and BMP actions at the follicle, possibly further reducing the influence of these ligands on steroid production discussed above. Alternatively, the increase in **bambi** expression may be a negative-feedback response to an increase in one or more TGFβi ligands considering BAMBI expression is induced in response to TGFβi family signaling in mammals (Sekiya et al., 2004).

Finally, the location of TGFβ member transcripts suggests different regulation and possibly actions from those in mammals. As mentioned, our data suggest the majority of **bmp4** and **bmp7** mRNA is located in the trout oocyte (Table 3), which differs significantly from mammalian reports that suggest they are predominately in the follicular cells (Erickson and Shimasaki, 2003). All other transcript locations were similar to reports in other vertebrates. For example, as previously reported in trout (Bobe et al., 2008), the location of **gdf9** transcripts appears to be similar to the mammal (Shimasaki et al., 1999), chicken (Johnson et al., 2005), and zebrafish (Liu and Ge, 2007) with the majority, if not all, of the mRNA expression reported in the oocyte and not the somatic cells. Similar to earlier reports on **inhba** subunits in the trout (Tada et al., 2002) all of the **inhba** subunits and **fst** mRNA appear to be most highly expressed in the somatic follicular cells, which corroborates the location reported in birds and mammals, (Chen and Johnson, 1996; Knight and Glister, 2006). Messenger RNA for **bambi** was also predominantly found in the follicular cells, however, comparative data in other species could not be found. In mammals, the expression of IGF-related transcripts appears to be highly variable, which is in line with the current finding that these transcripts were ubiquitously expressed in rainbow trout ovarian tissue. Lastly, the expression of all of the steroidogenic transcripts and star mRNA was primarily found in the somatic follicular cells, which is consistent with their function in steroidogenesis (Nakamura et al., 2005). Clearly, additional work must be completed to determine the roles and precise locations of important TGFβi ligands and their receptors in the trout ovary, but the current data indicate the presence of high quantities of **bmp4**, **bmp7** and **gdf9** transcripts in the oocytes consistent with their regulatory actions on the surrounding somatic cells. The disparate temporal patterns of expression among the TGFβi ligands, **fst**, and **bambi** support they are differentially regulated during ovarian development and serve multiple regulatory functions. While the temporal expression profile of TGFβ members reported for rainbow trout does not completely agree with what is known in mammals, they are certainly similar in many ways and provide a base for future functional studies in fish.

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**References**


