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Sex-specific expression, synthesis and localization of aromatase regulators in one-year-old Atlantic salmon ovaries and testes

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ABSTRACT

Transcripts for *dax1*, *foxl2*, *mis* and *sf1* are co-expressed in the somatic companion cells of teleost germ cells. These regulatory factors function, in part, to modulate the transcription of aromatase, particularly *cyp19a*, the terminal enzyme of estrogen biosynthesis. At least two separate aromatase loci exist in teleost fish that encode distinct isoforms. The activity of two forms, *cyp19a* and *cyp19b1*, is predominantly associated with the ovary and the brain, respectively. We isolated sequences that compose the proximal promoters of *cyp19a*, *cyp19b1* and *foxl2a*, to identify potential transcription factor binding motifs to define sex-specific regulatory profiles for each gene. We also provide evidence for the translation and immunological localization of DAX-1, FOXL2 and MIS to the endoplasmic reticulum and accumulation within secretory vesicles of the salmon oocyte. We found no evidence for the expression of CYP19A or CYP19B1 in the oocyte at the one-year-old stage. However, synthesis of both aromatases was localized to testicular germ and soma cells at this early stage of development. Production of these regulatory factors in the germ cells may serve to modulate the transcription and activity of endogenous aromatase and/or contribute to the differentiation of the neighbouring companion cells through secretory signaling.

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1. Introduction

Many of the genes involved in sexual determination and differentiation processes have been characterized in mammals (Swain and Lovell-Badge, 1999; Brennan and Capel, 2004), fish (Devlin and Nagahama, 2002; Guiguen et al., 2010), as well as other species (Morrish and Sinclair, 2002; DeFalco and Capel, 2009). The functions of many of these genes have been studied extensively during the short development window in which the bipotential gonadal primordium becomes male or female anlagen. However, little is known of the functions of many of these factors before or after gonadogenesis. In this report, we examine the DNA and protein expression of DAX-1, FOXL2A and MIS – factors implicated in the differentiation and maturation of the teleost gonad – at the one-year-old stage of development.

MIS is a secreted, intercellular glycoprotein belonging to the TGFβ family of growth and differentiation factors. MIS is best known for its canonical function of preventing differentiation of the female genital primordia through regression of the Müllerian duct mesoepithelium in mammalian XY embryos (Blanchard and Josso, 1974). Secreted from pre-Sertoli cells, MIS acts through a mesenchymal receptor complex and Smad signaling to mediate degeneration of the duct (Orvis et al., 2008). MIS expression has also been correlated with rapid expansion of the granulosa cell layer, similar to Sertoli cell proliferation observed during embryonic development (Ingraham et al., 2000). As well, MIS has been demonstrated to control the rate of proliferation of Leydig cells, particularly progenitor Leydig cells (Lee et al., 1999). Similarly, in the female gonad, the expression of MIS has been shown to modulate the growth and recruitment of primordial follicles (Durlinger et al., 2002). In medaka, the expression of MIS has been

Abbreviations: AR, androgen receptor; ARE, androgen response element; BMP-15, bone morphogenetic protein-15; CRE, cyclic AMP (cAMP) response element; CREB, cAMP response element-binding protein, cytochrome P450 superfamily aromatase A (CYP19A) and B (CYP19B); DAX-1, Dosage-sensitive sex reversal, adrenal hypoplasia congenital, critical region on the X-chromosome, gene-1; DM-domain proteins, doublesex- and male abnormal-3 (MAB-3)-related transcription (DMRT) factors; EGR-1, early growth response-1; ER, estrogen receptor; ERE, estrogen response element; FGF-8, fibroblast growth factor-8; FOXL2, forkhead box L2; GDF-9, growth differentiation factor-9; IGF-1, insulin-like growth factor-1; KLH, keyhole limpet hemocyanin; MBM plastic, methyl methacrylate/butyl methacrylate; MIS, Müllerian inhibiting substance; RXR/RAR, retinoid X receptor/retinoic acid receptor; SMAD, mothers against decapentaplegic; SF-1, steroidogenic factor-1; SOX, sex-determining region of the Y chromosome (SRY)-related, high-mobility group (HMG) box (SRY box); SP-1, stimulatory protein-1; TGFβ, transforming growth factor β; WT-1, Wilm's tumour-1.

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implicated in the maturation of the germ cells of both sexes (Morinaga et al., 2007; Shiraishi et al., 2008).

The expression of *mis* has been localized to mammalian (Teixeira et al., 2001), chicken (Oréal et al., 2002), trout (Vizziano et al., 2007) and medaka (Klüver et al., 2007) granulosa and Sertoli cells. Unlike in mammals where MIS is detected only in Sertoli cells as an early determinant in the male pathway, *mis* is expressed in both the male and female gonad of chicken, trout and medaka (Oréal et al., 2002; Klüver et al., 2007; Vizziano et al., 2007), well before sexual differentiation.

DAX-1 is an orphan nuclear receptor and the various potential roles that it serves in the differentiation and development of the gonad may be mediated through control of aromatase (CYP19) and/or estrogen receptors (Jeffs et al., 2001; Meeks et al., 2003). In mammals (Wang et al., 2001) and among teleosts (Nakamoto et al., 2007; Wang et al., 2007; Ijiri et al., 2008), DAX-1 and SF-1 are considered to be inextricably linked with the transcription of *cyp19a*. For example, the importance of the control of DAX-1 on SF-1-regulated genes (ie; genes involved in steroid hormone production) is shown in the overexpression of CYP19A in DAX-1-deficient Leydig cells that results in significant elevations in intratesticular estradiol levels (Wang et al., 2001). In mammals, *dax1* expression has been associated with Sertoli and Leydig cells (Jeffs et al., 2001) and granulosa and theca cells (Ikeda et al., 1996), but not germ cells (Meeks et al., 2003).

The forkhead domain transcriptional regulator, FOXL2, is one of the earliest genes expressed that is associated with differentiation of the ovary in many species (Cocquet et al., 2002; Loffler et al., 2003; Govoroun et al., 2004; Pannetier et al., 2006; Vizziano et al., 2007; Wang et al., 2007). The expression patterns of *foxl2* and *cyp19a* correlate with early ovarian differentiation for goat (Pannetier et al., 2006), chicken (Govoroun et al., 2004) and trout (Vizziano et al., 2007). Indeed, highlighting the importance of the synthesis of estrogens in the differentiation of the female gonad of lower vertebrates, both *cyp19a* and *foxl2* expression have been colocalized to the granulosa cells of medaka (mRNA; Nakamoto et al., 2006), tilapia (protein; Wang et al., 2007) and Japanese flounder (mRNA; Yamaguchi et al., 2007).

Transcripts of *foxl2* have been detected in mouse (Loffler et al., 2003) and chicken (Govoroun et al., 2004) somatic and germ cell components of the developing ovary. Transcripts for *foxl2* have also been detected in developing male somatic and germ cells at low levels in the mouse (Loffler et al., 2003). However, expression of FOXL2 has been localized to only the mammalian (Cocquet et al., 2002) and chicken (Govoroun et al., 2004) ovarian somatic cells. In the teleosts examined to date, detection of *foxl2* has been confined to the gonadal soma (Nakamoto et al., 2006; Wang et al., 2007; Yamaguchi et al., 2007).

Aromatase is the terminal enzyme in the steroidogenic pathway that converts androgens to estrogens. In teleost fish, at least two separate loci, *cyp19a* and *cyp19b*, encode distinct aromatase isoforms, most commonly associated with the ovary and the brain, respectively. Both DAX-1 and FOXL2, in a relationship with SF-1, are known to assemble at and activate transcription from both mammalian and teleost *cyp19a* promoters (Wang et al., 2001; Pannetier et al., 2006; Nakamoto et al., 2007; Wang et al., 2007; Yamaguchi et al., 2007). Also, MIS has been demonstrated to modulate the expression of *cyp19a* through signal transduction acting on cAMP-mediated pathways (Ingraham et al., 2000; Teixeira et al., 2001 and refs. therein in both). The receptor for MIS has been detected in each companion cell-type of ovarian and testicular germ cells (Ingraham et al., 2000; Teixeira et al., 2001), implying regulation by either autocrine or paracrine control.

In this report we show the expression of *dax1*, *foxl2a*, *mis* and *sf1*, genes inextricably linked with the regulation of aromatase expression and thus with gonad development. The main focus of this study was to use immunohistochemistry (IHC) to determine the localization and functionality of these genes within the various cell groups that comprise the ovaries and testes of one-year-old Atlantic salmon. To the best of our knowledge, IHC using antibodies for DAX-1, FOXL2A, MIS, CYP19A and CYP19B1 together for gonads at one developmental stage for both sexes has not previously been done. We provide immunological evidence for the localization of DAX-1, FOXL2A and MIS in the oocyte, particularly concentrated in the endoplasmic reticulum and cortical alveoli within the ooplasm, as well as to the surrounding granulosa cells. We found immunolocalization of both CYP19A and CYP19B1, as well as DAX-1 and MIS, to both the germinal and somatic cells of the testes. This is the first report to demonstrate the endogenous expression and localization of DAX-1, FOXL2A and MIS in male and female germ cells for any species. Production of these regulatory factors in the germ cells may serve to modulate the transcription and activity of endogenous aromatase and/or contribute to the differentiation of the neighbouring companion cells through secretory signaling.

2. Materials and methods

2.1. Animals and sampling

Treatment of the fish used in this study was in compliance with the regulations of the University of Victoria Animal Care Committee. Male and female Atlantic salmon (*Salmo salar*; Mowi stock) were fed a 1% Skretting fry feed diet and exposed to natural photoperiod (Microtek Research and Development, Sidney, BC, Canada). One-year-old fish were euthanized, followed by rapid dissection of ovarian or testicular tissues. The tissues were flash frozen on dry ice and immediately stored at −80 °C until RNA extraction or placed into fixative at 4 °C for subsequent protein work.

2.2. Rabbit polyclonal immunization, production and purification

Antibodies were prepared by ImmunoPrecise Antibodies Ltd. (Vancouver Island Technology Park, Victoria, BC, Canada). The immunization and sera collection were performed according to institutional guidelines for animal care and handling.

For salmonid DAX-1, FOXL2A, MIS, and the aromatase CYP19A and CYP19B1 antigens, antibodies to three different and unique peptide sequences were raised for each antigen. For all antibodies, 5.0 mg of peptide antigen was used: 4.0 mg coupled to KLH (keyhole limpet hemocyanin) and 1.0 mg free. Two New Zealand white rabbits (*Oryctolagus cuniculus*) per antigen were immunized and for all rabbits 1.0 mL pre-immune serum was collected for pre-immune controls. Terminal bleeds were done after the third boost. The primary IgGs were purified from the serum using a Protein-A column (Pierce, Rockford, IL, USA) and stored in phosphate buffered saline (PBS) pH 7.4. The antisera were subsequently assayed by ELISA and Western blotting and were used to determine antigenicity and working concentrations.

Of the possible 21 unique primary antibodies, 12 of them showed positive immunoreactivity to their original antigens. All of these primaries were screened for immunopositive results on ovary and testis sections from one-year-old Atlantic salmon (see below for procedure). For each antigen, the primary that showed the best immunopositive result was further purified using a KLH column to absorb out excess anti-KLH IgGs. These were: MIS = EGWGQESNLRKEVLTRC; DAX-1 = VKLIHRDDTTRFAKLLITC; FOXL2A = PPPVSSSNGGGLQFAC; CYP19A = LVDQKRRGLREADKLDHIN and CYP19B1 = SIVDDTSMSEALL.

2.3. Atlantic salmon light microscopy embedding

The following embedding procedures have been modified from those previously described (Torgersen et al., 2009; Dafoe et al., 2010). Sections of each gonad were fixed in freshly prepared 4% formaldehyde (from 16% EM grade formaldehyde, Cat # 15710) (Electron Microscopy Sciences (EMS), Hatfield, PA, USA) in 0.01 M PBS (tablets, P-4417) (Sigma-Aldrich, St. Louis, MO, USA). The samples were stored at 4 °C for at least 24 h. Further processing continued at this

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