Targeted cod (CodArray) gene chip: A genomic tool for evaluating the role of androgens on the growth of previtellogenic oocytes in Atlantic cod (*Gadus morhua*)

by

Trond M. KORTNER & Augustine ARUKWE (1)

**ABSTRACT** - Prior to vitellogenesis (previtellogenesis), androgens appear to play pivotal roles in stimulating oocyte growth. In this study, suppression subtractive hybridization (SSH) with subsequent array development was used to open a possible revealing window in understanding the functional aspects of oocyte development. We constructed a targeted cod cDNA array containing 200 differentially-expressed genes using SSH after *in vitro* exposure of previtellogenic gonadal tissue from Atlantic cod to a mixture of androgens. The targeted CodArray was hybridized using total RNA from cod previtellogenic oocyte cultures exposed to 11-ketotestosterone (11-KT) and testosterone (T) (0, 10 and 100 µM) for 5 and 10 days. Array analyses showed 0.5-3.5 -fold significantly altered transcript levels for a wide range of genes. Interestingly, T produced increased levels of zonadhesin and zona pellucida protein-2, whereas the levels of these genes were generally decreased after exposure to 11-KT. 11-KT produced apparent increases of cyclin-B, and suggests a control in the timing of the early embryonic cell cycle. Real-time PCR analysis confirmed the changes in expression for selected genes. In addition, quantitative histological analyses were used to investigate modulations of previtellogenic oocyte growth phases after androgen exposure. Our data show that both 11-KT and T are capable of inducing previtellogenic oocyte growth and development in *in vitro*, with 11-KT being the strongest modulator. Taken together, the present study suggests some novel roles of androgens on the growth and development of previtellogenic oocytes, indicating possible androgen control of early follicular and oocyte growth in the cod ovary.

Key words. - Previtellogenic oocytes - Androgens - Atlantic cod - Microarray - Histology.

**Introduction**

Prior to vitellogenesis (previtellogenesis), androgens are suggested to play an integral role in the regulation of oocyte growth and development. It has been demonstrated that 11-KT induces an increase in the diameter and development of previtellogenic eel oocytes (Rohr et al., 2001). In mammals, androgens also modify the intra-ovarian gene expression, as demonstrated by increased mRNA abundance of insulin-like growth factor-1 (IGF-1) and IGF-1-receptor (Vendola *et al.*, 1999) in follicles up to early antral stage. Nevertheless, the underlying molecular mechanism(s) involved in possible gene regulation resulting in the growth of previtellogenic oocytes has not been investigated, and were therefore addressed in the present study. Oogenesis is an integral aspect of reproduction that comprises multi-step processes resulting in egg laying and can be subdivided into previtellogenesis, vitellogenesis and final oocyte maturation. Oogenesis in Atlantic cod has been extensively studied; however, these investigations have mainly focused on the vitellogenic process. Thus, what is known today about previtellogenesis in cod (and teleosts in general) is in sharp contrast with the knowledge regarding the processes of vitellogenesis and final oocyte maturation. Like in other vertebrates, testosterone (T) is present in female teleost fish. In addition, the non-aromatizable androgen 11-ketotestosterone (11-KT) is usually present, and generally believed to be the most potent androgen in teleosts (Borg, 1994). However, aromatizable (T) and non-aromatizable (11-KT) androgens may have strikingly different effects (Borg, 1994), and previous studies do not describe exactly the roles or effects of androgens in the fish ovary. In the present study, using an *in vitro* previtellogenic oocyte culture technique that was based on an agarose floating method, we have evaluated the *in vitro* effects of the androgens 11-KT and T on fish oocyte growth and development using the Atlantic cod as model species. In addition, quantitative histological analysis was used to investigate if cod previtellogenic gonadal tissue shows any morphological changes after exposure to androgens.

**Methods**

The *in vitro* organ culture technique employed was based on the agarose floating method as described earlier (Kortner and Arukwe, 2007; Nader *et al.*, 1999). Cod previtellogenic gonadal tissue was cultured randomly in triplicates (n = 3) for 1, 5, 10 and 20 d with different concentrations of 11-KT and T (0 (ethanol solvent control), 1, 10, 100 and 1000 nM). A cDNA library was generated by performing SSH with androgen exposed tissues and solvent control samples. A cDNA array (CodArray) containing 200 gene clones with unique expression patterns (either up- or down-regulated) from the SSH was constructed. Total RNA was purified from tissues homogenized in Trizol reagent according to established procedures, and high quality RNA was used for cDNA synthesis. Hybridization of the array was performed using
Cy5/Cy3-labeled cDNA probes. Data from each array were log-transformed and normalized using the global loess method. PCR primers for amplification of gene-specific PCR products were designed from conserved regions of the studied genes. Real-time PCR was performed for evaluating gene expression profiles as described earlier (Kortner and Arukwe, 2007). Cod previtellogenic gonadal tissues were fixed in paraformaldehyde, embedded in paraffin blocks, sectioned and stained with Mayer’s haematoxylin and eosin. Tissues were examined under a microscope, and volume fractions of the different growth phases of oocytes were calculated by stereological point-counting (Freere and Weibel, 1967). The proportional volume fraction (calculated as percentage) occupied by each stage of oocyte development was then determined.

Results and discussion

The array analyses demonstrate that exposure of previtellogenic oocytes to androgens produced differential gene expression patterns, showing 0.5-3.5-fold significantly altered transcript levels from a diverse range of genes involved in hormonal regulation, signal transduction, cell growth control, intra-/extra-cellular structure, apoptosis/protein degradation, biometabolism, transcription, and transport/binding as well as a number of transcripts whose function in fish are yet to be identified. Interestingly, T produced increased levels of two genes associated with the zona radiata (zonadhesin and zona pellucida protein-2), whereas the levels of these genes were generally decreased after exposure to 11-KT. 11-KT treatment showed an apparent increase of cyclin-B, which is suggested to control the timing of early embryonic cell cycle. On the basis of the results obtained from the array analysis, zona pellucida protein-2 precursor (ZP2) and cyclin-B mRNA expression were quantified using real-time PCR with gene specific primer pairs. The primary criterion for selecting these genes for real-time PCR validation is because they belong to our targeted study objectives, namely growth and development of previtellogenic oocytes.

The expression of ZP2 in cod previtellogenic oocytes was modulated after exposure to different concentrations of 11-KT and T in a concentration-specific manner. T produced a 1.5-2-fold increase in ZP2 mRNA expression 10 days post-exposure compared to the solvent control (Fig. 1A), whereas groups exposed to 11-KT showed minor differences compared to the solvent control (data not shown). The expression of cyclin-B in cod previtellogenic oocytes was modulated after exposure to different concentrations of 11-KT in a concentration-specific manner at day 10 post-exposure; a general pattern of increase in cyclin-B mRNA expression was observed, reaching a 2.3-fold increase for samples exposed to 1000 µM 11-KT, compared to the control (data not shown). In contrast, no significant differences in gene expression between concentration groups exposed to T were observed at day 10 post-exposure (Fig. 1B). Quantitative histological analyses showed increased volume fractions of oocytes of secondary previtellogenic growth phase (Fig. 2) in relation to total gonadal tissue volume in tissues exposed to different concentrations of 11-KT and T. At day 10 post-exposure, a concentration-dependent pattern was observed, displaying a 2.6, 2.8 and 3.2-fold increases in secondary previtellogenic growth phase oocyte volume densities for tissues exposed to 10, 100 and 1000 µM 11-KT, respectively. The same pattern was observed in tissues exposed to T, where a 2.3, 1.8 and 2.0-fold increase in volume fractions of previtellogenic oocytes of secondary growth was observed for tissues exposed to 10, 100 and 1000 µM T, respectively.

Conclusions

Despite the apparently high nominal androgen concentration used in this study, it should be noted that only a fraction of the androgen reached the tissue using the agarose method. In addition, previous studies have also used comparable androgen concentrations (Braun and Thomas, 2003;
The present study provides the identification, sequencing and expression patterns of 200 androgen responsive genes in the previtellogenic cod ovary. Quantitative real-time polymerase chain reactions confirmed the changes in expression for selected genes that are believed to be involved in the growth and development of previtellogenic oocytes in the Atlantic cod. Furthermore, we show that androgens promote oocyte growth and development in cod previtellogenic gonadal tissues in vitro, which allow to hypothesise a role for androgen in the control of early follicular and oocyte growth in the cod ovary.

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