A novel osteopontin-like protein is expressed in the trout ovary during ovulation

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Abstract Using suppression subtraction hybridization between ovulatory and postovulatory trout ovaries, a down-regulated cDNA was obtained that presumably encodes a novel ovarian protein ('NOP'). NOP mRNA is present in the ovary during ovulation and down-regulated by 48 h postovulation, suggesting an important role for NOP during ovulation. Besides the ovary, NOP is also strongly expressed in the testis and at lower levels in the skin, gills, kidney and gastrointestinal tract. While the overall identity is not high, NOP shares several sequence similarities with mammalian and chicken osteopontins, including the percentage of aspartate, serine and alanine residues and the presence of a cell attachment motif.

Key words: Ovulation; Trout; Ovary; Osteopontin; Suppression subtraction hybridization

1. Introduction

Vertebrate ovulation has been compared to an inflammatory reaction [1]. As such, many factors could potentially be involved in the control of ovulation. In mammals, the process of ovulation has been thoroughly studied and many ovarian factors have been hypothesized to be involved [1]. Given the large number of potential ovulatory mediators, we have utilized subtractive cloning approaches to isolate transcripts that change during the periovulatory period. For example, a family of proteins that are highly up-regulated in the trout ovary at ovulation and have sequence similarities to serine protease inhibitors were isolated using conventional cDNA subtraction [2]. Using differential display PCR, a gastricsin-like protein was identified in the brook trout ovary that was highly up-regulated 4–10 days following ovulation [3].

While these subtractive approaches successfully identified gene products regulated during the periovulatory period, a more inclusive subtraction technique is suppression subtraction hybridization (SSH). Thus, in order to identify other factors that could play an important role in the fish ovary during the ovulatory period, we used SSH between ovulatory and postovulatory ovarian tissue to look for down-regulated mRNAs following ovulation in rainbow trout. From this subtraction, we obtained two down-regulated cDNAs encoding proteins present in high quantity in the ovary during ovulation that we previously identified using other subtraction techniques [2,4]. However, we also obtained a differentially expressed cDNA presumably encoding a protein with no significant similarity when blasted directly against the GenBank database, that we provisionally call a novel ovarian protein (NOP). Using an 'annotation-sorted similarity' search, some sequence similarity can be observed between NOP and several vertebrate osteopontins (OPNs). In the present paper, we describe this novel mRNA and its expression within the ovary during the periovulatory period.

2. Materials and methods

2.1. Animal and tissue collection

Investigations and animal care were conducted according to the guidelines specified by the University of Notre Dame Institutional Animal Care and Use Committee.

Mature rainbow trout (*Oncorhynchus mykiss*) and brook trout (*Salvelinus fontinalis*) were obtained during the reproductive season. Prior to collecting tissue, the reproductive stage of individual trout was determined by sampling follicles in vivo as previously described [5]. For both species, ovarian tissue was collected from ovulating females (20–100% of the ovary ovulated at the time of sampling), and 1, 2, 4–6 and 8–10 days after ovulation for both species. In addition, brook trout ovarian tissue was collected from females prior to germinal vesicle breakdown (GVBD). For in vitro incubations with phorbol esters, ovarian tissue was collected as previously described [6,7]. To collect ovarian tissue, trout were over-anesthetized in 2-phenoxyethanol and decapitated. The abdominal cavity was opened and the ovaries were removed, dissected, and aliquots were stored in liquid nitrogen before RNA extraction. In addition, from a female assayed at 48 h postovulation, different tissues were removed and aliquots stored in liquid nitrogen before RNA extraction. Testes were also removed from a spermiating male and aliquots were stored in liquid nitrogen.

2.2. In vitro incubation for phorbol ester stimulation

Large scale in vitro incubations of phorbol ester-stimulated follicles were performed as previously described [6,7]. Stimulated follicles received a combined treatment of 0.05 μg phorbol-12-myristate-13-acetate (PMA, Sigma, St. Louis, MO, USA)/ml and 0.05 μg A23187 (Calbiochem, La Jolla, CA, USA)/ml and separate groups were incubated for 6 or 12 h at 12°C under intermittent agitation. Following incubation, the ovarian tissue was processed for RNA as described below.

2.3. RNA extraction and Poly(A)$^+$ RNA isolation

Total RNA was extracted by homogenization in 1 ml of Tri Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) per 50 mg tissue [8,9]. Poly(A)$^+$ RNA was isolated using the PolyAtract mRNA Isolation System (Promega, Madison, WI, USA).

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Abbreviations: SSH, suppression subtraction hybridization; NOP, novel ovarian protein; PMA, phorbol-12-myristate-13-acetate; MBP, maltose binding protein

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2.4. SSH and Northern capture

SSH was performed using a kit (PCR Select, Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions. Ovarian mRNA from female rainbow trout sampled at ovulation and 4–8 days postovulation was used for the subtraction. For each reproductive stage, 0.4 μg of mRNA from each of five fish (2.0 μg total) was pooled to perform SSH analysis. Briefly, after reverse transcription and second strand synthesis, ovulatory and postovulatory cDNA was digested with *Rsa*I. An adaptor (adaptor 1) was ligated on both ends of a fraction of digested cDNA obtained at ovulation. This fraction was referred to as 'tester 1'. A different adaptor (adaptor 2R) was ligated on a second fraction of digested cDNA (referred to as 'tester 2') from the same ovulatory stage. Postovulatory cDNA was also digested with *Rsa*I and is referred to as the 'driver'. Initially, two separate hybridizations were performed at 68°C for 8 h using an excess of driver and either tester 1 or 2. These two hybridization mixtures were then pooled in the presence of an excess of driver and incubated at 68°C overnight. After hybridization, the mixture was diluted and used for PCR. After incubation at 75°C for 5 min and 94°C for 25 s, a primary PCR was performed (94°C, 10 s; 66°C, 30 s; 72°C, 1 min and 30 s; 27 cycles) using Advantage Polymerase Mix (Clontech) with a primer that was complementary to both adaptors 1 and 2R. A secondary PCR was then performed (94°C, 10 s; 68°C, 30 s; 72°C, 1 min and 30 s; 11 cycles) using a diluted aliquot of the primary PCR as a template, and primers complementary only to adaptor 1 (primer 1) and only to adaptor 2R (primer 2R). This PCR was performed in presence of [^{32}P]dATP (ICN, Costa Mesa, CA, USA). The resulting PCR mixture was then applied to a Centrisep Column (Princeton Separation, Adelphia, NJ, USA) to remove unincorporated reagents. After denaturation, this labeled PCR mixture was used to probe a Northern blot on which total RNA from different females sampled at ovulation and 4–8 days postovulation had been run in separate lanes. After washing under stringent conditions, the Northern blot was exposed to X-ray film for 48 h. The film and original Northern membrane were aligned and differentially regulated bands between 'ovulation' and '4–8 days postovulation' females were excised, soaked in water for 10 min and boiled for at least 10 min. After spinning, the supernatant was removed and the DNA was ethanol-precipitated (−70°C, overnight) in the presence of glycogen using 3 M sodium acetate and absolute ethanol. The samples were centrifuged to pellet the DNA and washed. An aliquot of the DNA was reamplified by PCR (94°C, 10 s; 68°C, 30 s; 72°C, 1 min and 30 s; 30 cycles) using primers 1 and 2R. The reamplified PCR products were then separated on an agarose gel, the bands were cut and cloned in TOPO pCR 2.1 (Invitrogen, San Diego, CA, USA) and the ligated plasmid was used to transform competent TOP 10 cells (Invitrogen). Inserts were sequenced as previously described [6] and were used to probe Northerns to substantiate that the band truly represented a differentially regulated cDNA as observed during the original Northern capture.

2.5. Cloning of full-length cDNA

Ovulatory rainbow and brook trout phorbol ester-stimulated cDNA libraries were constructed in ZAP Express (Stratagene, La Jolla, CA, USA). The rainbow trout library was screened under high stringency as previously described [6], using the cDNA band obtained from Northern capture. A number of positive plaques were obtained, rescreened once to homogenity, converted to pBK-CMV (Stratagene) phagemids by in vivo excision, and the phagemids were used to transfect XLOLR cells. Six clones were obtained and two of the largest were sequenced on both strands as previously described [6].

From the rainbow trout full-length cDNA, two primers were designed to amplify a 550 bp fragment using the brook trout cDNA library as a template. This band was cloned, sequenced and used to screen the brook trout library as described above. Five clones were obtained and three of the largest were sequenced on both strands as previously described [10].

2.6. Northern blot analysis

Northern blot analysis was performed using mRNA from brook trout tissue as previously described [6]. Brightstar nylon membranes (Ambion, Austin, TX, USA) were hybridized overnight at 42°C in ULTRAhyb buffer (Ambion). All Northerns were probed using the full-length brook trout NOP (btNOP) cDNA (accession # AF223388) obtained from library screening, and were washed under stringent conditions (15 min twice in 1× SSPE, 0.1% sodium dodecyl sulfate (SDS), 45°C and 15 min twice in 0.1× SSPE, 0.5% SDS, 65°C). Northern blots were exposed to phosphorimaging screens (Kodak, Rochester, NY, USA) and visualized using a Storm 840 phosphorimager (Molecular Dynamics, Sunnyvale, CA, USA). When necessary, radioactive RNA bands were quantified using Imagequant (Molecular Dynamics) and the results were analyzed statistically using an ANOVA followed by Tukey's test to evaluate significant differences in mRNA levels between reproductive stages.

2.7. Expression of btNOP recombinant fusion protein for antibody production

The presumed btNOP (accession # AF223388) open reading frame was amplified by PCR. This fragment was cloned into the pMAL-c2E (New England Biolabs, Beverly, MA, USA) expression vector as previously described [3] and used to transform competent *Escherichia coli* INVαF′ cells (Invitrogen). One of the clones was sequenced and found to contain no PCR artifacts. Expression in this system produces a fusion protein consisting of maltose binding protein (MBP) and the trout NOP protein separated by an enterokinase cleavage site.

A fusion protein was produced and purified using an amylose affinity column as previously described [4]. A fraction with the highest protein content and the least amount of contaminating protein was chosen for immunization.

2.8. Antibody production and purification

Rabbits were immunized subcutaneously with 500 μg of purified fusion protein mixed with Freund's complete adjuvant. Booster injections consisting of 500 μg of purified fusion protein mixed with Freund's incomplete adjuvant were given on days 14, 28 and 42. Immune serum was collected after exsanguination on day 52.

IgGs were purified from the immune serum and antibodies to MBP and copurified *E. coli* proteins were removed as previously described [4].

2.9. Immunocytochemistry

Ovarian tissues obtained from female brook trout sampled during ovulation and 5–8 days postovulation were fixed and embedded in paraffin as previously described [11]. Tissue sections (8 μm) were cut and placed on silane-treated microscope slides (Polysciences, Warrington, PA, USA).

Immunocytochemistry was performed using the ABC Staining System (Santa Cruz Biotechnology, Santa Cruz, CA, USA) according to the manufacturer's instructions as described before [3]. Primary antibody was diluted in blocking serum (10 μg/ml) and applied overnight at 4°C. To validate the specificity of the signal, adjacent sections were either incubated in the absence of the primary antibody or incubated with an antibody directed against an ovarian progastricsin [3] at the same dilution. The progastricsin antibody was also directed against a MBP fusion and produced using the same protocol. This antibody gives a specific signal on ovarian sections from 5 to 8 days postovulation but not during ovulation [3].

Adjacent sections were cleared in toluene, hydrated in decreasing ethanolic solutions and stained in hematoxylin and counterstained in eosin as previously described [11].

3. Results

3.1. SSH and Northern capture

From SSH and Northern capture, three down-regulated cDNAs were obtained. One of these cDNAs was a trout ovulatory protein ('TOP') mRNA which is known to be present in high quantity in the brook trout ovary and down-regulated after ovulation [2]. The second cDNA was a trout kallikrein-like mRNA that is also present in the ovary and is down-regulated in the postovulatory ovary [4].

A third down-regulated cDNA was obtained that had not been previously identified and was used to screen a rainbow trout cDNA library made from ovaries taken at the time of ovulation. The full-length cDNA obtained from this library screen presumably encodes a protein that has little or no homology to known proteins when directly blasted in Gen-

Fig. 1. Amino acid sequence alignment of brook trout (b trout; accession # AF223388) and rainbow trout (r trout; accession # AF204760) NOPs, and human (accession # X13694), mouse (accession # J04806) and chicken (accession # X56772) OPNs.

Bank. Therefore, this protein was provisionally called 'NOP'.

3.2. Brook and rainbow trout NOP cDNA and protein sequence

The full-length rtNOP cDNA (AF204760) obtained from library screening is 1545 bp in length. An ATG site is present at position 131 with an in-frame stop codon at position 1174. The ACAATGA sequence surrounding the start site between positions 128 and 134 resembles the preferred eukaryotic initiation sequence of ACCATGG [12]. The open reading frame presumably encodes a 347 amino acid protein with a calculated molecular mass of 36.0 kDa (Fig. 1).

The full-length btNOP cDNA (AF223388) obtained from library screening is 1568 bp in length. An ATG site was present at position 136 with an in-frame stop codon at position 1215. The ACAATGA sequence surrounding the start site between positions 133 and 139 resembles the preferred eukaryotic initiation sequence of ACCATGG [12]. The open reading frame presumably encodes a 359 amino acid protein with a calculated molecular mass of 37.3 kDa (Fig. 1). Overall, the nucleotide sequences of the two NOP cDNAs are highly similar (over 90%). However, the coding region of the btNOP is longer than rtNOP because of a single 12 amino acid addition in btNOP at amino acid 96 (Fig. 1).

The putative amino acid sequences of btNOP and rtNOP are extremely similar and are characterized by the presence of a high number of serine residues (16–17%) and the absence of cysteine and tryptophan residues. The btNOP and rtNOP amino acid sequences are also rich in alanine (11–12%), aspartic acid (11%), threonine (10%) and glutamic acid (10%). In addition, both rtNOP and btNOP contain a G-R-G-D-S motif identified as a cell attachment motif (Fig. 1).

While no significant sequence similarities were observed by directly blasting the rtNOP or btNOP sequences in GenBank, an 'annotation-sorted similarity' search on the Protein Information Resource website (http://pir.georgetown.edu) indicated some sequence similarities between NOP and several OPNs (20–25% identity) (Fig. 1).

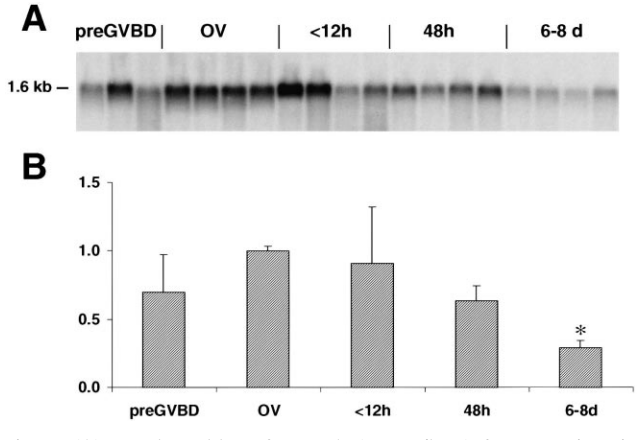

Fig. 2. (A) Northern blot of mRNA (0.5 μg/lane) from ovarian tissue of females sampled before GVBD (preGVBD), during ovulation (OV) and at various times postovulation: within 12 h (< 12 h), 48 h (48 h), 6–8 days (6–8 d). Each lane represents mRNA from a different female. (B) Graph representing the average intensity (± S.D.) of the 1.6 kb NOP transcript analyzed over the different reproductive stages using the results from the females shown in 'A'. *Significantly different from ovulation at $P < 0.05$.

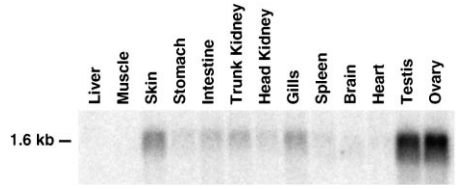

Fig. 3. Northern blot of mRNA (0.5 μg per lane) from different tissues sampled from a postovulatory female 48 h after ovulation and a testis from a spermiating male. The right lane contains mRNA from an ovary during ovulation.

3.3. Northern blots probed with btNOP cDNA

On Northern blots, the full-length btNOP cDNA hybridized predominantly with a 1.6 kb transcript (Figs. 2–4). The NOP mRNA was detected in the ovary before and during ovulation and the highest levels of expression were observed in females at the completion of ovulation. The mRNA was significantly down-regulated in the ovary 48 h postovulation and decreased even further by 5–8 days postovulation (Fig. 2). On Northern blots of mRNA from other tissues, the NOP mRNA was strongly detected in the testis and the ovulatory ovary (Fig. 3). In addition, it was also detected at low levels in several other tissues such as the kidney, gills and skin (Fig. 3). The size of the transcripts detected in all tissues appeared to be identical. On a Northern blot of PMA/A23187-stimulated follicles, btNOP mRNA was up-regulated in some females after 6 h. In contrast, after 12 h of in vitro incubation, a strong up-regulation was observed (Fig. 4). Interestingly, a higher size transcript was present in 12 h phorbol ester-stimulated samples.

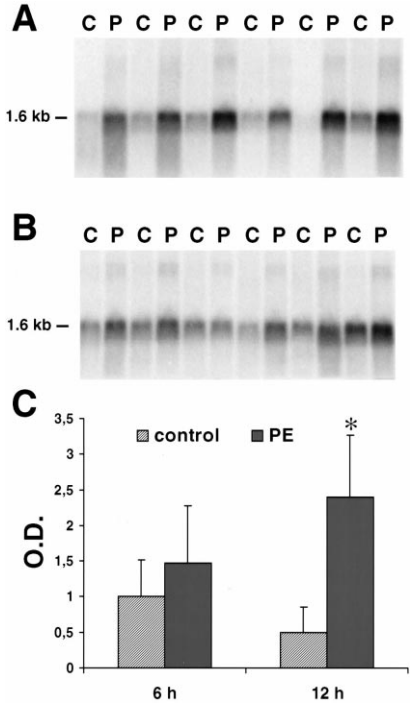

Fig. 4. Northern blot of mRNA (0.5 μg/lane) from ovarian follicles of six different females in the presence of dimethylsulfoxide vehicle (C) or treated (P) for 12 (A) and 6 (B) h in vitro with a combination of PMA and A23187. Graph (C) represents the average intensity (±S.D.) for control (C) and PMA/A23187 treatment (P) after 6 and 12 h of incubation. *Significantly different from control at $P < 0.05$.

3.4. Immunolocalization of NOP proteins in the brook trout ovulatory follicle

Ovarian sections from females sampled during ovulation were stained with hematoxylin and eosin. These sections clearly show distinct granulosa and theca layers in the follicles (Fig. 5). Prior to ovulation, the theca and granulosa layers in trout follicles are stretched to accommodate a large (~4 mm) oocyte. However, after ovulation the follicle shrinks dramatically and the granulosa then appears multilayered. Using immunocytochemistry, the NOP antibody produced a strong signal in many of the granulosa cells of ovulatory follicles (Fig. 5). In contrast, no signal was observed using the progastricsin control antibody.

The NOP antibody did not recognize any bands on Western blots of protein taken from ovaries throughout the periovulatory period.

4. Discussion

While a direct 'Blast' search against GenBank using the amino acid sequence of btNOP and rtNOP showed little homology with known proteins, an annotation-sorted similarity search detected sequence similarities between NOP and OPNs. When fully aligned, the overall identity between NOP and OPNs is not particularly strong at the amino acid level (20–25%, Fig. 1). However, the primary amino acid sequences of NOPs and OPNs share several common characteristics. For example, OPNs are rich in aspartic acid, glutamic acid and serine, similar to the percentage of these residues in the NOP sequences. OPNs contain a cell attachment motif (G-R-G-D-S) that is also present in NOP. Furthermore, OPNs contain an aspartic acid-rich domain up-stream of the cell attachment motif. In NOP, the region up-stream of the G-R-G-D-S domain is also rich in aspartic acid residues and the sizes of rtNOP (347 aa) and btNOP (359 aa) are close to the size of OPNs (~300 aa). The low identity between the amino acid sequences of OPN and NOP is consistent with the low identities observed between murine and other vertebrate OPNs (human, 50%; chicken, 29%).

NOP mRNA is detected in the ovary before GVBD and high levels of NOP mRNA are observed in the ovary during ovulation in all females assayed. The highest levels were observed in females within 24 h of ovulation. However, at this stage, half of the females had levels similar to those observed by 48 h postovulation. This suggests that levels of NOP mRNA peak immediately at the end of ovulation and then rapidly decrease. By 48 h postovulation, levels of NOP transcripts are significantly lower than during ovulation and are even lower by 5–8 days postovulation. The pattern of expression of the NOP cDNA in the periovulatory ovary strongly suggests that NOP could play a role in ovulation in salmonids. This hypothesis is also supported by the strong up-regulation of NOP by phorbol ester after 12 h of in vitro stimulation. The exogenous activation of protein kinase C by phorbol esters has an effect on several ovarian processes in fish involved in ovulation, including oocyte expulsion [13,14] and prostaglandin synthesis [15]. The up-regulation of NOP by phorbol ester is also consistent with an increase in OPN mRNA levels observed in mice after stimulation by phorbol esters [16].

Immunostaining of NOP proteins was clearly obtained on ovarian sections within the granulosa cells of the follicle.

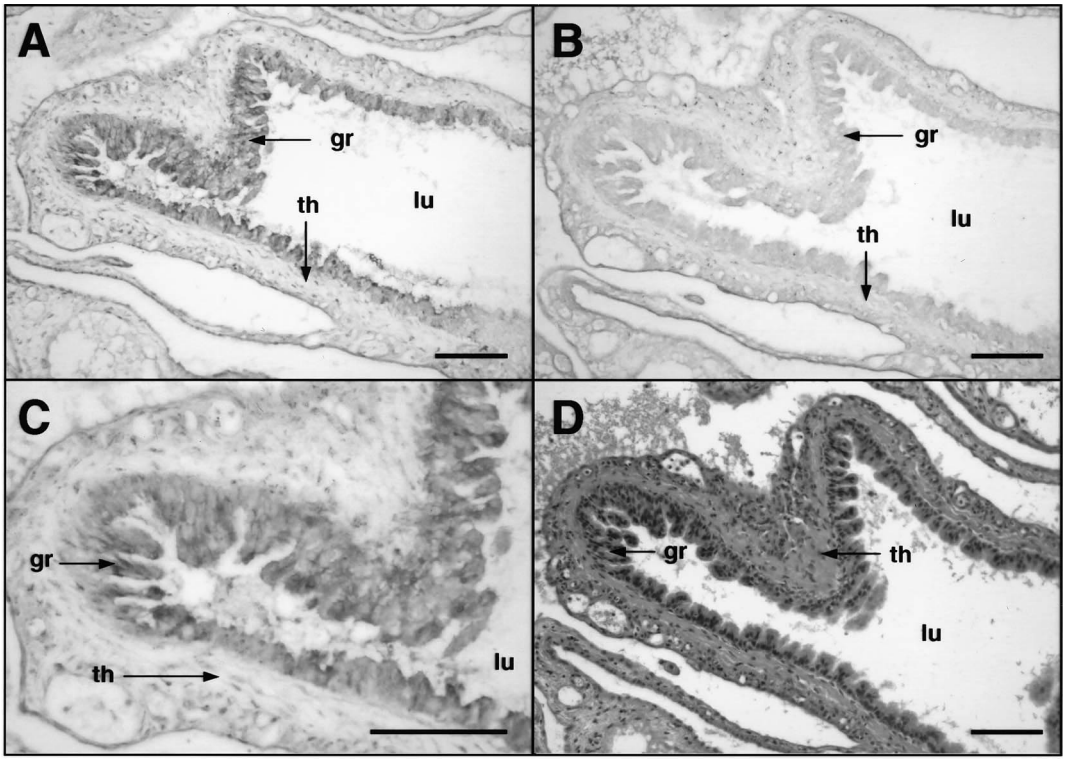

Fig. 5. Immunocytochemistry and hematoxylin–eosin-stained sections of brook trout ovarian tissue at the completion of ovulation. gr, granulosa; th, theca; lu, lumen. All bars represent 100 μm. A and C: Immunocytochemistry in the presence of the NOP antibody. B: Immunocytochemistry in the presence of the trout progastricsin control antibody. D: Hematoxylin and eosin-stained section.

However, we were unable to observe NOP immunostaining on Western blots of ovarian tissue and coelomic fluid using the same antibody. During the extraction of tissues for Western analysis, it is possible that conformational changes occurred in the protein that made the antibody unable to detect NOP on Western blots. Interestingly, Cancel and coworkers [17] failed to immunodetect OPN in the bull reproductive tract and suggested that posttranslational modifications might have compromised the ability of the OPN antibody to detect OPN in these tissues.

The NOP transcript was predominantly detected in the ovary and the testis suggesting a role in gonadal function. However, a signal was observed in several other tissues such as the skin, kidney, gills, intestine, stomach, spleen and brain. The tissue distribution of btNOP is also consistent with the tissue distribution of OPNs. OPN mRNA has been reported in mammalian bone cells, cartilage, fetal skin, brain, kidney, ovary, uterus and other tissues [18–22]. In addition, OPN mRNA and protein were detected in mouse testis and sperm [23].

Thus, while the exact identity of NOP is unknown, several characteristics of the sequence and expression suggest that it could be related to OPN. OPN is a multifunctional glycoprotein that promotes cell adhesion and migration [24]. Previous studies have suggested that OPN plays a role in bone resorption, tumorigenesis and metastasis. More recently, OPN has been implicated in a number of disease states associated with inflammation and tissue remodeling. Several functional features of OPNs are consistent with the presence of NOP in the ovary during and immediately following ovulation. For example, vertebrate ovulation has often been compared with an inflammatory reaction [1]. Interestingly, OPN can stimulate macrophages invasion [25] and T-cell activation [26] and recruitment [27]. Finally, the immunolocalization of NOP to cells in the granulosa layer that is confluent with the coelomic fluid is consistent with the presence of OPNs on many epithelial surfaces communicating with the external environment [28].

In conclusion, a novel ovarian protein was identified by SSH in trout. This protein shows sequence similarities with OPN suggesting that NOP could be the trout homolog of OPN. Furthermore, the temporal expression of the NOP mRNA suggests that NOP could play an important role in the ovary during the periovulatory period in fish.

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