Veterinary Parasitology (2017.1) 233:20-24.

The putative serine protease inhibitor (serpin) genes encoded on Echinococcus multilocularis genome and their expressions in metacestodal stage.

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The putative serine protease inhibitor (serpin) genes encoded on *Echinococcus multilocularis* genome and their expressions in metacestodal stage

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Abstract

Two putative serpin genes were identified in *Echinococcus multilocularis*, in addition to the already reported *serpinEmu*, and were designated as *serpin2Emu* and *serpin3Emu*. Western blot analysis using polyclonal antibodies against serpinEmu, putative serpin2Emu protein, and putative serpin3Emu protein indicated that all three proteins were localized in both intracellular and excretory-secretory (ES) fractions of *E. multilocularis* metacestodes. In addition, immune staining of parasite tissue indicated that all three proteins were localized at the germinal layer.

Keywords

Alveolar echinococcosis, *Echinococcus multilocularis*, Serine protease inhibitor, Metacestode

1. Introduction

Alveolar echinococcosis (AE) is a zoonotic infectious disease caused by the larval stage of the cestode *Echinococcus multilocularis.* In humans, metacestodes develop asexually like a tumor, mainly in the liver (Eckert and Deplazes, 2004). The mechanisms underlying infiltration into host tissue and immune evasion by larval stage parasites have been unknown.

Serine protease inhibitor (serpins) family proteins play diverse roles in the regulation of various biological functions such as cell development, and inflammation as well as function in the complement system (Gettins, 2002). The serpin family proteins are characterized by the presence of a single protein motif

termed the “reactive center loop” (RCL), and posess an inhibitory activity against serine proteases by an irreversible “suicide” mechanism (Gettins, 2002). A number of serpins have been identified in parasitic helminthes, suggesting their putative involvement in host immune regulation and parasite survival (Molehin et al., 2012).

An *E. multilocularis* serpin orthologue, oncospheral serpinEmu, and its inhibitory activity on elastase, plasmin, and trypsin have been reported (Merckelbach et al., 2003; Merckelbach and Ruppel, 2007). In the present study, we attempted to identify new genes of the serpin family in *E. multilocularis* using genome and gene data sets.

2. Materials and methods

2.1. Parasites and animals

E. multilocularis were maintained using mice (5-week-old female BALB/c, Japan SLC, Shizuoka, Japan). Female rabbits (Japanese white, Japan SLC, Shizuoka, Japan) of 8 weeks old were used for antibody production. We performed all experiments in accordance with the ethical guidelines for animal experiments in Asahikawa Medical University.

2.2. Identification and cloning of *E. multilocularis* serpin genes

We searched for orthologues of serpin genes within the gene data sets of *E. multilocularis* (Tsai et al., 2013, http://www.sanger.ac.uk) using tBLASTn. The amino acid sequences of serpin family, *Mus musculus* α1-antitrypsin (NCBI Ref. Seq.

No. NP_001239498.1), antithrombin III (NP_543120.1), *Caenorhabiditis elegans* SeRPin (NP_503315.1), *Schistosoma mansoni* Contrapsin (GenBank Acc. No. CCD 60352.1, Modha and Doenhoff, 1994), and the already reported *E. multilocularis* serpinEmu (CAD12372.2) were used for queries. Geneious 8. 1. 3 (Biomatters, Auckland, New Zealand) was employed for genetic analysis. The detection of the conserved domains, transmembrane domains, and signal sequences were performed using NCBI conserved domain database (Marchler-Bauer et al., 2015), TMHMM v. 2.0 (Krogh et al., 2001), and SignalP 4.1 (Petersen et al., 2011), respectively.

Total RNA isolation and cDNA synthesis were performed using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and the GeneRacer Kit (Thermo Fisher Scientific, MA, USA), respectively. The full-length coding sequences (CDSs) of serpin genes were amplified by PCR using Q5 High-Fidelity DNA Polymerase (New England Biolabs, MA, USA). Primer sets were designed for amplification of three genes: serpinEmu-F and R (5′-ATGTTTGCCAAAACTAGCTTCATTCC-3′ and 5′-CTACTTGGATTCGGGGTGAACAACA-3′), serpin2Emu-F or serpin2Emub-F and R (5′-ATGTTCATGGTACTTGTGGCCCA-3′ or 5′-ATGTTCATGGTACTTGTGGCCCAAAA-3′ and 5′-TTAGCGAGGATCAGCAATACGAGC-3′), and serpin3Emu-F and R (5′-ATGATTCCTCAACGTTTGAAGGAC-3′ and 5′-TCAGTTAATGGCGGGCAGTTT-3′). The PCR products were cloned into pGEM-T (Promega, WI, USA) vector, and sequencing was conducted using the BigDye Terminator v3.1 cycle sequencing kit and ABI genetic analyzer 3500 (Thermo Fisher Scientific, MA, USA).

2.3. Sequence alignment and phylogenetic analysis

101

102 The alignment of amino acid sequences of serpins in *E. multilocularis* and
103 other parasites was constructed using ClustalW. Following sequences were used;
104 *Brugia malayi* Bmserpin-1 (GenBank Acc. No. AAB42377.1, Yenbutr and Scott,
105 1995), Bmserpin-2 (AAB65744.1, Zang et al., 2000), *Haemonchus contortus*
106 Hc-serpin (ACP43576.1, Yi et al., 2010), *Clonorchis sinensis* CsSERPIN
107 (ABR68547.1, Kang et al., 2010), CsSERPIN2 (AHZ96593.1, Lei et al., 2013),
108 CsproSERPIN (ADI60059.1 Yang et al., 2009), *Paragonimus westermani*
109 PwSERPIN (ABV57466.1, Hwang et al., 2009), *S. mansoni* Contrapsin, Smpi56
110 (CCD60349.1, Ghendler et al., 1994), Sm_SerpC (CCD74817.1, Curwen et al., 2006),
111 Smp_062080 (CCD60354.1, Harrop et al., 2000), *S. japonicum* Sj_serpin
112 (AAA50230.1, Yan et al., 2005), SjB10 (AAG45932.1, Molehin et al., 2014), and *S.*
113 *haematobium* Sh_serpin (AAA19730.3, Blanton et al., 1994). We used the
114 neighbor-joining method for constructing the phylogenetic tree with MEGA6.

115

116 2.4. Antibody production

117

118 Histidine tag-fusion serpins were constructed using pET28a (+) vector (Merck
119 Millipore, Darmstadt, Germany). The recombinant proteins were then expressed by
120 *Escherichia coli* BL21 (DE3) (New England Biolabs, MA, USA). The codon usage
121 optimization for *E. coli* by OptimumGene™ (GenScript, NJ, USA) led recombinant
122 putative serpin2Emu (r-serpin2Emu) to successful expression. The recombinant
123 proteins were purified using TALON Metal Affinity Resin (Clontech, CA, USA). 2M
124 urea was used for solubilization of r-serpin2Emu and recombinant putative
125 serpin3Emu (r-serpin3Emu). Rabbits were immunized using each recombinant

126 protein. Antibodies were purified with HiTrap protein G HP Columns (GE
127 Healthcare, Buckinghamshire, UK). To avoid the cross reactions caused by the
128 antibodies against the N-terminal region of recombinant proteins, antibodies were
129 absorbed by recombinant *E. multilocularis* 18 kDa antigen (Sako et al., 2002)
130 expressed by pET28 a (+) vector.
131
132 2.5. Western blotting
133
134 The ES proteins were collected by incubation of homogenized parasites in
135 serum-free Dulbecco's Modified Eagle's Medium (DMEM, Sigma Aldrich, St. Louis,
136 USA) at 37 °C in 5% CO_2 overnight. The intracellular soluble proteins were
137 prepared by sonication of parasites in serum-free DMEM followed by centrifugation
138 and collection of supernatant.
139 SDS-PAGE and western blotting of ES and intracellular soluble proteins were
140 conducted as described by Sako et al. (2002) in individual gels under reducing
141 conditions. The antibodies against three recombinant proteins were diluted in 1%
142 casein in PBST (250 ng/mL), and used as the primary antibodies. Pre-immune
143 rabbit sera (1:100 dilution) were used as negative controls. The alkaline
144 phosphatase-conjugated anti-rabbit IgG (1:20000 dilution, Abcam, Cambridge, UK)
145 was used as the secondary antibody.
146
147 2.6. Immunostaining
148
149 Deparaffinized parasite tissue sections were treated with 3% H_2O_2, and
150 blocked by 5% bovine serum albumin (BSA) in PBS. The slides were incubated for 2

h with each rabbit antibody diluted in 1% BSA in PBS (1 μg/mL). Pre-immune rabbit sera (1:100 dilution) were used as negative controls. The HRP-conjugated anti-rabbit IgG (1:1000 dilution, Abcam, Cambridge, UK) was used as the secondary antibody. Detection was conducted with Takara DAB substrate (Takara, Shiga, Japan). Each of the sections was counterstained with hematoxylin.

3. Results

In this study, four *E. multilocularis* serpin genes were identified from genome and gene data sets. Of these four genes, EmuJ_001193100.1 was identical to the already reported *serpinEmu* (Merckelbach et al., 2003). *SerpinEmu* gene had a duplicated gene (EmuJ_001193200.1) with only 1 bp difference, resulting in a substitution of 344L to 344R. In addition, we identified two putative serpin genes, EmuJ_000824000.1 and EmuJ_000824100.1.

SerpinEmu was cloned and this clone was used for the following analyses. And the 5′-term alternative splicing variant of the EmuJ_000824000.1 was cloned, and was designated as "putative *serpin2Emu* (GenBank Acc. No. LC148049)." EmuJ_000824000.1 CDS was also cloned, and designated it as "putative *serpin2Emu-b* (LC148050)." We cloned EmuJ_000824100.1 and designated it as "putative *serpin3Emu* (LC148050)."

The signal sequence and transmembrane domain were not detected in all putative serpins. The serpin-specific RCL was detected from C-termini of them. As a result of phylogenetic analysis, tree indicated two major groups. SerpinEmu fall into Group A, in contrast, putative serpin2Emu and serpin3Emu fall into group B (Fig. 1).

Both r-serpinEmu and r-serpin2Emu were expressed as 47 kDa proteins, and r-serpin3Emu was expressed as 50 kDa, and cross reactivity among three antibodies was not observed (data not shown). We detected a 42 kDa band of native serpinEmu in intracellular fraction. In addition, a 55 kDa (Fig 2, white arrow) band and a weak 42 kDa band of serpinEmu were detected in the ES fraction (Fig 2, white arrowhead). In contrast, putative serpin2Emu and serpin3Emu proteins were detected in both ES and intracellular proteins as broad 50–60 kDa bands; of a larger size compared to the predicted size (Fig. 2, brackets). In addition, a smaller size bands than predicted (42 kDa and 45 kDa) of serpin2Emu and serpin3Emu were detected in the ES fraction (Fig. 2, black arrowheads).

SerpinEmu and serpin3Emu was detected in the germinal layer and the protoscolices (Fig. 3B and D, black and white arrows, respectively) of metacestodes. Serpin2Emu was detected at the germinal layer (Fig. 3C, black arrow). In addition, strong serpin2Emu expression was observed in the calcareous corpuscles (Fig. 3C, white arrowheads).

4. Discussion

Serpins of parasitic helminthes are classified into secretory and intracellular categories (Molehin et al., 2012). We detected serpinEmu in the metacestode ES and intracellular soluble fractions as a 42 kDa band. In addition, we detected a 42 kDa band of putative serpin2Emu and a 45 kDa band of putative serpin3Emu in the ES fraction. These main 42–45 kDa bands are supposed to be intact proteins. In addition, anti-r-serpinEmu antibodies recognized a 55 kDa protein in ES proteins, which was larger than the intact serpinEmu. Anti-r-serpin2Emu and

anti-r-serpin3Emu antibodies also recognized 50–65 kDa proteins in the ES and intracellular fractions. The inhibition of protease by serpin represents kinetic trapping, and the trapped complex is covalent and irreversible (Gettins, 2002). The larger bands recognized by antibodies against each serpin might represent the complex of serpins and their target proteins. In addition, it is reported that the conformational disorder of the protease–inhibitor complex causes a disruption by other enzymes (Gettins, 2002). Anti-r-serpin2Emu and anti-r-serpin3Emu antibodies recognized smeared bands; in addition each polyclonal antibody recognized several smaller bands of less than the expected size, which were supposed to be disrupted complexes.

All the three serpins were expressed in the germinal layer. In addition, strong expression of putative serpin2Emu protein in the calcareous corpuscles was observed. The various roles of calcareous corpuscles for the survival of cestodes have been discussed (Vargas-Parada and Laclette, 1999). The putative serpin2Emu might contribute to any function of calcareous corpuscles.

In conclusion, we identified putative *serpin2Emu* and *serpin3Emu* genes, and demonstrated the secretion of these gene products from metacestodes. Further analysis regarding functions and binding target molecules of these proteins is needed.

Acknowledgement

The authors gratefully acknowledge Dr. Minoru Nakao for helpful discussions and technical advice. This work was supported by JSPS Grant-in Aid for Scientific Research (no. 15K19083) and Grant in aid for Innovative Research in Life Science

from Asahikawa Medical University.

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Figure captions

Fig. 1. Phylogenetic tree of the amino acid sequences of serpins from *Echinococcus multilocularis* and other parasitic helminthes.

Fig. 2. Western blotting using antibodies against three *Echinococcus multilocularis* serpins. ES and intracellular soluble proteins were probed with pre-immune rabbit sera (lanes 1, 3, and 5), anti-r-serpinEmu antibody (lane 2), anti-r-serpin2Emu antibody (lane 4), and anti-r-serpin3Emu antibody (lane 6). White arrowheads: a 42 kDa of serpinEmu; white arrow: a 55 kDa of serpinEmu; black arrowheads: the smaller size bands of serpin2Emu and serpin3Emu; brackets: the 50-60 kDa of serpin2Emu and serpin3Emu.

Fig. 3. Immunostaining of metacestodes using antibodies against three *Echinococcus multilocularis* serpins. Pre-immune rabbit sera (A), anti-r-serpinEmu antibody (B), anti-r-serpin2Emu antibody (C), and anti-r-serpin3Emu antibody (D)

were used as first antibodies. Black arrows: germinal layer; white arrows: protoscolices; white arrowheads: calcareous corpuscles. Bar, 50 µm.

Highlights

1. We identified two new putative serpin genes in *Echinococcus multilocularis*.

2. Putative serpin proteins were localized both intracellularly and in ES fraction.

3. All three serpins were expressed in metacestodes, and their localizations differed.

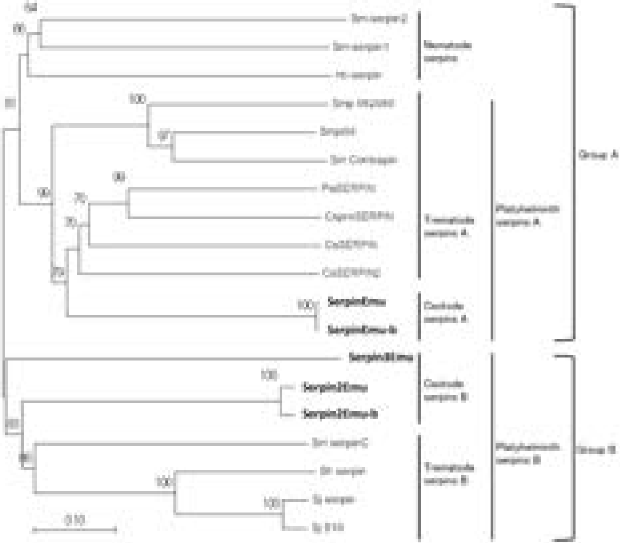
Nematode serpins
Trematode serpins A
Cestode serpins A
Platyhelminth serpins A
Group A
Serpin1Emu
Serpin1Emu-b
Serpin3Emu
Serpin2Emu
Serpin2Emu-b
Cestode serpins B
Trematode serpins B
Platyhelminth serpins B
Group B

Fig. 1

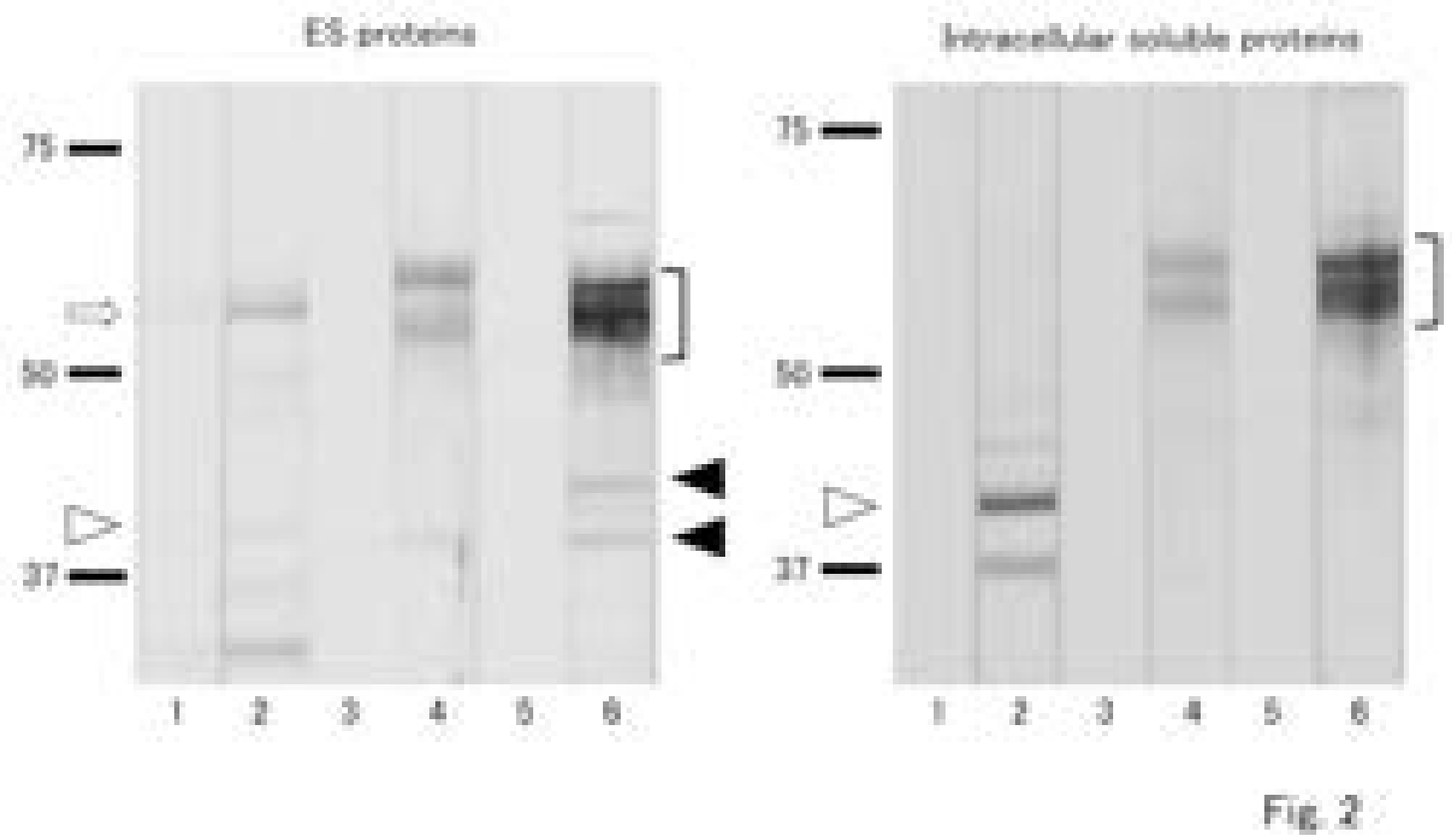
ES proteins
Intracellular soluble proteins
75
50
37
1
2
3
4
5
6

Fig. 2

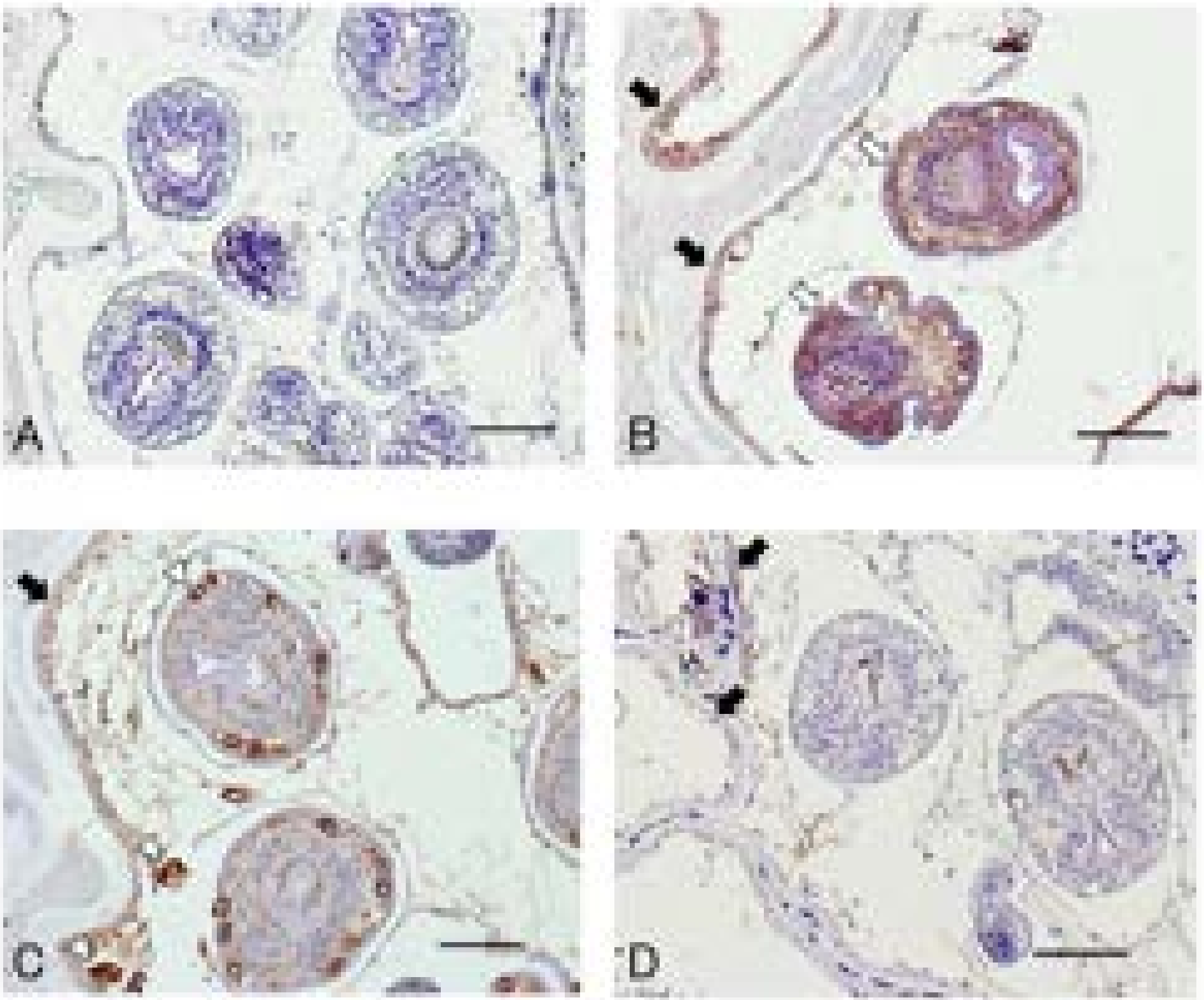

Fig. 3