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Expression of Epithin in the Mouse Preimplantation Development: 1. Colocalization with E-cadherin

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INTRODUCTION

Epithin is a member of mouse type II transmembrane serine proteases (TTSPs) which are emerging class of cell surface proteolytic enzymes that are transmembrane proteins containing both cytoplasmic N-terminal domains and C-terminal extracellular serine protease domain. They are ideally positioned to interact with other proteins on the cell surface as well as soluble proteins, matrix components, and proteins on adjacent cells. Cytoplasmic N-terminal domains of these membrane-spanning proteases suggest possible functions in intracellular signal transduction. These serine proteases have well characterized roles in diverse cellular activities, including blood coagulation, wound healing, digestion, and immune responses, as well as tumor invasion and metastasis (Hooper et al., 2001).

The cloning of epithin was done from a PCR-based subtractive cDNA library of isolated fetal thymic stromal cells (Kim et al., 1999). Northern analysis revealed that epithin mRNA was expressed in intestine, kidney, lung, thymus, and spleen, and enriched in the severe combined immunodeficiency-1 (SCID-1) thymus. The gene was mapped at 17 cM from the centromere on the mouse chromosome 9. Epithin has a multidomain structure containing a putative N-terminal transmembrane region, two CUB domains, four LDLRA repeats, and a C-terminal serine protease domain. Cho et al. (2001) suggested that translational processing is important for the release of the protein to carry out its functions in the extracellular environment by demonstrating N-terminal processing of epithin during protein synthesis and subsequent release of the processed form from the cell membrane into the culture medium, and by showing Gly149 in the N-terminal region as essential for both processing and release using site-directed mutagenesis. In addition, epithin colocalized with E-cadherin at cell-cell contact sites in 427 cells originated from thymic epithelial cells, suggesting a role in epithelial to mesenchymal transition (unpublished data).

These facts led us to examine whether both epithin mRNA and its protein are expressed in the mouse preimplantation embryos. Due to the limited availability of mouse embryos, we took advantages of the sensitive methods, such as RT-PCR and immunocytochemistry. In addition, we also examined the localization of epithin in relation to E-cadherin using double staining immunocytochemistry and confocal microscopy.

MATERIALS AND METHODS

Recovery and Culture of Embryos Fvb female mice with the age of 8 to 12 weeks were obtained from the Laboratory Animal Center in Seoul National University and maintained under 14 hr light and 10 hr dark photo-cycle (light on at 06:00) with water and food supplied *ad libitum*. Superovulation was induced by the intraperitoneal injection of 5 I.U. (international units) pregnant mare serum gonadotropin (PMSG, Sigma, St. Louis, MO) followed by the injection of human chorionic gonadotropin (hCG, Sigma, St. Louis, MO) 46 to 48 h later. To obtain embryos, females were mated overnight with Fvb males and checked for vaginal plugs in the next morning. Under these conditions, fertilization occurs about 12 h post-hCG. One cell zygotes were collected from

oviducts by tearing ampulla region with fine forceps in CZB medium containing hyaluronidase (1 mg/ml, H-3884, Sigma, St. Louis, MO) to remove the cumulus cells, and they were then cultured in CZB medium (Chatot et al., 1989) containing 0.5 % BSA (Sigma, St. Louis, MO) under paraffin oil in sterile culture dishes in an atmosphere of 5% CO2 in air at 37°C. On day 2 afternoon when embryos reached 4-cell stage, embryos were washed several times and transferred to CZB medium without EDTA but supplemented with glucose (1 mg/ml).

RNA Isolation and RT-PCR For epithin amplification, a pair of primers was designed based on the cDNA sequence (Accession number NM-011176). The sequence of upstream epithin primer set A (As) was 5′-CCT ACA ACC TGA CTT TCC TC-3′ and the downstream epithin primer set A (Aa) was 5′-CTG GGT CTG TGA CAG TGT CA-3′. These primers generated a PCR product that was 617 bp in length. The sequence of upstream epithin primer set B (Bs) was 5′-GCA GCA ACA GCA GCA AGA TT-3′ and the downstream epithin primer set B (Ba) was 5′-ATA TAC CTA CCG CTG CCA AA-3′. These primers generated a PCR product of 471 bp in length. Likewise, for E-cadherin amplification a pair of primers was designed based on the cDNA sequence (Accession number NM-009864). The sequence of the upstream E-cadherin primer was 5′-GCT GGA CCG AGA GAG TTA-3′ and the downstream E-cadherin primer was 5′-TCG TTC TCC ACT CTC ACA T-3′. These primers generated a PCR product that was 363 bp in length.

Ten embryos were added into each group. Total RNAs were isolated by acid guanidium thiocyanate-phenol-chloroform extraction method (Chomozynski and Sacchi, 1987). RT-PCR amplification was followed by Shim *et al.* (1997). PCR amplification conditions for both epithin and E-cadherin were as follows: initial denaturation at 94°C for 5 minutes was followed by 35 cycles of 94°C for 1 minutes, 57°C for 1 minutes, 72°C for 90 seconds and the final cycle had an extended incubation at 72°C for 7 minutes followed by decrease to 4°C. Six $\mu\ell$ of PCR products were analyzed on a 3% agarose gel electrophoresis. Gels were then stained with ethidium bromide and photographed under UV illumination. Co-amplification of RT-PCR with GAPDH as an internal control was performed.

Embryo Fixation and Immunocytochemical Staining Embryos were fixed in 3.7 % formaldehyde in PBS for 10 min at room temperature, neutralized with 50 mM NH4Cl in PBS for 10 min, and post-permeabilized with 0.25% Triton X-100 in PBS for 10 min. Immunocytochemical staining was performed by incubating the fixed samples with the anti-epithin antibody diluted 1:200 in PBS/Tween (PBS containing 0.1% Tween20 and 3% BSA) for 60 min, followed by an incubation in rhodamine-conjugated anti-rabbit Ig antibodies (KPL) diluted 1:500 (in PBS/Tween) for 60 min. Samples were observed under a Olympus confocal microscope.

Confocal Microscopy For cellular imaging, experiments were done using a confocal microscope (Olympus, FV500, Japan), mounted on an inverted microscope (Olympus, IX70, Japan) fitted with a 40X objective (numerical aperture 0.85). FITC-conjugated and TRITC-conjugated secondary antibodies were excited with 488 nm line of an Ar laser and 543 nm line of a HeNe Green laser, respectively. The each emitted fluorescence was passed through a 515 or 560 nm primary barrier filter and before it reached the photomultiplier tube. The laser intensity was minimized to prevent dye bleaching during the course of measurements. The fluorescence images were acquired in a slow mode (2.68 sec/frame).

RESULTS AND DISCUSSION

Epithin mRNA expression in mouse preimplantation embryos were determined by RT-PCR. The results showed that specific PCR fragments of the epithin gene were detected using two different

sets of primers (Fig. 1). The mRNA level of epithin seems more or less constant in all groups tested from 1-cell zygote to blastocyst. These results indicate that epithin mRNA is expressed in mouse preimplantation embryos.

To examine the expression of epithin at the protein level in mouse preimplantation embryos, we carried out immunofluorescent staining using polyclonal Ab raised against epithin C-terminal region that corresponds to the extracellular domain. As shown in Fig. 2, epithin proteins were detected in the blastomere membrane throughout the cleavage stages from 1-cell zygote to blastocyst. Notably, epithin was strongly stained at the blastomere junction of the compacted 8-cell embryos or later stage embryos (Fig. 2D, 2E). These results are consistent with the fact that epithin is a transmembrane protein containing extracellular domain and suggest the possible role in cell adhesion at the adhering regions of blastomeres. Epithin transcripts detected in 1-cell zygotes represent the maternal mRNA of epithin, while those detected in 2-cells or later stage embryos represent epithin mRNA mainly from zygotic gene expression. And epithin protein could also be detected from 1-cell zygote to blastocyst stage in the membrane of blastomere. However, epithin staining was stronger in the blastomere junction of 2-cell embryo and later stage embryos, suggesting its role in cell adhesion. One-cell zygote that does not have blastomere contacts showed even distribution of epithin protein in the whole membrane. Due to the onset of zygotic gene activation at 2-cell embryo, even distribution of epithin protein in 1-cell zygote membrane represents the maternal origin. This pattern of epithin distribution in mouse preimplantation embryo is similar to that of E-cadherin, which led us to test for interaction(s) with E-cadherin.

Next, we tested for any interaction(s) between epithin and E-cadherin by using double staining immunocytochemistry and confocal microscopy. As shown in Figure 3, colocalization of epithin protein with E-cadherin is evident in the blastomere junction of morula stage embryo. E-cadherin plays a central role in compaction process by forming hemophilic dimer between the membranes of different blastomeres. Therefore, colocalizational results indicate a possible functional link between epithin and E-cadherin in blastomeric cell adhesion, possibly in compaction processes, during mouse preimplantation development.

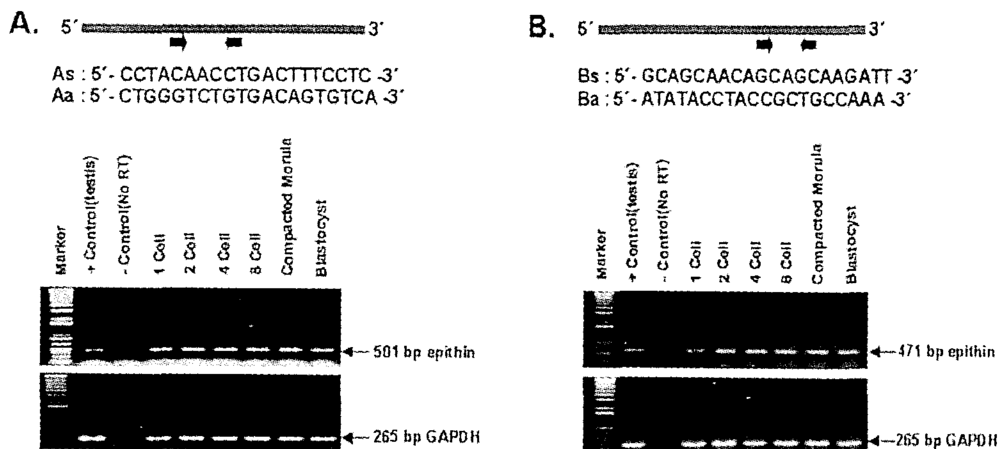

Figure 1. RT-PCR analysis of epithin mRNA expression in the mouse preimplantation embryos. Total RNA isolated from the various stages of embryos was reverse transcribed and PCR amplification was performed. EtBr-stained gels of RT-PCR products were photographed. The GAPDH was used as a control. **A.** The result of RT-PCR using primer set A(As and Aa). Amplified PCR fragments were indicated by arrows. **B.** The result of RT-PCR using primer set B (Bs and Ba). Amplified PCR fragments were indicated by arrows.

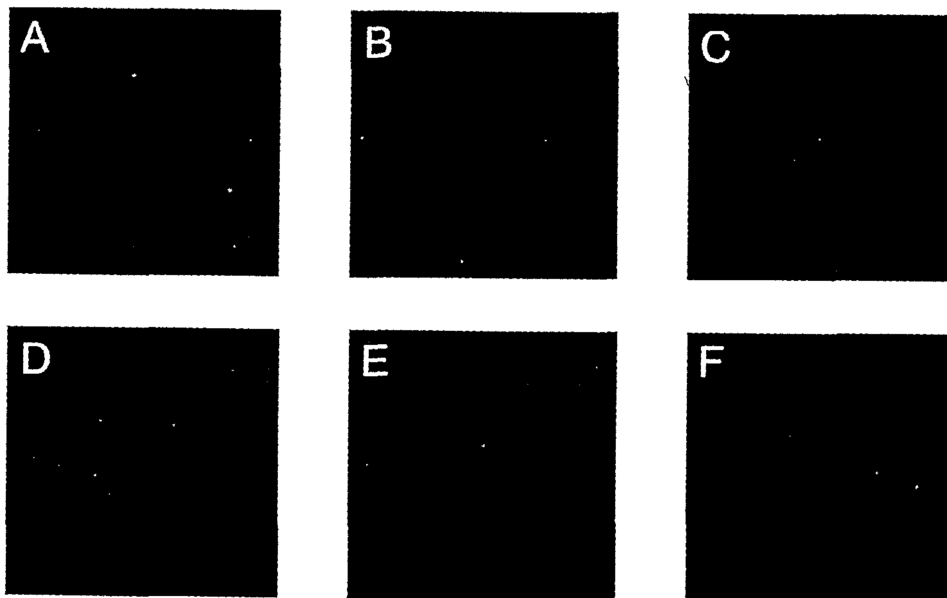

Figure 2. Expression and localization of the epithin protein in mouse preimplantation embryos. One-cell zygotes collected from oviduct were cultured, and embryos at each stage were fixed and stained with epithin polyclonal Ab, followed by TRITC-conjugated secondary Ab. (A) 1-cell zygote (B) 2-cell embryo (C) 4-cell embryo. (D) 8-cell embryo. (E) Morula (F) Blastocyst. Magnification was x400.

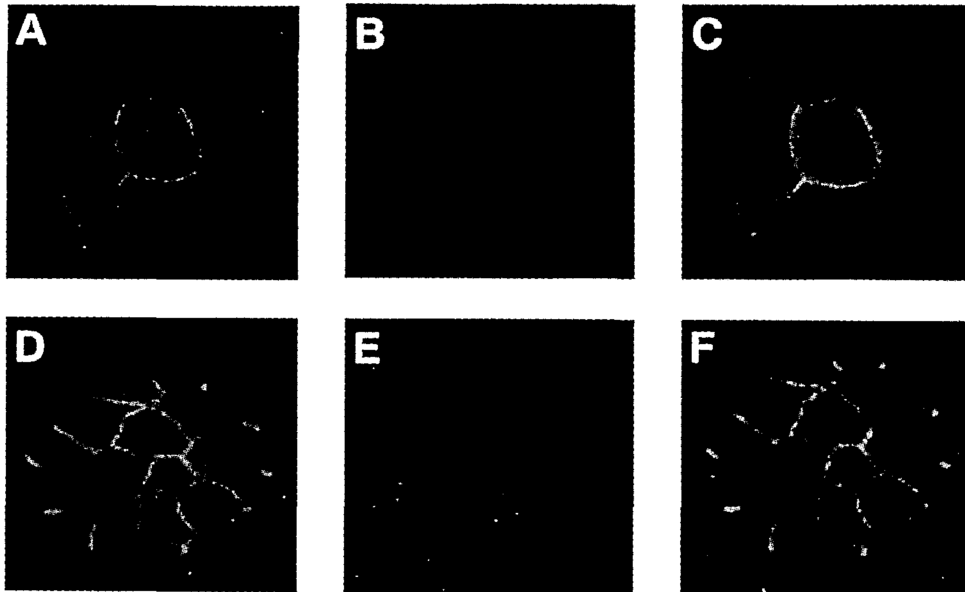

Figure 3. Colocalization of epithin and E-cadherin in the blastomere junction of the morula embryos. The compacted 8-cell embryos (A, B, C) or morula (D, E, F) embryos were immunostained with specific antibodies against epithin (A, B) and E-cadherin (C, D) and observed with confocal microscope. E and F are merged images.