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Developmental Biology 281 (2005) 134–144

DEVELOPMENTAL BIOLOGY

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Expression of epithin in mouse preimplantation development: Its functional role in compaction

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Received for publication 16 June 2004, revised 26 January 2005, accepted 25 February 2005

Abstract

The preimplantation development of mammalian embryo after fertilization encompasses a series of events including cleavage, compaction, and differentiation into blastocyst. These events are likely to be associated with substantial changes in embryonic gene expression. In the present study, we explored the expression patterns and function of epithin, a mouse type II transmembrane serine protease, during preimplantation embryo development. RT-PCR analysis showed that epithin mRNAs were detectable during the cleavage stages from a 1-cell zygote to the blastocyst. Immunocytochemical studies revealed that epithin protein was expressed at blastomere contacts of the compacted 8-cell and later embryonic stages. Epithin colocalized with E-cadherin at the membrane contacts of the compacted morula-stage embryo as revealed by double-staining immunocytochemistry and confocal microscopy, respectively. Post-transcriptional epithin gene silencing by RNA interference (RNAi) resulted in the blockade of 8-cell in vitro-stage embryo compaction and subsequent embryonic deaths after several rounds of cell division. These results strongly suggest that epithin plays an important role in the compaction processes that elicit the signal for the differentiation into trophectoderm and inner cell mass.

Keywords: Epithin; Gene expression; Mouse preimplantation embryo; Compaction; E-cadherin

Introduction

One of the most critical events in preimplantation development is the successful cellular progress which involves the major morphogenic changes in the embryos with progressive cleavage. Particularly, compaction is unique in that the spherical blastomeres flatten onto one another resulting in a spherical mass of cells known as a morula and is concomitant with the first developmental transition events in mammals. As a result of compaction, differentiation into blastocyst is initiated after forming an outer epithelial cell layer that surrounds the inner cell mass and blastocoel (Gallicano, 2001). These events are likely to be regulated by marked changes in the profile of embryonic gene expression, in which gene expression is spatiotemporally linked with any of the morphogenic transitions and subsequent differentiation (Ko et al., 2000; Stanton and Green, 2001; Stanton et al., 2003).

Epithin, a mouse type II membrane serine protease, is cloned from a PCR-based subtractive cDNA library of isolated fetal thymic stromal cells (Kim et al., 1999). Epithin mRNA is expressed in intestine, kidney, lung, thymus, and spleen and is enriched in the thymus of severe combined immunodeficiency-1 (SCID-1) mice (Kim et al., 1999). 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 (Bass and Ellis, 2002; Hooper et al., 2001; Wu, 2001). Cho et al. (2001) reported that translational processing is important for the release of the protein and activity in the extracellular environment. Gly149 in the N-terminal region for the N-terminal processing of epithin during protein synthesis and its subsequent release from the cell membrane into the culture medium is essential for both processing and release using site-directed mutagenesis (Cho et al., 2001). In

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doi:10.1016/j.ydbio.2005.02.026

addition, epithin is colocalized with E-cadherin at cell–cell contact sites in thymic epithelium cells, suggesting a possible role in E-cadherin-mediated cell adhesion (unpublished results). Recently, List et al. (2002) reported the gene knockout of Matriptase/MT-SP1, the human orthologue of epithin. Their offsprings develop to term but die within 48 h of birth. All epidermal surfaces of the newborn mice are grossly abnormal; their skins were dry, red, shiny, and wrinkled. The pups suffer a rapid and fatal dehydration due to the malfunction of their epidermal barrier. It seems that the Matriptase/MT-SP1 null embryos, which originated from the fertilization of gametes from the heterozygotes, might have achieved compaction via the MT-SP1 protein translated from its mRNA of maternal origin present in oocytes from the heterozygotes. In this regard, the contribution of the epithin and its functional role in mouse preimplantation development remain to be clarified.

Cell adhesion plays a critical role in the differentiation of the trophectoderm epithelium and the morphogenesis of the blastocyst (Bloor et al., 2002; Watson and Barcroft, 2001). In mouse preimplantation embryo development, E-cadherin-mediated cell adhesion initiates during compaction at the 8-cell stage and is regulated post-translationally via protein kinase C and other signaling molecules (Goval et al., 2000; Pey et al., 1998). E-cadherin adhesion organizes the epithelial polarization of blastomeres at compaction. Subsequently, the proteins of the epithelial tight junction are expressed and assemble at the apicolateral contact region between outer blastomeres in three phases, culminating at the 32-cell stage when blastocoel cavitation begins. Cell adhesion events also coordinate the cellular allocation and spatial segregation of the inner cell mass (ICM) of the blastocyst, and the maintenance of epithelial trophectoderm and nonepithelial phenotypes during early morphogenesis (Fleming et al., 2001).

These facts, together with the colocalization of epithin with E-cadherin in the thymic cells, attracted our attention toward the possible involvement of epithin in mouse preimplantation development. In this regard, the present study was performed to examine whether both epithin mRNA and its protein are expressed in the preimplantation embryos of mice using RT-PCR and immunocytochemistry, respectively. Furthermore, we explored the possible role of epithin in mouse preimplantation development using RNA interference analysis that can eliminate the possible effects of the maternally originated epithin mRNA as well.

Materials and methods

Harvesting and culture of embryos

Female mice of the Fvb strain ranging from 8–12 weeks of age were obtained from the Laboratory Animal Center at Seoul National University and maintained under a 14 h light and 10 h dark photo-cycle (lights 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 an injection of human chorionic gonadotropin (hCG, Sigma) 46 h later. Females were immediately caged with Fvb males and checked for vaginal plugs in the following morning. One-cell zygotes were collected from oviducts by tearing the ampulla region with fine forceps in CZB medium containing hyaluronidase (1 mg/ml, Sigma), washed with fresh CZB media, and then cultured in CZB medium (Chatot et al., 1989) containing 0.5% BSA (Sigma) under paraffin oil in sterile culture dishes in an atmosphere of 5% CO_2 in air at 37°C. In the late afternoon of day 2 when embryos reached a 4-cell stage, they were washed several times and transferred to CZB medium lacking EDTA, but supplemented with glucose (1 mg/ml).

RNA isolation and RT-PCR

Total RNAs were isolated by the acid guanidium thiocyanate–phenol–chloroform extraction method (Chomozynski and Sacchi, 1987; Shim et al., 1996, 1997). Ten embryos were pooled in a microfuge tube containing 300 μl of solution D (4 M guanidium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% *N*-lauryl sarcosine, and 0.1 M 2-mercaptoethanol) on ice and immediately plunged into liquid nitrogen for storage until use. After thawing, 0.1 volume of 2 M sodium acetate (pH 4.0), 1 volume of water-saturated phenol, and 0.2 volume of chloroform–isoamyl alcohol (49:1) were added. After vortexing, the mixture was incubated on ice for 10 min. Total RNA was then recovered after a centrifugation at 12,000 rpm for 30 min at 4°C by precipitation from the supernatant with 1 volume of isopropanol. The pellet was washed twice with 75% ethanol and dissolved in distilled water. Unless otherwise mentioned, diethyl pyrocarbonate (DEPC)-treated water was used in all experiments.

Isolated RNAs were denatured in the presence of 100 pmol random hexanucleotide in a final volume of 10.75 μl at 75°C for 10 min. After a brief centrifugation at 4°C, 9.25 μl of master mix [200 U RNaseH-MMLV reverse transcriptase, 4 μl dNTP mix (2.5 mM each), 0.25 μl RNasin (26 U/μl), and 4 μl of 5X RT buffer (50 mM Tris–HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, and 10 mM dithiothreitol)] were added and further incubated at 37°C for 1 h. The RT reaction was terminated by raising the temperature to 95°C for 5 min.

PCR amplification was carried out with 2 μl of RT reaction mixture in 40 μl of PCR reaction solution containing 4 μl of 10× PCR buffer (Promega), 3.2 μl dNTP mix (2.5 mM each), and 1 U Ex-Taq polymerase (Promega). In each reaction, 10 pmol of PCR primers was added. Samples were then overlaid with 40 μl of mineral oil and subjected to amplification on a Gene Amp PCR System 2400 (Perkin Elmer). PCR amplification conditions for both

epithin and E-cadherin were as follows: initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 57°C for 1 min, and polymerization at 72°C for 90 s. The final cycle had an extended incubation at 72°C for 7 min, followed by a decrease to 4°C. Six microliters of PCR products was electrophoresed on a 3% agarose gel. Gels were stained with ethidium bromide and photographed under UV illumination (Kaiser, Germany). The intensity of the bands was measured by Bio-Profile Bio-1D Windows application program and quantitated. Amplification of GAPDH was performed as an internal control.

For epithin amplification, a pair of primers was designed based on its cDNA sequence (GenBank accession number NM-011176). The sequence of upstream epithin primer set A (As) was 5′-TTG AGG AGG GTG TGG AGT T-3′ and the downstream epithin primer set A (Aa) was 5′-AGG CCA CCA CAG ATG TTG GC-3′. These primers generated a PCR product with 501 bp. 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′ which generated a PCR product with 471 bp. Likewise, for E-cadherin amplification, a pair of primers was designed based on its cDNA sequence (GenBank 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 PCR products of 363 bp in length.

We used the constitutively expressed glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene as a control. The primers consist of upstream primer 5′-CCA CAG TCC ATG CCA TCA CT-3′ and downstream primer 5′-CTG CTT CAC CAC CTT CTT GA-3′. This primer set produced 265 bp GAPDH transcripts using RT-PCR conditions as described above.

Double-stranded RNA preparation

Double-stranded RNAs (dsRNAs) were prepared as described previously with some modifications (Sonn et al., 2004). To generate a template for transcription in vitro, gel-purified PCR products of epithin, 617 base pairs, using an upstream primer of 5′-CCT ACA ACC TGA CTT TCC TC-3′ and a downstream primer of 5′-TGA CAC TGT CAC AGA CCC AG-3′, were subcloned into the pGEM-T Easy vector. Sense and antisense cRNAs were synthesized in vitro using T7 and SP6 RNA polymerases (Roche), respectively, followed by the digestion of templates with DNase. Both of the cRNA products were extracted with phenol/chloroform and ethanol-precipitated. To anneal sense and antisense RNAs, equimolar quantities of sense and antisense RNAs were mixed in the annealing buffer (10 mM Tris, pH 7.4, 0.1 mM EDTA) to a final concentration of 2 μM each, heated at 94°C for 90 s, and cooled down to room temperature for 3–4 h. To avoid the presence of contaminating single-strand cRNAs in the dsRNA samples, the preparations were treated with 2 μg/ml RNase T1 (Calbiochem) and 1 μg/ml RNase A (Sigma) for 30 min at 37°C. The dsRNAs were then treated with 140 μg/ml proteinase K (Sigma), phenol extracted, ethanol precipitated, washed in 75% ethanol, and dissolved in water. Formation of dsRNA was confirmed by migration on an agarose gel together with the single-stranded RNAs; each dsRNA's mobility on the gel shifted compared to the single-strand cRNA. Double-stranded RNAs were diluted with water to a final concentration of 2–4 mg/ml and stored at −70°C until use.

Embryo fixation and immunocytochemical staining

Embryos were fixed in 3.7% formaldehyde in PBS for 10 min at room temperature, neutralized with 50 mM NH_4Cl in PBS for 10 min, and permeabilized with 0.25% Triton X-100 in PBS for 10 min (Kim et al., 2002). Immunocytochemical staining was performed by incubating the fixed samples with anti-epithin antibody diluted 1:200 in PBS/Tween (PBS containing 0.1% Tween 20 and 3% BSA) for 30 min, followed by an incubation in rhodamine-conjugated anti-rabbit IgG antibodies (KPL) diluted 1:500 (in PBS/Tween) for 60 min. Anti-epithin antibody was a polyclonal antibody raised against the epithin C-terminal region which corresponds to its extracellular domain (Cho et al., 2001). Monoclonal anti-E-cadherin antibody was purchased from BD Transduction Laboratories and diluted 1:500 before use. Samples were observed under an Olympus confocal microscope.

Confocal microscopy

For cellular imaging, we used a confocal microscope (Olympus, FV500), mounted on an inverted microscope (Olympus, IX70) fitted with a 40× 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 emitted fluorescence was passed through a 515 or 560 nm primary barrier filter before it reached the photomultiplier tube. The laser intensity was minimized to prevent dye bleaching during the course of measurements. Fluorescent images were acquired in a slow mode (2.68 sec/frame). The images were processed using Adobe Photoshop 5.5 software to enhance the contrast between immunostained and background signals.

Microinjection of dsRNA

Microinjection of dsRNAs was carried out as previously described (Sonn et al., 2004). Briefly, microinjections of dsRNAs were performed as follows: the holding and the injection pipettes were fabricated from prewashed borosilicate glass tubes with an outer diameter of 1.0 mm and inner

diameter of 0.75 mm (Humagen Fertility Diagnostic, Inc., Charlotteville, VA). The injection pipette was prepared on a micropuller (P-97, Sutter Instrument Co., Navato, CA) by pulling a capillary containing a filament which facilitates its loading of solutions by capillary action. Holding pipettes were made by cutting and fire-polishing a pulled pipette on a microforge (Narishige), followed by S-shaping on a small flame.

Micromanipulation of embryos was performed according to a standard procedure (Nagy et al., 2002). One-cell embryos were placed in HEPES-buffered CZB medium containing 20 mM HEPES and 5 mM sodium bicarbonate under light mineral oil (all from Sigma, St. Louis, MO). A holding pipette was used to keep the 1-cell embryos stationary during manipulation. An injection pipette loaded with dsRNA solution was inserted into the cytoplasm of each zygote, followed by the microinjection of ~10 pl dsRNA using a constant flow system (Transjector, Eppendorf). After microinjection, embryos were cultured in CZB medium as described previously.

Statistical analysis

Data for epithin RNAi analyses were statistically evaluated using Student's *t* test or one-way analysis of variance followed by Fisher's least significant difference test for a post hoc comparison. Statistical significance was set at $P < 0.05$.

Results

Expression of epithin mRNA in mouse preimplantation embryos

To examine epithin expression at the mRNA level in preimplantation mouse embryos, we performed RT-PCR. First, we did control experiments to ensure the optimal number of PCR cycles and the same amount of RNA input/lane. There was a linear increase of PCR products in PCR cycles (from 25 to 40 cycles) and as a function of RNA input (from 5 to 20 embryos) (Fig. 1A). Moreover, PCR products were verified by subcloning and sequencing analysis as predicted on the bases of cDNA sequence data. Specific PCR products of the epithin gene were detected with two different sets of primers as shown in Figs. 1B and C and were more or less constant from the germinal vesicle (GV) oocytes to blastocysts.

Localization of epithin protein in mouse preimplantation embryos

To determine epithin protein expression in mouse preimplantation embryos, we used immunofluorescent staining with a polyclonal anti-epithin antibody. As shown in Fig. 2, epithin was detected in the blastomere membrane throughout the cleavage stages from a 1-cell zygote to the blastocyst. Notably, epithin reactivity was strong at the blastomere contacts of compacted 8-cell or later stage embryos (Figs. 2D and E). These results are consistent with the fact that epithin is a transmembrane protein with an extracellular protease domain. In addition, the distribution patterns of epithin were similar to those of E-cadherin, suggesting the possible role in cell adhesion at blastomere contacts. We then tested for localizational relationship between epithin and E-cadherin using double-staining immunocytochemistry and confocal microscopy. Fig. 3 shows the colocalization of epithin with E-cadherin at the blastomere contacts of a compacted 8-cell embryo.

Role of epithin in preimplantation development

To examine the possible role of epithin in mouse preimplantation embryonic development, RNA interference analyses were performed; then both RT-PCR and double immunostaining were used to verify the interference procedure; the morphological changes during preimplantation development were observed. Fig. 4 shows the percentage of embryos that developed to blastocysts following injection of epithin dsRNA. About 79% (72 of 92 embryos) and 35% (31 of 87 embryos) of zygotes that had been injected with 0.4 and 4.0 pg of epithin dsRNA, respectively, developed into blastocysts, while none (0 of 83 embryos) did in the group in which 20 pg of epithin dsRNA was injected. This dose-dependent effect of epithin dsRNA injection upon blastocyst development, compared to 20 pg of GFP dsRNA injection group (75%, 60 of 80 embryos) as a negative control, indicates an epithin-specific RNA interference.

In the next experiment, the 20 pg of epithin dsRNAs dosage (with the highest rate of RNAi in Fig. 4) was chosen and the E-cadherin RNAi (20 pg) group was added as a positive control; their cavitation rates were observed. Fig. 5A is the representative transmission images of each stage embryo injected with either E-cadherin or epithin dsRNA, respectively. As shown in Fig. 5B, about 31% (21 of 93 embryos) of the E-cadherin RNAi group developed into blastocysts, while none (0 of 81 embryos) in the epithin RNAi group did; negative controls of non-injection, vehicle injection, and GFP RNAi showed 85% (89 of 104 embryos), 79% (67 of 84 embryos), and 77% (76 of 98 embryos), respectively. The E-cadherin RNAi and epithin RNAi embryos became aberrant at the 8-cell stage and compaction did not occur (Figs. 5A and B).

The expression of E-cadherin and epithin was determined in RNAi embryos that had been injected with the corresponding specific dsRNA. RT-PCR analysis of epithin dsRNA-injected embryos detected no epithin mRNA, while that of the E-cadherin dsRNA-injected embryos resulted in barely detectable E-cadherin mRNA (Fig. 6A). However, epithin dsRNA injection did not affect E-cadherin mRNA

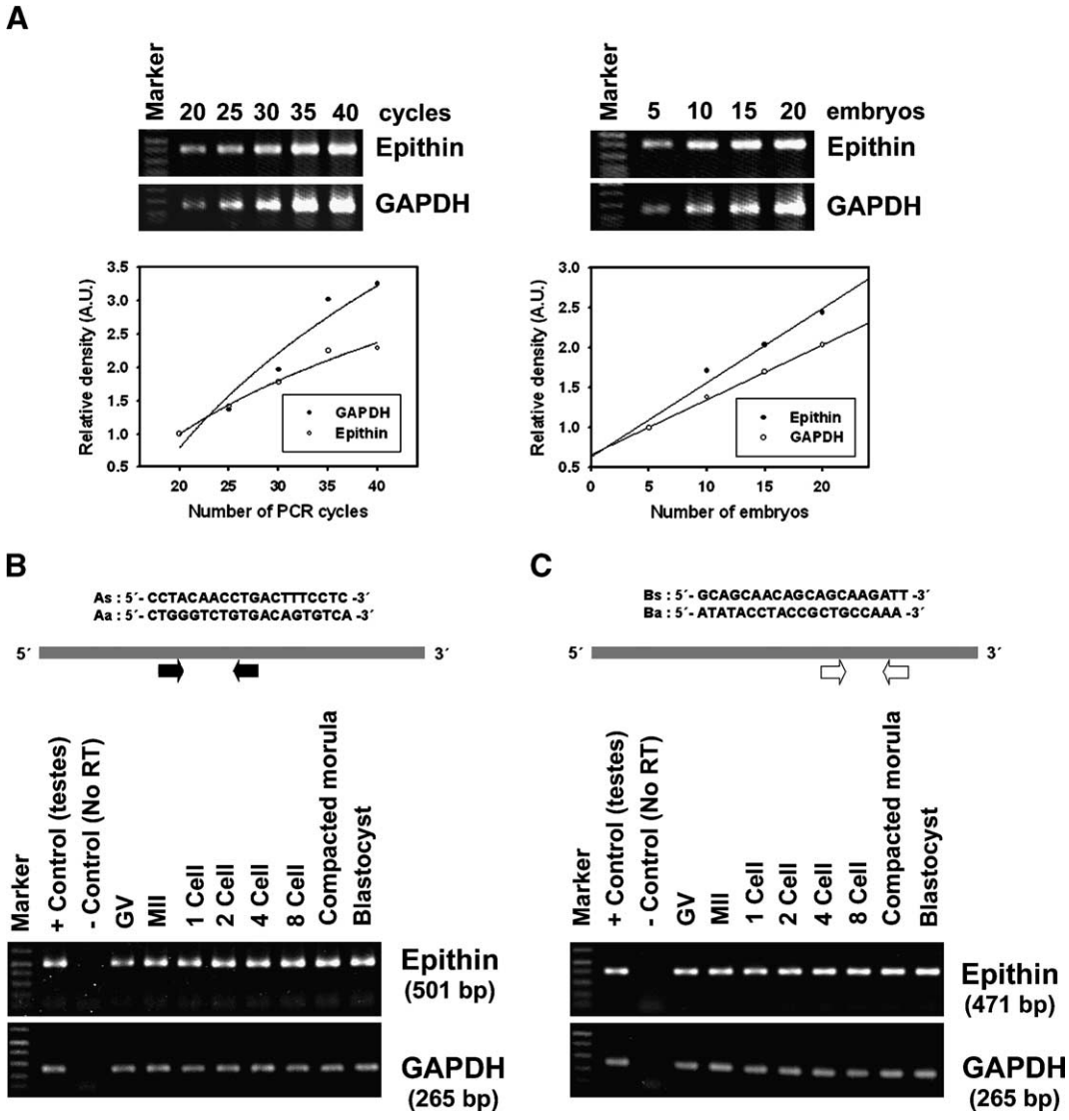

Fig. 1. RT-PCR analysis of epithin mRNA expression in mouse preimplantation embryos. Total RNAs isolated from various embryonic stages were reverse transcribed and PCR-amplified. Ethidium bromide-stained gels of RT-PCR products were photographed. (A) Optimization of RT-PCR analysis according to the number of PCR cycles and embryos for survey efficient RNA input with constant 10 embryos and 30 cycle repetition, respectively. Each relative intensity of epithin and GAPDH signals was expressed as relative to 20 PCR cycles and 5 embryos using arbitrary unit (A.U.) below the images. (B) Result of RT-PCR using epithin primer set A. Amplified PCR products of 501 bp are indicated by arrow, which were subcloned and in vitro transcribed for epithin ablation after dsRNA preparation; GAPDH was used as a procedural control. (C) Result of RT-PCR using epithin primer set B. Amplified PCR products of 471 bp are indicated by arrow; GAPDH was used as control. Abbreviations: No RT, no reverse-transcriptase; GV, germinal vesicle; MII, metaphase of meiosis II.

expression, and vice versa, indicating the specificity of RNA interference. A GFP RNAi group was added as a negative control and it showed no change in mRNA expression of either E-cadherin or epithin. For RT-PCR analysis of the epithin RNAi group, primer set B was used to avoid detecting the injected dsRNA that was prepared by in vitro transcription of subclone from RT-PCR products using primer set A. Most of the epithin dsRNA-injected embryos failed to undergo compaction at the 8-cell stage. Fig. 6B is a representative of the immunocytochemical staining of 8-cell stage embryos injected with vehicle as a control (upper panels), E-cadherin dsRNA (middle panels) or epithin dsRNA (lower panels), respectively. Epithin expression was blocked by the epithin-specific RNAi, and E-cadherin expression was reduced by E-cadherin-specific RNAi. In Fig. 6B which represents epithin staining in an E-cadherin-specific RNAi embryo, epithin has an abnormal distribution with a reduced E-cadherin expression. These results might suggest the possible interaction between epithin and E-cadherin during compaction processes in the 8-cell stage embryos.

In order to further verify the functional interaction between these two molecules, we performed RNA interference experiments by injecting both epithin dsRNA and E-cadherin dsRNA at the same time (double RNAi) and examined the inhibition of cavitation rates. As shown in Fig.

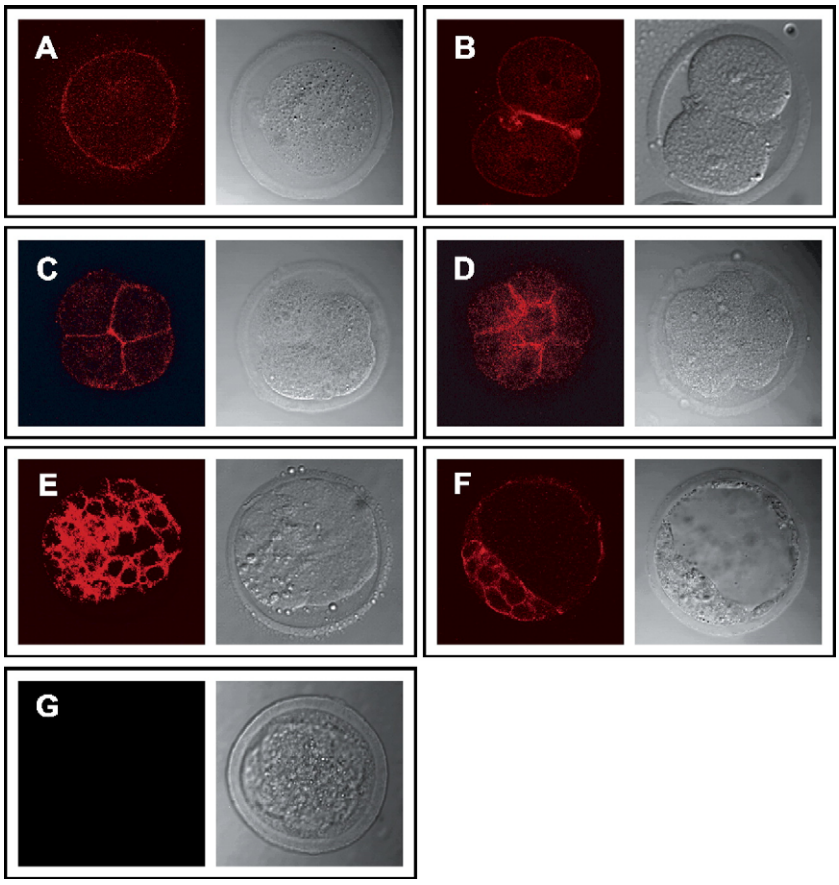

Fig. 2. Expression and localization of epithin protein in mouse preimplantation embryos. One-cell zygotes collected from oviducts were cultured, and embryos at each stage were fixed and stained against an epithin polyclonal antibody. Epithin immunostained fluorescence images are on the left and transmission microscopic ones are on the right. (A) 1-cell zygote. (B) 2-cell stage embryo. (C) 4-cell stage embryo. (D) 8-cell stage embryo. (E) Compacted 8-cell stage embryo. (F) Blastocyst. (G) 8-cell embryo stained with 2nd antibody only as a negative control (400× magnification).

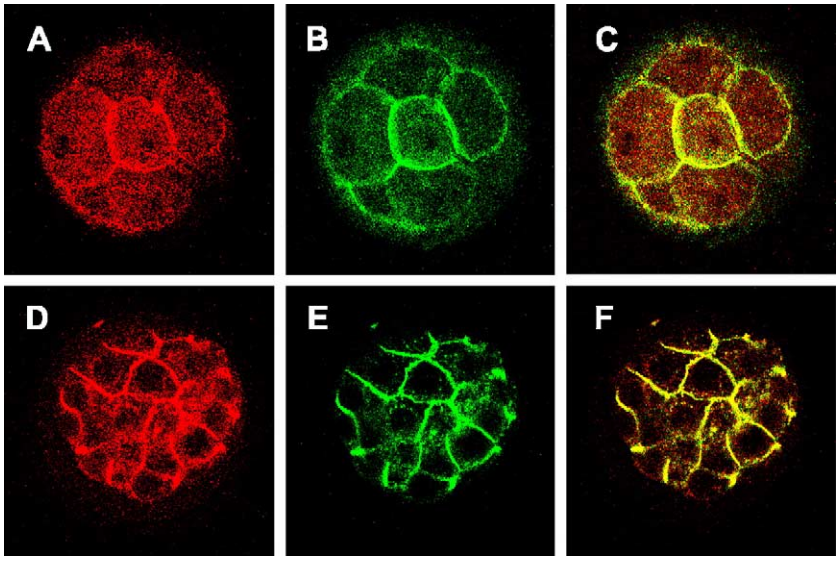

Fig. 3. Colocalization of epithin and E-cadherin in the blastomere contacts of morula embryos. Compacted 8-cell (A–C) or morula (D–F) embryos were immunostained with specific antibodies against epithin (A, D) or E-cadherin (B, E) and observed with confocal microscopy. Panels C and F are merged images (400× magnification).

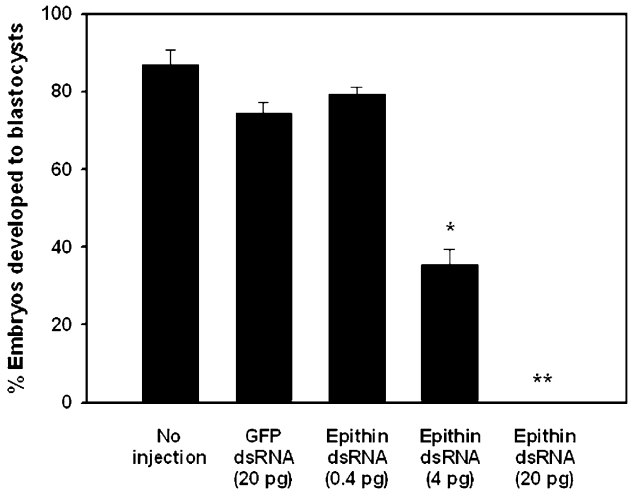

Fig. 4. Injection of epithin dsRNA blocked mouse early embryo development. Epithin dsRNA was prepared and injected into 2 PN (pronuclei) stage embryos at indicated amounts. Injected embryos were cultured for 4 days and embryos that had developed into blastocysts were counted. GFP dsRNA (20 pg) was injected as a negative control. Four independent experiments were performed, represented as a percentage, and analyzed statistically. * and ** indicate $P < 0.05$ and $P < 0.005$, respectively.

7A, 17 of 90 embryos (about 19%) of the double RNAi group developed to blastocysts, while 33 of 78 embryos (42%) of E-cadherin RNAi group and 28 of 76 embryos (37%) of epithin RNAi group developed to blastocysts; negative controls of GFP RNAi group showed 89% cavitation rate (75 of 84 embryos). The double RNAi group was statistically significant ($P < 0.01$) when compared with either epithin RNAi group or E-cadherin RNAi group. Fig. 7B shows markedly reduced expression of both epithin and E-cadherin proteins in the double RNAi-injected embryos that failed to undergo compaction at the 8-cell stage. The synergistic inhibition of cavitation by double RNAi supports the possible functional interaction between epithin and E-cadherin during compaction process.

Taken together, our results indicate that epithin-specific RNAi not only inhibits its expression but also causes a blockade of the 8-cell stage compaction during mouse preimplantation embryonic development, possibly through interaction with E-cadherin.

Discussion

In the present study, we explored the expression patterns of epithin and its possible role in mouse preimplantation development. We first observed that epithin mRNA and protein were present in mouse preimplantation embryos using RT-PCR and immunocytochemistry. In fact, epithin transcripts detected in 1-cell zygotes might represent maternal RNA, while those detected in 4–8 cell or later-stage embryos might depict epithin mRNA mainly from zygotic gene expression. One-cell zygotes which lack blastomere contacts showed an even distribution of epithin at the plasma membrane. Considering the onset of zygotic epithin gene activation, epithin detected in 1-cell zygotes and 2-cell embryos represented proteins of maternal origin. In addition, epithin staining was stronger in the blastomere contacts of 2-cell embryos and those in later stages.

Another important observation is that the colocalization of epithin with E-cadherin was evident in blastomere contacts of the morula stage embryo. It is well-known that the basic adherens junction (i.e., E-cadherin/beta-catenin/alpha-catenin/actin) is present and very active in the compaction process (Watson and Barcroft, 2001). A severe and well-documented abnormality before implantation is observed when adherens junction proteins, such as E-cadherins, are missing due to gene targeting (Riethmacher et al., 1995) or due to antibody treatment (Ohsugi et al., 1997). E-cadherin plays an important role in the compaction process by forming homophilic dimers between the membranes of different blastomeres (Watson and Barcroft, 2001). Thus, colocalization of epithin and E-cadherin at blastomere contacts suggests their involvement in blastomeric cell adhesion.

Regarding colocalization of epithin with E-cadherin in blastomere contact sites in 2–8 cell embryos, it should be noted that their formation and the direct association of E-cadherin with the cytoskeleton would be important during embryogenesis primarily because all of the components necessary for assembling these junctions are present by the 2-cell stage, although typical adherens junctions exist in embryos (Ohsugi et al., 1999; Pey et al., 1998). However, certain stimulation or signaling at the 8–16 cell stage might be required for compaction to occur despite the presence of all the necessary components. In fact, compaction will take place earlier if embryos are induced to do so in vitro using activators of PKC (Goval et al., 2000; Ohsugi et al., 1993; Pey et al., 1998; Winkel et al., 1990). Accordingly, evidence suggests that PKC interacts, most likely by phosphorylation, with beta-catenin to promote its binding to E-cadherin, alpha-catenin, and actin to form the junction (Goval et al., 2000; Pey et al., 1998). In vivo, compaction takes place with acute, strictly regulated timing. This regulation appears to be due to tyrosine phosphorylation of beta-catenin, which in its tyrosine phosphorylated form functions to keep E-cadherin/beta-catenin adhesion complexes nonfunctional until used for adhesion during compaction and blastocyst formation (Ohsugi et al., 1999). Such possible interaction of epithin with E-cadherin/beta-catenin adhesion complexes awaits further intensive studies.

Above all, the present study strongly suggests that epithin may play an important role in compaction process. First, we found that ablation of epithin gene expression using RNAi technique resulted in the inability of cell adhesion at the 8-cell stage and a subsequent embryonic lethality after several rounds of cell division. This phenotype resulted from epithin RNAi is quite similar to that observed in E-cadherin RNAi. Furthermore, there was a synergistic reduction of cavitation rate by double RNAi experiments (Fig. 7A) suggesting the functional interaction

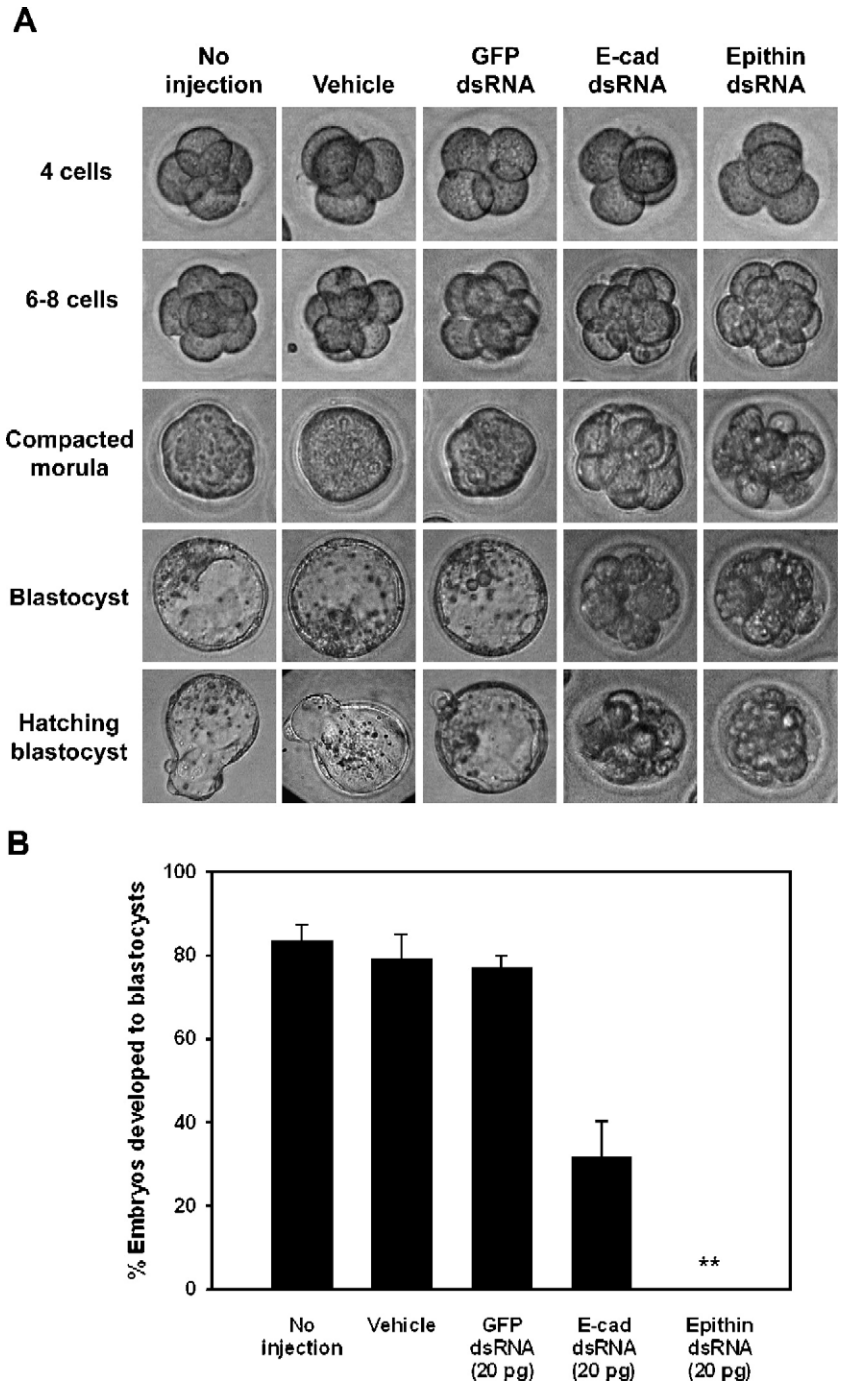

Fig. 5. Phenotypes of epithin RNAi embryos. (A) Morphological changes in mouse preimplantation development following epithin dsRNA injection into zygotes. *x* and *y* axis represent the experimental group and the developmental stages, respectively. Note a lack of compaction at the 8-cell stage in both E-cadherin RNAi and epithin RNAi groups. (B) The percentage of embryos that developed into blastocysts following injection of epithin dsRNA, which is graphical representation of the results shown in panel A. Incidence of cavity formation after injection of epithin dsRNA into the zygote is represented as a percentage in four independent experiments. GFP RNAi groups represent negative controls, and E-cadherin RNAi groups are positive controls. The amounts of GFP dsRNA, E-cadherin dsRNA, and epithin dsRNA were 20 pg.

between epithin and E-cadherin. Consistent with our data, it has been previously reported that E-cadherin RNAi resulted in the reduced cavity formation during mouse preimplantation development (Wianny and Zernicka-Goetz, 1999). In fact, research directed at characterizing the patterns of gene expression during early development has shown that the embryo is initially under maternal control and is later superseded by new transcriptional activity provided by the activation of the embryonic genome. Thus, RNA interference, which can be explained as a post-transcriptional gene silencing or knockdown, is a suitable way to selectively reduce the expression of maternal mRNA and to examine

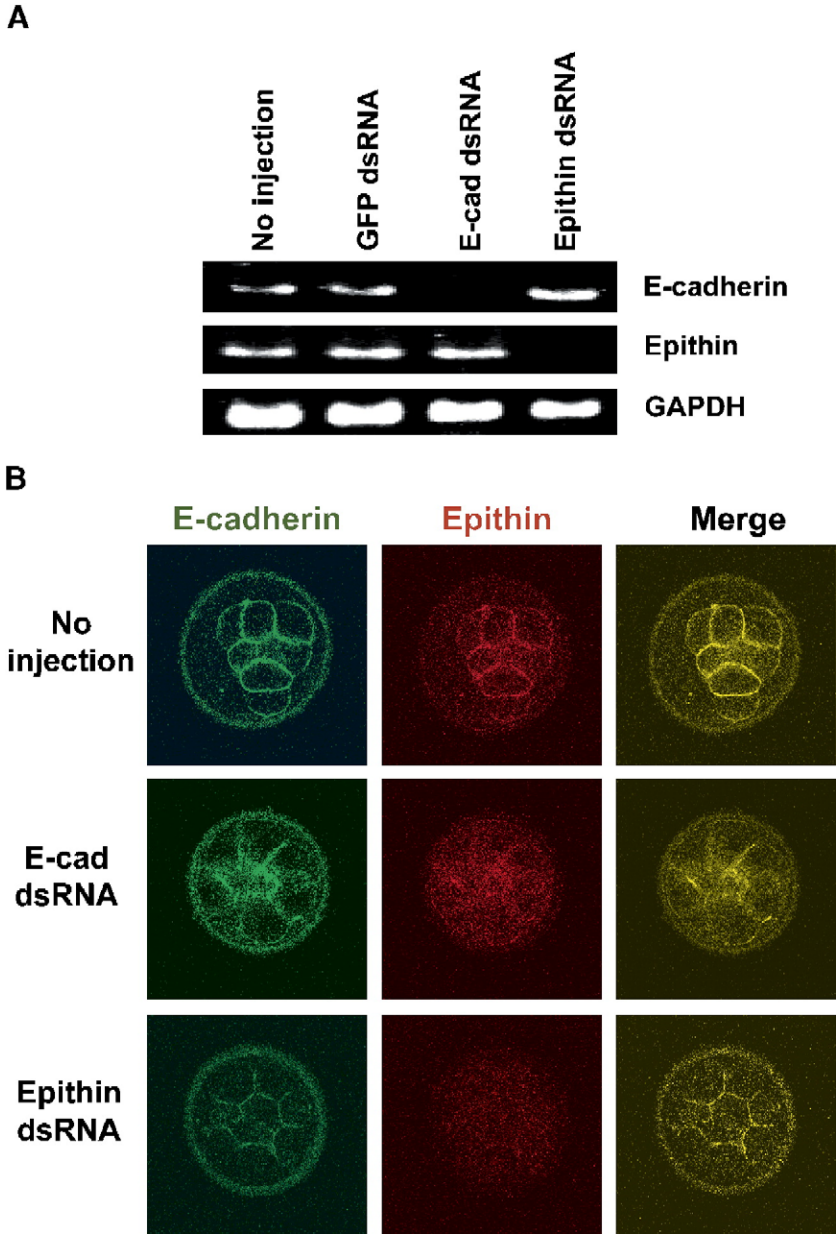

Fig. 6. Specific reduction of epithin expression by RNAi. Injection of epithin dsRNA into zygotes reduced epithin in both protein and RNA levels. Expression of E-cadherin and epithin genes was determined using 8-cell embryos that were injected with the dsRNAs at the 1-cell zygote stage and cultured in vitro for 2 days. (A) The mRNA levels of both E-cadherin and the epithin genes were determined with RT-PCR. Sizes of specific PCR fragments are indicated on the right side of the gels. (B) Reduction in the protein levels was determined by immunocytochemical analyses. Early 8-cell embryos injected with none as a control (upper panels), E-cadherin dsRNA (middle panels) and epithin dsRNA (lower panels) were immunostained with antibodies against E-cadherin (FITC-labeled green color) and epithin (TRITC-labeled red color) (400× magnification).

gene function during preimplantation embryo development (Svoboda et al., 2000).

Second, we found that the distribution of epithin was notably abnormal in the E-cadherin RNAi group, suggesting a possible interaction between epithin and E-cadherin proteins for proper localization and functioning during compaction. In addition, the lethality observed in epithin RNAi embryos that fail to compact is consistent with the reports that E-cadherin null embryos that fail to compact lead to death at the 8–16-cell stage (Larue et al., 1994). Interestingly, this phenotype appears to be relatively similar to the ablation of other components of adherens junctions (i.e., alpha-catenin and beta-catenin) (Haegel et al., 1995; Torres et al., 1997).

Although the mechanisms by which epithin, a type II transmembrane serine protease, may cooperate with E-cadherin in compaction process remains to be elucidated, our results indicate the possibility that epithin appears to interact with E-cadherin, but not as a protease. The ablation of epithin by RNAi results in the blockade of compaction; if

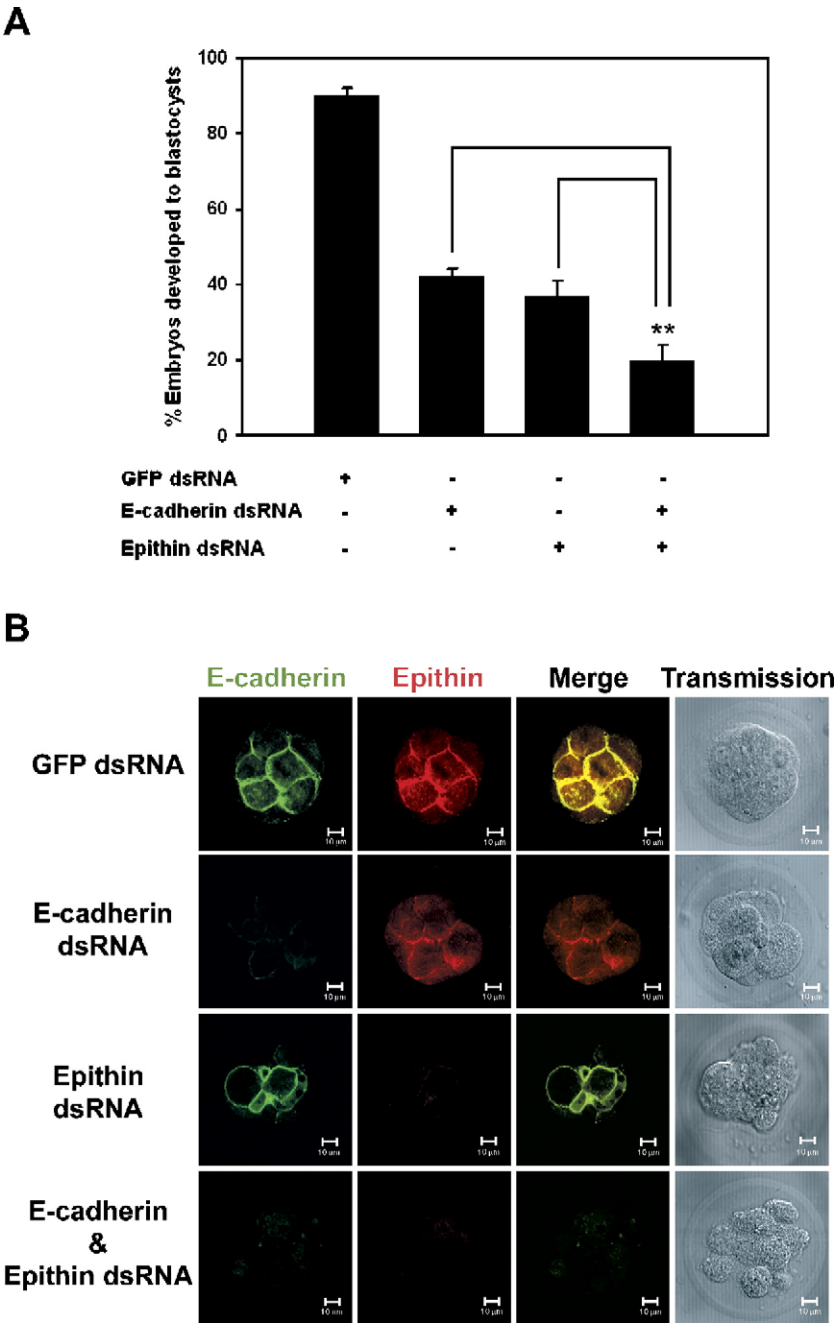

Fig. 7. Possible functional interaction between epithin and E-cadherin in compactional processes. Embryos were injected with both E-cadherin dsRNA and epithin dsRNA (4 pg each) at the 1-cell zygote stage and cultured in vitro for 2–3 days. (A) The percentage of embryos that reached blastocyst stage following injection of proper dsRNA is graphically represented. Incidence of cavity formation after injection of both epithin and E-cadherin dsRNAs into the zygote is represented as a percentage of four independent experiments. GFP RNAi group represents a negative control. The amount of GFP dsRNA injected was 20 pg as shown in Fig. 3. Note a synergistic reduction in the cavitation rate in double RNAi group. $^{**}P < 0.01$, significantly different from E-cadherin RNAi and epithin RNAi groups. (B) Changes in the protein levels of the double RNAi embryos were analyzed by immunocytochemistry. Eight- to sixteen-cell embryos injected with GFP dsRNA as a negative control (upper panels), E-cadherin dsRNA (middle panels) or epithin dsRNA (lower panels) or both were immunostained with antibodies against E-cadherin (FITC-labeled green color) and epithin (TRITC-labeled red color). Magnification was ×400.

epithin functions as a protease and its substrate is E-cadherin, colocalization and the RNAi results contradict one another. It is possible that the protease substrate of epithin might be another protein(s) or epithin itself, indicating autocatalysis (Cho et al., 2001). In fact, epithin has domains other than its protease, such as the CUB, and LDLRA domains. It appears then that these domains might interact with E-cadherin during the compaction process. In this regard, further studies such as high-resolution immunoscanning electron microscopy or immuno-coprecipitation experiment would be required to substantiate the direct interaction between epithin and E-cadherin.

In conclusion, our study demonstrates that epithin mRNA and its protein are not only expressed temporally in preimplantation mouse embryos but play an important role in compaction probably via an interaction with E-cadherin.

Acknowledgments

This work was supported by the grants from BRC of the 21st century Frontier Program in Neuroscience and the Korean Brain Science Research Program, Korea Science and Engineering Foundation by the Korea Ministry of Science and Technology. S. Sonn and J.-H. Park are recipients of the Brain Korea 21 fellowship of the Ministry of Education and Human Resources. We greatly appreciate Biomedical English Editing Service, Portland, Oregon, USA, for their professional editing of the manuscript.

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