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Expression of Epithin in the Mouse Preimplantation Development: 2. Functional Role in Compaction

Inkoo Khang, Seungkeun Sonn, Kunsoo Rhee, Dongeun Park, Kyungjin Kim
School of Biological Sciences, Seoul National University, Seoul 151-742, KOREA

INTRODUCTION

Epithin, the type II transmembrane serine protease (TTSP), was shown to be expressed in mouse preimplantation embryos in the preceding study. In addition, colocalization with E-cadherin that plays a central role in cell adhesion (compaction) suggested a possible functional link between epithin and E-cadherin in blastomeric cell adhesion, possibly in compaction processes, during mouse preimplantation development.

Cell adhesion plays a critical role in the differentiation of the trophoectoderm epithelium and the morphogenesis of the blastocyst. In the mouse embryo, E-cadherin mediated adhesion initiates at compaction at the 8-cell stage, and is regulated post-translationally via protein kinase C and other signaling molecules. E-cadherin adhesion organizes 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 (trophoectoderm) and non-epithelial (ICM) phenotypes during early morphogenesis (Fleming et al., 2001).

In the present study, we attempted the analysis of epithin function in mouse preimplantation development by inactivating epithin expression using RNA interference technique. We. hereby, provide another evidence that RNA interference works in mammalian system.

MATERIALS AND METHODS

Double Stranded RNA Preparation To generate template for transcription in vitro, total RNA were reverse transcribed with Superscript II reverse transcriptase (Gibco BRL). For epithin PCR amplification, primer set C was designed based on the cDNA sequence (Accession number NM-011176). The sequence of upstream epithin primer set C (Cs) was 5′-CCT ACA ACC TGG CTT TCC TC-3′ and the downstream epithin primer set C (Ca) was 5′- GAG GAG GAG CTT GCA AGA CT-3′. These primers generated a PCR product that was 501 bp in length. For E-cadherin PCR amplification, same primers that were used for RT-PCR analysis in previous section were used. 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 3 minutes, 57°C for 30 seconds, 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. All PCR reactions were performed in Gene Amp PCR System 2400 (Perkin Elmer). Gel purified PCR products were cloned into the pGEM-T easy plasmid. RNAs were synthesized using the T7 and SP6 RNA polymerase (Roche). DNA templates were removed with DNase treatment. The RNA 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 (10mM Tris, pH 7.4, 0.1mM EDTA) to a final concentration of 2M each, heated for 1min 30 seconds at 94°C, and incubated at room temperature

for several hours. To avoid the presence of contaminating single strand RNA in the dsRNA samples, the preparations were treated with 2 g/ml RNase T1 (Calbiochem) and 1 g/ml RNase A (Sigma) for 30 min at 37°C. The dsRNAs were then treated with 140 g/ml proteinase K (Sigma). The dsRNA was phenol extracted, ethanol precipitated, washed in 75% ethanol and then dissolved in water. Formation of dsRNA was confirmed by migration on an agarose gel: for each dsRNA, the mobility on the gel was shifted compared to the single-stranded RNAs. RNAs were diluted with water to a final concentration of 24 mg/ml. Samples were stored at -70°C prior to use.

Micromanipulation Microinjection was performed as follows; Both the holding pipettes and the injection pipettes were fabricated from prewashed borosilicate glass tubes (Humagen Fertility Diagnostic, Inc., Charlotteville, VA), 1.0 mm outer diameter and 0.75 mm inner diameter. The injection pipette was prepared by pulling a capillary containing a filament inside on micropuller (P-97, Sutter Instrument Co., Navato, CA). The holding pipette was made by breaking a pulled pipette and fire-polishing on a microforge (Narishige). The 1-cell embryos were placed in HEPES-buffered CZB medium containing 20 mM HEPES (Sigma, St. Louis, MO) and 5 mM sodium bicarbonate (Sigma, St. Louis, MO) under pre-equilibrated washed mineral oil (Sigma, St. Louis, MO) for 10 min prior to micromanipulation. A holding pipette was used to hold the 1-cell embryos stationary during manipulation. The injection pipette loaded with dsRNA solution that were microinjected into the cytoplasm of the zygotes using a constant flow system (Transjector, Eppendorf). Each zygote was injected with ~10 pl dsRNA. After microinjection, oocytes and embryos were cultured in CZB medium supplemented with 5 mg/ml BSA, at 37 °C in a 5% CO_2 atmosphere.

Embryo Fixation and Immunocytochemical Staining and Confocal Microscopy These procedures were performed in a same way as described in the preceding report.

Statistical Analysis Data for epithin RNAi analysis were statistically evaluated using Students t-test or one-way analysis of variance followed by Fishers least significant difference test for a *post hoc* comparison. Statistical significance was set at $P < 0.05$.

RESULTS AND DISCUSSION

To examine the role of epithin in mouse preimplantation development, RNA interference analysis was performed using epithin dsRNA prepared by annealing equimolar concentration of *in vitro* transcribed mRNA of both sense and antisense strand which were subcloned into pGEM-T easy vector using RT-PCR products from primer set C (Cs : 5'-cct aca acc tga ctt tcc tc-3' and Ca: 5'-gaa gag ggg ctt gca aga ct-3'). ~10 pl of epithin dsRNA stock (2 mg/ml) were injected into the zygote, resulting in the injection of ~20 pg epithin dsRNA into each zygote. Figure 1 represents the percentage of embryos developed to blastocysts following injection of epithin dsRNA. About 79 % and 35 % of zygotes injected with 0.4 and 4.0 pg of epithin dsRNA respectively developed to blastocyst, while none developed to blastocyst in a group that 20 pg of epithin dsRNA were injected. The dose-dependent effect of epithin dsRNA injection on the ratio of blastocyst development, together with GFP dsRNA injection group (75%) as a negative control, indicate the specific RNA interference with epithin.

In the next experiment, zygotes were injected with dsRNAs of E-cadherin or epithin and their development was observed. About 31% of embryos with E-cadherin dsRNA were developed to blastocysts(Fig. 2A, 2B). Development of these embryos were blocked at the early 8-cell stage in which compaction did not occur(Fig. 2C). Reduction in the expression of E-cadherin and epithin was determined in embryos that had been injected with specific dsRNA. RT-PCR analysis of epithin dsRNA-injected embryo showed no epithin mRNA detection, while that of E-cadherin

dsRNA-injected embryo showed barely detectable E-cadherin mRNA (Fig. 3A). However, epithin dsRNA injection did not affect E-cadherin mRNA expression, and vice versa. GFP dsRNA group was added as a (-) control and showed change in mRNA expression of neither E-cadherin nor epithin. For RT-PCR analysis of epithin dsRNA injected group, the primer set B were used to avoid the possible detection of the injected dsRNA that was made by using primer set A. Figure 3B represents immunocytochemical staining of embryos blocked at 8-cell stage when dsRNA of either E-cadherin or epithin were injected, respectively. Epithin protein expression was totally blocked by epithin-specific RNAi, while E-cadherin protein expression was markedly reduced by E-cadherin-specific RNAi. These results indicate that epithin RNAi blocked its protein expression in mouse preimplantation embryo and caused the block of development at 8-cell and provide another evidence that RNA interference works in mammalian system.

In the present study, RNAi analysis of epithin function demonstrate that ablation of epithin protein result in the block of preimplantation development at 8-cell, indicating the role in compaction together with E-cadherin. Thus, block at 8-cell detected by morphological observation after epithin RNA interference suggest that epithin play an important role in compaction. In addition, epithin appears to interact with E-cadherin not as a protease, because the ablation of epithin by RNAi resulted in the blockade of compaction. If epithin functions as a protease to cut E-cadherin, colocalization and RNAi results are contradictory each other. Considering the fact that there are many domains in epithin other than protease domain, such as CUB domain, LDLRA domain etc, we are currently dissecting the function of each domain using dominant-negative mutants of epithin.

In conclusion, our study demonstrates for the first time that epithin mRNA and its proteins are expressed in mouse preimplantation embryos and epithin play an important role in compaction process together with E-cadherin.

A. Embryos developed to blastocysts

Exp.	Control	GFP dsRNA (20 pg)	Epithin dsRNA (0.4 pg)	Epithin dsRNA (4.0 pg)	Epithin dsRNA (20 pg)
1	17/20	15/20	21/25	9/20	0/21
2	22/25	19/25	16/20	8/23	0/18
3	29/30	16/20	16/21	5/19	0/20
4	18/23	10/15	20/26	9/25	0/24
Total	86/98	60/80	73/92	31/87	0/83
%	87 %	75 %	79 %	35 %	0 %

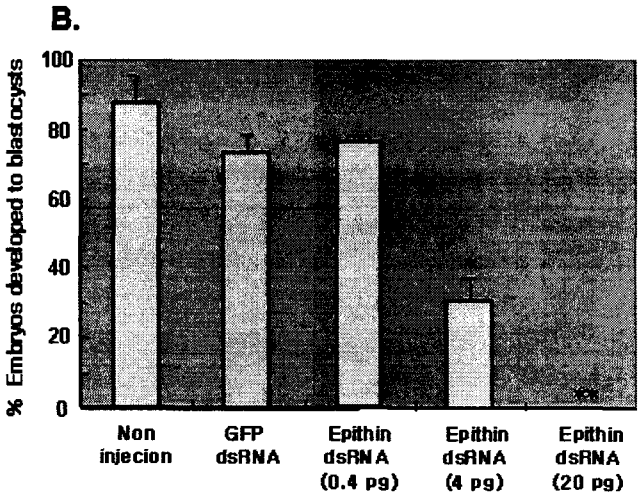
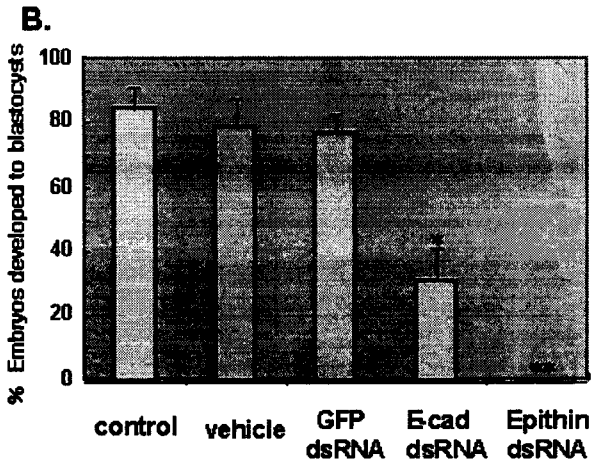


Figure 1. Injection of epithin dsRNA blocked mouse early embryo development. Epithin dsRNA that corresponds to the set of epithin cDNA was prepared and injected into the fertilized one-cell embryos at indicated amounts. The injected embryos were cultured for four days and the number of embryos that had developed to blastocysts was counted. GFP dsRNA was injected as a control. Four experiments were performed (A) and analyzed statistically (B). * and ** indicate $p<0.05$ and $p<0.005$, respectively.

A. Embryos developed to blastocysts

Exp.	Control	Vehicle	GFP dsRNA	E-Cad dsRNA	Epithin dsRNA
1	37/40	19/22	16/20	13/26	0/22
2	17/20	20/22	17/23	2/26	0/25
3	16/22	5/20	25/30	6/18	0/19
4	19/22	13/20	18/25	8/23	0/15
Total	89/104	67/84	76/98	29/93	0/81
%	85 %	79 %	77 %	31 %	0 %



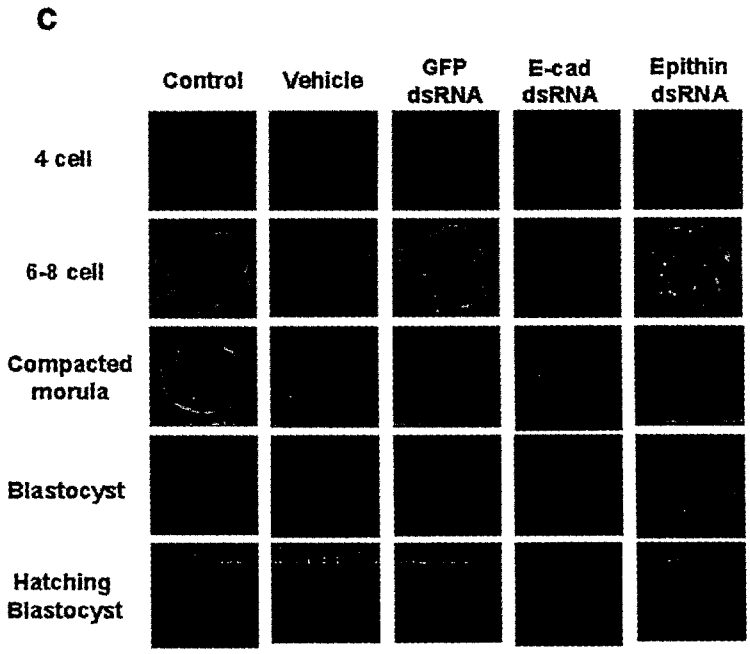


Figure 2. Phenotypes obtained by injection of epithin dsRNA into zygotes. Incidence of cavity

formation after injection of epithin dsRNA into the zygote. A. Table shows the percentage of embryos developed to blastocysts following injection of epithin dsRNA. GFP dsRNA injection was performed as a negative control. B. Graph represents the results shown in table. C. Morphological changes in mouse preimplantation development following epithin dsRNA injection into zygote. X- and Y-axis represent the experimental group, and the developmental stages, respectively. Note the block at 8-cell stage in E-cadherin dsRNA group and epithin dsRNA group.

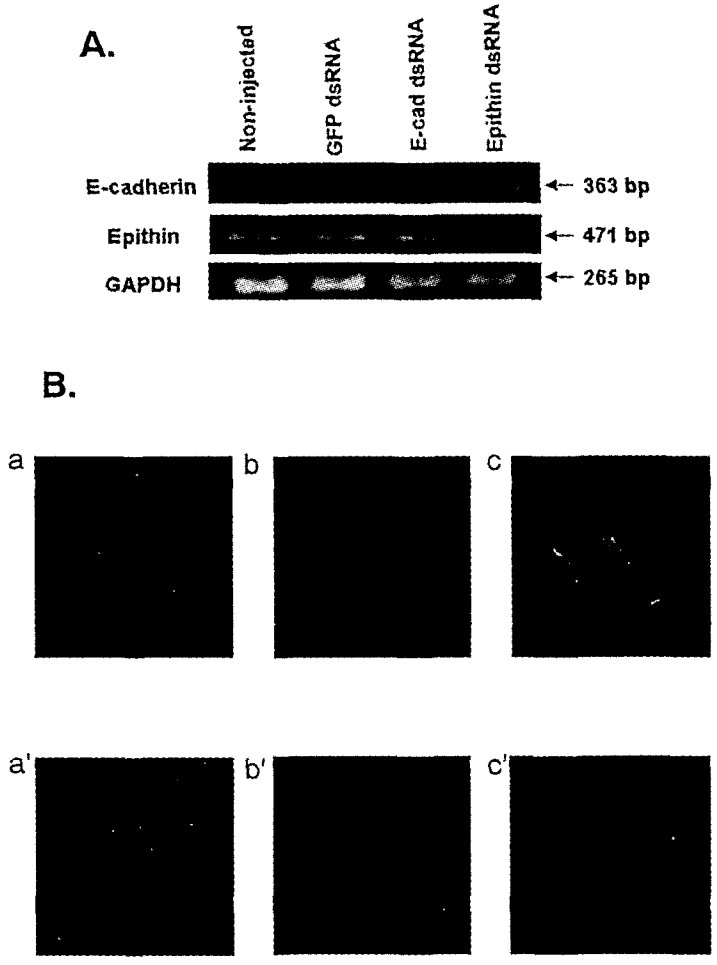


Figure 3. Specific reduction of epithin expression by RNAi. Injection of epithin dsRNA into the zygote reduced epithin expression both in protein and RNA levels. Expression of the E-cadherin and the epithin genes were determined in 8-cell embryos in which dsRNAs of these genes had been injected 38 days ago. (A) The mRNA levels of the E-cadherin and the epithin genes were determined with the RT-PCR methods. The sizes of the specific PCR fragments were indicated in the right side of the gels. (B) Reduction in the protein levels was determined with the immunocytochemical analysis. Early 8-cell embryos had been injected with dsRNA of E-cadherin (a, b, c) and epithin (a', b', c') were immunostained with antibodies against E-cadherin (a, a') and Epithin (b, b').