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Knockdown of the tetraspan protein epithelial membrane protein-2 inhibits implantation in the mouse

Madhuri Wadehra [a,b,1], Molina Dayal [c,d,1,2], Monica Mainigi [c], Teri Ord [c], Ramaswamy Iyer [b], Jonathan Braun [a,b,*], Carmen J. Williams [c,d,*]

[a] *Molecular Biology Institute, Department of Pathology and Laboratory Medicine, University of California at Los Angeles, Los Angeles, CA 90095, USA*
[b] *Jonsson Comprehensive Cancer Center, Department of Pathology and Laboratory Medicine, University of California at Los Angeles, Los Angeles, CA 90095, USA*
[c] *Center for Research on Reproduction and Women's Health, University of Pennsylvania, Philadelphia, PA 19104, USA*
[d] *Division of Reproductive Endocrinology and Infertility, Department of Obstetrics and Gynecology, University of Pennsylvania, Philadelphia, PA 19104, USA*

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Abstract

The establishment of pregnancy requires a successful molecular interaction between the trophectoderm cells of the blastocyst stage embryo and the endometrial cells of the uterus. These interactions are complex and require synchronous development and coordinated endocrine, paracrine, and autocrine communication. In this study, we demonstrate that the tetraspan protein epithelial membrane protein-2 (EMP2) is involved in these molecular interactions during implantation. EMP2, which is highly expressed in the uterus, translocates from an intracellular location to the apical surface of the endometrial epithelium during the window of implantation and is expressed in decidualized stromal cells. We developed plasmid constructs that utilized either ribozyme-mediated or short hairpin RNA-mediated mechanisms to target endometrial EMP2 mRNA for destruction. These constructs were transfected into the mouse uterus on day 1 of pregnancy using the technique of in vivo reproductive tract gene transfer. Reduction in EMP2 expression by either method resulted in a significant decrease in the number of implantation sites in the treated uterine horns as compared to control horns. These studies indicate a previously unknown function of tetraspan proteins in implantation and could provide a molecular framework for the development of therapeutic modalities for both contraception and fertility.

Keywords: Epithelial membrane protein-2; Tetraspan; Implantation; Endometrium; Protein trafficking; GAS3/PMP22

Introduction

The "window of implantation" is defined as the time of the menstrual cycle when the uterus supports blastocyst attachment and implantation (Psychoyos, 1986; Robertson et al., 2001). In humans, this period encompasses days 20 to 24 of a regular 28-day menstrual cycle, which in the mouse corresponds to 4–5 days postcoitum (pc) (Suzuki et al., 2000). The window of implantation is characterized by morphological alterations in the appearance of the uterine epithelium that include loss of microvilli and flattening of the apical membranes (Murphy, 2004). In addition, the epithelial cells develop apical surface protrusions known as "uterodomes" whose presence correlates well with the period of endometrial receptivity (Adams et al., 2001). These morphological alterations occur in response to the effects of progesterone on an estrogen-primed endometrium and do not require the presence of an implanting blastocyst (Png and Murphy, 2000).

Although some molecules involved in implantation have been identified, including Hox proteins, integrins, and selectins (Bagot et al., 2000; Genbacev et al., 2003; Lessey, 2002; Paria et al., 2002), a unifying mechanism to explain the coordinate expression of specific surface proteins during the implantation

* Corresponding authors. J. Braun, is to be contacted at Pathology and Lab Medicine, 13-222 Center for Health Sciences, Geffen School of Medicine at UCLA, CA 90095, USA. Fax: +1 310 825 5674. C.J. Williams, Center for Research on Reproduction and Women's Health, 1313 BRB II/III, 421 Curie Blvd., Philadelphia, PA 19104-6080, USA. Fax: +1 215 573 7627.
E-mail addresses: jbraun@mednet.ucla.edu (J. Braun), cjwill@mail.med.upenn.edu (C.J. Williams).
[1] Authors contributed equally to the work.
[2] Current address: Division of Reproductive Endocrinology, Fertility and IVF, George Washington University, 2150 Pennsylvania Ave., NW, 6th Floor, Washington, DC 20037, USA.

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window remains evasive. In previous work using cultured fibroblast and B cell lymphoma cell lines, we demonstrated that epithelial membrane protein-2 (EMP2), a tetraspan protein in the GAS3/PMP22 family, can regulate the cell surface expression of a number of proteins including integrins α5β1 and α6β1 and class I major histocompatibility complex proteins (Wadehra et al., 2002, 2003a, 2004). These EMP2-regulated alterations in cell surface protein expression are accompanied by corresponding alterations in cell–matrix and cell–cell interactions.

Given the relatively high level of expression of EMP2 in the uterus (Wang et al., 2001) and the connection between EMP2 and the expression of various cell surface proteins, we hypothesized that EMP2 might have a significant role during implantation. Here, we report that, in the mouse, EMP2 is most highly expressed on the apical endometrial surface during the window of implantation. To determine if EMP2 had an essential function during implantation, we utilized two different strategies to "knock down" EMP2 expression in the mouse uterus. Plasmid constructs were developed that utilized either ribozyme-mediated or short hairpin RNA-mediated mechanisms to target EMP2 mRNA for destruction. These constructs were transfected into the mouse uterus on day 1 of pregnancy using the technique of in vivo reproductive tract gene transfer (Daftary and Taylor, 2003). Both of the constructs successfully inhibited expression of endometrial EMP2 protein by day 5 of pregnancy, and both resulted in a significant decrease in the ability of blastocysts to implant. These studies strongly suggest a requirement for EMP2 in implantation in the mouse.

Materials and methods

Animals

All animals used in this study were maintained in accordance with the National Academy of Science *Guide for the Care and Use of Laboratory Animals*, with a controlled light schedule (14L:10D) and controlled temperature range. Female CF-1 mice (Harlan Sprague–Dawley, Indianapolis, Indiana) were housed individually with single B6D2F_1/J males (Jackson Laboratory, Bar Harbor, ME) and checked each morning for a vaginal plug. The day of the vaginal plug was recorded as day 1 pc. For some experiments, females were superovulated by IP administration of 5 IU pregnant mare's serum gonadotropin (PMSG; Calbiochem, San Diego, California) followed 48 h later by 5 IU human chorionic gonadotropin (hCG; Sigma, St. Louis, Missouri) and then housed with males overnight as above. Females without evidence of a vaginal plug the following morning were not utilized for subsequent experiments.

Antibodies

Rabbit polyclonal antibodies were generated against murine EMP2 (mEMP2) or human EMP2 (hEMP2); these antisera have been described previously (Wadehra et al., 2003b; Wang et al., 2001). The corresponding preimmune sera or pooled rabbit sera were used as controls.

Immunohistochemistry

Uteri were fixed in 4% paraformaldehyde or 10% formalin then paraffin-embedded and sectioned. Antigen exposure was accomplished by incubating the slides at 95°C for 20 min in 0.1 M citrate, pH 6.0. Immunohistochemistry was performed using primary mEMP2 antiserum (1:250) or the same dilution of either the corresponding preimmune control serum or pooled rabbit sera (Vector Labs Inc., Burlingame, CA). Detection was performed using either a Vectastain ABC kit (Vector Labs Inc, Burlingame, California) or a DAKO LSAB2 kit (Dako, Carpenteria, California) according to the manufacturers' instructions. Microtome sections were also processed for conventional histologic assessment by staining with hematoxylin and eosin (Fischer Scientific, Allentown, PA).

Plasmid constructs

Ribozyme constructs

A murine EMP2 hammerhead ribozyme (mEMP2-RZ1) has been described previously (Wadehra et al., 2002). This ribozyme sequence is inserted into pEGFP-N3 and will be referred to as pEGFP-mRZ1. To construct a human EMP2-specific ribozyme, the following two sets of complementary 42 bp oligonucleotides were obtained from Invitrogen Life Technologies (Carlsbad, California).

hRZ1a: 5′-GAAAGGACGAAACGGTGGCAAACTTGTTTATTG-CAGCTTAT-3′
hRZ1s: 5′-GGACTCATTAGGACAATGCCTAACAACAACAATTG-CATTCAT-3′
hRZ2a: 5′-GAAAGGACGAAACCGCCTGCAAACTTGTTTATTG-CAGCTTAT-3′
hRZ2s: 5′-GGACTCATCAGCAGGCCACCAAACAACAACAATTG-CATTCAT-3′

These oligonucleotides contain the 22 bases of the hammerhead ribozyme conserved catalytic core, a small region of pEGFP-N3 (Clontech) sequence, and two 10-nucleotide recognition domains (underlined; based on nucleotides 256–276 and 376–396 of the human EMP2 sequence; GenBank accession no. NM001424). The ribozyme sequences were introduced into pEGFP-N3 using PCR and will be referred to as pEGFP-hRZ1 and pEGFP-hRZ2.

shRNA constructs

A PCR-based strategy (PCR SHAGging) for generating RNA polymerase III (U6 snRNA promoter)-driven constructs expressing 29 bp short hairpin RNA (shRNA) targeting mouse or human EMP2 was employed (www.cshl.org/public/SCIENCE/hannon.html). The human U6 promoter from pGEM-Zeo-U6 (generous gift of Gregory Hannon, Cold Spring Harbor Laboratory, Cold Spring, NY) was amplified using a forward primer 5′-GATTTAGGTGACAC-TATAG-3′ (SP6 promoter sequence) and one of two reverse primers (shRNA sequences underlined, U6 promoter sequences in bold):

Mouse EMP2 (targets nucleotides 260–288; GenBank accession no. NM007929): 5′-AAAAAAAACACGATGCGGAAGACAATAATGAAGAC-CAAGCTTCGCCTTCATCATTGTCTTCCACATCGTGTC**GGTGTTTCGT-CCTTTCCACAA**-3′; human EMP2 (targets nucleotides 508–536; GenBank accession no. NM001424): 5′-AAAAAAA GAAGCCGCAATCATAACACAC-AGACACG CAAGCTTCCATGTCTGTGTGTCATGATTGCGGCCTCC-**GGTGTTTCGTCCTTTCCACAA**-3′. PCR was performed using puReTaq Ready-To-Go PCR Beads (Amersham Biosciences, Piscataway, NJ) in a 25-μl reaction as follows: an initial denaturation step at 95°C for 5 min followed by 30 cycles at 95°C for 30 s, 52°C for 30 s, and 72°C for 20 s, and then 72°C for 5 min. The PCR product was TA-cloned into pCR II (Invitrogen, Carlsbad, CA). The resulting constructs will be referred to as m-shRNA and h-shRNA for the vectors targeting mouse or human EMP2, respectively.

Cell lines and transfection

HEC1A cells (ATCC, Manassas, VA) were cultured in McCoy's 5a medium (Invitrogen Life Technologies) supplemented with 10% fetal calf serum (Hyclone Laboratories, Logan, Utah), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 U/ml streptomycin (all from Invitrogen Life Technologies). RL95-2 cells (ATCC) were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium supplemented with 10 mM HEPES, 2.0 g/l sodium bicarbonate, 0.005 mg/ml insulin, 10% fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 U/ml streptomycin.

HEC1A and RL95-2 cells were transfected with pEGFP, pEGFP-hRZ1, or pEGFP-hRZ2 using FuGENE 6 (Roche Molecular Biochemicals, Indianapolis, Indiana). Stable HEC1A clones were selected using Geneticin (800 μg/ml, Invitrogen Life Technologies); these stable cell lines will be referred to as HEC1A-GFP, HEC1A-hRZ1, and HEC1A-hRZ2, respectively. Stable clones were similarly generated for RL95-2 cells. NIH 3T3 cells stably expressing a FLAG-tagged murine EMP2 were established and cultured as previously described (Wang et al., 2001). The cells were transfected with pCRII, m-shRNA, or h-shRNA using FuGENE 6 (Roche) according to the manufacturer's instructions.

Western blot analysis

Cells were washed in PBS, collected, and resuspended in Laemmli buffer. When appropriate, the cell extracts were treated with peptide N-glycosidase F (New England Biolabs, Beverly, Massachusetts) to deglycosylate the proteins as previously described (Wadehra et al., 2002). The lysates were separated on a 12.5% SDS-PAGE gel, and proteins were transferred to nitrocellulose. Protein loading and transfer were detected on nitrocellulose membranes by Ponceau S staining (Sigma). EMP2 was detected using primary hEMP2 (1:2000) or mEMP2 (1:1000) antisera, as appropriate, and secondary horseradish-peroxidase-conjugated goat anti-rabbit IgG (Transduction Laboratories, Lexington, Kentucky). Actin was detected using primary monoclonal anti-β actin (Sigma cat# A-5441; 1:12,000 dilution) and secondary horseradish-peroxidase-conjugated sheep anti-mouse IgG (Amersham). The secondary antibodies were detected using ECL detection reagents (Amersham).

Blastocyst attachment assay

HEC1A-GFP, HEC1A-hRZ1, HEC1A-hRZ2 cells, and nontransfected HEC1A cells were grown to confluence in Costar 24-well tissue culture plates (Corning Inc., Corning, NY). Blastocyst stage mouse embryos were flushed from the uteri of superovulated, mated females and then cultured in vitro in KSOM medium (Specialty Media, Phillipsburg, NJ) to allow hatching. Cells were washed with Dulbecco's PBS (Invitrogen Life Technologies) and then cultured in medium not containing G418. A single hatched blastocyst was placed in each well. After 3 days of culture, attachment of each blastocyst to the underlying cells was examined using transmitted light microscopy. A blastocyst was considered "attached" if no movement was seen with gentle shaking of the plate. Either free-floating or mobile blastocysts were considered "not attached".

Inhibition of uterine EMP2 expression and evaluation of implantation in vivo

Liposome-mediated plasmid transfection in vivo was a modification of a method described previously (Bagot et al., 2000; Daftary and Taylor, 2003). DNA–liposome preparations were made immediately prior to the transfection surgery by mixing the appropriate plasmid DNA with FuGENE 6 (Roche Molecular Biochemicals, Indianapolis, Indiana) in a 1:3 (μg:μl) ratio as per the manufacturer's instructions. The quantity of plasmid DNA used for each experiment is indicated in the text or figure legends. After incubation for 15 min, sterile PBS was added to the DNA–liposome mixture such that the DNA to be injected was in a final volume of 100 μl, and the mixture was drawn into a 1 ml syringe. In the afternoon of day 1 pc, females were anesthetized and the distal portion of each uterine horn was identified via small flank incisions (Hogan et al., 1994). The uterine horns each were slowly injected via a 30-G needle with 100 μl of the appropriate DNA–liposome mixture. Luminal placement of the injected fluid was confirmed by observation of slight distension of the visible portion of the uterine horn.

The details of the mechanics of this method are worth mentioning here. In test experiments on CF1 mice in which the entire reproductive tract was exposed via an abdominal incision, the capacity of a uterine horn was only about 20 μl. Injected fluid above this volume leaked rapidly into the vagina. This finding indicated that the maximal dose of plasmid DNA to which the uterine horn could be exposed was approximately 20% of the original amount in the DNA–liposome mixture. However, it is unlikely that even a 20 μl fluid volume would remain in the uterine horn long-term, so perhaps a better way to consider the method is that it is a brief exposure to a specific concentration of DNA. In other words, the uterine epithelium was exposed briefly to a flow of 100 μl of fluid containing 0.20, 0.10, or 0.05 μg DNA/μl at the 20, 10, and 5 μg plasmid DNA doses, respectively. When injections were performed with Coomassie Blue dye to track the injected fluid, no dye was observed to enter the contralateral uterine horn. However, because of the possibility of crossover of the fluid into the opposite uterine horn, the first horn injected was the "treatment" horn, and the second horn was injected with the control DNA so that any crossover fluid would be washed out of the control horn. This method was chosen over using different mice for each type of DNA so that each mouse could serve as its own internal control.

For experiments to assess the efficacy of the transfected plasmids in reducing EMP2 expression, females were euthanized 4 days after transfection (day 5 pc) and the uteri were processed for immunohistochemistry using mEMP2 antisera or the corresponding preimmune control. For the implantation experiments, females were euthanized 1 week later (day 8 pc), and the number of visible implantation sites in each uterine horn was counted. Unless otherwise indicated, a total of at least 10 females were evaluated in at least three independent experiments. Mice that had vaginal plugs and surgery that were not pregnant at all were excluded from the analysis.

Statistical methods

Differences in blastocyst attachment in vitro were evaluated by Chi-square tests, and differences in the numbers of implantation sites in vivo were evaluated by a Wilcoxon matched pairs test using Prism 4 (GraphPad Software, San Diego, CA).

Results

Expression of EMP2 in the mouse uterus

To determine if EMP2 could be involved in implantation, EMP2 expression and localization in the uterus were examined in non-hyperstimulated, mated mice before (day 1 pc) and during (day 4 pc) the "window of implantation" (Fig. 1). On day 1 pc, EMP2 was expressed in both the luminal and glandular endometrial epithelium in the supranuclear region of the intracellular compartment, in a pattern consistent with a Golgi/endoplasmic reticulum (ER) distribution (Figs. 1A–C). At this time, no EMP2 staining was detected in the endometrial stroma. On day 4 pc, EMP2 was still expressed mainly in the supranuclear region of the intracellular compartment of the luminal and glandular epithelium, but the staining was more diffuse (Figs. 1D–F). In the luminal epithelium, EMP2 was found localized to the apical cell surface; this localization pattern was present in glandular epithelium as well but was not as dramatic. In addition, EMP2 was expressed in the stroma by day 4 pc.

EMP2 expression was also examined in the early post-implantation period. On day 6 pc, the decidualized stromal cells at the site of implantation expressed relatively high levels of EMP2 (Fig. 2). EMP2 expression in decidual cells of the inter-implantation sites was similar to that around the implanting embryos (data not shown). In addition, high EMP2 expression was noted in embryonic trophectoderm cells (Figs. 2C, D). The decidual expression of EMP2 persisted through day 7 pc (Figs. 2E, F), but it was decreased qualitatively at this time as compared to day 6 pc. These experiments established that, during early pregnancy, the peri-implantation period was the

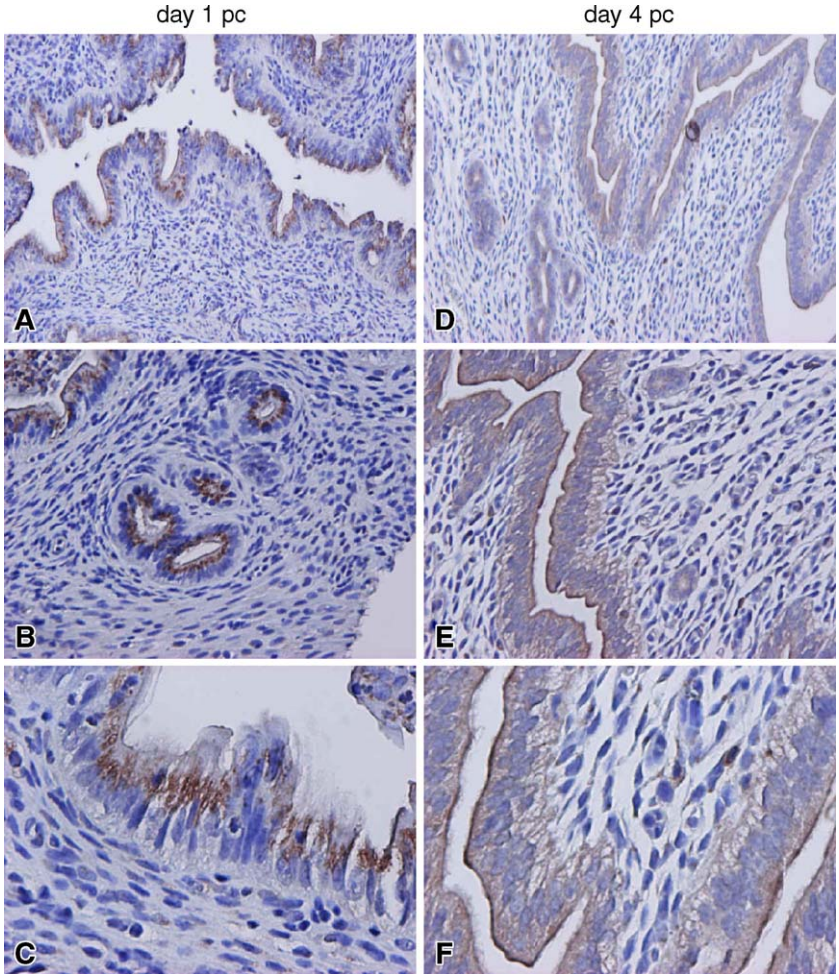

Fig. 1. EMP2 expression and localization in the mouse uterus before implantation. Mouse uteri were obtained on day 1 pc (A–C) or day 4 pc (D–F) and then fixed and processed for immunohistochemistry using mEMP2 antiserum. Magnification: 100× (A, D); 200× (B, E); 400× (C, F).

time of maximal EMP2 expression in both the endometrial epithelial and stromal cells.

Because we wanted to use ovarian hyperstimulation to synchronize the mouse estrous cycles in later experiments (to assist in the timing of mating and surgical procedures and to increase the number of possible implantation sites per uterine horn), we needed to determine if EMP2 expression was similar in normally cycling and hyperstimulated mice. Uteri were collected at 24-h intervals after initiating hyperstimulation treatment and ending day 5 pc. On day 1 after PMSG treatment, EMP2 was expressed on the apical side of the nuclei of the luminal epithelium, similar to unstimulated mice, but some EMP2 was also seen on the apical cell surface (Fig. 3B). By 48 h after PMSG, the overall level of EMP2 staining was diminished, although some staining was present in the supranuclear region (Fig. 3C). On days 1 and 2 pc, EMP2 staining increased in the supranuclear region and was found throughout the cytoplasm of the epithelial cells (Figs. 3D, E). On day 3 pc, EMP2 staining was present diffusely throughout the cytoplasm of the epithelial cells and had begun to accumulate at the apical surface of the luminal epithelium (Fig. 3F). By days 4–5 pc, EMP2 staining was dramatically increased at the apical cell surface of luminal and glandular epithelium (Figs. 3G, H). Similar to non-hyperstimulated mice, EMP2 expression in the endometrial stroma was noted at this time. At all time points, the glandular epithelium exhibited a staining pattern similar to that of the luminal epithelium (data not shown). These data demonstrated that, in both normally cycling and hyperstimulated mice, EMP2 had the temporal and spatial expression pattern in the endometrium consistent with that of the protein involved in implantation.

EMP2 expression and modulation in human endometrial cell lines

Human endometrial carcinoma cell lines have been used previously as in vitro models of uterine epithelium for the study of implantation (Hohn et al., 2000; Thie and Denker, 2002; Tinel et al., 2000). We hypothesized that if EMP2 were important for implantation then it would be expressed in these cell lines and that its expression could be modulated prior to using the cells for in vitro attachment assays. We chose two cell lines for these in vitro assays: HEC1A cells, which support trophoblast attachment in the presence of serum, and RL95-2 cells, which efficiently support trophoblast attachment in the presence or absence of serum (Thie et al., 1995). Endogenous

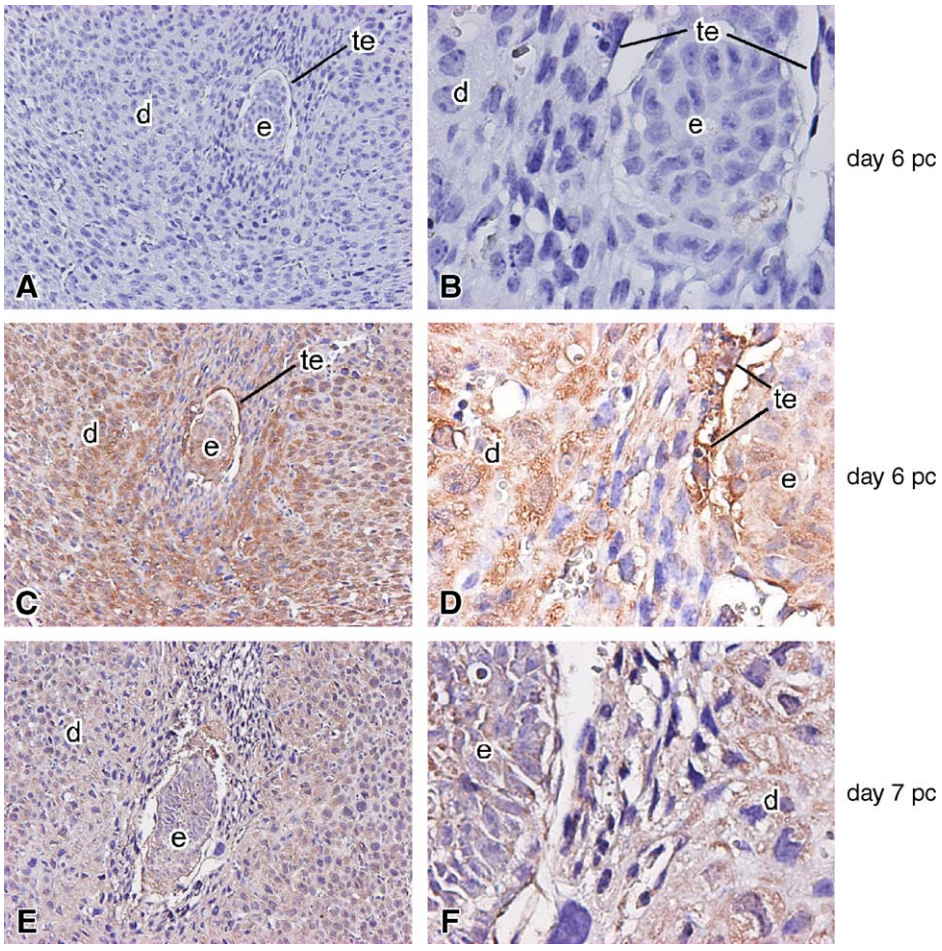

Fig. 2. EMP2 expression and localization in the mouse uterus after implantation. Mouse uteri were obtained on day 6 pc (A–D) and day 7 pc (E–F) and then fixed and processed for immunohistochemistry using control pooled rabbit sera (A, B) or mEMP2 antiserum (C–F). e, embryo; d, decidua; te, trophectoderm cells. Magnification: 100× (A, C, E); 400× (B, D, F).

EMP2 expression was determined by immunoblotting in HEC1A and RL95-2 cells. Although both cell lines expressed EMP2, the level of EMP2 in RL95-2 cells was much higher (Fig. 4A).

We previously developed a ribozyme-containing vector that targeted mouse EMP2 for destruction (Wadehra et al., 2002). For this study, we generated a similar ribozyme designed to target human EMP2 and tested its effect on expression of EMP2 in HEC1A and RL95-2 cells. HEC1A cells were stably transfected with a vector containing one of two human EMP2-specific ribozymes (HEC1A-hRZ1, HEC1A-hRZ2) or a vector control (HEC1A-GFP). Although both of the ribozyme constructs reduced EMP2 expression, the hRZ1 construct was somewhat more effective than hRZ2 (Fig. 4B). The efficacy of the hRZ1 construct was confirmed in RL95-2 cells as well by performing a transfection with the pEGFP vector control or pEGFP-hRZ1. The transfection efficiency after selection in both cultures was estimated at 80% based on counts of the number of cells expressing EGFP. Immunoblot analysis revealed an obvious decrease in EMP2 expression in the cells over-expressing EGFP-hRZ1 when compared to EGFP alone (Fig. 4C). These results documented that the hRZ1 and hRZ2 constructs encoded functional ribozymes.

Effect of modulating EMP2 expression on blastocyst attachment in vitro

To determine if alterations in the level of EMP2 expression in HEC1A cells affected their ability to support blastocyst adhesion, we performed in vitro attachment assays using intact hatched mouse blastocysts. Although it was a heterologous system, mouse blastocysts were utilized for these assays (rather than JAR spheroids made from choriocarcinoma cells) to provide a closer approximation to the blastocyst–endometrium interaction that occurs in vivo. Blastocysts were placed onto confluent monolayers of untransfected HEC1A, HEC1A-GFP, HEC1A-hRZ1, or HEC1A-hRZ2 cells, and evaluated for attachment to the underlying cells. HEC1A cells expressing either of the ribozyme constructs had significantly reduced blastocyst attachment when compared to the controls (Table 1). Whereas 42% (31/74) of the blastocysts attached to HEC1A-GFP cells, only 4% (3/68) blastocysts attached to the HEC1A-hRZ1 cells. Of note, there was a correlation between the level of EMP2 expression and blastocyst attachment—intermediate EMP2 expression in the HEC1A-hRZ2 cells (Fig. 4B) was associated with an intermediate level of inhibition of blastocyst attachment (25%, 18/71). Untransfected HEC1A and HEC1A-

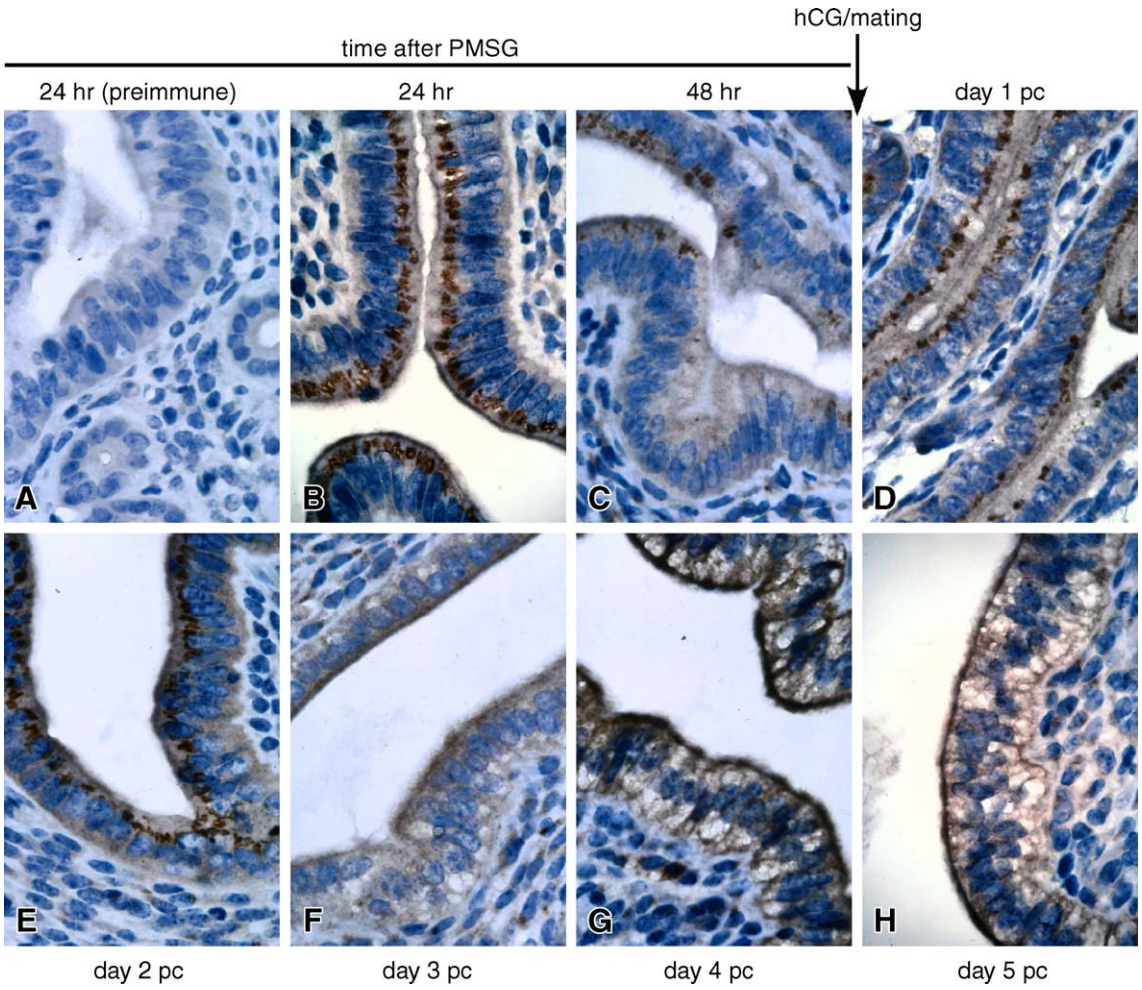

Fig. 3. EMP2 expression and localization in the mouse uterus after hyperstimulation. Mouse uteri were obtained at the indicated times after PMSG or hCG administration and mating then fixed and processed for immunohistochemistry using either preimmune serum (A) or mEMP2 antiserum (B–H). Magnification: 400×.

GFP cells displayed similar attachment rates, suggesting that transfection of these cells did not alter their adhesive properties. To confirm these findings, we performed similar experiments using RL95-2 cells. Reduction of EMP2 expression in RL95-2 cells by transfection with hRZ1 yielded results similar to those obtained using HEC1A cells, with a 50% reduction in blastocyst attachment compared to GFP-transfected control cells (data not shown). These data demonstrated that a reduction of EMP2 expression in human endometrial epithelial cells effectively inhibited the attachment of intact mouse blastocysts in vitro.

Effect of modulating EMP2 expression on blastocyst attachment in vivo

To determine if EMP2 was involved in implantation in vivo, we utilized the mouse ribozyme construct to reduce EMP2 protein levels in the mouse endometrium during the peri-implantation period. These experiments were based on previous work demonstrating that liposome-mediated gene transfection is an effective method for in vivo gene transfer to the mouse reproductive tract and can be used to define functional roles for proteins involved in implantation (Bagot et al., 2000). The uterine horns of hyperstimulated, mated mice were transfected on day 1 pc with ribozyme-containing or control plasmids. The rationale for this timing for the transfection was that it would maximize the time available for turnover of pre-existing EMP2 protein to occur following mRNA destruction induced by the ribozyme, thereby achieving the greatest degree of protein knockdown possible without disrupting the mating process. Furthermore, it maximized the time for recovery of the uterine epithelium from any potential disruptive effects of the transfection procedure. In each experiment, the opposite uterine horn in the same mouse was used as a control to eliminate any bias due to possible differences in the numbers of implanting embryos or differences in nonspecific effects of anesthesia or the surgical procedure itself. There was a dose-dependent decrease in EMP2 expression in the luminal epithelium by day 5 pc in response to transfection with either 5 or 20 μg pEGFP-mRZ1 (Figs. 5B, C). Control uterine horns transfected with either 5 or 20 μg pEGFP (Figs. 5E, F) expressed EMP2 at levels similar to that of horns transfected with diluent and FuGENE 6 (no DNA; Fig. 5A). These data confirmed that the mRZ1 ribozyme effectively decreased expression of EMP2 in the endometrium in vivo.

The effect of reducing EMP2 expression on implantation was assessed on day 8 pc, 7 days after the transfection surgery.

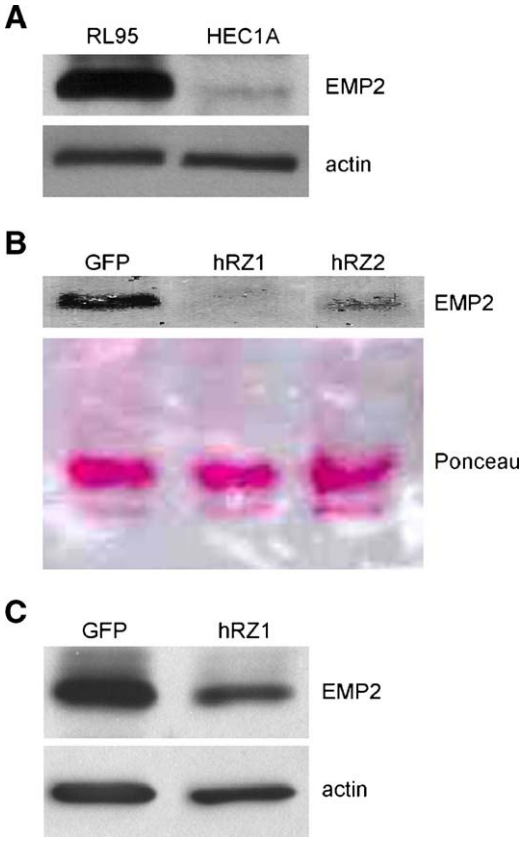

Fig. 4. Expression and modulation of EMP2 expression in human endometrial cells. (A) Immunoblot analysis of EMP2 expression in human endometrial cell lines. Equivalent amounts of cell lysates (30 μg) from RL95-2 and HEC1A cells, as indicated, were immunoblotted with hEMP2 antiserum (upper panel). The same blot was reprobed using a monoclonal anti-actin antibody (lower panel). (B) Immunoblot analysis of EMP2 expression in stably transfected HEC1A-GFP, HEC1A-hRZ1, and HEC1A-hRZ2 cells, as indicated (upper panel). The same blot was stained using Ponceau S (lower panel). (C) Immunoblot analysis of EMP2 expression in RL95-2 cells transfected with either pEGFP or pEGFP-hRZ1, as indicated (upper panel). The same blot was reprobed for actin (lower panel).

An example of the appearance of the uterine horns at this time is shown (Figs. 6A, B). There were striking differences in the number of implantation sites in the pEGFP-mRZ1-transfected uterine horns as compared to the pEGFP-transfected horns (Fig. 6C). No visible implantation sites were observed in uterine horns transfected with 20 μg pEGFP-mRZ1, whereas multiple implantation sites were present in the control horns transfected with the same amount of pEGFP. An intermediate number of implantation sites was seen when 5 or 10 μg pEGFP-mRZ1 was used as compared to the same quantity of pEGFP, suggesting a dose-dependent response.

There were no significant differences between the number of implantation sites in control nontransfected and pEGFP-transfected uterine horns, however, there was a trend toward a reduction in implantation sites at the higher doses of pEGFP (compare the no DNA control to 5, 10, or 20 μg GFP; Fig. 6C). It is possible that this reduction was caused by migration of the ribozyme into the control horn. Alternatively, it is possible that the higher doses of DNA could induce a nonspecific endometrial inflammatory response. We do not believe that this reduction in implantation sites alters the conclusions of this study because the control horns were transfected with the same amount of DNA and liposome as the experimental horns, and therefore the nonspecific effects should be similar in the two groups.

To ensure that the effect on implantation was due to the decrease in EMP2 protein and not a nonspecific effect of expressing a functional ribozyme, mouse uterine horns were transfected with 20 μg of the human EMP2-specific ribozyme (pEGFP-hRZ1) that cannot target mouse EMP2 (because the EMP2 recognition sequences in the human ribozyme differ from those in the mouse ribozyme). There was no significant difference between the number of implantation sites in the pEGFP-hRZ1-transfected and uterine horns transfected with a pEGFP control plasmid (mean ± SEM of 4.1 ± 1.9 and 6.6 ± 1.8, respectively, $N = 8$, $P = 0.25$).

Although these experiments strongly suggested that EMP2 was required for implantation, we wanted to corroborate this finding using an alternate method of decreasing EMP2 expression in vivo. Plasmid constructs containing short hairpin RNA sequences driven by the human U6 RNA polymerase III promoter were generated to target either mouse or human EMP2. The mouse shRNA plasmid (m-shRNA) was tested for efficacy in vitro using NIH 3T3 cells overexpressing mouse EMP2. This construct was found to reduce EMP2 expression 48 h after transfection more than two-fold when compared to cells transfected with either pCRII alone or h-shRNA, even though the constructs were only transiently expressed (Fig. 7). This finding verified that the m-shRNA sequence chosen was effective and that the control h-shRNA sequence had no effect on mouse EMP2 expression in these cells.

To determine if the shRNA constructs would similarly affect expression of EMP2 in vivo, uterine horns of hyperstimulated and mated mice were transfected on day 1 pc with either m-shRNA or h-shRNA (control). Again, the opposite uterine horn in the same mouse was used for the control plasmid to eliminate confounding effects of using different mice for different constructs. In response to transfection with 10 μg m-shRNA, there was a significant decrease in EMP2 expression in the luminal epithelium by day 5 pc when compared to horns transfected with 10 μg h-shRNA (Fig. 8). These data confirmed that the mouse EMP2-specific shRNA construct effectively decreased expression of EMP2 in vivo. On day 8 pc, the mean number of implantation sites in the m-shRNA horns was

Table 1
Blastocyst attachment to HEC1A cells expressing different levels of EMP2

	Attached	Not attached
No DNA	39/78 (50%)	39/78 (50%)
GFP	31/74 (42%)	43/74 (58%)
hRZ1 [a]	3/68 (4%)	65/68 (96%)
hRZ2 [b]	18/71 (25%)	53/71 (75%)

[a] Significant differences between the hRZ1 and GFP cells (Chi-square, $P < 0.0001$).

[b] Significant differences between the hRZ2 and GFP cells (Chi-square, $P < 0.05$).

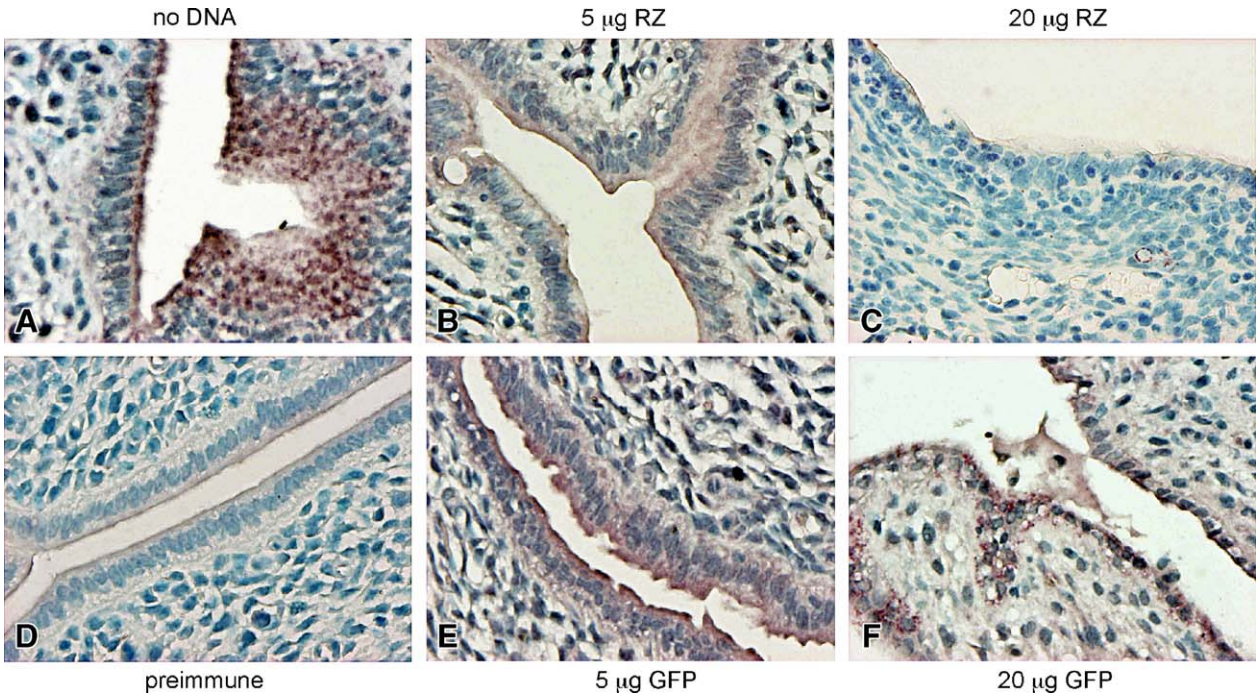

Fig. 5. Effect of ribozyme transfection on EMP2 expression in mouse endometrium. Uterine horns were transfected on day 1 pc with PBS/FuGENE alone (A, D) or the indicated amounts of pEGFP-mRZ1 (B, C, RZ) or pEGFP (E, F, GFP). The uterine horns were removed on day 5 pc and processed for immunohistochemistry using mEMP2 antiserum (A–C, E, F) or the corresponding preimmune serum (D). Magnification: 400×.

significantly lower than that in the control horns ($P < 0.01$; Wilcoxon matched pairs test) (Fig. 9A).

To ensure that the results we obtained using the hyperstimulated mouse model accurately reflected the situation in mice cycling normally, we repeated the in vivo transfection experiments on non-hyperstimulated mice. Females were housed singly with males until the morning a vaginal plug was observed. On the afternoon of day 1 pc, in vivo transfection of the uterine horns was performed using 10 μg or 20 μg m-shRNA. As before, the contralateral horn in each female was injected with the same amount (10 μg or 20 μg) of control h-shRNA plasmid. As expected, the mean number of implantation sites in the control horns of normally cycling mice was lower than that seen in hyperstimulated mice. When only 10 μg shRNA was transfected, we did not observe a significant difference in the number of implantation sites between the uterine horns. However, similar to our results with hyperstimulated mice, normally cycling mice had an obvious reduction in implantation sites in the horns treated with 20 μg m-shRNA targeting EMP2 as compared to h-shRNA (Fig. 9B). Together with the results obtained using the ribozyme constructs, these data using the shRNA constructs suggest an absolute requirement for EMP2 expression for successful implantation in the mouse.

Discussion

Implantation is the end result of a tightly orchestrated series of interactions between a receptive uterus and a mature blastocyst (Norwitz et al., 2001). Although some endometrial molecules involved in controlling these interactions have been identified (Lessey, 2002; Paria et al., 2002), a unifying mechanism remains evasive. Here, we demonstrate that EMP2 is expressed at the endometrial surface and in decidualized stromal cells in the peri-implantation period and that its expression is likely required for mouse implantation. Combined with studies of EMP2 and related proteins in other cell types, these findings lead us to hypothesize that EMP2 affects expression of endometrial cell surface proteins that are required for successful embryo attachment and early steps of invasion. This idea is supported by our data demonstrating that down-regulation of EMP2 expression in endometrial cells inhibits their ability to support mouse blastocyst attachment in vitro and implantation in vivo.

In addition to its endometrial expression pattern, EMP2 is also expressed in the embryonic trophectoderm on day 6 pc (Fig. 2). These findings are consistent with our studies of preimplantation embryos in which EMP2 is most highly expressed in the trophectoderm of the expanded and hatched blastocyst stages (Wadehra, Braun, and Williams, unpublished results). The function of EMP2 in the embryo itself is the subject of another study. We would like to point out, however, that in the studies reported here the attaching/implanting embryos were not exposed to the ribozyme or shRNA plasmids because the in vivo transfections were performed on day 1 pc when the embryos were located in the oviducts; embryos do not enter the uterine horns until between day 3 and 4 pc. Because we observed a clear decrease in EMP2 protein levels in the endometrium in response to the active ribozyme or shRNA treatments by day 4 pc, we interpret our results to be an effect of the plasmid constructs on endometrial and not embryo EMP2 expression.

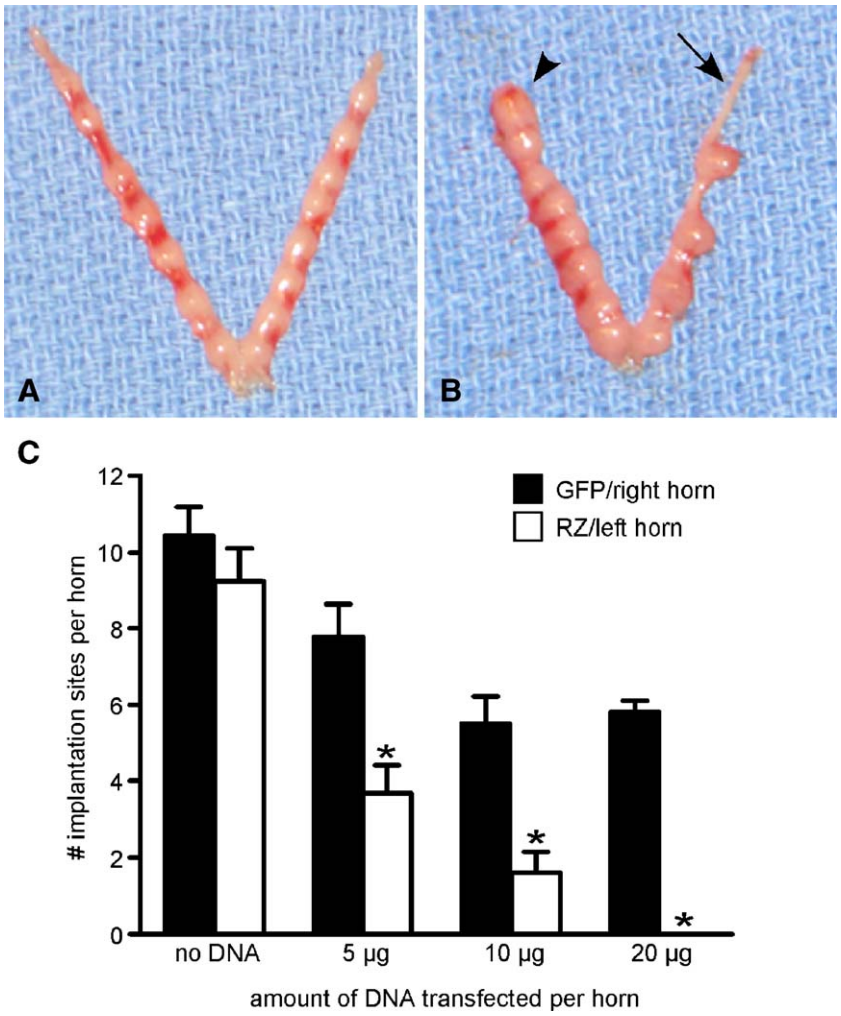

Fig. 6. Effect of ribozyme-mediated inhibition of EMP2 expression on implantation in vivo. (A) Appearance of nontransfected uterine horns on day 8 pc. (B) Appearance of uterine horns on day 8 pc after transfection on day 1 pc with control (arrowhead) or active (arrow) construct. (C) Uterine horns were transfected with pEGFP (GFP), pEGFP-mRZ1 (RZ), or not transfected (no DNA), and implantation sites per horn were counted on day 8 pc. The graph shows the mean number of implantation sites ± SE in uterine horns transfected with the indicated amount of DNA. Implantation sites in at least 10 uterine horns were counted for each group. Significant differences in the numbers of implantation sites between GFP- and RZ-transfected horns were observed at all doses (*$P < 0.01$). GFP-transfected or control nontransfected right horn, black bars; RZ-transfected or control nontransfected left horn, white bars.

A role for EMP2 as a trafficking molecule in the endometrium could explain its importance in implantation. In several other cell types, changes in EMP2 expression and localization cause pleiotropic changes in the proteins expressed on the plasma membrane (Wadehra et al., 2002, 2003a). Of note, EMP2 regulates the expression of integrins,

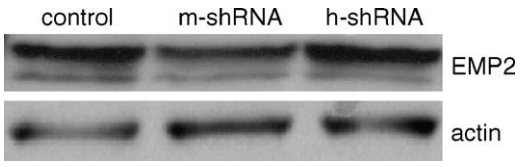

Fig. 7. Effect of shRNA transfection on EMP2 expression. NIH 3T3 cells stably overexpressing mouse EMP2 were transfected with h-shRNA or m-shRNA, as indicated. After 48 h, the cells were collected and protein extracts immunoblotted using hEMP2 antiserum. The blots were stripped and reprobed with actin to document even protein loading (lower panel).

membrane proteins whose cyclic regulation appears to be critical for implantation (Lessey et al., 2000). In NIH 3T3 cells, increasing the expression of EMP2 upregulates the surface expression of integrin $\alpha6\beta1$ while downregulating $\alpha5\beta1$, resulting in an increase in the ability of these cells to adhere to laminin (Wadehra et al., 2002). In the uterus, the decidualized stroma responds to the implanting embryo by upregulating the expression of laminin and integrins $\alpha1\beta1$, $\alpha4\beta1$, and $\alpha v\beta3$ (Sutherland et al., 1993; Thomas et al., 2002). Based on our observation that EMP2 expression in the endometrial stroma was upregulated at the time of implantation (Figs. 1 and 2), it is possible that these alterations in stromal integrin expression are mediated by EMP2. In fact, we demonstrated that, in human endometrial cells, expression of integrin $\alpha v\beta3$ is directly correlated with the level of EMP2 expression (Wadehra et al., 2005). Furthermore, knockdown of EMP2 using in vivo transfection of ribozyme or shRNA constructs resulted in a significant

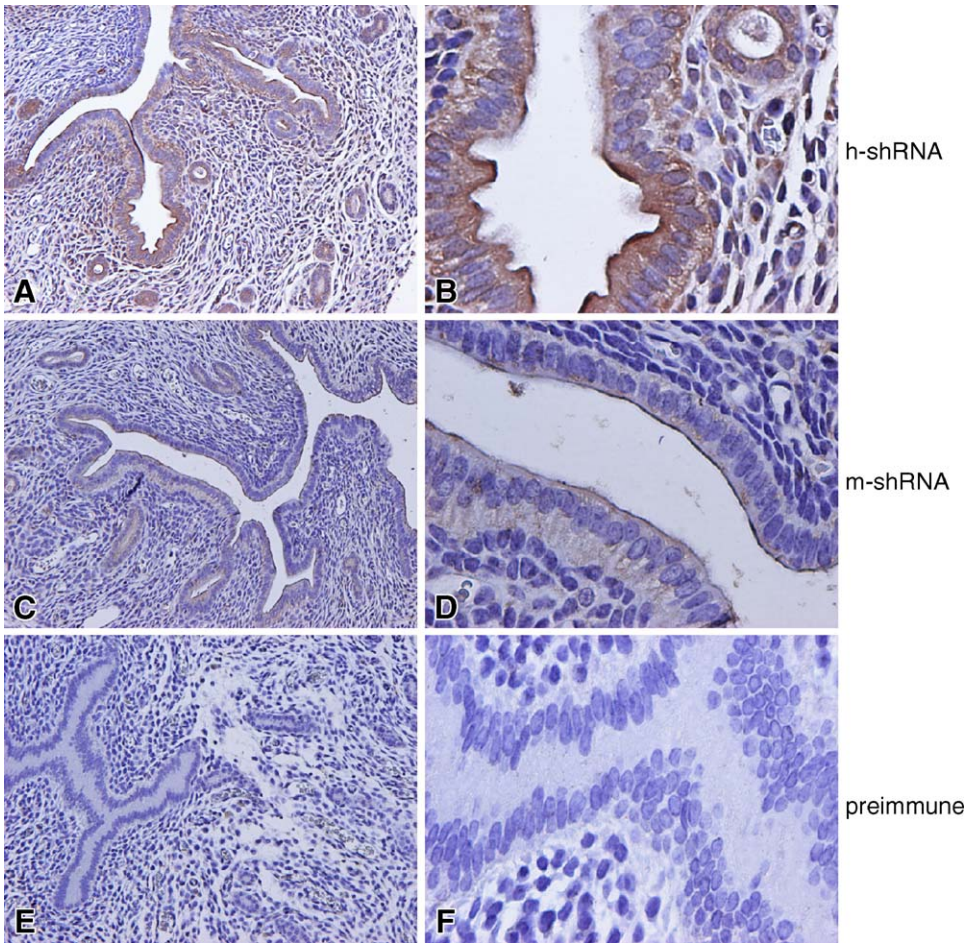

Fig. 8. Effect of shRNA transfection on EMP2 expression in mouse endometrium. Uterine horns were transfected on day 1 pc with h-shRNA (A, B) or m-shRNA (C–F). The uterine horns were removed on day 5 pc and processed for immunohistochemistry using mEMP2 antiserum (A–D) or the corresponding preimmune antiserum (E, F). Magnification: 100× (A, C, E); 400× (B, D, F).

decrease in integrin β3 expression in the mouse endometrium (Wadehra et al., 2005). Together with the data reported here, these results suggest that EMP2 expression is required for the appropriate expression of integrin αvβ3 (and potentially other endometrial molecules) during the implantation window in vivo.

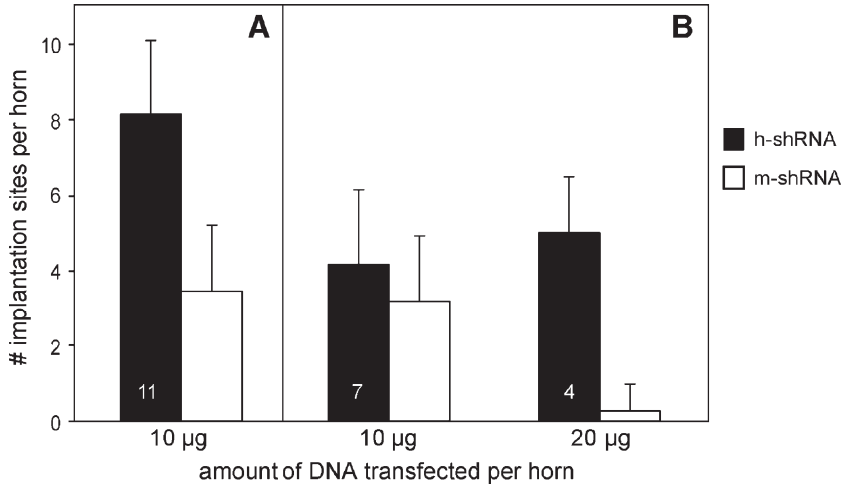

Fig. 9. Effect of shRNA transfection on implantation. Uterine horns were transfected day 1 pc with the indicated amount of either h-shRNA or m-shRNA. Implantation sites per horn were counted on day 8 pc. The graphs show the mean number of implantation sites ± SE in uterine horns transfected with the indicated construct. The number of pairs of uterine horns counted in each group is indicated at the base of the black bars. (A) Hyperstimulated females. (B) Normally cycling females.

Although EMP2 may affect implantation indirectly by its effects on expression of other proteins, it also could be involved in the morphological reorganization of the endometrial cell surface that includes membrane flattening and formation of uterodomes, also known as the "plasma membrane transformation" (Murphy, 2004). In HEK 293 cells, overexpression of EMP2 (or related GAS3/PMP22 family proteins) causes "blebbing", a process characterized by the protrusion from the cell surface of large membrane bound vesicles (Hagmann et al., 1999; Wilson et al., 2002). In nontransfected HEK 293 cells, blebbing occurs in response to activation of the $P2X_7$ purinergic receptor, a protein reported to physically interact with EMP2 and to depend on EMP2 for its actions (Wilson et al., 2002). The connection of EMP2 with a signaling pathway that results in major morphological changes of the cell membrane in cultured cells suggests that EMP2 could be involved in a similar signaling pathway in the endometrium that results in transformation of the epithelial plasma membrane structure.

One other tetraspan protein, CD9, which is distantly related to EMP2, is involved in mammalian reproduction. $CD9^{-/-}$ mice display a defect in sperm–egg binding and fusion at fertilization (Le Naour et al., 2000; Miyado et al., 2000). Importantly, these mice also display a defect in sperm transport into the oviduct, suggesting that sperm adhesion to the endometrial and/or oviductal epithelium is impaired. CD9 is expressed in human and bovine endometrium and interacts with specific integrin subunits in these tissues based on co-localization and immunoprecipitation assays (Park et al., 2000; Xiang and MacLaren, 2002). In contrast to EMP2, CD9 expression does not vary with the human menstrual cycle, so it is unlikely that CD9 specifically regulates the expression of endometrial surface proteins important for implantation (Park et al., 2000). However, together with the results presented above, these findings support a role for tetraspan proteins including EMP2 and CD9 in regulating the adhesive properties of the female reproductive tract epithelium.

The inability to synchronize processes involved in embryo–endometrium interactions results in a failure of implantation and may account for up to 75% of pregnancies lost (Norwitz et al., 2001). It is interesting to speculate that disruption of protein trafficking or epithelial remodeling due to inappropriate EMP2 expression in the endometrium may explain some of these losses. In any case, understanding the cell biological processes associated with EMP2 and other tetraspan proteins in the female reproductive tract may provide the scientific underpinnings for the generation of novel therapeutic modalities designed to alleviate infertility, enhance fetal health, and improve contraceptive methods.

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