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Adherens Junction Proteins in the Hamster Uterus: Their Contributions to the Success of Implantation¹

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ABSTRACT

The adherens junction (AJ) is important for maintaining uterine structural integrity, composition of the luminal environment, and initiation of implantation by virtue of its properties of cell-cell recognition, adhesion, and establishment of cell polarity and permeability barriers. In this study, we investigated the uterine changes of AJ components E-cadherin, beta-catenin, and alpha-catenin at their mRNA and protein levels, together with the cellular distribution of meprinbeta, phospho-beta-catenin, and active beta-catenin proteins, in hamsters that show only ovarian progesterone-dependent uterine receptivity and implantation. By *in situ* hybridization and immunofluorescence, we have demonstrated that uterine epithelial cells expressed three of these AJ proteins and their mRNAs prior to and during the initial phase of implantation. Immunofluorescence study showed no change in epithelial expression patterns of uterine AJ proteins from Days 1 to 5 of pregnancy. With advancement of the implantation process, AJ components were primarily expressed in cells of the secondary decidual zone (SDZ), but not in the primary decidual zone (PDZ). In contrast, we noted strong expression of beta-catenin and alpha-catenin proteins in the PDZ, but not in the SDZ, of mice. Taken together, these results suggest that AJ proteins contribute to uterine barrier functions by cell-cell adhesion to ensure protection of the embryo. In addition, cleavage of E-cadherin by meprinbeta might contribute to weakening uterine epithelial cell-cell contact for blastocyst implantation. We also report that the nuclear localization of active beta-catenin from Day 4 onward in hamsters implies that beta-catenin/Wnt-signal transduction is activated in the uterus during implantation and decidualization.

adherens junction proteins, catenins, decidua, decidualization, E-cadherin, hamster, implantation, mouse, uterus, Wnt

INTRODUCTION

Following mating, the cyclic uterus of hamsters and mice enters into the state of pregnancy on the second day, and that is marked by proliferation of the uterine epithelial cells [1]. Further uterine epithelial cell proliferation does not occur from

Day 3 of pregnancy onward [1, 2]. However, these cells undergo other changes such that they acquire competency for attachment of the blastocyst [3]. It is unclear how embryonic development and growth inside the uterine lumen and its subsequent implantation on the luminal epithelial surface for continuation of pregnancy are influenced by the changes that occur in the uterine luminal epithelium. It is possible that the simple columnar epithelial cells of the uterus maintain the luminal environment by their selective permeability and intercellular transport properties. The adherens junction (AJ) is a hallmark of epithelial cells [4]. Adherens junctions are positioned in the lateral surface of the apical region of epithelial cells, and they maintain the polarity and structural integrity of the epithelial cell layer by bringing two plasma membranes of neighboring epithelial cells together. In addition, AJs play an important role in a variety of biological and pathological processes, from morphogenesis to tumor progression [4]. The AJ consists of a transmembrane component with adhesive function, and cytoplasmic adaptor proteins that bind to the actin cytoskeleton. E-cadherin is the most prominent transmembrane Ca²⁺-dependent adhesion molecule in epithelial cells [5]. β -Catenin binds directly to E-cadherin and links it to α -catenin, which in turn binds to the actin cytoskeleton [4].

Embryo implantation consists of a series of sequential events beginning with trophoblast attachment and penetration, and uterine stromal cell proliferation and differentiation into decidual cells. Adhesion and attachment between the uterine epithelium and embryonic trophoblast are thought to be mediated by cell adhesion molecules. Among the various adhesion molecules, E-cadherin is the best characterized, and it is expressed in both the uterine luminal epithelium and the trophoblast [6–8]. Although apical expression of E-cadherin in the luminal epithelium may be required for attachment of the embryonic trophoblast to the luminal epithelium, alteration of intercellular E-cadherin between adjacent luminal epithelial cells is required for trophoblast invasion [9]. Loss or alteration of E-cadherin has been demonstrated in adenocarcinomas of many tissues, and cleavage of E-cadherin has been described as one of the cellular mechanisms for tumor penetration [10]. In this regard, it has been demonstrated that metalloproteases MMP3, MMP7, ADAM10, and meprin β cleave E-cadherin and impair cell-cell adhesion [11, 12]. Meprins are multimeric zinc metalloproteases composed of alpha (α) and beta (β) subunits. Although the two meprin subunits share $\approx 42\%$ identity in their amino acid sequences, they differ significantly in their cellular location and substrate specificity. Dimers containing meprin β are membrane-bound proteases [13]. One recent study showed that the meprin β weakens intercellular adhesion by processing E-cadherin [10]. Thus, cleavage of E-cadherin by meprin β may be a means to reduce E-cadherin-mediated cell adhesion in the luminal epithelium during implantation for trophoblast invasion.

It has been suggested that the embryo-induced deciduum around the implantation site is an active barrier that performs a

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number of important functions [8, 14–16]: 1) a protective role in controlling trophoblast cell invasion, 2) a role in preventing immunological rejection of the embryo, and 3) a nutritive role for the developing embryo. The deciduum has two distinct zones: a densely packed stromal zone immediately surrounding the embryo termed the *primary decidual zone* (PDZ), and a broad decidual zone encircling the PDZ termed the *secondary decidual zone* (SDZ) [17]. We previously demonstrated in mice that the PDZ cells express AJ and tight junction components for the formation of temporary AJ and tight junction barriers for the safeguard of the embryo by blocking the passage of microorganisms, immune cells and large molecules like antibodies and drugs from the mother [8, 15]. However, implantation strategies and regulatory processes are not similar across species. In contrast to mice, where both ovarian progesterone and estrogen secretions are needed for implantation, implantation in hamsters takes place only in the presence of ovarian progesterone, similar to rabbits, rhesus monkeys, guinea pigs, and possibly humans (reviewed in Reese et al. [3]). Thus, as part of our continuing effort to understand the contribution of junctional proteins to the process of implantation, we performed a comprehensive study to understand the relative importance of AJ proteins, E-cadherin, and catenins, in the uterus during the first 6 days of pregnancy in hamsters that have progesterone-dependent implantation. The cell-type-specific expression profile of uterine AJ proteins and their association suggest that noteworthy differences may exist between the hamster and mouse in molecular mechanisms controlling the embryo implantation process.

MATERIALS AND METHODS

Materials

Mouse monoclonal anti-E-cadherin antibody (catalog no. 610182) was purchased from BD Biosciences (San Jose, CA). Rabbit polyclonal anti- α -catenin (catalog no. 71-1200) and anti-total β -catenin (catalog no. 06-734) antibodies were from Zymed Laboratories Inc. (San Francisco, CA) and Millipore (Billerica, MA), respectively. Mouse monoclonal antibodies against active β -catenin (catalog no. 05-665, clone 8E7) dephosphorylated at serine 37 or threonine 41 residues, and rabbit polyclonal antibodies against phospho- β -catenin (catalog no. 9561) phosphorylated specifically at serine 33, serine 37, and threonine 41 residues in exon 3 were obtained from Millipore (Temecula, CA) and Cell Signaling Technologies (Danvers, MA), respectively. Goat anti-mouse meprin β subunit antibody (catalog no. AF3300) was purchased from R&D Systems Inc. (Minneapolis, MN). Goat anti-rabbit (catalog no. 62-6111 [fluorescein isothiocyanate {FITC} conjugated]; catalog no. 81-6114 [TRITC conjugated]) and goat anti-mouse (catalog no. 81-6511 [FITC conjugated]) were obtained from Zymed Laboratories. Normal mouse (sc-2025), rabbit (sc-2027), and goat (sc-2028) immunoglobulin Gs (IgGs) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Animals

Adult virgin male and female golden hamsters (*Mesocricetus auratus*) and CD1 mice were purchased from Charles River Laboratory (Wilmington, MA). They were housed on a 12L:12D cycle and fed commercial chow ad libitum. All animal maintenance, handling, and procedures were performed in accordance with National Institutes of Health standards for the care and use of experimental animals and were approved by the Vanderbilt University Medical Center Institutional Animal Care and Use Committee. Single female hamsters that showed at least three regular estrous cycles were caged with a fertile male for mating. Microscopic finding of spermatozoa in the vaginal smear on the next morning was designated as Day 1 of pregnancy [1]. Female mice were mated with fertile males to induce pregnancy (Day 1 of pregnancy = vaginal plug) [18]. Hamsters on Days 1–6 of pregnancy were euthanized at 0830–0900 h. Entire uterine horns were removed on Days 1 to 4. On Day 4 of pregnancy, the whole uteri were collected in the morning (0900 h) without flushing. On Day 5, implantation sites of hamsters and mice were collected after an intravenous injection of Chicago Blue B dye solution (1% dye solution in saline). On Day 6, implantation sites are distinct and blue dye injection is not

required. Uterine tissues were cut into small pieces, flash frozen, and stored at -80°C until processed for cryosectioning and protein extraction [19–21].

Cloning of the Hamster-Specific Partial cDNAs for E-Cadherin (*Cdh1*), β -Catenin (*Ctnnb1*), and α -Catenin (*Ctnna1*)

RT-PCR was employed to generate the hamster-specific cDNA clones for *Cdh1*, *Ctnnb1*, and *Ctnna1*. The published sequences used to design the primers were of mouse origin. The primers were: *Ctnnb1* (GenBank accession no. BC048153.1, spanning nucleotides 965-1548, size 584 bp, 5'-ACTGTTCTACGCCATCACGAC-3' [sense] and 5'-CCTCTATGCCACCCACTTG-3' [antisense]), *Ctnna1* (GenBank accession no. NM_009818.1, spanning nucleotides, 2295–2567, size 273 bp, 5'-GTGATCAGTGCTGCCAAGAA-3' [sense] and 5'-TGTCTGCACAACAGCATTC-3' [antisense]), and *Cdh1* (GenBank accession no. NM_009864.2, spanning nucleotides 1809–2202, size 394 bp, 5'-GCACATATGTAGCTCTCATCA-3' [sense] and 5'-CCTTCACAGTCACACACATG-3' [antisense]). Uterine total RNA (1 μg) from a Day 4 pregnant hamster was reverse transcribed, and RT products were amplified by PCR for 35 cycles using the following cycle parameters: 94°C , 45 sec; 55°C , 30 sec; and 72°C , 1 min 30 sec. The RT-PCR products were cloned into pCR-II-TOPO cloning vector (3.9 kb) using a TOPO TA Cloning kit, version K2 (Invitrogen Corp., Carlsbad, CA), and nucleotide sequences of the clone were determined to verify the identity and orientation of the clones. The GenBank accession numbers for the resulting hamster *Cdh1*, *Ctnnb1*, and *Ctnna1* cDNA fragments are EU856102, EU856101, and EU856103, respectively. Nucleotide sequences of these partial cDNA clones showed more than 90% sequence similarities with that of the GenBank nucleotide database for mice, rats, and humans.

RNA Probe Preparation

Plasmids bearing hamster and mouse cDNAs were exacted, purified, and linearized (hamster *Cdh1*: *HindIII*/T7 for antisense, *NotI*/SP6 for sense; hamster *Ctnna1*: *KpnI*/T7 for antisense, *XhoI*/SP6 for sense; and hamster *Ctnnb1*: *XhoI*/SP6 for antisense, *BamHI*/T7 for sense) to generate antisense and sense riboprobes, which were transcribed using appropriate RNA polymerases and labeled with ^{35}S for in situ hybridizations. All labeled sense and antisense cRNA probes used for hybridizations had specific activities of approximately 2×10^9 dpm/ μg [20, 21].

In Situ Hybridization

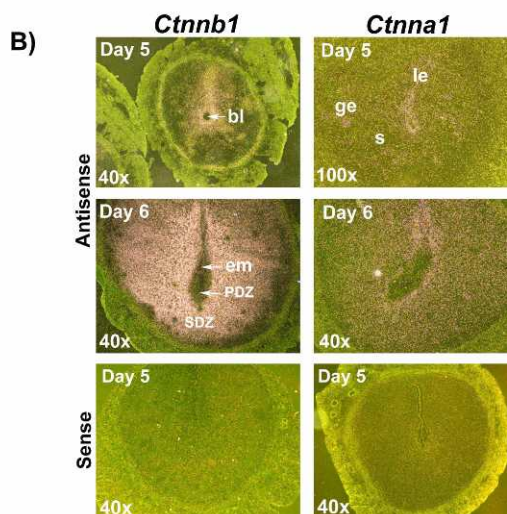
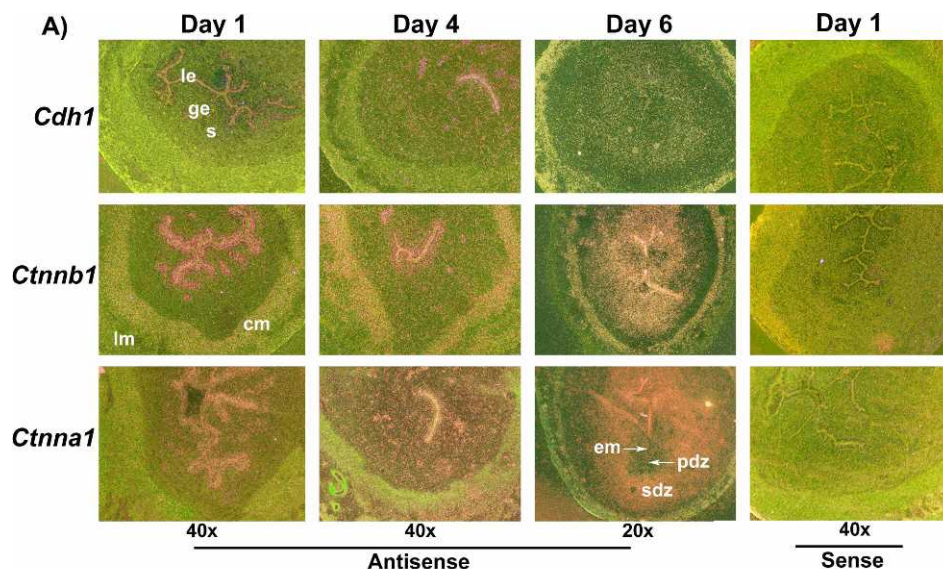
The protocol was followed as previously described by our group [20, 21]. Briefly, frozen uterine sections were mounted onto poly-L-lysine-coated slides and fixed in cold 4% paraformaldehyde solution in PBS for 15 min on ice. After prehybridization, sections were hybridized to ^{35}S -labeled antisense probes at 45°C for 4 h in 50% formamide hybridization buffer. Sections were also hybridized with ^{35}S -labeled sense probes as negative control. After hybridization and washing, sections were incubated with RNase A at 37°C for 20 min. RNase A-resistant hybrids were detected by autoradiography using Kodak NTB-2 liquid emulsion (Eastman Kodak Co., Rochester, NY). The slides were then stained with hematoxylin and eosin.

Indirect Immunofluorescence

Immunofluorescence was performed as described previously [19, 22]. In brief, cryosections (12 μM) were mounted onto poly-L-lysine-coated glass slides, fixed in cold 4% paraformaldehyde for 10 min, washed in PBST (0.01% Tween 20 in PBS), blocked for 10 min with serum (10%) from which secondary antibodies were generated and incubated overnight with appropriate primary antibodies at appropriate dilutions at 4°C . Slides were next washed and incubated with FITC- or TRITC-conjugated second antibodies for 1 h at room temperature. At the end of the reaction, washed sections were counterstained with 0.01% 4'-diamidino-2-phenylindole dihydrochloride (DAPI) and mounted using Fluoromount-G (catalog no. 0100-01; SouthernBiotech, Birmingham, AL). To verify the binding specificities of primary antibodies, negative control studies were performed in which equal amounts of nonspecific mouse, rabbit, or goat immunoglobulins were used instead of primary antibodies. The fluorescence was visualized by fluorescence microscopy using a Nikon Eclipse 80i microscope (Tokyo, Japan) equipped with an X-cite series 120 PC fluorescence illumination system [7].

To confirm the colocalization of AJ proteins E-cadherin and β -catenin on Days 4, 5, and 6 of pregnancy, double immunofluorescence staining was performed with a similar procedure as that described above. Here, two primary antibodies were added from two different species. Images were captured using a Nikon Eclipse TE2000-E [7].

FIG. 1. In situ hybridization of *Cdh1*, *Ctnnb1*, and *Ctnna1* mRNAs in early pregnant hamster and mouse uteri. **A)** Cross sections of hamster uteri from Days 1, 4, and 6 demonstrated cell-specific localization of *Cdh1*, *Ctnnb1*, and *Ctnna1* mRNAs. **B)** Cross sections of implant sites from mouse Days 5 and 6 demonstrated cell-specific localization of *Ctnnb1* and *Ctnna1* mRNAs. Dark field photomicrographs were representative of a single experiment using uterine sections from two to three animals in the same slide. No positive signals were observed when sections were hybridized with the sense probes. bl, blastocyst; cm, circular muscle; em, embryo; ge, glandular epithelium; le, luminal epithelium; lm, longitudinal muscle; PDZ, primary decidual zone; s, stroma; SDZ, secondary decidual zone.



RESULTS

Cdh1, *Ctnnb1*, and *Ctnna1* mRNAs Are Expressed in a Temporal and Cell-Specific Manner in the Peri-implantation Uterus of the Hamster and Mouse

We performed in situ hybridization studies to examine the expression pattern of *Cdh1*, *Ctnnb1*, and *Ctnna1* mRNAs in hamster uterine sections from Days 1, 4, and 6 of pregnancy. Expression of *Cdh1* and *Ctnna1* was mainly observed in the luminal and glandular epithelia on Days 1 and 4 of pregnancy (Fig. 1A). Uterine stromal cells on these days show some expression of *Ctnna1* above background levels. In Day 6 implantation sites, these mRNAs were primarily expressed in the luminal epithelial and SDZ cells, with little or no expression in the PDZ cells. *Ctnnb1* mRNA expression was distinct in the luminal and glandular epithelia and circular muscle layer on Days 1 and 4 of pregnancy. It was also expressed in subepithelial stromal cells on Day 4. On Day 6, *Ctnnb1* expression was concentrated in the luminal epithelial cells, decidual cells of both the PDZ and SDZ, and the circular muscle layer. There is little or no expression in peripheral undifferentiated stromal cells above the circular muscle layer. It appears that expression patterns of *Cdh1*, *Ctnnb1*, and *Ctnna1*

mRNAs and their products were more or less similar, suggesting their efficient cell-specific translation in the hamster uterus (Fig. 1A). Sections from Day 1 uterus showed no specific signals when hybridized with sense probes.

Cell-specific *Cdh1* mRNA localization in implantation sites on Days 5 and 6 of pregnancy in mice was previously reported. Although the luminal epithelial cell showed distinct accumulation of *Cdh1* mRNA, stromal cells surrounding the implanting blastocyst showed low levels of this mRNA [8]. Thus, we report here only the expression patterns of *Ctnnb1* and *Ctnna1* mRNAs on these days. We observed *Ctnnb1* mRNA expression in stromal cells immediately surrounding the implanting blastocyst on Day 5 in addition to its expression in luminal and glandular epithelia (Fig. 1B). On Day 6, *Ctnnb1* expression was mainly localized in the SDZ cells, whereas there was little or no expression in the PDZ cells. The expression of *Ctnna1* was found in the luminal epithelium on both Days 5 and 6. Uterine stromal cells of Day 5 implantation sites showed *Ctnna1* expression at lower levels than *Ctnnb1*, but not restricted to any specific area. *Ctnna1* expression pattern was similar to *Ctnnb1* at the implantation sites of Day 6; *Ctnna1* was primarily expressed in the SDZ cells, with little or no expression in the PDZ cells. Sections from Day 5 implantation sites showed no specific signals when hybridized with sense probes.

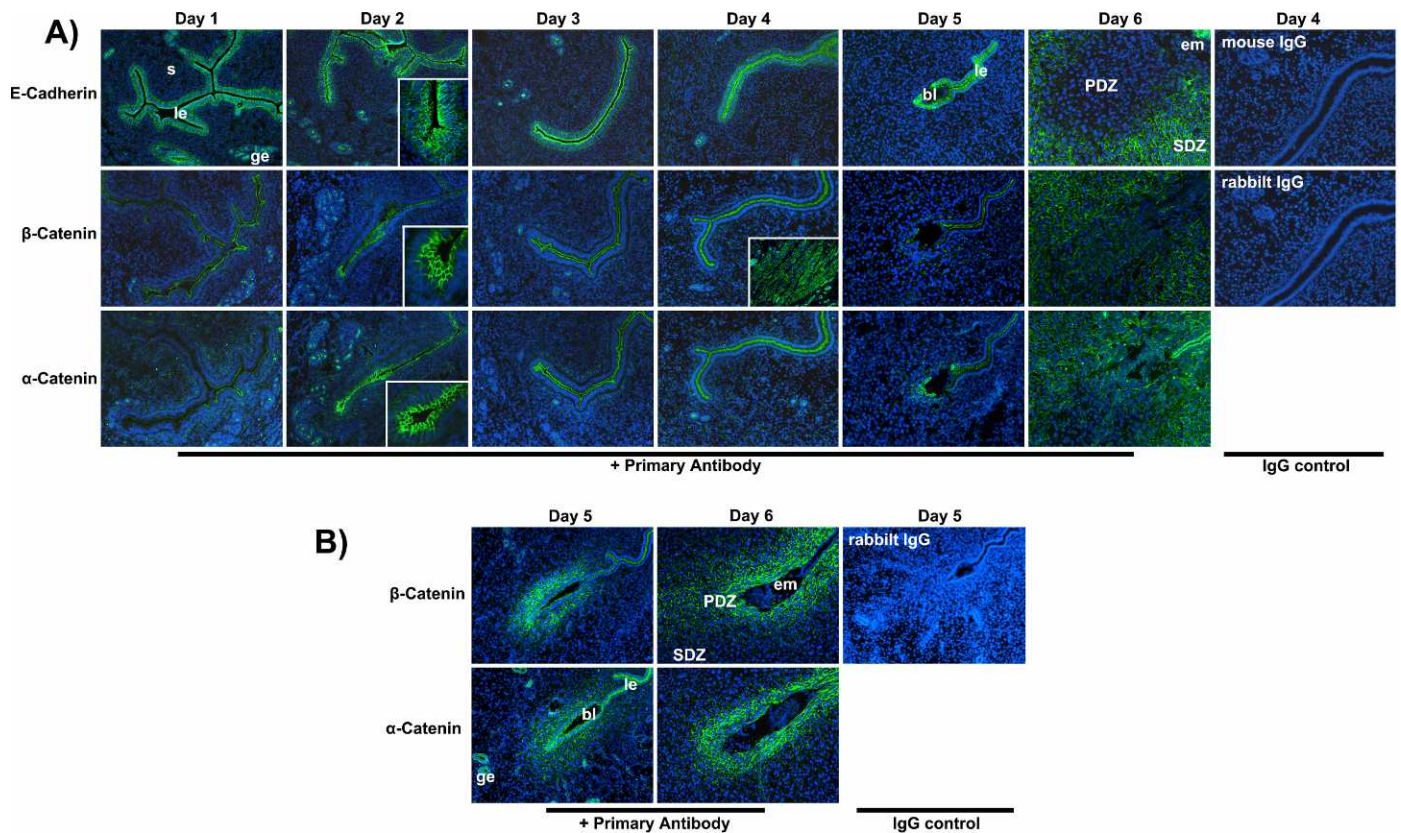


FIG. 2. Immunolocalization (FITC; green) of E-cadherin, total β -catenin, and α -catenin in early pregnant hamster and mouse uteri. **A)** Uterine cross sections from Days 1 to 6 of pregnancy in hamsters showed cell-specific localization of E-cadherin, total β -catenin, and α -catenin proteins. **B)** Uterine cross sections from mouse Days 5 and 6 implant sites showed cell-specific localization of total β -catenin and α -catenin proteins. The nuclei were stained with DAPI (blue). The photomicrographs (original magnification $\times 100$) shown are representative of at least five to seven independent experiments. No specific immune staining was observed in negative control sections. To show the specificity of total β -catenin and α -catenin immunolocalization, negative control pictures from Day 4 uterus in Figure 2A and Day 5 implant site in Figure 2B were presented, because both antibodies were raised in the same species (rabbit). Insets (original magnification $\times 400$) showed immunolocalization of proteins in the epithelium (Day 2) or myometrium (Day 4). bl, blastocyst; em, embryo; ge, glandular epithelium; le, luminal epithelium; PDZ, primary decidual zone; s, stroma; SDZ, secondary decidual zone.

E-Cadherin, Total β -Catenin, and α -Catenin Proteins Are Expressed in a Temporal and Cell-Specific Manner in the Peri-implantation Uterus of the Hamster and Mouse

E-cadherin, total β -catenin, and α -catenin localizations were detected at cell-cell contacts as well as free apical borders of the luminal and glandular epithelium from Days 1 to 4 (Fig. 2A) of pregnancy. Subepithelial stromal cells showed α -catenin expression. Although there was little or no E-cadherin and α -catenin immunostaining in the myometrium, total β -catenin staining was distinct in the circular muscle layer (inset in the Day 4 uterine section). We also observed E-cadherin, and total β -catenin and α -catenin expression in the blood vessels of stromal and muscle compartments (data not shown). After initiation of implantation on Day 5, apical and lateral membranous localization of these proteins persisted in the luminal epithelium surrounding and away from the implanting blastocyst (Fig. 2A). Stromal cells surrounding the Day 5 implantation chamber showed low levels of total β -catenin and α -catenin, but not E-cadherin expression. On Day 6, membranous localization of E-cadherin, and total β -catenin and α -catenin was distinct in the SDZ cells surrounding the implantation site. However, low levels of total β -catenin and α -catenin, but not E-cadherin, expression were noticed in the PDZ (Fig. 2A). Slides containing uterine sections from Days 1 to 6 of pregnancy showed no specific immunolocalization when stained in the presence of mouse or rabbit IgGs omitting

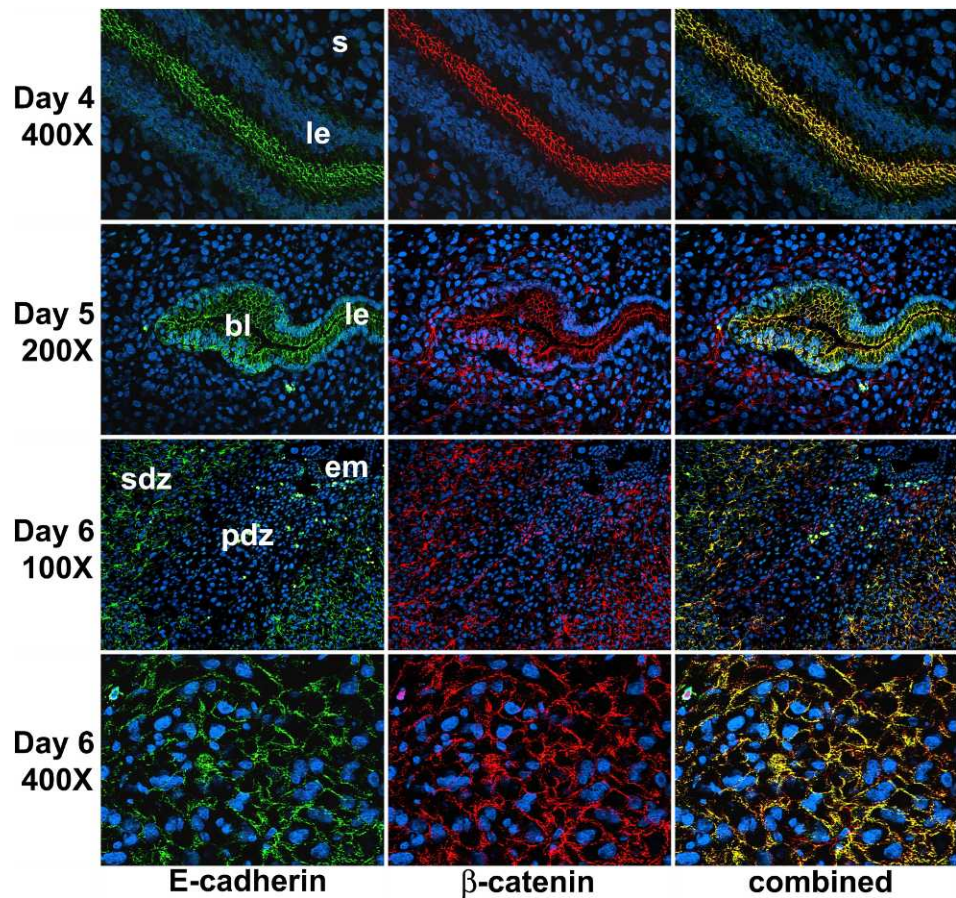
primary antibodies (representative pictures from Day 4 pregnant uterus are presented in Fig. 2).

To compare our observation of AJ protein expression at the implantation sites of hamsters with that of mice, we examined the immunofluorescence expression patterns of α -catenin and total β -catenin in Day 5 and Day 6 implantation sites in mice (Fig. 2B). E-cadherin protein localization on Day 5 and Day 6 implantation sites of mice was previously demonstrated. Abundant accumulation of this protein was reported in cells of the PDZ on Day 6 [8]. We observed intense membranous total β -catenin and α -catenin staining in stromal cells of the PDZ surrounding the implanting blastocyst on these days in addition to their expression in luminal epithelial cells. There were little or low levels of these two proteins in the SDZ cells. Negative control slides containing sections from Days 5 and 6 implantation sites showed no specific immunolocalization when stained in the presence of nonspecific rabbit IgG.

Colocalization of E-Cadherin and β -Catenin in Cells of the Uterus on Day 4 and Implantation Sites on Days 5 and 6 of Pregnancy in Hamsters

Double immunostaining for E-cadherin and total β -catenin was performed to examine the colocalization of these two proteins in uterine luminal epithelial cells of Days 4 and 5, and decidual cells of Days 5 and 6 (Fig. 3). The merged images of FITC-labeled E-cadherin and TRITC-labeled total β -catenin

FIG. 3. E-cadherin (FITC; green) and total β -catenin (TRITC; red) double labeling in the same uterine sections on Days 4 to 6 of pregnancy in hamsters. The nuclei were stained for DAPI (blue). The photomicrographs shown are representative of at least three independent experiments. bl, blastocyst; em, embryo; le, luminal epithelium; PDZ, primary decidual zone; s, stroma; SDZ, secondary decidual zone.



showed yellow lines, suggesting their colocalization in Days 4 and 5 uterine luminal epithelial cells, and in Day 6 decidual cells of the SDZ. In contrast, there was no colocalization of E-cadherin and β -catenin in the PDZ cells of Days 5 and 6 because E-cadherin is not expressed in these cells (Fig. 3).

Meprin β Expression in the Uterine Epithelium on Days 1 to 5 of Pregnancy in Hamsters

It has been shown that meprin β -expressing MDCK and caco-2 cells displayed reduced strength of cell-cell contacts due to proteolytic processing of endogenous E-cadherin [10]. Thus, we examined the expression pattern of meprin β in uterine

sections from Days 1 to 5 of pregnancy. Although immunohistochemical staining showed the presence of meprin β in the basal layer of luminal epithelial cells from Days 1 to 5 of pregnancy, we detected meprin β expression at lateral borders and apical domains of luminal epithelial cells on Day 4 and Day 5 implantation sites (Fig. 4). Uterine sections from Day 4 of pregnancy showed no specific immunolocalization when primary meprin β antibody was replaced with goat IgG. Meprin β colocalization with E-cadherin could not be performed because of the fact that addition of two primary antibodies together gave no specific immunolocalization of either of these two proteins. This finding suggests that meprin β gains access to the substrate repertoire present in the lateral and

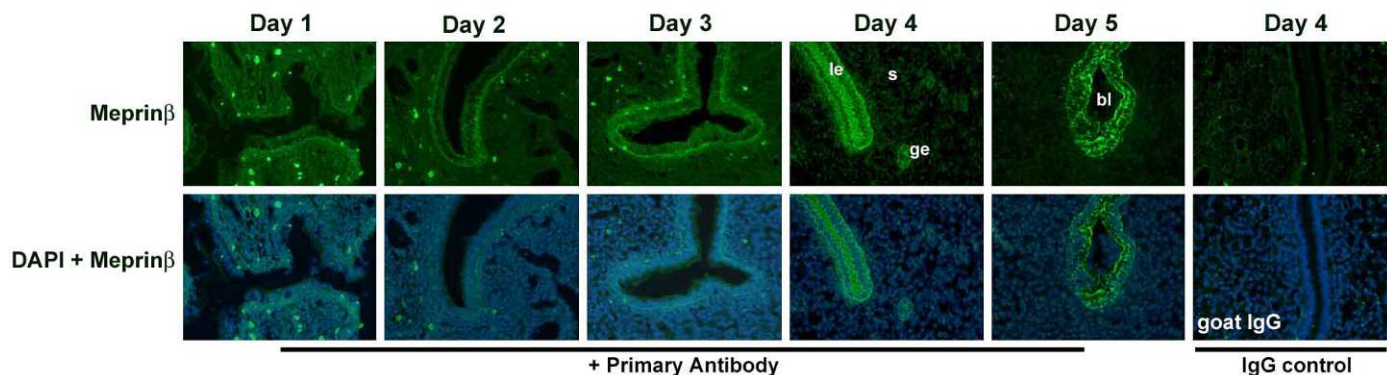


FIG. 4. Immunolocalization (FITC; green) of meprin β in cross sections of pregnant hamster uteri from Days 1 to 5. The nuclei were stained for DAPI (blue). The photomicrographs (original magnification $\times 200$) shown are representative of at least three to four independent experiments with sections from three animals. bl, blastocyst; ge, glandular epithelium; le, luminal epithelium; s, stroma.

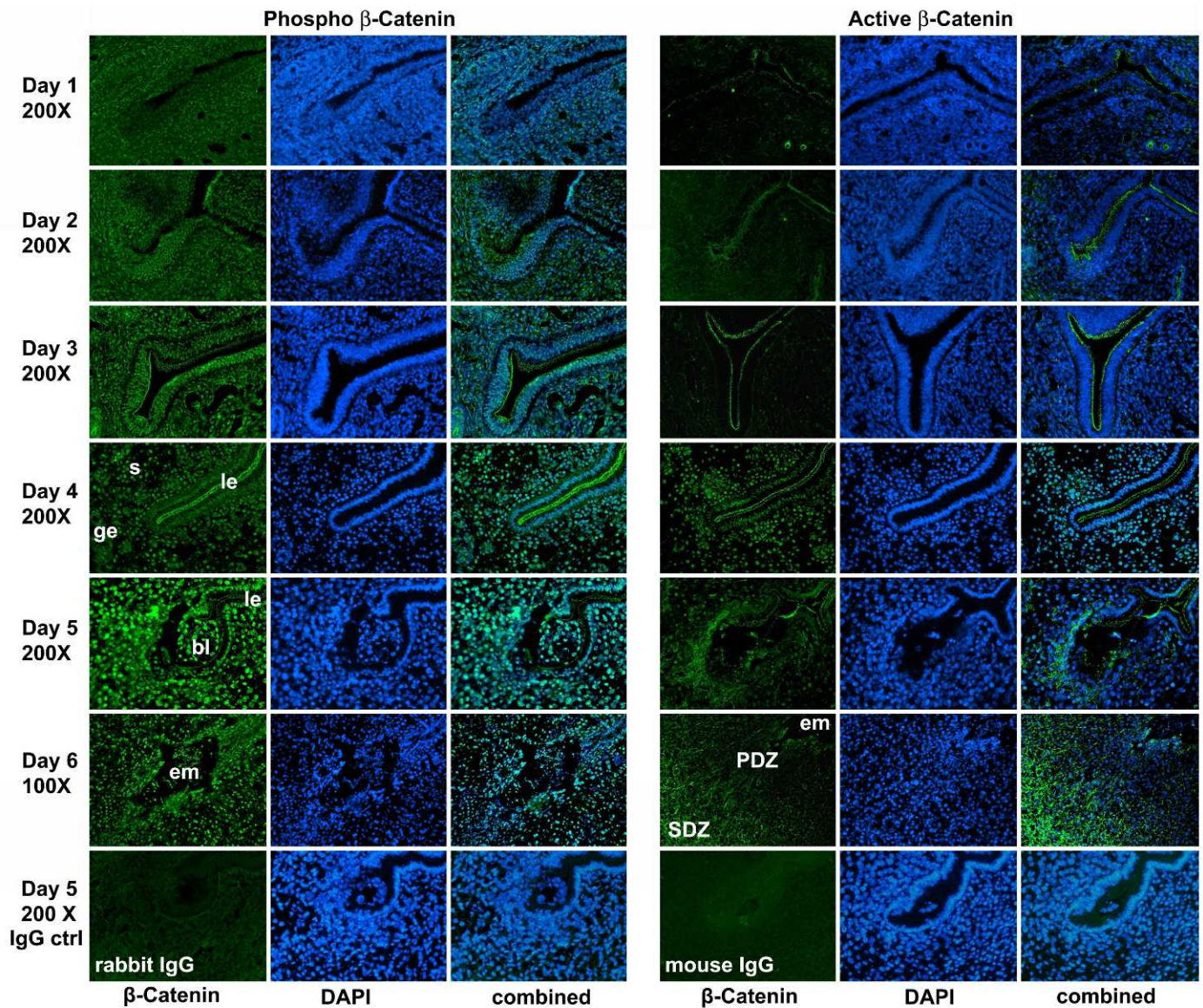


FIG. 5. Immunolocalization (FITC; green) of phospho- β -catenin and active β -catenin in uterine cross sections from Days 1 to 4 and implantation sites from Days 5 and 6 of pregnancy in hamsters. The nuclei were stained with DAPI (blue). The photomicrographs shown are representative of at least three to four independent experiments, with sections from more than one animal in the same slides. No or low staining was observed in negative control (ctrl) sections. bl, blastocyst; em, embryo; ge, glandular epithelium; le, luminal epithelium; PDZ, primary decidual zone; s, stroma; SDZ, secondary decidual zone.

apical membrane of the luminal epithelial cells on Days 4 and 5 of pregnancy.

Active β -Catenin and Phospho- β -Catenin Are Expressed in a Temporal and Cell-Specific Manner in the Peri-implantation Uterus of the Hamster

The phosphorylation status of β -catenin determines whether it will form a complex with E-cadherin, will degrade, or will translocate to the nucleus for activation of genes. Thus, to better understand the overall roles of uterine β -catenin during the peri-implantation period in hamsters, we analyzed the uterine expression patterns of active (nonphosphorylated) and inactive (phosphorylated) β -catenins juxtaposed with total β -catenin expression patterns. We observed by immunofluorescence staining that phosphorylated and dephosphorylated (active) β -catenins were expressed distinctively in the hamster uterus

during early pregnancy (Days 1 to 6). As shown in Figure 5, on Days 1 and 2 of pregnancy phosphorylated β -catenin was mainly localized in the nuclei of both luminal epithelial cells and stromal cells. From Day 3 onward, phosphorylated β -catenin was clearly detected in the apical surface of uterine epithelium, and its expression level in nuclei was substantially upregulated in both the stroma and the epithelium. By contrast, active β -catenin predominantly accumulated in the apical surface of uterine epithelium in all days of early pregnancy (Days 1 to 6). Active β -catenin expression in the uterus was also noticed in the nuclei of stromal cells on Days 4, 5, and 6 of pregnancy. It is worth noting that on Day 6 of pregnancy, active β -catenin was highly expressed in the membrane of decidualizing stromal cells in the SDZ. Negative control sections from Day 5 implantation sites showed no specific immunostaining when mouse monoclonal active β -catenin and rabbit polyclonal phospho- β -catenin antibodies were replaced with mouse and rabbit IgGs,

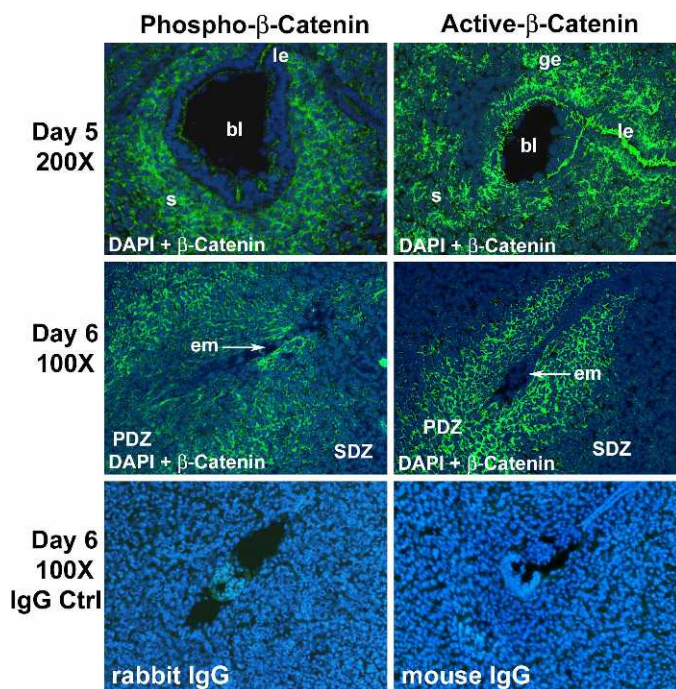


FIG. 6. Immunolocalization (FITC; green) of phospho- β -catenin and active β -catenin in uterine cross sections from implantation sites of Days 5 and 6 of pregnancy in mice. The nuclei were stained with DAPI (blue). The photomicrographs shown are representative of at least two to three independent experiments, with sections from more than one animal in the same slides. No or low staining was observed in negative control sections. bl, blastocyst; em, embryo; ge, glandular epithelium; le, luminal epithelium; PDZ, primary decidual zone; s, stroma; SDZ, secondary decidual zone.

respectively. These results suggest that in addition to β -catenin's role in E-cadherin/catenin complex formation, it is also involved in the regulation of nuclear functions in the uterus.

Active β -catenin and Phospho- β -catenin Expression at Day 5 and Day 6 Implantation Sites of Mice

Active- β -catenin and phospho- β -catenin were differentially expressed at the implantation sites (Fig. 6) compared with the expression pattern of total β -catenin. On Day 5, phospho- β -catenin showed apical membranous staining in the luminal epithelial cells, whereas its expression is both cytoplasmic and membranous in stromal cells surrounding the implanting embryo. Almost no nuclear staining of phospho- β -catenin was observed either in the stroma or in the luminal epithelium. Stromal cells away from the implantation chamber also showed phospho- β -catenin expression, but at a lower level. On Day 6 of pregnancy, phospho- β -catenin expression was noticed in cells of both the PDZ and SDZ, but staining intensity was stronger in the SDZ than in the PDZ (Fig. 6). Active β -catenin expression at the Day 5 implantation site was found in both uterine luminal and stromal cells (Fig. 6). In luminal epithelium, active β -catenin was strongly expressed in the apical region of cells. Low levels of active β -catenin protein were also observed in the nuclei of epithelial cells. Stromal cells surrounding the implanted blastocyst showed membranous, cytoplasmic, and nuclear active β -catenin. By contrast, active β -catenin staining was primarily noticed in the membrane and to some extent in the cytoplasm of the PDZ on the Day 6 implantation site. Cells of the SDZ showed lower levels of membranous staining than cells of the PDZ. A few

cells of the PDZ or SDZ showed nuclear expression of active β -catenin (Fig. 6). Negative control sections from Day 6 implantation sites when incubated with either mouse or rabbit IgGs instead of specific primary antibodies showed no specific immunostaining.

DISCUSSION

The present investigation analyzed the uterine cell-type-specific expression patterns of AJ proteins E-cadherin, β -catenin, and α -catenin during early pregnancy in hamsters where implantation takes place only in the presence of ovarian progesterone [3, 23]. We observed that there were no noticeable variations in the expression patterns of E-cadherin, α -catenin, and total β -catenin proteins in uterine luminal epithelial cells before implantation (Days 2 to 4) in hamsters. The coexpression studies indicate that E-cadherin is functionally linked to catenins. Studies in the human uterus also showed no difference in epithelial plasma membrane E-cadherin, α -catenin, and β -catenin protein expression by immunohistochemistry throughout the menstrual cycle [24–26]. Thus, we are not surprised to see little or no change in the AJ protein expression pattern in the uterine luminal epithelium during early pregnancy. This is also consistent with the report that relatively few ultrastructural changes are observed in the uterine epithelium compared with the blastocyst in hamsters prior to implantation [27]. The lack of changes in the patterns of AJ protein on these days eliminates the possibility that these proteins are markers of uterine receptivity. The uterine lumen in hamsters remains open the first 3 days of pregnancy, but the luminal space is gradually reduced afterward and is closed by the middle of Day 4, when embryo-uterine interaction takes place to initiate implantation in this species [28]. It is, however, unknown whether or not persistent apical expression of AJ proteins in two opposite layers of the luminal epithelia may gradually bring them closer to reduce the luminal space for implantation.

Blastocysts are completely enclosed by the uterine folds during the apposition stage and initiate contact at a few points in the apical surface of luminal epithelial cells [27]. We observed similar patterns of E-cadherin, β -catenin, and α -catenin protein expression in the uterine luminal epithelium surrounding the implanted embryo in Day 5 implantation sites as observed prior to implantation. We also found coexpression of E-cadherin and β -catenin in this epithelial cell layer on Day 5. These results suggest that when blastocyst attachment occurs, the AJ in the uterine epithelium surrounding the implanted blastocyst remains much the same as prior to implantation. In support of these findings, studies have demonstrated that either attenuation of uterine E-cadherin expression or neutralization of uterine E-cadherin functions causes impairment of normal implantation in rats [9] and mice [29], respectively. Thus, the intact cell-cell contacts in the uterine luminal epithelium are necessary to form the uterine chamber and to initiate the attachment process of implantation. However, studies by Murphy [30] demonstrated that toward the time of implantation, the epithelial cells are less firmly attached to each other. The involvement of metalloproteases in the control of cell adhesiveness via cleavage of E-cadherin has been demonstrated [10]. Studies in epithelial cell lines have established that the ≈ 97 -kDa fragment of E-cadherin was specifically generated by meprin β , but not by ADAM10 or MMP7 [10, 31, 32]. The parallel correlation between the expression of meprin β and E-cadherin in uterine epithelial cells around the time of implantation suggests that processing of E-cadherin by meprin β may be a means to weaken cadherin-

mediated cell-cell contacts during implantation. This is also consistent with our observation of nuclear accumulation of active β -catenin in the epithelial layer from Day 4 onward.

One of the important observations of our study is that unlike mice, which showed strong expression of total β -catenin and α -catenin proteins and their mRNA in stromal cells immediately surrounding the Day 5 implanted blastocysts, neither E-cadherin nor total β -catenin and α -catenin proteins were expressed in stromal cells surrounding the implanting blastocyst on Day 5 of pregnancy in hamsters. We have further demonstrated that in mice, β -catenin and α -catenin proteins are highly expressed in the membranes of the PDZ cells in Day 6 implantation sites. In contrast to mice, we do not find any expression of E-cadherin protein and mRNAs in the hamster PDZ cells, although we see low levels of α -cadherin and total β -cadherin proteins in these cells. These results suggest that unlike mice, where the PDZ cells behave like epithelial cells by expressing AJ proteins [8, 15, 33, 34], the PDZ cells of the hamsters may not be able to form AJs in the absence of E-cadherin. Thus, the PDZ cells in hamsters are perhaps loosely connected with each other and more easily engulfed by the invading trophoblast. In this regard, it is also worth noting that the trophoblast of hamsters is more invasive than that of mice [3]. Interestingly, however, we only noticed strong expression of all three AJ proteins and their mRNAs in cells of the SDZ, but we also found that E-cadherin and β -catenin are colocalized in these cells. These discrepancies between mice and hamsters could possibly be attributed to differences in the hormonal regulation of implantation between these two rodents. Our findings also suggest that similar to the PDZ in mice, perhaps cells of the SDZ of hamsters are close contacts with each other with the formation of junctions. Thus, the SDZ of the hamster may play roles in limiting trophoblast cell migration and passage of harmful maternal immune cells, antibodies, germs, and chemicals to the embryo, similar to the PDZ in mice.

Another important finding of this study is that active β -catenin and phospho- β -catenin were regulated differentially in the uterus during the preimplantation period in hamsters. As shown in Figure 5, active β -catenin expression patterns in the membrane of uterine luminal epithelial cells and Day 6 SDZ cells resembled the expression patterns of total β -catenin, suggesting its usual role in cell-cell adhesion in the epithelium. However, clear nuclear expression of active β -catenin was observed in both the epithelial and stromal cells of the hamster uterine epithelium from Day 4 onward, suggesting the involvement of WNT/ β -catenin signaling pathway in uterine receptivity, implantation, and stromal cell decidualization in hamsters. WNT/ β -catenin signaling is now recognized as a critical pathway in the regulation of growth, proliferation, differentiation, and adhesion [35, 36]. Although one study showed inhibition of both the blastocyst and uterine β -catenin during the window of implantation [37], another study showed an essential role for WNT/ β -catenin signaling in ensuring blastocyst competency to implantation [38]. It has been demonstrated that WNT1 stabilizes β -catenin binding to the cell adhesion protein cadherin, and thereby influences cell boundary formation [39, 40]. Thus, we suggest that in hamsters, coordinate action of WNT/ β -catenin may help transient cell-cell contact formation in the SDZ to create a cellular boundary in this zone to avoid excessive penetration of trophoblast cells. However, one of the surprising findings is that phospho- β -catenin was primarily immunodetected in the nuclei of uterine cells in addition to its localization in the cytoplasm. Nuclear expression of phospho- β -catenin was strong at Day 5 and Day 6 implantation sites. The finding of phospho- β -catenin in the nucleus is peculiar because studies

suggest that the phosphorylated form of the protein should be degraded in the cytoplasm [41], but it is not without precedent; phospho- β -catenin is almost exclusively localized in the nuclei of breast carcinoma [42] and colorectal cancer tissues [43]. The reason for nuclear phospho- β -catenin localization is currently unknown. One recent study showed that the nuclear localization of phospho- β -catenin showed a correlation with BCL2, a well-established antiapoptotic factor [42, 44]. Thus, it is possible that nuclear β -catenin signaling has an antiapoptotic effect. More investigation is required in order to elucidate the mechanism by which phospho- β -catenin is localized in the nucleus of uterine cells, and whether even if phosphorylated, it is able to serve as a transcriptional activator affecting uterine cell behavior. In contrast to hamsters, phospho- β -catenin and active β -catenin expression patterns were somewhat different at the implantation site of mice. Very little or no nuclear localization of these two forms of β -catenin was observed either in the epithelium or stromal cells at the implantation sites of Days 5 and 6. This inverse relationship of nuclear expression of β -catenin at the implantation site between hamsters and mice suggests that nuclear signaling of β -catenin is perhaps more important in implantation and decidualization processes in hamsters than its involvement in the implantation processes in mice. Furthermore, an almost parallel membranous expression of α -catenin, active β -catenin, and total β -catenins in cells of the antimesometrial PDZ of Day 6 implantation sites indicates their possible participation of α -catenin and β -catenin in AJ formation among the PDZ cells in mice.

Overall, although AJ proteins are initially needed for embryo-uterine adhesion during implantation, weakening of AJ-mediated cell-cell adhesion in the luminal epithelium surrounding the implanted blastocyst may facilitate trophoblast penetration. We also observed that although AJ proteins are mainly localized in the SDZ cells of Day 6 hamster implantation sites, their localization was in the PDZ cells in Day 6 implantation sites of mice, suggesting species-specific and cell-type-specific expression of these proteins. Although it is not clear whether or not E-cadherin and catenins form the same type of AJ in between cells of the SDZ as observed in epithelial cells, active β -catenin expression in both the membrane and nucleus of the SDZ cells in hamsters suggests that perhaps β -catenin-mediated WNT signaling helps in creating a transient decidual boundary between the embryo and mother by stabilizing the decidual cell-cell adhesion. These findings provide evidence that in hamsters the SDZ serves as a temporary barrier as well as a boundary at the maternal-embryonic interface.

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REFERENCES

1. Zhang Q, Paria BC. Importance of uterine cell death, renewal, and their hormonal regulation in hamsters that show progesterone-dependent implantation. *Endocrinology* 2006; 147:2215–2227.
2. Huet-Hudson YM, Andrews GK, Dey SK. Cell type-specific localization of c-myc protein in the mouse uterus: modulation by steroid hormones and analysis of the periimplantation period. *Endocrinology* 1989; 125:1683–1690.
3. Reese J, Wang H, Ding T, Paria BC. The hamster as a model for embryo implantation: insights into a multifaceted process. *Semin Cell Dev Biol* 2008; 19:194–203.
4. Hartsock A, Nelson WJ. Adherens and tight junctions: structure, function and connections to the actin cytoskeleton. *Biochim Biophys Acta* 2008; 1778:660–669.

5. Kemler R, Ozawa M, Ringwald M. Calcium-dependent cell adhesion molecules. *Curr Opin Cell Biol* 1989; 1:892–897.
6. Jha RK, Titus S, Saxena D, Kumar PG, Laloraya M. Profiling of E-cadherin, beta-catenin and Ca(2+) in embryo-uterine interactions at implantation. *FEBS Lett* 2006; 580:5653–5660.
7. Wang H, Ding T, Brown N, Yamamoto Y, Prince LS, Reese J, Paria BC. Zonula occludens-1 (ZO-1) is involved in morula to blastocyst transformation in the mouse. *Dev Biol* 2008; 318:112–125.
8. Paria BC, Zhao X, Das SK, Dey SK, Yoshinaga K. Zonula occludens-1 and E-cadherin are coordinately expressed in the mouse uterus with the initiation of implantation and decidualization. *Dev Biol* 1999; 208:488–501.
9. Li Q, Wang J, Armant DR, Bagchi MK, Bagchi IC. Calcitonin down-regulates E-cadherin expression in rodent uterine epithelium during implantation. *J Biol Chem* 2002; 277:46447–46455.
10. Huguenin M, Muller EJ, Trachsel-Rosmann S, Oneda B, Ambort D, Sterchi EE, Lottaz D. The metalloprotease meprinbeta processes E-cadherin and weakens intercellular adhesion. *PLoS ONE* 2008; 3:e2153.
11. Noe V, Fingleton B, Jacobs K, Crawford HC, Vermeulen S, Steelant W, Bruyneel E, Matrisian LM, Mareel M. Release of an invasion promoter E-cadherin fragment by matrilysin and stromelysin-1. *J Cell Sci* 2001; 114:111–118.
12. Maretzky T, Reiss K, Ludwig A, Buchholz J, Scholz F, Proksch E, de Strooper B, Hartmann D, Saftig P. ADAM10 mediates E-cadherin shedding and regulates epithelial cell-cell adhesion, migration, and beta-catenin translocation. *Proc Natl Acad Sci U S A* 2005; 102:9182–9187.
13. Bertenshaw GP, Norcum MT, Bond JS. Structure of homo- and hetero-oligomeric meprin metalloproteases. Dimers, tetramers, and high molecular mass multimers. *J Biol Chem* 2003; 278:2522–2532.
14. Bell SC. Decidualization and associated cell types: implications for the role of the placental bed in the materno-fetal immunological relationship. *J Reprod Immunol* 1983; 5:185–194.
15. Wang X, Matsumoto H, Zhao X, Das SK, Paria BC. Embryonic signals direct the formation of tight junctional permeability barrier in the decidualizing stroma during embryo implantation. *J Cell Sci* 2004; 117:53–62.
16. Brosens JJ, Gellersen B. Death or survival—progesterone-dependent cell fate decisions in the human endometrial stroma. *J Mol Endocrinol* 2006; 36:389–398.
17. Enders AC. The implantation chamber, blastocyst and blastocyst imprint of the rat; a scanning electron microscope study. *Anat Rec* 1975; 182:137–149.
18. Paria BC, Huet-Hudson YM, Dey SK. Blastocyst's state of activity determines the "window" of implantation in the receptive mouse uterus. *Proc Natl Acad Sci U S A* 1993; 90:10159–10162.
19. Wang X, Wang H, Matsumoto H, Roy SK, Das SK, Paria BC. Dual source and target of heparin-binding EGF-like growth factor during the onset of implantation in the hamster. *Development* 2002; 129:4125–4134.
20. Ding T, Song H, Wang X, Khatua A, Paria BC. Leukemia inhibitory factor ligand-receptor signaling is important for uterine receptivity and implantation in golden hamsters (*Mesocricetus auratus*). *Reproduction* 2007; 135:1–14.
21. Khatua A, Wang X, Ding T, Zhang Q, Reese J, DeMayo FJ, Paria BC. Indian hedgehog, but not histidine decarboxylase or amphiregulin, is a progesterone-regulated uterine gene in hamsters. *Endocrinology* 2006; 147:4079–4092.
22. Wang X, Su Y, Deb K, Raposo M, Morrow JD, Reese J, Paria BC. Prostaglandin E2 is a product of induced prostaglandin-endoperoxide synthase 2 and microsomal-type prostaglandin E synthase at the implantation site of the hamster. *J Biol Chem* 2004; 279:30579–30587.
23. Greenwald GS. Endocrinology of the pregnant hamster. In: Siegel HI (ed.), *The Hamster: Reproduction and Behavior*. New York: Plenum Publishing Corp; 1985:53–72.
24. van der Linden PJ, de Goeij AF, Dunselman GA, Erkens HW, Evers JL. Expression of cadherins and integrins in human endometrium throughout the menstrual cycle. *Fertil Steril* 1995; 63:1210–1216.
25. Nei H, Saito T, Yamasaki H, Mizumoto H, Ito E, Kudo R. Nuclear localization of beta-catenin in normal and carcinogenic endometrium. *Mol Carcinog* 1999; 25:207–218.
26. Fujimoto J, Ichigo S, Hori M, Tamaya T. Expression of E-cadherin, alpha and beta-catenin mRNAs in ovarian endometriosis. *Eur J Obstet Gynecol Reprod Biol* 1996; 67:179–183.
27. Parkening TA. An ultrastructural study of implantation in the golden hamster. I. Loss of the zona pellucida and initial attachment to the uterine epithelium. *J Anat* 1976; 121:161–184.
28. McLennan JG. Ultrastructural studies of early nidation in pregnancy and pseudopregnancy. *Am J Obstet Gynecol* 1974; 120:319–334.
29. Liu G, Zhang X, Lin H, Wang H, Li Q, Ni J, Zhu C. Effects of E-cadherin on mouse embryo implantation and expression of matrix metalloproteinase-2 and -9. *Biochem Biophys Res Commun* 2006; 343:832–838.
30. Murphy CR. Junctional barrier complexes undergo major alterations during the plasma membrane transformation of uterine epithelial cells. *Hum Reprod* 2000; 15(suppl 3):182–188.
31. Feng J, Woessner JF Jr, Zhu C. Matrilysin activity in the rat uterus during the oestrous cycle and implantation. *J Reprod Fertil* 1998; 114:347–350.
32. Kim J, Kang SG, Kim JI, Park JH, Kim SK, Cho DJ, Kim H. Implication of ADAM-8, -9, -10, -12, -15, -17, and ADAMTS-1 in implantational remodeling of a mouse uterus. *Yonsei Med J* 2006; 47:558–567.
33. Tung HN, Parr MB, Parr EL. The permeability of the primary decidual zone in the rat uterus: an ultrastructural tracer and freeze-fracture study. *Biol Reprod* 1986; 35:1045–1058.
34. Parr MB, Tung HN, Parr EL. The ultrastructure of the rat primary decidual zone. *Am J Anat* 1986; 176:423–436.
35. Nusse R. The Wnt gene family in tumorigenesis and in normal development. *J Steroid Biochem Mol Biol* 1992; 43:9–12.
36. Nusse R, Varmus HE. Wnt genes. *Cell* 1992; 69:1073–1087.
37. Li J, Zhang JV, Cao YJ, Zhou JX, Liu WM, Fan XJ, Duan EK. Inhibition of the beta-catenin signaling pathway in blastocyst and uterus during the window of implantation in mice. *Biol Reprod* 2005; 72:700–706.
38. Xie H, Tranguch S, Jia X, Zhang H, Das SK, Dey SK, Kuo CJ, Wang H. Inactivation of nuclear Wnt- β -catenin signaling limits blastocyst competency for implantation. *Development* 2008; 135:717–727.
39. Hinck L, Nelson WJ, Papkoff J. Wnt-1 modulates cell-cell adhesion in mammalian cells by stabilizing beta-catenin binding to the cell adhesion protein cadherin. *J Cell Biol* 1994; 124:729–741.
40. Li HY, Chang SP, Yuan CC, Chao HT, Ng HT, Sung YJ. Establishment of an efficient method to quantify embryo attachment to endometrial epithelial cell monolayers. *In Vitro Cell Dev Biol Anim* 2002; 38:505–511.
41. Daugherty RL, Gottardi CJ. Phospho-regulation of Beta-catenin adhesion and signaling functions. *Physiology (Bethesda)* 2007; 22:303–309.
42. Nakopoulou L, Mylona E, Papadaki I, Kavantzias N, Giannopoulou I, Markaki S, Keramopoulos A. Study of phospho-beta-catenin subcellular distribution in invasive breast carcinomas in relation to their phenotype and the clinical outcome. *Mod Pathol* 2006; 19:556–563.
43. Chung GG, Provost E, Kielhorn EP, Charette LA, Smith BL, Rimm DL. Tissue microarray analysis of beta-catenin in colorectal cancer shows nuclear phospho-beta-catenin is associated with a better prognosis. *Clin Cancer Res* 2001; 7:4013–4020.
44. Kirkin V, Joos S, Zornig M. The role of Bcl-2 family members in tumorigenesis. *Biochim Biophys Acta* 2004; 1644:229–249.