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REGULAR ARTICLE

Adam12 plays a role during uterine decidualization in mice

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Abstract In mouse, decidualization is characterized by the proliferation of stromal cells and their differentiation into specialized type of cells (decidual cells) with polyploidy, surrounding the implanting blastocyst. However, the mechanisms involved in these processes remain poorly understood. Using multiple approaches, we have examined the role of Adam12 in decidualization during early pregnancy in mice. Adam12 is spatiotemporally expressed in decidualizing stromal cells in intact pregnant females and in pseudopregnant mice undergoing artificially induced decidualization. In the ovariectomized mouse uterus, the expression of Adam12 is upregulated after progesterone treatment, which is primarily mediated by nuclear progesterone receptor. In a stromal cell culture model, the expression of Adam12 gradually rises with the progression of stromal decidualization, whereas the attenuated expression of Adam12 after siRNA knockdown significantly blocks the progression of decidualization. Our study suggests that Adam12 is involved in promoting uterine decidualization during pregnancy.

Keywords Adam12 · Stromal cells · Decidualization · Uterus · Implantation · Mouse (CD-1)

Introduction

Decidualization involves complex regulatory mechanisms to ensure the normal progression of postimplantation embryo-uterine development (Dey et al. 2004; Lim et al. 2002; Tranguch et al. 2005). In mice, decidualization is characterized by the proliferation of stromal cells and their differentiation into specialized type of cells (decidual cells) with polyploidy, surrounding the implanting blastocyst; this forms the primary decidual zone (pdz) closely encompassing the implanting embryo by day 5 of pregnancy (Das et al. 1999). This process is followed by the cessation of proliferation and apoptosis of stromal cells in the pdz, with new rounds of stromal cell proliferation outside the pdz by day 6 and the formation of the secondary decidual zone (sdz; Tan et al. 2002). This pattern persists through days 7 and 8 of pregnancy, and then the cells in sdz also undergo apoptosis to accommodate the growing embryo. Genomic endoreduplication of decidualizing stromal cells might ensure the increase of protein synthesis to provide nutritional support to the developing embryo before the establishment of the placenta (Tranguch et al. 2005). Decidualizing stromal cells form incomplete tight junctions serving as a filter to prevent the passage of large molecules and have paracrine communication with trophoblast (Wang et al. 2004). In addition, angiogenic factors produced by the decidual cells might regulate trophoblast differentiation, invasion, and placentation (Hess et al. 2007). However, the mechanisms

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involved in the massive tissue remodeling during decidualization remain poorly understood.

By microarray analyses, we have previously observed that Adam12 is distinctly upregulated at the site of implantation on days 5 and 7 of pregnancy (unpublished data), suggesting Adam12 plays potential roles during decidualization. Adam12, which belongs to a disintegrin/metalloprotease family, is known to be involved in regulating muscle cell fusion as a transmembrane protein (Yagami-Hiromasa et al. 1995), and in the activity of cell surface integrins through the connection of its extracellular disintegrin-like and cysteine-rich domains (Kawaguchi et al. 2003). Moreover, Adam12 might induce the activation of some growth factors, including epidermal growth factor (EGF; Zahnow 2006), heparin-binding EGF-like growth factor (HB-EGF; Shi et al. 2000) and insulin-like growth factor binding protein 3 (IGFBP-3; Loechel et al. 2000), by mediating the shedding of EGF receptor ligands, HB-EGF, and IGFs, respectively, as an active metalloprotease, and then activate subsequent downstream signaling, which involves many cellular events including proliferation, differentiation, and migration. In addition, Adam12 is greatly up-regulated in tumor cells in a variety of human cancer types (Carl-McGrath et al. 2005; Frohlich et al. 2006; Iba et al. 1999; Kveiborg et al. 2005; Le Pabic et al. 2003; Lendeckel et al. 2005). With regard to the role of Adam12 in the female reproductive tract during pregnancy, early studies have demonstrated that Adam12 is highly expressed in human placenta (Ito et al. 2004). However, the physiological relevance of Adam12 during implantation and decidualization remains to be further explored.

Using multiple approaches, we have examined the role of Adam12 in mouse uteri during early pregnancy. We have observed that Adam12 is expressed in the decidualizing stromal cells, and that its expression is upregulated with the progression of the decidualization process in both intact mice and cultured uterine stromal cells. Furthermore, attenuation of Adam12 expression by its specific short interfering RNA (siRNA) largely blocks the progression of stromal cell decidualization, reducing expression of the decidualization marker genes. This observation suggests that Adam12 is involved in promoting uterine decidualization during pregnancy.

Materials and methods

Animals and treatments

Adult CD-1mice, aged 6–8 weeks and weighing 20–25 g, were purchased from Vital River (Beijing, China). All experiments involving animals were fed laboratory chow and water under a constant photoperiod (12:12-h light–dark cycle) at a monitored ambient temperature of 22°C. The Guidelines for the Care and Use of Animals in Research (Chinese Academy of Sciences) were followed. Virgin females were mated with fertile or vasectomized males of the same strain to induce pregnancy or pseudopregnancy, respectively. The presence of vaginal plugs was used to designate day 1 of pregnancy or pseudopregnancy. Mice were killed at 0900 hours on various days of pregnancy to collect uteri. Pregnancy on days 1–4 was confirmed by recovering embryos from the reproductive tracts, and the implantation sites on day 5 of pregnancy were identified by intravenous injection of 0.1 ml 1% trypan blue in saline 5 min before the mice were killed.

To induce delayed implantation, pregnant mice were ovariectomized at 0800 hours to 0900 hours on day 4 of pregnancy, Delayed implantation was maintained with a daily s.c. injection of progesterone (P_4; 2 mg/mouse; P6149, Sigma) from days 5–7 of pregnancy. To initiate delayed implantation, estradiol-17β (E_2; 100 ng/mouse; E2257, Sigma) was given to P_4-primed delayed-implantation mice on day 7 of pregnancy. P_4 and E_2 were dissolved in sesame oil (S3547, Sigma). The mice were killed to collect uteri after 24 h and 48 h of E_2 treatment.

Artificial decidualization was induced by intraluminal injections of sesame oil (10 μl/mouse) in one uterine horn on day 4 of pseudopregnancy, whereas the contralateral horn without any infusion served as the control. The mice were killed for collection of uteri from days 5 to 7 of pseudopregnancy, and the decidual cell reaction was confirmed by increased uterine weight and histological examination.

To examine the effects of P_4 on the expression of Adam12 in the uterus, ovariectomized mice were treated with s.c. injections of sesame oil (0.1 ml/mouse), P_4 (2 mg/mouse), or P_4 and RU486 (prolactin [PR] antagonist; 2 mg/mouse; M8046, Sigma). Mice were killed, and the uteri were collected at 0, 1, 2, 4, 6, 12, and 24 h after each treatment.

Isolation of uterine stromal cells and induction of decidualization in vitro

Cells were isolated by using a previously described method (Tan et al. 2004) with some modifications. Briefly, uterine horns from mice on day 4 of pregnancy were dissected longitudinally to expose the uterine lumen and cut into 3–5 mm pieces. After being washed with phosphate-buffered saline (PBS), the tissues were placed in PBS containing 0.25% trypsin (27250018, Invitrogen) for 1 h at 4°C followed by 1 h at room temperature. After these digestion steps, the tissues were immediately diluted in PBS containing 10% charcoal-stripped fetal bovine serum (FBS; F6765, Sigma) to terminate digestion and mixed gently to remove epithelial clumps by pipetting up and down more

than 50 times with a 1-ml pipette. The remaining tissues were washed twice in PBS and then placed in PBS containing 0.5% collagenase Type II (17101015, Invitrogen) for 30 min at 37°C. At the end of digestion, the tissues were immediately diluted in PBS containing 10% FBS. After being washed twice in PBS, the tissues were mixed thoroughly by pipetting up and down more than 50 times with a 1-ml pipette until the supernatant became turbid with dispersed stromal cells. The stromal cells were purified through a 70-μm nylon filter and then centrifuged, and the pellet was washed twice with fresh phenol-red-free culture medium (1:1, v/v; DMEM/Ham's F-12; 11039021, Invitrogen) before primary culture. Cells were plated at 2×10^5 cells per 6-well cell culture plate, containing phenol-red-free culture medium (DMEM/Ham's F-12, 1:1) with 10% charcoal-stripped FBS, 10 U/ml penicillin–streptomycin solution (15140122, Invitrogen). After incubation for 1 h, the unattached cells were removed by several washes with fresh phenol-red-free culture medium (DMEM/Ham's F-12, 1:1), and cell culture was continued after the addition of fresh phenol-red-free culture medium (DMEM/Ham's F-12, 1:1) containing 10% charcoal-stripped FBS, 10 U/ml penicillin–streptomycin solution, 10 nM E_2, and 1 μM P_4.

Immunohistochemistry

Mouse uteri were cut into small pieces, fixed in Bouin's solution for 24 h, dehydrated, and embedded in paraffin. Paraffin-embedded endometrial sections (5 μm) were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked by incubating the sections in 3% peroxide in methanol for 10 min at room temperature. After three washes in PBS, nonspecific binding was blocked in PBS with 5% bovine serum albumin (BSA) for 1 h at room temperature followed by incubation with rabbit anti-Adam12 primary antibody (sc-25579, Santa Cruz Biotechnology) diluted in blocking solution (1:70) overnight at 4°C. After three washes in PBS, the sections were incubated with secondary antibody (horseradish-peroxidase-conjugated goat anti-rabbit IgG; ZB-2301, Zhongshan Biotechnology) diluted in blocking solution (1:100) for 1 h at 37°C. The secondary antibody was detected with 3, 3′-diaminobenzidine solution (ZLI-9033, Zhongshan Biotechnology). For some sections, primary antibody was replaced with normal rabbit IgG (1:70; I5006, Sigma) as negative controls.

Immunocytochemistry

The cells were fixed in 4% paraformaldehyde for 15 min at room temperature and permeated by using 0.2% Triton X-100 for 10 min at room temperature. After three washes in PBS, nonspecific binding was blocked in PBS with 5% BSA for 1 h at room temperature, and then the cells were incubated overnight with mouse anti-vimentin (1:500; ab20346, abcam) or mouse anti-cytokeratin (1:300; ab6401, abcam) primary antibody diluted in blocking solution at 4°C. After three washes in PBS, the cells were incubated with secondary antibody (fluorescein isothiocyanate [FITC]-conjugated goat anti-mouse IgG; ZB-0312, Zhongshan Biotechnology) diluted in blocking solution (1:200) for 1 h at 37°C, followed by counterstaining with propidium iodide (PI; 2 μg/ml in PBS) for 10 min at 37°C. Stained cells were analyzed by using an epifluorescence microscope. For some cells, primary antibody was replaced with normal mouse IgG (1:200, I5381, Sigma) as negative controls.

siRNA transfections

Stromal cells were cultured in 2 ml antibiotic-free normal growth medium supplemented with FBS per 6-well cell culture plate for 24 hours, according to the manufacturer's instructions. For each transfection, the medium was removed from wells, and 1 ml siRNA Transfection Medium (sc-36868, Santa Cruz Biotechnology) were added to each well containing 8 μl Adam12-siRNA duplex (sc-41415, Santa Cruz Biotechnology) or negative Control-siRNA (sc-37007, Santa Cruz Biotechnology) and 8 μl siRNA Transfection Reagent (sc-29528, Santa Cruz Biotechnology). After 8 h, the Transfection Medium was replaced, and the cells were cultured with normal medium for a further 72 h.

Bromodeoxyuridine incorporation assay

Cells were cultured for 8 h in the presence of 20 μM bromodeoxyuridine (BrdU; B9285, Sigma) after being transfected with Control-siRNA and Adam12-siRNA for 48 h. The cells were then fixed in 4% paraformaldehyde for 10 min at room temperature, permeated by using 0.5% Triton X-100 for 10 min at room temperature, washed three times with PBS, denatured with 2 N HCl for 30 min, and neutralized with 0.1 M sodium borate for 30 min at 37°C. After a blocking step with 5% BSA in PBS for 1 h at room temperature, mouse anti-BrdU antibody (1: 100; ZM-0013, Zhongshan Biotechnology) was applied and incubated overnight at 4°C. A secondary antibody (FITC-conjugated goat anti-mouse IgG; ZB-0312, Zhongshan Biotechnology) diluted in blocking solution (1:200) was then applied, and incubation continued in a dark chamber for 1 h at 37°C, followed by counterstaining with PI (2 μg/ml in PBS) for 10 min at 37°C. Samples were examined by using an epifluorescence microscope.

Western blot analysis

Proteins were extracted from the pooled uterine tissues or from cells at various stages after treatment. A total of 20 μg

protein extracts was separated by SDS-polyacrylamide gel electrophoresis, with samples being loaded onto each lane of 12% polyacrylamide gels. After this separation, proteins in the gels were blotted onto polyvinylidene difluoride membranes (Amersham, Arlington Heights, Ill., USA). The membranes were blocked with 5% nonfat milk in TRIS-buffered saline containing 0.1% Tween 20 at room temperature and then probed with the following primary antibodies at 4°C overnight: rabbit anti-Adam12 (1:1000; ab28225, abcam), goat anti-Adam9 (1:1000; BAF949, R&D Systems), rabbit anti-PR (1:200; sc-538, Santa Cruz Biotechnology), mouse anti-cyclin D3 (1:1000; #2936, Cell Signaling), and rabbit anti-β-actin (1:500; sc-1616-R, Santa Cruz Biotechnology). After incubation with secondary antibodies, viz., horseradish-peroxidase-conjugated rabbit anti-goat IgG (ZB-2306, Zhongshan Biotechnology), horseradish-peroxidase-conjugated goat anti-rabbit IgG (ZB-2301, Zhongshan Biotechnology), and horseradish-peroxidase-conjugated goat anti-mouse IgG (ZB-2305, Zhongshan Biotechnology) diluted in blocking solution (1:100), the membranes were subjected to enhanced chemiluminescence (ECL detection system; sc-2048, Santa Cruz Biotechnology).

RNA preparation and quantitative reverse transcription plus polymerase chain reaction

Total RNA was isolated from the stromal cells at 24, 48, and 72 h by using the TRIzol reagent (10296010, Invitrogen) according to the manufacturer's instructions. Total RNA (2 μg) was reverse-transcribed into single-stranded cDNA by using Moloney Murine Leukemina (M-MuLV) virus reverse transcriptase (M0253, New England BioLabs) and 2 μl Oligo (dT) primers (CD106, TianGen Biotechnology). cDNA (1 μl) and 0.5 μM primers were adjusted with RNase-free water to a volume of 11 μl followed by the addition of 9 μl SYBR Green master mix (FP202, TianGen Biotechnology). Primers for the detection of decidual prolactin-related protein (dPRP), Adam12, and β-actin (Spandidos et al. 2008) were as follows: dPRP, sense 5′-TTA TGG GTG CAT GGA TCA CTC C-3′, antisense 5′-CCC ACG TAA GGT CAT CAT GGA T-3′; Adam12, sense 5′-TGG GAC CAG AGA GGA GCT TAC-3′, antisense 5′-GTT GCA CAG TCA GCA CGT CT-3′; β-actin, sense 5′-TGG AAT CCT GTG GCA TCC ATG AAA C-3′, antisense 5′-TAA AAC GCA GCT CAG TAA CAG TCC G-3′; they were used under the following conditions: 95°C/1 min; 95°C/15 s, 60°C/1 min for 40 cycles. The quantitative reverse transcription plus polymerase chain reaction (qRT-PCR) was performed with the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, Calif., USA).

Statistical analysis

Each experiment was performed at least three times. One-way analysis of variance followed by a least-significant-difference test was used for statistical comparisons among multiple groups. For statistical comparisons between two groups, an independent-sample *t*-test was used. Statistically ignificant differences were determined at $P<0.05$.

Results

Expression of Adam12 in mouse uterus during implantation and artificially induced decidualization

We first examined Adam12 protein expression in the implantation uterus to confirm our previous observations. Western blot analyses revealed an increase expression of Adam12 protein in the uterus with the initiation of implantation (day 5 vs. days 1–4) and the progression of uterine decidualization (days 6–7; Fig. 1a, b; $P<0.001$). We then analyzed the spatiotemporal expression of Adam12 in mouse uterus on days 1–8 of pregnancy by immunohistochemistry. Adam12 was visualized in the myometrium on days 1–8 of pregnancy, but its expression remained at low levels in the uterine epithelium and stromal on days 1–4 and in the inter-implantation tissue on days 5–8 of pregnancy (Fig. 1c–j). By contrast, immunohistochemical localization of Adam12 was detected in the decidualizing stromal cells surrounding the implanted embryo on days 5–8. To explore the correlation of the presence of Adam12 in the implanting uterus with the initiation of attachment, we next examined Adam12 expression in the delayed implanting uteri. We observed that Adam12 was reinduced in the stromal cells surrounding the activated blastocyst (Supplemental Fig. 1a–d). To confirm whether the induction of Adam12 expression in the decidualizing stromal cells was influenced by implanting blastocysts, we examined the expression of Adam12 in artificially induced decidualization. As observed in intact pregnant mice, Adam12 was consistently expressed in the myometrium (Fig. 1k–p). Furthermore, no Adam12 immunostaining signals occurred in the mouse uterus on day 5 of pseudopregnancy, its expression only becoming apparent in the artificially induced decidualizing stromal cells on days 6–8 of pseudopregnancy (Fig. 1k–p). These results collectively indicated that Adam12 was involved in the stromal cell decidualization.

Expression of Adam12 is regulated by P_4 in mouse uterus

Since progesterone is the primary hormone essential for normal decidualization, we examined the influence of P_4 on uterine Adam12 expression in mice. Western blot analyses

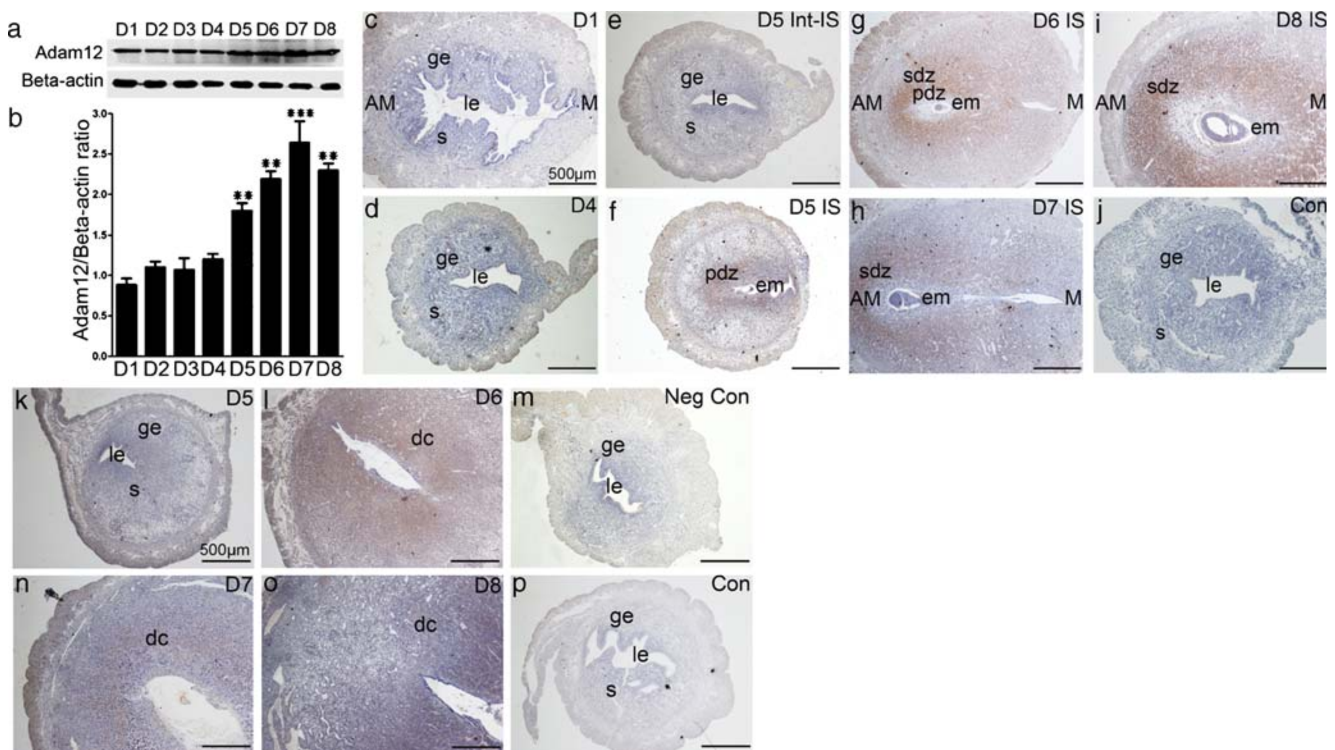

Fig. 1 Expression of Adam12 during early pregnancy and artificially induced decidualization. **a** Western blot analysis of Adam12 protein in mouse uterus on days 1–8 (*D1–D8*) of pregnancy. **b** Densitometric values from Western blot analyses of Adam12 protein in mouse uterus on days 1–8 (*D1–D8*) of pregnancy (mean±SE of three independent experiments). $^{**}P<0.001$, $^{***}P<0.0001$ as compared with D1 of pregnancy. **c–j** Immunostaining of Adam12 protein in mouse uterus on days 1, 4, 5, 6, 7, and 8 of pregnancy. **k–p** Immunostaining of Adam12 protein in mouse uterus in induced decidualization on days 5, 6, 7, and 8 of pseudopregnancy (*AM* antimesometrial pole, *Con* control, *em* embryo, *ge* glandular epithelium, *Int-IS* interimplantation site, *IS* implantation site, *le* luminal epithelium, *M* mesometrial pole, *pdz* primary decidual zone, *s* stroma, *sdz* secondary decidual zone)

revealed that P_4 significantly induced the expression of Adam12 compared with sesame oil treatment (Fig. 2a), with peak induction at 6 h post-treatment ($P<0.001$; Fig. 2b), whereas this induction was significantly blocked by co-treatment of PR antagonist, RU486 (Fig. 2c). These results implied that the up-regulation of Adam12 after P_4 treatment was nuclear PR-dependent.

Expression of Adam12 increases during decidualization of stromal cells in culture

To explore the role of Adam12 in the decidualization of mouse uterine stromal cells, we established a mouse primary stromal cells culture system according to the procedure described previously (Tan et al. 2004). The purity of isolated uterine stromal cells was monitored by immunofluorescence staining of vimentin and cytokeratin; epithelial cell contamination in isolated uterine stromal cells was apparently low (Fig. 3a–d). At 24–72 h after P_4 and E_2 treatment, stromal cells exhibited morphological characteristics of mature decidual cells in culture (Fig. 3e–h). qRT-PCR and Western blot analysis of decidualization marker genes, including PR (protein), cyclin D3 (protein; Fig. 3j) and dPRP (mRNA; $P<0.05$, Fig. 3k; Das et al. 1999; Orwig et al. 1997; Tan et al. 2002; Vallejo et al. 2005), revealed a similar upregulation of these genes in cultured decidualizing stromal cells. In this regard, we observed that the expression of Adam12 was gradually induced at both the mRNA and protein levels together with the progression of decidualization of stromal cells in culture ($P<0.05$, Fig. 3i, j), reinforcing the idea that Adam12 plays a role during stromal decidualization.

Adam12 plays a role during decidualization of stromal cells

To validate the contribution of Adam12 on the decidualization of stromal cells, we applied siRNA to knockdown Adam12 expression in cultured stromal cells. As shown in Fig. 4a, Adam12 mRNA in stromal cells was decreased after transfection with Adam12 siRNA in comparison with Control-siRNA ($P<0.05$). This reduction was correlated with an approximate 50% reduction in Adam12 protein expression in Adam12-siRNA-treated cells when examined at 72 h post-treatment ($P<0.001$; Fig. 4b, c), but not Adam9 (Fig. 4b, d). Consequently, the expressions of decidualization marker genes, viz., dPRP (mRNA), PR, and cyclin D3 (protein) were decreased respectively by ~40%, ~30%,

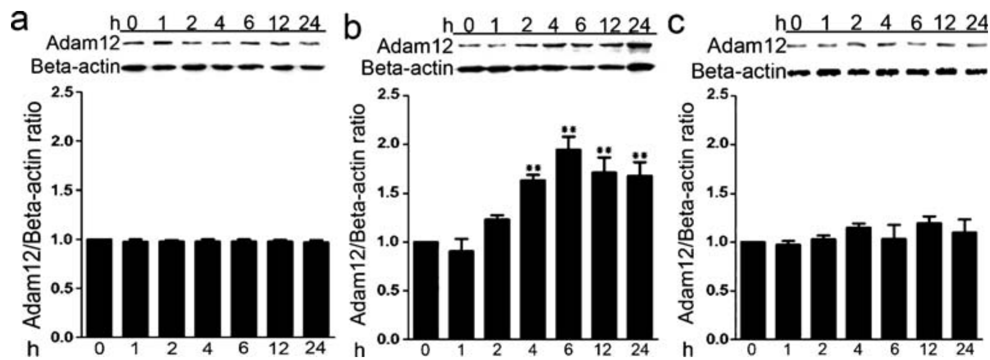

Fig. 2 Western blot analysis of Adam12 in mouse uterus after steroid hormone treatments. **a** *Top* Expression of Adam12 in mouse uterus at 1, 2, 4, 6, 12, and 24 h after sesame oil treatment. *Bottom* Densitometric values from Western blot analyses. **b** *Top* Expression of Adam12 in mouse uterus at 1, 2, 4, 6, 12, and 24 h after P_4 treatment. *Bottom* Densitometric values from Western blot analyses. **c** *Top* Expression of Adam12 in mouse uterus at 1, 2, 4, 6, 12, and 24 hours after P_4 plus RU486 treatment. *Bottom* Densitometric values from Western blot analyses. Mean±SE of three independent experiments, **$P<0.001$ as compared with 0 h

and ~30% in the Adam12 knockdown group in comparison with Control-siRNA ($P<0.05$; Fig. 4e–h). These results demonstrated that knockdown of the *adam12* gene blocks the decidualization process of stromal cells, and that Adam12 is essential for normal stromal decidualization.

Discussion

The normal decidualization of uterine stromal cells is necessary for successful embryo implantation, because this process provides nutrition to the developing embryo, protects it from maternal immune responses, and regulates trophoblast invasion into the endometrium (Tranguch et al. 2005). However, the complexity of this dynamic process makes it difficult to understand the molecular mechanism governing uterine stromal cell decidualization. In our study, the decidualization-specific expression pattern of Adam12 in stromal cells during normal pregnancy indicates that Adam12 plays a role in the decidualization of stromal cells. Moreover, Adam12 is expressed in decidualizing stromal cells under artificially induced decidualization, indicating

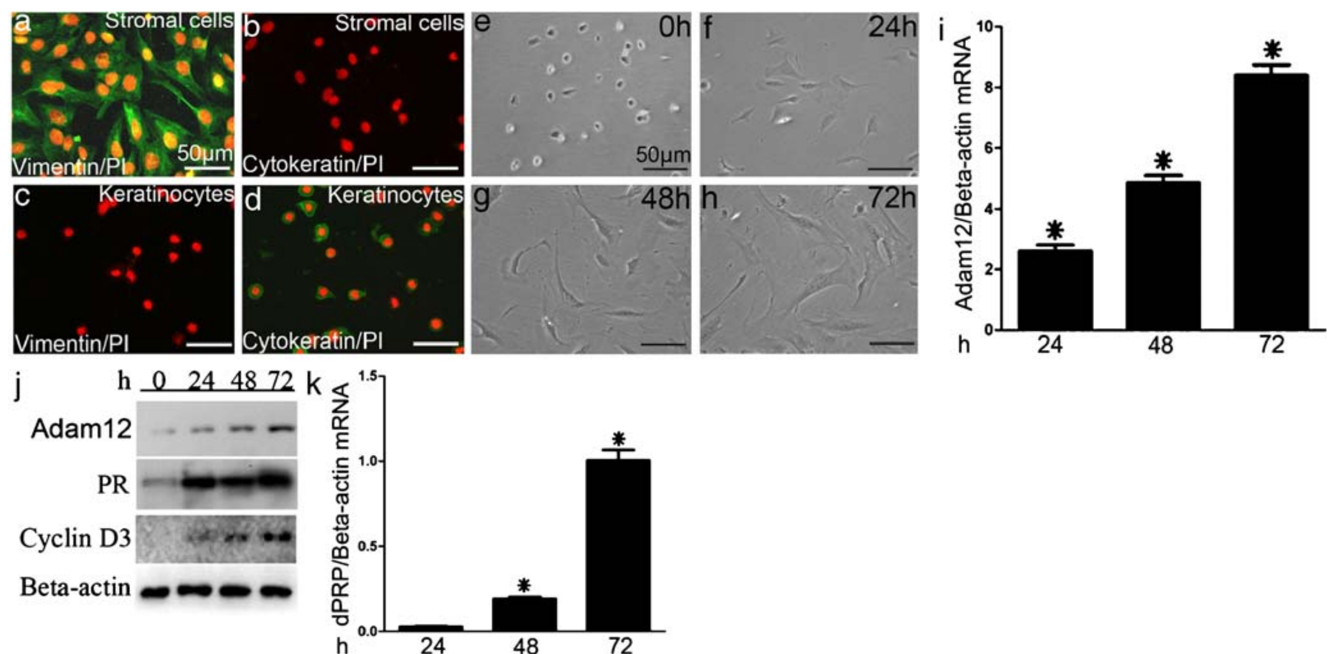

Fig. 3 Mouse uterine stromal cells undergo decidualization in primary culture. Decidualizing stromal cells were collected at 24-h intervals after P_4 and E_2 treatments. **a–d** Immunocytochemical analysis of vimentin and cytokeratin protein in primary stromal cells (*PI* propidium iodide). **e–h** Morphology of stromal cells cultured up to 72 h. **i** qRT-PCR analysis to monitor Adam12 mRNA expression in stromal cells cultured up to 72 h (mean±SE of three independent experiments). *$P<0.05$ as compared with 0 h. **j** Western blot analysis of Adam12, PR and cyclin D3 proteins in the stromal cells cultured up to 72 h. **k** qRT-PCR analysis to monitor dPRP mRNA expression in stromal cells cultured up to 72 h (mean±SE of three independent experiments). *$P<0.05$ as compared with 0 h

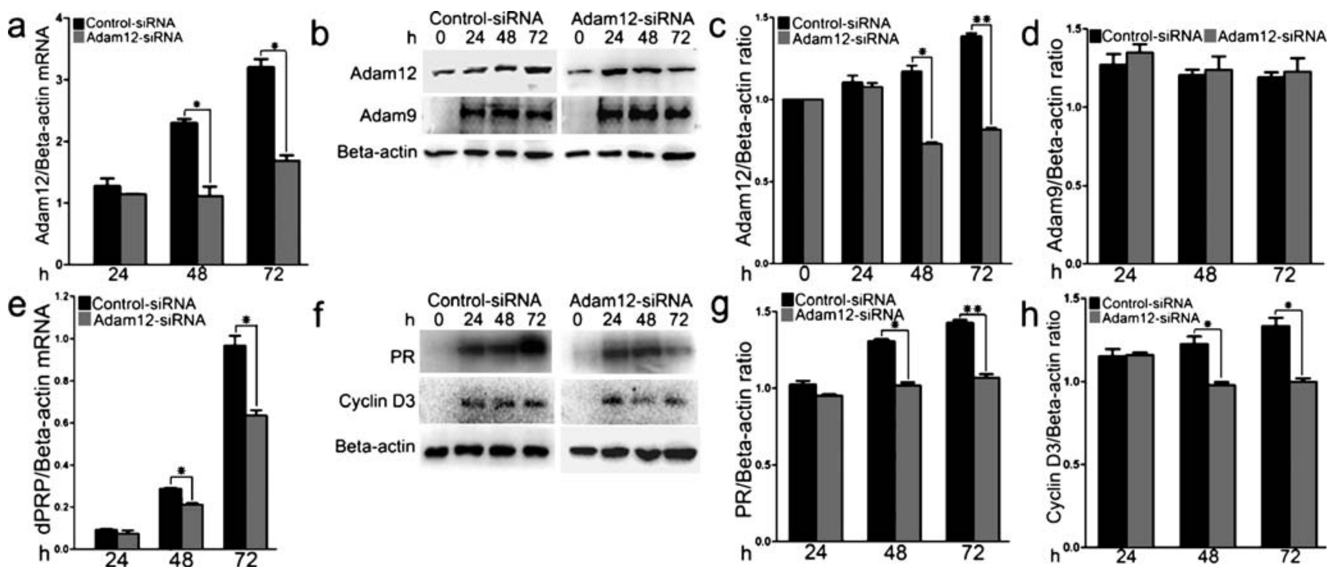

Fig. 4 Inhibition effect of siRNA-mediated down-regulation of Adam12 on decidualizing stromal cells. **a** qRT-PCR analysis to monitor Adam12 mRNA expression in the decidualizing stromal cells cultured up to 72 h (mean±SE of three independent experiments). $^{*}P<0.05$ as compared with Control-siRNA treatment. **b** Western blot analysis of Adam12 and Adam9 proteins in the decidualizing stromal cells cultured up to 72 h. **c** Densitometric values from Western blot analyses of Adam12 protein in the decidualizing stromal cells cultured up to 72 h (mean±SE of three independent experiments). $^{*}P<0.05$, $^{**}P<0.001$ as compared with Control-siRNA treatment. **d** Densitometric values from Western blot analyses of Adam9 protein in the decidualizing stromal cells cultured up to 72 h (mean±SE of three independent experiments). **e** qRT-PCR analysis to monitor dPRP mRNA expression in the decidualizing stromal cells cultured up to 72 h (mean±SE of three independent experiments). $^{*}P<0.05$ as compared with Control-siRNA treatment. **f** Western blot analysis of PR and cyclin D3 proteins in the decidualizing stromal cells cultured up to 72 h. **g** Densitometric values from Western blot analyses of PR protein in the decidualizing stromal cells cultured up to 72 h (mean±SE of three independent experiments). $^{*}P<0.05$, $^{**}P<0.001$ as compared with Control-siRNA treatment. **h** Densitometric values from Western blot analyses of cyclin D3 protein in the decidualizing stromal cells cultured up to 72 h (mean±SE of three independent experiments). $^{*}P<0.05$ as compared with Control-siRNA treatment

that the expression of Adam12 is not directly influenced by the implanting blastocyst.

In rodents, P_4 is specifically required for the decidualization process of stromal cells (Dey et al. 2004). We have shown that the expression of Adam12 in uteri is upregulated by P_4 treatment in ovariectomized mice, further pointing toward a potential role of Adam12 during decidualization. Using an induced decidualization culture system of mouse primary stromal cells, we have observed that the expression of Adam12 is greatly increased during the decidualization process. Observations that transfection the stromal cell with a siRNA selectively targeted to Adam12 mRNA noticeably reduce the expression level of Adam12 mRNA and protein, thereby blocking the progression of stromal cell decidualization, provide direct evidence that Adam12 is involved in regulating uterine decidualization. However, the molecular mechanism by which Adam12 regulates stromal cell decidualization warrants further investigation. Unexpectedly, the expression of Adam9, which was used to demonstrate that Adam12 siRNA was specific, was immediately upregulated after in vitro decidualization. This indicates that Adam9 is also involved in regulating uterine decidualization, but the functional significance of Adam9 in uterine decidualization is unclear. The disintegrin/metalloprotease family may thus play a more significant role in uterus decidualization.

Given the multiple functions of Adam12, several possible hints for its involvement in decidualization have been obtained. Adam12 has been previously suggested to be involved in regulating the activity of cell surface integrins, the subsequent reorganization of the actin cytoskeleton, and the attachment between the cell and extracellular matrix (ECM) components through the connection of its extracellular disintegrin-like and cysteine-rich domains (Kawaguchi et al. 2003). Cell-cell and cell-ECM interactions play an important role in the decidualization of stromal cells (Pafilis et al. 2007). Therefore, Adam12 might participate in the decidualization of stromal cells by indirectly regulating cell-cell and cell-ECM interactions. Whereas several proline-rich regions in Adam12 cytoplasmic domains contain some consensus binding sites for Rous sarcoma oncogene Src homology-3 domain-containing proteins (Seals and Courtneidge 2003), the binding of Adam12 to Src by its cytoplasmic domain might further mediate Src activity and the subsequent downstream signaling that stimulates cell proliferation and/or differentiation (Suzuki et al. 2000). Impairment of the decidualization of stromal cells in the

Src-deficient mouse indicates that Src is indispensable for decidualization in this species (Shimizu et al. 2005). The overlapping expression of Adam12 and Src indicates that Adam12 participates in the proliferation of stromal cells during decidualization by activating Src. Src activity induces Stat3 activity and further mediates cell proliferation (Garcia et al. 2001). Stat3 target genes are involved cell cycle regulation, including cyclin D3 (Leeman et al. 2006). Uterine stromal cell decidualization is characterized by stromal cell proliferation and their differentiation into decidual cells with polyploidy (Das 2009). D-type cyclins including cyclin D3 are the best known regulators of mammalian cell proliferation and the transition of the G1/S phase (Grana and Reddy 1995; Herzinger and Reed 1998). Cyclin D3 has been demonstrated to be important for the process of decidualization (Das et al. 1999). After Adam12-siRNA treatment, cyclin D3 expression decreases in comparison with the control. By using BrdU incorporation assay, we have shown that Adam12 inhibits the proliferation of stromal cell after Adam12-siRNA treatment ($P<0.05$, supplemental Fig. 2a–c); Adam12 might therefore function in decidualization through the regulation of cell cycle process. On the other hand, Adam12 might also promote cell proliferation by inducing the activation of growth factors including EGF (Zahnow 2006), HB-EGF (Shi et al. 2000), IGFBP-3 (Loechel et al. 2000), and transforming growth factor-β (Atfi et al. 2007). These growth factors are closely associated with stromal cell decidualization (Dey et al. 2004). Thus, Adam12 might be involved in the proliferation of stromal cells during the decidualization of stromal cells by activating these growth factors and subsequent downstream signaling in a paracrine manner.

Overall, our study provides multiple lines of evidence showing potential roles of Adam12 in uterine decidualization during early pregnancy in mice.

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