Expression of the Metastasis Suppressor KAI1 in Decidual Cells at the Human Maternal-Fetal Interface

Regulation and Functional Implications

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At the human maternal-fetal interface, the decidua forms a dense matrix that is believed to limit trophoblast invasion. We investigated whether the metastasis suppressor KAI1 (CD82) is expressed at the maternal-fetal interface. Immunohistochemistry showed strong expression of KAI1 in decidual cells, whereas trophoblast cells were negative for KAI1. In luteal phase endometrium, KAI1 was present in decidualizing endometrial stromal cells. We investigated whether KAI1 expression in endometrial stromal cells is regulated by the decidualizing stimuli cAMP and progesterone or by the cytokine interleukin (IL)-1 β . Western blot analysis revealed induction of KAI1 protein by cAMP analog, but not by progesterone, in a delayed fashion. In contrast, IL-1 β rapidly stimulated KAI1 expression at the transcript level and at the protein level. Cultured decidual cells from term placenta expressed a basal level of KAI1 protein that was elevated on cAMP stimulation. Silencing of KAI1 by RNA interference attenuated expression of decorin, a decidual product implicated in limiting trophoblast invasion. This study shows for the first time the expression of KAI1 in decidual cells at the human maternal-fetal interface, where the metastasis sup-

pressor might participate in intercellular communication with trophoblast cells and the control of trophoblast invasion. (Am J Pathol 2007; 170:126–139; DOI: 10.2353/ajpath.2007.060175)

Successful pregnancy requires coordinate progression of decidualization, placenta formation, and embryo development. Decidualization is a differentiation process of the endometrium, the mucosa lining the uterine lumen. In humans, decidualization occurs independently of the presence of a conceptus during the second half of the menstrual cycle and is controlled by progesterone- and cAMP-dependent events and modulated by cytokines, such as interleukin-1 β (IL-1 β), in a complex crosstalk of endocrine, paracrine, and autocrine signals.^{1–3} Decidualization transforms the fibroblastoid endometrial stromal cells into decidual cells in preparation for blastocyst implantation. Decidual cells are characterized by an enlarged polygonal shape, abundant secretion of marker proteins like decidual prolactin (dPRL) and insulin-like growth factor-binding protein 1 (IGFBP-1) and altered expression of extracellular matrix proteins.¹ The transformation of the endometrium into the decidua is also associated with a massive influx of leukocytes. In the late secretory phase of the menstrual cycle, they account for 30 to 40% of cells in the stromal compartment. The largest leukocyte population in the endometrium consists of uterine NK (uNK) cells.^{4,5}

The decidua forms a dense cellular matrix that is believed to generate a local cytokine environment promoting trophoblast attachment and to act as a physical barrier limiting trophoblast invasion.^{6,7} In humans,

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implantation and placentation involve breaching of the endometrial luminal epithelium by the trophoblast, invasion of the underlying maternal decidua, and formation of floating and anchoring chorionic villi.⁸ The unique structure of the human fetal-maternal interface is established by proliferation and differentiation of cytotrophoblast stem cells in the anchoring villi along various lineages. On the one hand, they form the terminally differentiated multinucleated syncytiotrophoblast lining the villus; on the other hand, they differentiate into extravillous trophoblast cells (EVTs).⁹ EVT cells are highly invasive, intrude the maternal tissue from the columnar tip of the anchoring villus, and further differentiate into interstitial and endovascular trophoblast cells. The latter penetrate the walls of maternal arterioles and eventually replace the maternal endothelial lining, remodel and widen the arterioles, and divert maternal blood flow to the intervillous space.^{6,8,10}

Hemochorial placentation as described above is critically dependent on the highly invasive nature of EVT cells, which reach as far as the first third of the myometrium.¹⁰ However, invasion must be tightly controlled in a temporal and spatial fashion to accommodate the needs of the growing embryo without compromising the mother's health. Remarkably, extravillous trophoblast shares several features with a malignant tumor: it has a high proliferative and invasive potential, it is immunologically tolerated by the host, and it disseminates into the host's vasculature. Nonetheless, unopposed proliferation and malignant transformation of trophoblastic cells are rare events, and metastases are not formed. This suggests that strict control mechanisms inherent in fetally derived cells and/or implemented by the invaded maternal tissue are in operation to ensure normal development.

Attachment to and penetration of the maternal tissue by the trophoblast involves the action of metalloproteinases (MMPs) to degrade extracellular matrix proteins as well as cell-cell communication via cell surface proteins to support adhesion and migration.¹¹ Thus, as cytotrophoblast cells differentiate, they change their repertoire of cell adhesion molecules, cadherins, and integrins.⁹ Decidual cells in turn express tissue inhibitors of MMPs (TIMPs), extracellular matrix proteins, and adhesion molecules that ascertain directed and limited invasion of the trophoblast.^{12,13} Additional decidual activities limiting trophoblast invasion include the secretion of transforming growth factor β (TGF β), which inhibits MMP production,^{14,15} and the production of decorin, a proteoglycan deposited in the extracellular matrix, which may serve as TGF β storage.¹⁶

Conceivably, metastasis suppressor proteins might also be involved in controlling trophoblast invasion. One such cell surface protein, KAI1 (named after the Chinese term for anticancer, "kang ai"), was originally described as a metastasis suppressor in prostate cancer¹⁷ but has subsequently been recognized as a general suppressor of the metastatic phenotype in many cancer types including those of the liver, colon, esophagus, pancreas, lung, bladder, ovary, cervix, and breast. Although KAI1 does not affect primary tumor growth, its loss of expression has been correlated with clinical progression of those tumors to metastasis.¹⁸ Structurally, KAI1 (CD82) belongs to the family of tetraspanin proteins (formerly called TM4SF proteins). They span the

plasma membrane four times such that the N and C termini are located intracellularly, and a small (EC1) and a large (EC2) extracellular loop are formed. The EC2 of the tetraspanins contains at least two disulfide bonds and a variable region that is the site of interaction with other transmembrane proteins.¹⁹ Such lateral interactions occur between tetraspanins and laminin-binding integrins, various members of the Ig superfamily (CD2, CD3, CD4, CD8, and MHC class I and II), proteoglycans, growth factor receptors and their ligands, eg, epidermal growth factor receptor (EGFR) and proHB (heparin binding)-EGF, and other tetraspanins. The extended network resulting from such interactions is also referred to as the tetraspanin web.¹⁹ KAI1 plays an important role in T-cell activation, triggers cytoskeletal changes, and enhances homotypic cell aggregations involving intercellular adhesion molecule 1.^{20,21} The mechanism of KAI1-mediated metastasis suppression is not fully understood, but the ability of KAI1 to interact with membrane proteins like EGFR, β 1 integrins, and E-cadherin seems to play a role in this context.^{18,22,23} Association of KAI1 with EGFR leads to enhanced receptor internalization and desensitization. Loss of KAI1 expression in epithelial tumors, particularly with concomitant EGFR overexpression, is predicted to promote EGF-induced signaling.²² In general, reduced KAI1 expression is associated with increased motility, reduced cell-cell interactions, and altered adhesion to extracellular matrix components.²⁴ Forced overexpression of KAI1 in a human lung carcinoma cell line significantly suppressed cell invasion and promoted TIMP-1 production, which in turn reduced MMP-9 activity.²⁵ Restoration of KAI1 expression in a metastatic prostate cancer cell line inhibited integrin-mediated cell migration, invasion, and activation of the tyrosine kinase receptor c-Met.²⁶ Recently, a new concept for KAI1-mediated metastasis suppression has evolved. When KAI1-expressing cancer cells disseminate into the vasculature, they interact with an endothelial cell-surface protein, the Duffy antigen receptor for chemokines (DARC). Direct binding of KAI1 to DARC inhibits tumor cell proliferation and induces senescence. In contrast, tumor cells not expressing KAI1 can proliferate at distant sites and give rise to overt metastasis.^{27,28}

Although it is known that KAI1 is expressed in a wide range of tissues, its presence in the utero-placental unit has not previously been investigated. The present study was designed to examine the expression pattern of KAI1 at the maternal-fetal interface and thus its potential implication in the control of trophoblast invasion. KAI1 expression was investigated by immunohistochemistry on paraffin-embedded placenta and endometrium samples and by Western blot analysis of isolated decidual cells from term placenta and cultured endometrial stromal cells in response to cAMP, progesterone, and IL-1 β stimulation.

Materials and Methods

Tissue Collection and Cell Culture

Paraffin-embedded placenta samples (including 15 from the first, 10 from the second, and 10 from the third trimester) and endometrium samples (10 from the prolifer-

ative phase and 10 from the secretory phase) were selected after histological review from the files of the Department of Gynecopathology, University Clinic Hamburg-Eppendorf.

Primary cultures of human endometrial stromal cells (ESCs) were prepared from anonymous uterine biopsy samples obtained from premenopausal women at the time of hysterectomy for benign gynecological disorders. Purified ESCs were prepared as previously described²⁹ and maintained in basal medium: phenol red-free Dulbecco's modified Eagle's medium/Ham's F12 supplemented with 10% steroid-depleted dialyzed fetal calf serum (FCS; PAA Laboratories, Cölbe, Germany), 100 U/ml penicillin, 100 μ g/ml streptomycin, and 0.2% Primocin (InvivoGen, San Diego, CA). Primary cultures of decidual cells were obtained by dissecting the decidua from term placenta, followed by enzymatic digestion and maintenance in the same medium as described for ESCs. The tissue material for primary cultures of ESCs was obtained from the Department of Gynecopathology, University Clinic Hamburg-Eppendorf, and term placenta for preparation of decidual cells was from the Department of Obstetrics and Gynecology, University Clinic Rostock. The local research and ethics committees approved this study, and patient consent was obtained before tissue collection. The MDA-MB-231 human breast adenocarcinoma cell line was maintained in Dulbecco's modified Eagle's medium supplemented with 10% FCS, 100 U/ml penicillin, and 100 μ g/ml streptomycin.

Magnet-Assisted Transient Transfection

An expression vector for human KAI1, pcDNA/KAI1, was kindly provided by K. Milde-Langosch (University Clinic Hamburg-Eppendorf). It was generated as follows: human KAI1 cDNA (nucleotides 181 to 985 in GenBank accession no. NM_002231.2) was amplified by polymerase chain reaction (PCR) from cDNA prepared from T47D breast cancer cells using primers incorporating a 5' *Hind*III and a 3' *Bam*HI site. The PCR product was partially digested with *Hind*III and *Bam*HI to retain the internal *Bam*HI site and inserted into the respective sites of the eukaryotic expression vector pcDNA3.1(+) (Invitrogen, Karlsruhe, Germany). For the preparation of protein extracts, MDA-MB-231 cells were plated at 10^6 cells/well in six-well plates 6 hours before transient transfection with pcDNA/KAI1 using a modified magnet-assisted transfection procedure. In brief, 0.5 μ l of Lipofectamine 2000 (Invitrogen) was added to 14.5 μ l of OPTIMEM (Invitrogen), and 225 ng of DNA was diluted in 28.5 μ l of OPTIMEM. Both solutions were mixed and incubated for 15 minutes at room temperature. Then 0.125 μ l of MA Lipofection Enhancer (IBA, Göttingen, Germany) was added, and incubation continued for another 15 minutes at room temperature. In the meantime, medium on the cells was changed to 2 ml of OPTIMEM. The DNA mixture was added to the cells, and the culture plates were placed on a magnet plate (IBA) at 37°C for 15 minutes. After removal of the magnet plate, cells were incubated for 16 hours before protein extraction.

For immunofluorescence analysis, MDA-MB-231 cells were plated in eight-well chamber slides (BD Biosciences, Heidelberg, Germany), and magnet-assisted transfection was performed as above, but the amount of vector DNA and the volume of all reagents were divided by a factor of 30. Cells were incubated in 250 μ l of medium for 48 hours before fixation.

Protein Isolation and Western Blot Analysis

Primary cultures of endometrial stromal or term decidua cells were treated with 0.5 mmol/L 8-bromo-adenosine cAMP (8-Br-cAMP; Biolog, Bremen, Germany) or 250 nmol/L progesterone (Sigma, Deisenhofen, Germany) in basal medium. For stimulation with IL-1 β (Strathmann Biotec, Hamburg, Germany), primary cultures were maintained under serum-free conditions in OPTIMEM. MDA-MB-231 cells were treated with 8-Br-cAMP in medium containing 10% FCS. Whole-cell extracts were prepared using the PARIS kit (Ambion, Huntingdon, Cambridgeshire, UK) or radioimmunoprecipitation assay buffer [phosphate-buffered saline with 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, and Complete protease inhibitors (Roche Applied Science)]. Nuclear and cytosolic protein extracts were prepared as previously described.³⁰ Proteins were electrophoresed on 10% SDS-polyacrylamide gels (NuPage Bis-Tris; Invitrogen) and transferred by tank blotting onto polyvinylidene difluoride Immobilon membranes (Millipore, Eschborn, Germany). For electrophoresis and transfer under nonreducing conditions, RIPA lysates were added to an equal volume of loading buffer without β -mercaptoethanol (10 mmol/L Tris, 10% SDS, 20% glycerol, and 0.01% bromophenol blue), and antioxidant was omitted from the NuPAGE transfer buffer (Invitrogen). For reducing conditions, 25% β -mercaptoethanol was included in the loading buffer, and electrotransfer was performed in the presence of 0.25% antioxidant. For detection of KAI1, the following monoclonal antibodies were used: G2 (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:200 dilution under reducing conditions and TS82b (Diacclone, Besançon, France) at 1:500 dilution under nonreducing conditions. Additional antibodies against KAI1 were tested: rabbit polyclonal antibodies H-173 and C-16 and goat polyclonal antibody N-14 (all from Santa Cruz Biotechnology). Monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (clone 6C5; HyTest, Turku, Finland) was used at 1:10,000 dilution, and rabbit polyclonal IGFBP-1 antibody (H-120; Santa Cruz Biotechnology) was used at 1:200 dilution. Mouse monoclonal antibody against decorin (clone 115402; R&D Systems, Wiesbaden, Germany) was used at 2 μ g/ml. Immunodetection was performed with the enhanced chemiluminescence system (SuperSignal; Pierce, Bonn, Germany).

siRNA Transfection

Primary cultures of endometrial stromal cells were seeded in 12-well plates. When cells had reached con-

Table 1. Oligonucleotides Used as Primers for RT-PCR Analysis

mRNA		Oligonucleotide	Position*	References
dPRL	s	5'-GACAGAGACACCAAGAAGATCGGAACATA-3'	-136/-107 (Exon 1a)	GenBank accession no. M58594, V00566 ²⁹
GAPDH	as	5'-CAAGGGGGCCACGCTCTGGCA-3'	66/86	GenBank accession no. HUMGAPDH
	s	5'-GGAGTCCACTGGCGTCTTCAC-3'	288/308	
	as	5'-GAGGGGCCATCCACAGTCTTCT-3'	554/575	
KAI1	s	5'-AGAAGTGGGCCCTGTGACC-3'	-61/-43	GenBank accession no. NM_002231
	as	5'-TTGCCCATGTTGAAGTAGAAGAG-3'	313/335	
L19	s	5'-GGGATTTGCATTTCAGAGATCAG-3'	367/388	GenBank accession no. NM_014763 ⁶²
	as	5'-GGAAGGGCATCTCGTAAG-3'	531/548	

*Positions are given relative to the first nucleotide of the ATG translational start codon.
s, sense; as, anti-sense.

fluency, medium was changed to 0.8 ml of OPTIMEM with or without 0.5 mmol/L 8-Br-cAMP and/or 10^{-6} mol/L progesterone. Per well, 80 pmol of short interfering RNA (siRNA) oligonucleotides targeting KAI1 (set of three oligonucleotides; Stealth Select RNAi; Invitrogen) and 1 μ l of MATra-A (IBA) were mixed in 40 μ l of OPTIMEM and added to the cells after a 15-minute incubation at room temperature. Parallel cultures received 80 pmol of non-targeting siRNA oligonucleotides (Stealth RNAi negative control oligonucleotides, medium GC content; Invitrogen). Culture plates were placed on a magnet plate (IBA) at 37°C for 15 minutes. After removal of the magnet plate, cells were incubated for 22 hours before medium was changed to basal medium with 8-Br-cAMP and progesterone as above. Seventy-two hours later, monolayers were harvested in 100 μ l of RIPA buffer, of which 10 μ l was loaded per lane for Western blot analysis.

RNA Extraction and Reverse Transcriptase (RT)-PCR

RNA was extracted from cultured cells with peqGold RNAPure reagent (Peqlab, Erlangen, Germany) according to the manufacturer's protocol, but the aqueous phase obtained after chloroform extraction was subjected to an additional purification step by phenol/chloroform/isoamylalcohol extraction. One microgram of RNA was used for oligo(dT)-primed cDNA synthesis with the ImProm-II Reverse Transcription System (Promega, Mannheim, Germany). Of the resulting 20 μ l of cDNA, 0.5 μ l was used per semiquantitative PCR reaction. Twenty microliters of PCR reaction mix contained 1 $\times$ Taq buffer (Promega), 0.2 mmol/L dNTPs, 2 pmol of sense and antisense primers, 1 mol/L betain, and 0.1 μ l of Taq DNA polymerase (Biotherm; GeneCraft, Münster, Germany). The oligonucleotides used as primers are listed in Table 1. All programs started with a denaturation step at 95°C for 4 minutes and terminated with an elongation step at 72°C for 10 minutes. Specific amplification conditions were as follows: for KAI1 cDNA: 22 cycles of 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 90 seconds; GAPDH cDNA: 20 cycles of 95°C for 30 seconds, 65°C for 30 seconds, and 72°C for 30 seconds; and L19 cDNA: 25 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 60 seconds. For amplification of dPRL

cDNA, a touchdown program was applied: three cycles each with decreasing annealing temperatures of 95°C for 30 seconds, 67/65/63/61/59/57°C for 30 seconds, and 72°C for 90 seconds, followed by 30 cycles of 95°C for 30 seconds, 54°C for 30 seconds, and 72°C for 90 seconds. PCR products were resolved in 2% agarose gels, stained with SYBR Gold (Molecular Probes; Invitrogen) and visualized in a Typhoon 8600 Imager (Amersham Biosciences, Freiburg, Germany).

Immunohistochemistry

Serial sections of 4 to 6 μ m were cut from formalin-fixed, paraffin-embedded tissues, mounted on aminopropyl-triethoxysilane-coated slides, deparaffinized in xylene, and rehydrated in graded alcohol to Tris-buffered saline (50 mmol/L Tris, pH 7.4, and 150 mmol/L NaCl). For the detection of KAI1, the slides were microwaved for 20 minutes in antigen unmasking solution (Linaris, Wertheim, Germany) and washed with phosphate-buffered saline (PBS) after cooling down for 20 minutes. Endogenous peroxidase activity was blocked by a 10-minute incubation in a solution of 0.75% H₂O₂ in methanol, followed by 20 minutes in normal goat serum (1:50 in PBS). After 15 minutes of avidin and biotin blocking (Linaris), slides were incubated with anti-KAI1 mouse monoclonal antibody G2 (Santa Cruz Biotechnology) at a dilution of 1:50. Slides were then reacted with biotinylated secondary antibody (Linaris) for 30 minutes. Staining was performed with the Vectastain Universal Elite ABC kit (Linaris) using diaminobenzidine as the substrate. Sections were counterstained with hematoxylin (Hemalaun Meyer; Merck, Darmstadt, Germany), dehydrated, and mounted (Eukitt; Labo-Med, Leipzig, Germany). Additional KAI1 antibodies tested for immunohistochemical application were rabbit polyclonal antibody C-16 (Santa Cruz Biotechnology) and monoclonal antibody clone 5B5 from two suppliers (Novocastra, Newcastle on Tyne, UK; and Labvision/Neomarkers, Fremont, CA). Procedures for antigen retrieval and details for the detection of cytokeratin 7 (CK7), vimentin, CD56, and CD68 are listed in Table 2. Histological and immunohistochemical evaluation was performed independently by two pathologists.

Table 2. Antibodies Used for Immunohistochemistry

Antibody (clone)	Supplier	Used as marker for	Pretreatment for antigen retrieval	Dilution
Cytokeratin 7 (OV-TL 12/30)	DakoCytomation	Trophoblast, epithelial cells	Trypsin without ethylenediamine tetraacetic acid, 15 minutes, 37°C	1:200
Vimentin (V9)	DakoCytomation	Decidua, mesenchymal cells	Antigen unmasking solution (Linaris), 20-minute microwave	1:1000
CD56/NCAM (123C3)	Zymed	Uterine NK cells, endovascular trophoblast	TEC buffer (2 mmol/L Tris, pH 7.8, 1.7 mmol/L ethylenediamine tetraacetic acid, and 1 mmol/L sodium citrate), 20-minute microwave	1:100
CD68 (PG-M1)	DakoCytomation	Macrophages	Antigen unmasking solution, 20-minute microwave	1:500

Immunofluorescence

Primary endometrial stromal cells or MDA-MB-231 cells were plated in eight-well chamber slides (BD Biosciences). Stromal cells were treated with 0.5 mmol/L 8-Br-cAMP for the periods indicated in the figure legends, and MDA-MB-231 cells were transfected with KAI1 expression vector as described above. Monolayers were fixed with 4% paraformaldehyde for 10 minutes at room temperature, followed by 10-minute incubation with 0.2% Triton X-100 if indicated. After washing with PBS, nonspecific binding was blocked with normal goat serum. Primary antibodies were diluted in PBS and left on the cells for 1 hour at room temperature. After washing with PBS, secondary antibody diluted in PBS/2% normal goat serum was added for 1 hour. Slides were mounted in ProLong Antifade (Molecular Probes) containing 0.1% 4,6-diamidino-2'-phenylindole for nuclear counterstain, and observed in a CKX41 microscope (Olympus) equipped with a 40× confocal water immersion objective (Apochromat 40×/1.2 W Korr.; Carl Zeiss, Jena, Germany) and a cooled digital camera (CC-12; Olympus, Hamburg, Germany). The following antibodies to KAI1 were applied: rabbit polyclonal antibody H-173 (1:50; Santa Cruz Biotechnology), mouse monoclonal antibodies BL2, TS82b (1:50; Diaclone), and G2 (1:100; Santa Cruz Biotechnology). A mouse monoclonal antibody and a rabbit polyclonal antibody to pan-cadherin (Abcam, Cambridge, UK) were used at 1:500 and 1:100 dilution, respectively. Secondary antibodies were Cy2- and Cy3-conjugated anti-mouse and anti-rabbit IgG (1:100; Dianova, Hamburg, Germany).

Results

Expression of KAI1 at the Maternal-Fetal Interface

Immunohistochemistry was performed on paraffin-embedded placenta samples with a monoclonal antibody (G2) to KAI1. To characterize the decidual and trophoblastic cell populations as well as macrophages present at the maternal-fetal interface, additional staining was performed with antibodies against vimentin, cytokeratin 7 (CK7), and CD68, respectively. CD56 (neural cell adhe-

sion molecule) was used as a marker for uNK cells, which have a CD56^{bright} phenotype, as opposed to peripheral blood NK cells, which are mainly CD56^{dim}.⁴ CD56 is also present on endovascular trophoblast.³¹

Figure 1 shows the immunohistochemical analysis of second trimester placenta. Strong expression of KAI1 was observed at the maternal-fetal interface, where KAI1 was localized to the decidual cells (Figure 1A), which are also positive for vimentin (Figure 1B) and negative for CK7 (Figure 1C). KAI1 showed clear plasma membrane localization in decidual cells (Figure 1G). Trophoblast cells (both villous and extravillous), identified by CK7 staining in Figure 1C, were negative for KAI1. Some additional KAI1-expressing cells could be identified as CD56-positive uNK cells (compare with Figure 1E) and CD68-positive macrophages (compare with Figure 1F; also present in the villous mesenchymal core), as has been previously reported in the literature.³² The same pattern of plasma membrane staining in decidual cells was obtained with a second monoclonal KAI1 antibody (clone 5B5), whereas antibody C-16 gave artifactual cytoplasmic staining in EVT and cytotrophoblast cells (not shown). Expression of KAI1 in decidual cells was observed in all analyzed samples but was more readily detected in samples of the first and second trimester, which contain more decidual tissue. Trophoblast cells of all lineages were consistently negative for KAI1.

Expression of KAI1 in the Endometrium

To investigate whether KAI1 expression in decidual cells was initiated under maternal control in the endometrium of the menstrual cycle preceding the establishment of pregnancy, we performed immunohistochemistry on endometrial biopsies. In the normal human endometrium, KAI1 expression was observed throughout the cycle with strongest expression in the late secretory phase. Macrophages and uNK cells, highly abundant in the stromal compartment of the late secretory phase, were strongly positive for KAI1 (Figure 2A, compare with E and F, respectively). Vimentin-positive (Figure 2B) and CK7-negative (Figure 2C) decidualized endometrial stromal cells, identified in the vicinity of blood vessels, also displayed, albeit weaker, KAI1 expression (Figure 2A). The protein was localized to the plasma membrane (Figure

Figure 1. KAI1 expression at the maternal-fetal interface. Paraffin-embedded tissue from second trimester pregnancy was analyzed by immunohistochemistry. **A:** Strong immunostaining for KAI1 (antibody G2) in the large decidua cells (D), which were also positive for vimentin (**B**). In addition, the mesenchymal core (MC) of the floating villi is vimentin-positive (**B**). **C:** Cytokeratin-7 (CK7) staining identified trophoblast cells. The extravillous trophoblast (EVT) emanated from the column of an anchoring villus (AV) and invaded the decidua (EVT, **arrow**). The villous trophoblast (VT, **arrows**) lined the anchoring and the floating villi (FV). All CK7-positive trophoblast cells were negative for KAI1 (compare with **A**). **D:** Negative control (omission of first antibody). **E:** Immunostaining for CD56 identified uNK cells. **F:** Immunostaining for CD68 identified macrophages. **G:** A decidua cell (**arrow**) showing clear membrane localization of KAI1. All images show tissue from one representative individual. Magnification: $\times 100$ (**A–F**); $\times 200$ (**G**).

Figure 2. KAI1 expression in the human endometrium. Paraffin-embedded tissue from the secretory phase of the menstrual cycle was analyzed by immunohistochemistry, using the same antibodies as indicated in Figure 1. **A:** KAI1 was present in the enlarged decidualizing endometrial stromal cells (S, **arrow**) near endometrial vessels (V) and in infiltrating leukocytes (see also **E** and **F**). **B:** Vimentin staining of endometrial stroma. **C:** CK7 identified luminal (LE, **arrow**) and glandular epithelia (GE, **arrow**), which were KAI1-negative. Endometrial stroma was CK7-negative. **D:** Negative control (omission of first antibody). **E:** CD56 immunostaining for uNK cells. **F:** CD68 immunostaining for macrophages. **G:** A decidualized endometrial stromal cell (**arrow**) in the vicinity of a vessel showing clear membrane localization of KAI1. **A** through **F** show tissue from one representative individual and were taken at $\times 100$ magnification; **G** shows tissue from a different individual and was taken at $\times 200$ magnification.

2G). In addition, low expression levels of KAI1 were also found in endometrial glands of the proliferative phase endometrium (not shown).

Inducible Expression of KAI1 in Cultured Endometrial Stromal and Decidual Cells

Decidualization of human ESCs is dependent on a persistent elevation of the intracellular cAMP concentration and is fine-tuned by progesterone-dependent signals.^{1,33} We hypothesized that KAI1 expression as seen *in vivo* in decidualized ESCs might be regulated by cAMP and/or progesterone. We also wished to assess subcellular localization of KAI1 in cultured cells because a recent report described a translocation of KAI1 from the membrane to the nucleus in cancer cells on treatment with etoposide, a DNA-damaging chemotherapeutic agent known to induce p53 expression. Furthermore, the authors reported coimmunoprecipitation of KAI1 and p53.³⁴ We have demonstrated previously that cAMP, but not progesterone, treatment of ESCs results in induction and nuclear accumulation of p53.³⁵ Cytosolic and nuclear proteins were extracted from ESCs that had been left untreated or treated with cAMP analog, progesterone, or a combination thereof for 3 days. As a reference, we prepared cytosolic extracts from MDA-MB-231 human breast cancer cells that had been transfected to overexpress KAI1. Western blot analysis revealed induction of KAI1 protein in ESCs by cAMP but not by progesterone. The addition of progesterone seemed to reverse the induction by cAMP. The protein was exclusively found in the cytoplasmic/microsomal preparation and was absent from the nuclear fraction (Figure 3A). It migrated at an apparent molecular weight of approximately 43 kd. Overexpressed protein isolated from MDA-MB-231 cells covered a wider range. A previous report showed that electrophoretic mobility of KAI1 is not affected by the absence or presence of reducing agent but depends on the extent of N-glycosylation. Migration has been observed between 45 and 90 kd; N-glycanase treatment reduces the apparent molecular weight to 28 kd.³⁶ Induction of KAI1 protein expression by cAMP, but not by progesterone, was confirmed on ESC cultures from a second individual (Figure 3B). Again, progesterone partially antagonized cAMP-mediated induction of KAI1 protein levels. Up-regulation of KAI1 protein was a consistent response to the cAMP stimulus: it was observed in seven of eight individual ESC preparations; KAI1 remained below the level of detection only in one preparation (data not shown).

We also prepared primary cultures of decidual cells from term placenta. Whole-cell extracts from untreated cultures or cultures on 3-day treatment with cAMP analog were subjected to Western blot analysis. In untreated term decidual cells, we found a basal level of KAI1 protein. This was elevated on cAMP stimulation (Figure 3C).

We wondered whether cAMP-dependent induction of KAI1 was a general phenomenon or specific to endometrial/decidual cells. Nontransfected MDA-MB-231 breast cancer cells were maintained in serum-supplemented

Figure 3. Western blot analysis of KAI1 in cultured endometrial and decidual cells. **A:** ESCs were cultured in the absence or presence of 8-Br-cAMP (0.5 mmol/L) and/or progesterone (250 nmol/L) for 3 days before cytosolic (CE) and nuclear extracts (NE) were prepared. As a size reference, cytosolic extract was prepared from MDA-MB-231 cells that had been transfected with pcDNA/KAI1 expression vector. Per lane, 30 μ g of protein was loaded. The blot was immunodetected with KAI1 antibody G2, then stripped and re-probed with GAPDH antibody as a loading control. Migration of size markers is indicated in kilodaltons. **B:** ESCs from a different individual were treated with cAMP and/or progesterone as described in **A**, and whole-cell extracts (30 μ g/lane) were subjected to Western blot analysis with a different KAI1 antibody (TS82b). After stripping, the blot was re-probed with GAPDH antibody. **C:** Whole-cell extract was prepared from term decidual cells that had been cultured in the absence or presence of 8-Br-cAMP (0.5 mmol/L) for 3 days, and 10 μ g of protein per lane was loaded. The blot was immunodetected with KAI1 antibody (G2), stripped, and detected with GAPDH antibody. Migration of molecular size markers is indicated in kilodaltons.

medium and exposed to increasing doses of 8-br-cAMP for 3 days. Surprisingly, Western blot analysis revealed a marked dose-dependent reduction of KAI1 protein as opposed to the induction seen in samples from cultured ESCs, which were included for comparison (Figure 4A). The reduction in KAI1 expression in MDA-MB-231 cells occurred primarily at the protein level because it was not reflected by a similar decrease in KAI1 mRNA (Figure 4B). Regulation of KAI1 expression by cAMP thus seems to be cell type specific.

We then investigated the kinetics of KAI1 induction by cAMP in ESCs. As shown in Figure 5A, KAI1 protein became faintly detectable after 2 days of treatment with cAMP but was clearly induced after 3 days. An extended time course showed cAMP induction of KAI1 protein after 3, 6, and 10 days (Figure 5B). Induction of the decidual phenotype by cAMP was confirmed by immunodetection of the decidualization marker IGFBP-1 in treated but not in untreated cells at all time points. From parallel cultures, we prepared mRNA for semiquantitative RT-PCR analysis. In contrast to KAI1 protein, KAI1 mRNA was not markedly regulated by cAMP, suggesting that expression is controlled mainly at a posttranscriptional level. Decidualization was underpinned by the pronounced induction of dPRL mRNA expression in cAMP-treated cells. No

Figure 4. KAI1 expression in response to cAMP in breast cancer cells. **A:** MDA-MB-231 cells were treated with increasing doses of 8-Br-cAMP for 3 days and analyzed for KAI1 expression by Western blot with antibody TS82b in comparison with untreated and 8-Br-cAMP-treated ESCs (30 μ g total protein/lane). The blot was stripped and reprobed with GAPDH antibody. **Lanes 1 and 3,** no treatment; **lanes 2 and 6,** 0.5 mmol/L 8-Br-cAMP; **lane 4,** 0.1 mmol/L 8-Br-cAMP; and **lane 5,** 0.25 mmol/L 8-Br-cAMP. **B:** KAI1 mRNA levels from the same MDA-MB-231 cultures as shown in **A, lanes 3 to 6,** were assessed by semiquantitative RT-PCR. The cDNA for ribosomal protein L19 was amplified as a loading control.

expression of the decidual marker gene was detectable in untreated cells.

One of the few known soluble ligands that stimulates KAI1 expression is the cytokine IL-1 β .³⁷ Cytokines are important local modulators of endometrial stromal cell function.^{1,2} IL-1 β is produced by various cell types in the human endometrium of the late secretory phase and in the decidua of early pregnancy, and it seems to be the major conceptus-derived factor inducing full decidualization in the baboon.^{2,38} Because the KAI1 promoter is a transcriptional target of nuclear factor- κ B (NF- κ B) as the downstream effector of IL-1 β signaling,³⁷ we investigated the effect of IL-1 β on KAI1 expression in ESCs after 1, 4, and 24 hours of stimulation both at the mRNA and at the protein level. An induction of KAI1 transcripts was detectable within 1 hour with increasing doses of IL-1 β (1, 5, and 20 ng/ml), was more pronounced after 4 hours, and seemed to regress toward 24 hours of stimulation (Figure 6B). With the higher doses, accumulation of KAI1 protein in the same individual cell preparation was visible within 1 hour, and all doses increased the protein levels within 4 hours and more strongly within 24 hours (Figure 6A). The massive time-dependent induction of KAI1 protein in response to IL-1 β treatment was reproduced in another individual culture (Figure 6C). It has to be noted that this extent of response was only seen under serum-free conditions (data not shown). Taken together, induction of KAI1 expression in ESCs by IL-1 β was a rapid and transcriptional event.

When establishing Western blot analysis for KAI1, we tested a range of different antibodies by comparing whole-cell extracts from nontransfected with those from transfected, KAI1-overexpressing MDA-MB-231 or COS-7 cells. Monoclonal antibody G2, which had also been used for immunohistochemistry (Figures 1 and 2),

Figure 5. Time course of cAMP-induced KAI1 expression in ESCs. **A:** Cultured ESCs were incubated in the absence or presence of 8-Br-cAMP for 1, 2, or 3 days. Five micrograms of total protein (extracted with the PARIS kit) were subjected to Western blot analysis with KAI1 antibody (TS82b), followed by detection of GAPDH. **B:** Cultured ESCs from a different individual were maintained in the absence or presence of 8-Br-cAMP for 3, 6, or 10 days, before whole-cell protein extracts (in RIPA buffer) and RNA were isolated. Western blot analysis was performed on parallel blots (20 μ g protein/lane) with antibodies against KAI1 (TS82b) and IGFBP-1. The asterisk denotes a nonspecific band detected with the IGFBP-1 antibody. After stripping, the top blot was reprobed with GAPDH antibody. **C:** Semiquantitative RT-PCR was performed to analyze KAI1, dPRL, and GAPDH expression on the corresponding RNA samples from the same cultures as in **B**.

gave reliable results. Monoclonal antibody TS82b was also specific but recognized KAI1 only under nonreducing conditions (Figure 5). This phenomenon has been described for other monoclonal KAI1 antibodies that apparently recognize a conformational epitope dependent on disulfide bridging in the extracellular loops.³⁶ Antibody H-173 recognized KAI1 but resulted in high background staining, whereas antibodies C-16 and N-14 strongly reacted with nonspecific bands but failed to detect KAI1. Because we wished to investigate subcellular localization of KAI1 by immunofluorescence studies, we first assessed the specificity of the commercial KAI1 antibodies for this application using transiently transfected MDA-MB-231 cells overexpressing KAI1. Monoclonal antibodies G2 and BL2 and polyclonal antibody H-173 readily reacted with transfected cells and showed extranuclear localization of KAI1. Contours of all cells, whether transfected or not, were visualized by concomitant staining with pan-cadherin antibody (Figure 7). These antibodies and TS82b, which had been used for immunodetection on Western blots, were then used on primary cultures of ESCs that had been plated on cover slides and treated with cAMP for 10 days. All antibodies gave essentially the same pattern of punctate extranuclear structures and plasma membrane staining. Neither by immunofluorescence analysis nor by Western blotting did we find any indication for nuclear localization of KAI1, in contrast to the report by Wu et al³⁴ who had used a polyclonal antibody from Santa Cruz Biotechnology (without further specification) for Western analysis, immunofluorescence, and immunohistochemistry.

Figure 6. Time- and dose-dependent induction of KAI1 expression in ESCs by IL-1 β . **A:** Cultured ESCs were incubated with increasing doses of IL-1 β (lanes 2, 5, and 8, 1 ng/ml; lanes 3, 6, and 9, 5 ng/ml; and lanes 4, 7, and 10, 20 ng/ml) for 1, 4, or 24 hours or were left untreated (lane 1). Total protein (6 μ g/lane) was analyzed by Western blotting with KAI1 antibody TS82b. The blot was stripped and reprobed with GAPDH antibody. **B:** From the same cultures as indicated in **A**, RNA was extracted and analyzed for KAI1 and L19 expression levels by semiquantitative RT-PCR. **C:** ESCs prepared from a different individual were exposed to IL-1 β (20 ng/ml) for 0, 1, 4, or 24 hours. Total protein (6 μ g/lane) was analyzed with KAI1 and GAPDH antibodies as indicated above.

Effect of KAI1 Silencing in ESCs

To dissect the role of KAI1 in decidualization, we silenced KAI1 expression in ESCs by RNA interference. In cells transfected with a negative control siRNA, the same pattern of KAI1 induction was observed as shown above in nontransfected cells: basal KAI1 protein was low and not increased by addition of progesterone alone. Treatment with 8-Br-cAMP elicited a marked induction that was slightly reduced on further addition of progesterone (Figure 8). The decidualization marker IGFBP-1 was markedly up-regulated by the cAMP analog but not affected by progesterone. Next to IGFBP-1, microarray analyses have revealed decorin as one of the most strongly up-

Figure 7. Detection of KAI1 in cultured cells. **a, c, and e:** MDA-MB-231 cells were transiently transfected with pcDNA/KAI1, fixed with paraformaldehyde, and permeabilized with Triton X-100. Dual immunofluorescence was performed with KAI1 antibody followed by Cy3-labeled secondary antibody (red) and pan-cadherin antibody followed by Cy2-labeled secondary antibody (green). **b, d, f, and g:** Primary cultures of ESCs were treated with 8-Br-cAMP for 10 days, fixed with paraformaldehyde, and subjected to immunofluorescence with KAI1 antibodies and Cy3-labeled secondary antibody (red). The following antibodies were used: KAI1 antibodies G2 (**a** and **b**), BL2 (**c** and **d**), H173 (**e** and **f**), and TS82b (**g**); rabbit pan-cadherin antibody (**a** and **c**); and mouse pan-cadherin antibody (**e**). Nuclei were counterstained with 4,6-diamidino-2'-phenylindole.

regulated genes in decidualizing stromal cells.³⁹ We also observed a massive induction of decorin production on treatment with 8-Br-cAMP but not progesterone (Figure 8). Decorin is produced as a core protein migrating as a doublet around 46 kD apparent molecular mass. The mature proteoglycan decorin is formed by attachment of a glycosaminoglycan chain⁴⁰ and migrates between 70 and 100 kD in our cell system. Silencing with siKAI1 effectively abrogated KAI1 protein expression in all cultures. Interestingly, the cAMP-mediated induction of both IGFBP-1 and decorin core protein was blunted, and most notably, expression of mature decorin proteoglycan was ablated on knockdown of KAI1 (Figure 8).

In summary, KAI1 expression *in vivo* was shown in decidualized endometrial stromal cells of the late secretory phase and in decidual cells at the maternal-fetal interphase. In primary cell cultures, KAI1 was demonstrated to be up-regulated in ESCs in response to cAMP in a delayed fashion consistent with the kinetics of cAMP-induced decidualization, whereas IL-1 β elicited a rapid induction of KAI1 expression. Silencing of KAI1 in ESCs

Figure 8. Effect of KAI1 silencing. Primary cultures of ESCs were transfected with nontargeting siRNA oligonucleotides (si-negCo) or with siRNA oligonucleotides targeting KAI1 (si-KAI1) and stimulated for 4 days with 8-Br-cAMP and/or progesterone, as indicated. Equal amounts of whole-cell extract were electrophoresed under nonreducing conditions to detect KAI1 by Western blotting. The blot was stripped and reprobed with GAPDH antibody as a loading control. For detection of decorin, aliquots of the same samples were separated and blotted under reducing conditions (core, core protein doublet; PG, mature proteoglycan). The blot was sequentially stripped and reprobed with IGFBP-1 and GAPDH antibodies.

exposed to a decidualization stimulus resulted in a marked reduction of IGFBP-1 and decorin protein levels.

Discussion

Trophoblast tissue has the capacity to invade the maternal decidua using similar mechanisms as those used by malignant tumors. However, as opposed to malignant invasion, trophoblast invasion is strictly limited. Knowledge of the factors regulating this process is of paramount importance for understanding and potentially controlling both physiological (embryonic implantation) and pathological (malignant tumor) invasion. Several mechanisms seem to be inherent in EVT to limit their own invasiveness: EVTs produce TIMPs that control activity of self-secreted MMPs; deeply invaded EVTs lose coordinated expression of integrins to match the extracellular matrix ligands in the surrounding tissue, and they fuse to form multinucleated trophoblastic giant cells, which are no longer invasive. A subset of EVTs undergoes programmed cell death. Induction of apoptosis may also be triggered by decidua-derived tumor necrosis factor α . Corticotropin-releasing hormone, produced both by the trophoblast and the maternal decidua, has been shown to down-regulate expression of the carcinoembryonic antigen-related cell adhesion molecule 1, a cell surface molecule implicated in the invasive potential of EVTs.^{41,42}

In this study, we investigated for the first time the expression pattern of the metastasis suppressor KAI1 in the utero-placental unit. As shown by immunohistochemistry, strong expression of KAI1 was observed at the maternal-fetal interface, where KAI1 was expressed by the decidual cells and localized to the plasma membrane. All populations of trophoblast cells (villous and extravillous), identified by CK7 staining, were negative for KAI1. In the endometrium of the menstrual cycle, KAI1 was localized to decidualizing endometrial stromal cells. Furthermore, macrophages and uNK cells, highly abundant between decidual cells in early pregnancy and in the stromal compartment of the secretory phase endometrium, were strongly positive for KAI1.

Our observations indicate that expression of KAI1 is initiated in the endometrial stromal cells in the secretory phase of the menstrual cycle coinciding with decidualization. Like the decidualization process, which in humans is independent of a blastocyst-derived signal, the induction of KAI1 also seems to be a maternally driven event and may serve to render the endometrial lining ready to cope with the invading trophoblast in the event of pregnancy. Induction of KAI1 expression in response to a decidualizing stimulus could be reciprocated in cultured ESCs. We demonstrated that KAI1 protein expression in ESCs is induced by cAMP in a delayed but persistent fashion. Up-regulation was apparent after 2 to 3 days and was maintained for at least 10 days. It coincided with the expression of dPRL mRNA and IGFBP-1 protein, which are well-established markers of decidualization. A persistent elevation of intracellular cAMP in stromal cells is essential for acquisition and maintenance of the decidual phenotype.^{1,43} In the late secretory phase of the menstrual cycle, endometrial stromal cells are exposed to a variety of local and endocrine factors that bind to G-protein coupled receptors and stimulate the production of cAMP, including prostaglandin E₂, relaxin, corticotropin-releasing hormone, and the gonadotropins luteinizing hormone and follicle stimulating hormone.¹ In pregnancy, the decidua is exposed to vast amounts of human chorionic gonadotropin (hCG) secreted by the syncytiotrophoblast, and signaling through the luteinizing hormone/hCG receptor involves cAMP as a second messenger.⁴⁴ All of these ligands are candidates for cAMP-dependent induction of decidual KAI1 expression, which we demonstrated *in vivo* in secretory phase endometrium and in the decidua of pregnancy at least up to the second trimester. We also showed that in cultured decidual cells isolated from term placenta, basal KAI1 expression was present and could further be stimulated by cAMP treatment. Progesterone alone did not induce KAI1 protein expression in endometrial stromal cells, although this ovarian steroid is the key orchestrator of decidualization *in vivo*. However, this observation is consistent with the realization that, at the cellular level, persistent stimulation of the protein kinase A pathway is the crucial process driving decidualization, whereas progesterone serves to modulate the response and is required for long-term maintenance of the decidual phenotype.^{1,45}

From a technical viewpoint, we noticed that numerous commercially available antibodies to KAI1 gave artifac-

tual results in Western blot applications and that adequate sample preparation was critical. Reducing agents may destroy the epitopes recognized by certain monoclonal antibodies. A recent report showing nuclear translocation of KAI1 and, moreover, direct interaction of nuclear KAI1 and p53 by coimmunoprecipitation and immunoblot detection³⁴ should be considered with caution. We found no indication of nuclear localization of KAI1 in our system, neither by Western blot analysis of nuclear extracts nor by immunofluorescence or immunohistochemistry.

The present knowledge on the regulation of KAI1 expression is limited. It has been shown that a KAI1 promoter/reporter gene construct can directly be activated by the tumor suppressor protein p53, and a p53 response element was identified 860 bp upstream of the transcriptional start site in the KAI1 gene.⁴⁶ This response element was later found to be neighbored by binding motifs for AP2 and AP1 transcription factors, and loss of wild-type p53 and/or loss of AP2 or the AP1 protein junB correlated with a down-regulation of KAI1 mRNA levels in a series of prostate cancer cell lines.⁴⁷ Yet the relevance of p53 for KAI1 expression is still controversial; a different group could not detect KAI1 up-regulation on DNA damage-induced p53 activation.⁴⁸ It is probably the combination of AP2, AP1, and p53 that leads to high-level KAI1 promoter activity.⁴⁹ We recently reported that during cAMP-induced decidualization, wild-type p53 is massively up-regulated in human ESCs.³⁵ It was therefore tempting to speculate that p53 might be the key to induction of KAI1 expression in decidualizing cells. However, although the level of KAI1 protein was clearly regulated in response to cAMP, no corresponding change was seen in KAI1 mRNA levels. Furthermore, the induction of KAI1 protein by cAMP only manifested after 2 to 3 days of stimulation. The cAMP-mediated induction of KAI1 protein therefore seems to occur predominantly at a posttranscriptional level, a phenomenon that we had also observed for the increase in p53 protein, which mainly results from protein stabilization.³⁵ Interestingly, cAMP-dependent regulation of KAI1 protein levels seems to be a cell type-specific phenomenon. In a breast cancer cell line, we observed a marked down-regulation of KAI1 by cAMP in contrast to the stimulation in ESCs and decidual cells. Yet again, altered KAI1 expression was mainly seen at the level of the protein. A nontranscriptional mode of KAI1 regulation in response to cAMP is also consistent with the outcome of a genome-wide search for cAMP-responsive genes that did not return CD82/KAI1.⁵⁰

In contrast, the KAI1 promoter is a target of NF- κ B, a transcription factor downstream of IL-1 β signaling.⁵¹ When NF- κ B p50 occupies its binding site in the KAI1 promoter, it recruits the coactivator Tip60 or the repressor β -catenin. The transcriptional outcome depends on the relative cellular levels of Tip60 versus β -catenin. Relative overexpression of β -catenin in metastatic cells could thus explain the loss of KAI1 expression in metastatic progression and the observed failure of metastatic prostate, breast, or colon cancer cells to up-regulate KAI1 in response to IL-1 β .⁵² Notably, conceptus-derived IL-1 β has

been implicated in the induction of endometrial stromal IGFBP-1 expression during decidualization in the primate.³⁸ In our primary culture system of human ESCs, we observed a rapid and pronounced increase in KAI1 protein levels in response to IL-1 β that could be accounted for by transcriptional induction. Maternally derived or trophoblast-derived IL-1 β , in conjunction with hCG, may thus contribute to the maintenance of KAI1 expression in the decidua of pregnancy.

Trophoblast invasion involves proteolysis and remodeling of the maternal decidua. In addition to the MMPs, the plasminogen system contributes to these processes. Binding of urokinase plasminogen activator (uPA) to its membrane-anchored receptor (uPAR) leads to pericellular conversion of plasminogen to the active serine protease plasmin. This system is negatively controlled by the plasminogen activator inhibitor type 1.¹² In the placental bed, the interstitial EVT is positive for uPA and plasminogen activator inhibitor type 1 but does not express uPAR. Decidual cells in turn are uPAR positive but are uPA-negative in those areas that are invaded by the EVTs. Terminally differentiated trophoblast giant cells are both uPA- and uPAR-negative.⁵³ Interestingly, KAI1 has been observed to reduce the proteolytic function of uPAR. In a model system of human normal breast cells stably transfected with KAI1, expression of this tetraspanin did not affect the level of uPAR but reduced its ligand binding. KAI1 caused a redistribution of uPAR to focal adhesions in conjunction with the integrin $\alpha 5 \beta 1$, resulting in loss of uPA binding.⁵⁴ It is conceivable that one of the functions of KAI1 in decidual cells at the trophoblast invasion front is to reduce plasmin activation, which would result from interaction of the decidual uPAR with trophoblast-derived uPA. Decidual KAI1 would thus limit local proteolytic activity.

Tetraspanins act as molecular facilitators by their lateral association with other transmembrane proteins, thus modulating growth factor signaling and processes of adhesion, migration, and differentiation.¹⁹ It is less clear if they can also function as cell surface receptors for secreted soluble proteins, extracellular matrix components, or surface molecules on opposing cells.¹⁹ Such a role is supported by findings on the tetraspanin CD9, which has been claimed to interact with fibronectin via the EC2⁵⁵ and to act as a receptor for pregnancy-specific glycoprotein 17, a secretory product of the mouse placenta. Binding of pregnancy-specific glycoprotein 17 to CD9 on macrophages stimulates secretion of various interleukins, prostaglandin E₂, and TGF β .⁵⁶ Binding of a monoclonal anti-CD82 antibody to KAI1/CD82 on T lymphocytes, apparently acting as a pseudo-ligand, has been shown to induce downstream effects on activation and cytoskeletal reorganization.⁵⁷ Most recently, KAI1 expressed on tumor cells was shown to interact with DARC, a cell-surface protein on vascular endothelial cells. This association inhibits the proliferation of disseminating tumor cells and induces senescence.^{27,28} Future investigations should address whether KAI1 expressed by decidual cells can participate in intercellular communication with trophoblast cells by cell-cell contact. We report here that silencing of KAI1 in ESCs exposed to a decidualization stimulus

results in reduced expression of the decidualization marker IGFBP-1. This implies that KAI1 expression is intimately linked with the process of differentiation and not merely a consequence of it. Furthermore, we demonstrate that expression of decorin, another product highly up-regulated on decidualization, depends on the presence of KAI1. Although the underlying mechanism remains to be elucidated, this observation has interesting implications. Decidual decorin is deposited in the extracellular matrix and has been proposed to serve as a storage of inactive TGF β , which is released by proteolytic activity of EVTs to exert its anti-invasive function.¹⁶ Decorin itself prevents metastatic spreading of breast cancer *in vitro* and *in vivo* and inhibits tumor xenograft growth, probably by down-regulation of EGFR tyrosine kinase signaling.^{58,59} This occurs by direct binding of decorin to the EGFR, followed by receptor internalization and degradation.⁶⁰ It is interesting to note that HB-EGF stimulates differentiation of cytotrophoblast to EVTs and enhances migration and invasion,⁶¹ whereas decorin inhibits migration and invasiveness of EVTs.¹⁶ Collectively, our data imply that KAI1 is involved in decidual transformation of ESCs and that decidual KAI1 expression is important to sustain decorin production, which in turn serves to control trophoblast invasion.

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