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Evidence of a limited contribution of feto-maternal interactions to trophoblast differentiation along the invasive pathway

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Abstract

Trophoblast differentiation is a key event in human placental development. During extravillous trophoblast (EVT) differentiation, stem cells from the anchoring villi detach from their basement membrane and proliferate to form aggregates called trophoblast cell columns (TCCs). They subsequently invade the decidua and differentiate into interstitial and endovascular trophoblasts. The influence of the decidua on EVT differentiation is controversial. We therefore compared the pattern of trophoblast differentiation marker expression in viable intrauterine and tubal pregnancies, as decidual cell markers (prolactin [PRL] and insulin-like growth factor binding Protein-1 [IGFBP1]) were only expressed in endometrial implantation sites. Extravillous trophoblast differentiation in anchoring villi from uterine and ectopic pregnancies exhibited a comparable phenotypical switch: α6 integrin subunit, E-cadherin, EGF receptor, Ki 67 and connexin 40 were localized in the proximal part of the TCC, while α5βl and αl integrins, c-erb B2, hPL and HLA-G were expressed by invasive cytotrophoblasts. The cyclin-dependent kinase inhibitors pl6 and p57 were mainly detected in invasive cytotrophoblasts some distance from the columns. However, the TCC was markedly longer in tubal pregnancy than in intrauterine pregnancy. These findings suggest that the decidua is not necessary to trigger EVT invasion, but that it is likely to limit the extent of the TCC and to accelerate the onset of EVT migration.

Key words: deciduas; fallopian tube; implantation; trophoblast

INTRODUCTION

Successful human placentation necessitates a series of events involving cellular and molecular interactions between specialized fetal and maternal cells. Two major morphologically and functionally distinct cytotrophoblast populations can be identified in human placenta. Villous cytotrophoblasts are polarized immotile cells that fuse to form the overlying syncytium in direct contact with maternal blood. Extravillous cytotrophoblast (EVT) cells derive from cytotrophoblasts (CTBs) that rest on a basement membrane surrounding the tips of anchoring villi. Extravillous trophoblasts proliferate and differentiate as they leave their basement membrane and form cellular aggregates (Le. trophoblast cell columns: TCC). When they become invasive, individual cytotrophoblasts invade the endometrium and, eventually, its arterial system (1). The human placenta is characterized by extensive invasion of EVTs into the maternal uterus, allowing trophoblasts to be in direct contact with maternal blood (hemochorial placentation) (2, 3). The EVTs switch from a proliferative to an invasive phenotype is governed by the sequentially regulated expression of a pleiad of genes (4-9). Extravillous trophoblast invasion proceeds mechanisms similar to those used by malignant cells to invade host tissues (10,11).

In normal pregnancy, EVTs are in close contact with maternal decidual cells derived from the differentiation of endometrial stromal cells under the influence of progesterone. Decidualization is a feature unique to those species that exhibit invasive hemochorial placentation (12). The extent of decidualization in mammals is closely related to the degree of trophoblast invasiveness into the uterus (13). The most pronounced trophoblast invasion and decidual changes occur in humans. Decidualization is characterized (1) by the presence of swollen endometrial stromal cells (14), limited by a basement-membrane-like material (15, 16) (2), by the acquisition of specific endocrine activities, and (3) by the presence of numerous immune cells (17).

Decidualization is generally regarded as an endometrial barrier that limits trophoblast invasion and protects the uterus from irreversible damage as a result of excessive hemochorial placentation (18-20). Others consider that

the decidua promotes trophoblast migration (12, 21). Several *in vitro* studies have attempted to evaluate the potential role of isolated decidual components in trophoblast activities (19, 21-23). However, *in vitro* experimental settings fail to mimic the complex *in vivo* situation. Ectopic pregnancy occurs in tissues that are classically considered to exhibit impaired or absent decidualization (2, 24-26).

In this study, we first compared the expression of specific markers of decidualization (prolactin and IGFBP-1) in intrauterine and tubal pregnancies. We then examined the distribution of several markers of EVT proliferation, invasion and differentiation in normal and ectopic pregnancies in order to determine the possible contribution of the decidua to their expression by the EVTs. Despite the absence of decidualization markers in tubal pregnancy, the patterns of cadherins, integrins, connexins, epidermal growth factor receptor (EGF-R), c-erb-B2, human placental lactogen (hPL), human leukocyte antigen-G (HLA-G) and cyclin-dependant kinase inhibitors (pl6, p57) were identical to those of intrauterine pregnancies. However, the length of the TCC was considerably lower in intrauterine pregnancy.

These data indicate that the acquisition of the EVT invasive phenotype occurs in the absence of decidua. They are however, consistent with a role of maternal decidual in limiting the size of the TCC and promoting the dissociation of cell aggregates and thereby the spreading of individual EVTs.

MATERIALS AND METHODS

Tissue sources

Ectopic implantation sites were collected during 60 laparoscopic salpingectomies performed for tubal pregnancy. Age-matched first-trimester placentas were obtained by aspiration during legal elective abortion for non-medical reasons. In addition, full-thickness implantation sites in one hysterectomy specimen (10 weeks) were also studied. The protocol was approved by our local university Ethics Committee. The gestational age and viability of tubal pregnancies were determined by vaginal ultrasonography immediately before surgery (measure of the crown-rump length and fetal heart activity) and were also calculated from the time of embryo transfer during IVF procedures (Table 1). Specimens were frozen in liquid nitrogen and stored at -70°C until use, or fixed in neutral buffered 10% formalin before embedding in paraffin.

Among the 60 pregnant tubes collected by salpingectomy, only six fulfilled the criteria of evolutive pregnancy. In these latter pregnancies, anchoring villi were observed with no evidence of necrosis, marked inflammatory infiltration or hemorrhage. In aborted ectopic pregnancies, typical anchoring villi could not be detected in all cases.

The length of the trophoblast cell column was compared at tubal and intrauterine implantation sites. Using Olympus Micro-image 3.0.1 software (Omnilabo, Aartselaar, Belgium), we measured the distance separating the fetal mesenchyme core from the mesenchyme of the maternal decidua (for example, see → in Figs 1B3 and B4). Tubal pregnancies (n = 6) were compared with placental tissue obtained either by aspiration (n = 10) or by hysterectomy (one gravid specimen at 10 weeks of pregnancy). One hundred columns attached to the maternal site were measured. Statistical analysis was performed using the Mann-Whitney test. P-values less than 0.05 were considered to denote significance.

Morphological studies

Antibodies

The sources of the first-step antibodies are indicated in Table 2. Rhodamine-labeled rabbit antimouse and swine antirabbit antibodies were purchased from Dako (Glostrup, Denmark) (Table 2).

Immunohistochemistry on frozen specimens

Frozen sections (6 µm thick) were prepared using a Leica CM3050S cryostat and collected on 3-aminopropyltriethoxysilan (Sigma, St Louis, MO) -coated glass microscope slides (Labonord, Belgium).

Sections were fixed in acetone (-20°C) for 10min (for integrins α1, x5β1 and α6 and E-cadherin staining), in 4% paraformaldehyde for 20min (for HLA-G, Ki-67, cyclin-dependent kinase inhibitors pl6 and p57, EGF-R and c-erb B2 staining) or in methanol at -20°C for 10min (for connexin 32, 40 and 43 staining).

After fixation, slides were washed three times in phosphate-buffered saline (PBS) for 5min. Non-specific binding sites were blocked by placing the slides in 10% bovine serum albumin (BSA, Sigma) for 30min. For EGF-R, c-erb B2 and connexins 32, 40 and 43, the blocking solution consisted of 2% BSA and 1% Triton X100 in PBS. After three washes in PBS, the slides were incubated for 1 h with an appropriate dilution of the primary antibody (in 1% BSA) or overnight at 4°C (for connexins 32, 40 and 43). After five further washes, the second antibody conjugated to rhodamine was applied for 30 min and the slides were washed three times in PBS for 5 min. After incubation with the different antibodies, nuclei were labeled with 4',6-diamidino-2-phenylindole (DAPI, 1µgmL^{-1}) for 20 min, washed for 5 min in PBS and mounted with Aquapolymount anti-fading solution (Agar, UK). All incubations and washes were performed at room temperature. Appropriate controls were prepared with the same procedure but omitting the primary antibody or replacing the primary antibody with a non-specific IgG antibody at the same concentration. The controls were always negative. Samples were examined with an Olympus IX50 fluorescence microscope equipped with appropriate filters (Omnilabo, Aartselaar, Belgium).

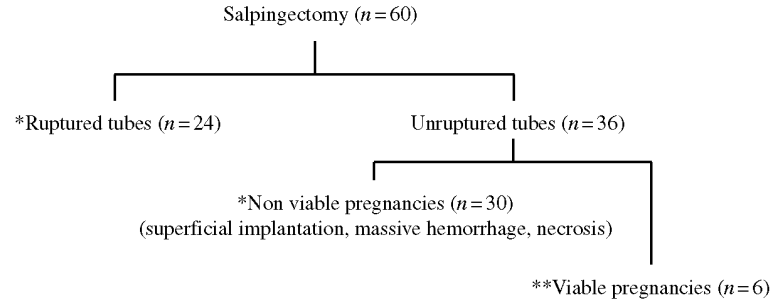


Table 1: *Salpingectomy*

No.	Implantation site	Circumstances of diagnosis	Gestational age (weeks)	HCG (IU mL^{-1})
1	Ampulla	Asymptomatic	11	58,000
2	Ampulla	Abdominal pain	8	9,640
3	Ampulla	Abdominal pain	8	13,100
4	Ampulla	IVF follow up	7	8,600
5	Ampulla	IVF follow up	6	7,000
6	Ampulla	IVF follow up	6	7,600

*Cases excluded from detailed immunohistochemistry after histopathological evaluation. **Cases selected for precise analyses and immunohistochemistry. *n* = 60.

Immunohistochemical staining of IGFBP-1, hPL and cytokeratin 7

Paraffin sections (6 µm thick) were de-waxed in xylene and rehydrated in PBS. Endogenous peroxidase activity was quenched by incubation with 3% hydrogen peroxide in PBS for 10 min. Non-specific antibody binding was blocked by incubation for 30 min in blocking reagent containing 10% BSA in PBS. The primary antibodies (Table 2) were diluted in 1 % BSA and incubated with the sections for 1 h at room temperature. After washing, a peroxidase-conjugated swine anti-rabbit or rabbit antimouse antibody was applied for 30 min at room temperature. The slides were then incubated with a solution of diaminobenzidine and 0.03% hydrogen peroxide, then washed in running tap-water and counterstained with hematoxylin. Sections were mounted in Eukitt (Labonord, Belgium), examined and photographed under an Olympus IX50 microscope. Controls were prepared as described above.

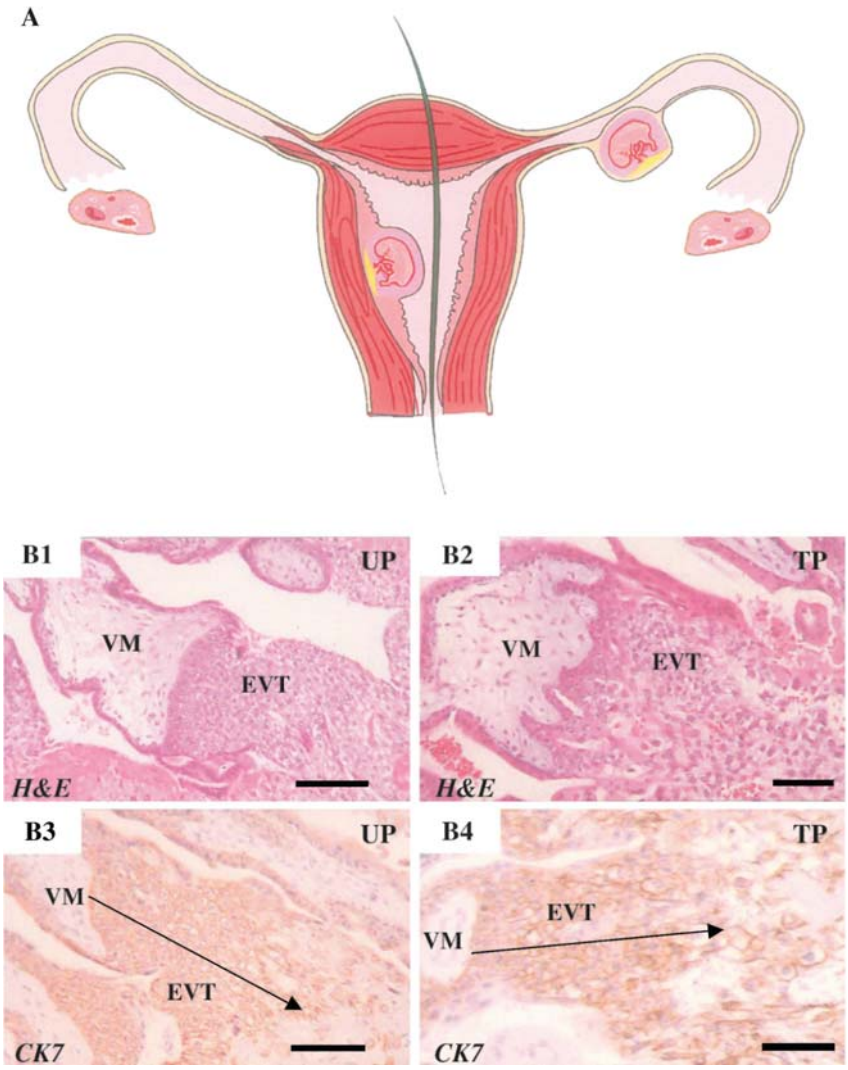
Prolactin RT-PCR

Tubal implantation sites (*n* = 6) containing floating and anchoring villi and the tubal wall were identified histologically on hematoxylin-stained frozen slides. Ten 10-µm thick frozen sections were prepared in a Leica CM3050S cryostat and placed in lysis buffer (Rneasy Mini Kit, Qiagen, GmbH, Hilden, Germany) for RNA extraction. Using the same procedure, RNA was also extracted from normal implantation sites of five

intrauterine first-trimester pregnancies and from six uterine decidua samples from the patients who had tubal pregnancies (n = 6). Prolactin gene expression (PRL) was also examined in endometrial tissue from the proliferative phase (negative controls, n = 10) and the late luteal phase (positive controls, n = 10).

For RNA purification, samples were lyzed, then homogenized and processed as recommended by the manufacturer.

Fig. 1: *(A) First-trimester uterine and fallopian tube pregnancies. (B) First-trimester anchoring villous with extravillous trophoblasts (EVTs) forming the cell column at the fetal-maternal interface in uterine pregnancy (UP) and viable tubal pregnancy (TP) (B1 and B2: hematoxylin and eosin staining).* Scale bar = 100µm. Immunoperoxidase labeling with anticytokeratin 7 antibody stained all trophoblast populations, including the villous trophoblast and EVTs (B3 and B4). Long → denote the direction of EVT migration. VM = villous mesenchyme; EVT = extravillous trophoblast of the cell column. Scale bar = 100 µm.



Prolactin gene expression mRNA and 28S rRNA were measured in aliquots of total RNA (10 ng) by RT-PCR. RT-PCR was carried out using the GeneAmp Thermostable rTth reverse transcriptase RNA PCR kit (Perkin Elmer, Branchburg NJ) and two pairs of primers (Eurogentec, Liège, Belgium). The sequence of the sense primer for PRL was δ'-GGTGACCCTTCGAGACCTGTT-S' and that of the antisense primer 5'-GGAAGAAGTGTGGCAGCTGTT-3'. The sequence of the sense primer for 28S rRNA was 5'-GTTCACCCAC-TAATAGGGAACGTGA-3', and that of the antisense primer 5'-GATTCTGACTTAGAGGCGTTCAGT-3'. Reverse transcription was performed at 70° C for 15min, followed

by 2min of incubation at 95° C to denature the RNA-DNA heteroduplexes. Amplification started at 94°C for 15 s, 30 s at 60° and 15 s at 72°C for 35 cycles for PRL (for 28S rRNA the annealing temperature was 20 s at 68° C and extension was 10 s at 72° C for 19 cycles) and terminated by 2 min at 72° C The RT-PCR products were resolved on 10% acrylamide gels and analyzed using a Fluor-S Multilmager (Bio-Rad, Hercules, CA) after staining with Gelstar dye (FMC Bioproducts, Rockland, ME). The expected sizes of the PRL mRNA and 28S rRNA were 150 bp and 212 bp, respectively. RT-PCR was performed three times on each sample.

Table 2: *Antibodies used for immunohistochemistry*

Antigens	mAbs	Species	Isotype	Dilution	Suppliers
Cytokeratin 07	OV-TL 12/30	Mouse	IgG1	1:200	Dako
Vimentin	V9	Mouse	IgG1	1/200	Dako
hPL	A0137	Rabbit	-	1:300	Dako
hPRL	-	Rabbit	-	1:200	Dr Parlow-NIDDK
hPRL	A0569	Rabbit	-	1.200	Dako
hPRL	NCL-PRO	Mouse	lgG3	1:200	NovoCastra
IGFBP-1	6303	Mouse	-	1:100	Medix Biochemica
α6 subunit	SP2/0	Mouse	lgG2b	1:50	Immunotech (Cappel)
α5β1	P1D6	Mouse	lgG3	1:50	Dako
subunit	TS2/7	Mouse	IgG1	1:50	T Cell Diagnostic
E-Cadherin	SHE 78-7	Mouse	lgG2	1/100	Zymed
EGF-R	-	Mouse	IgG1	1:100	Genzyme
c-erbB_2	9G6	Mouse	IgG1	1:10	Calbiocem
Connexin 40	-	Rabbit	-	1:50	Dr D. Gros, Marseille
Connexin 32	-	Mouse	IgG1	1:100	Zymed
Connexin 43	-	Mouse	IgG1	1:100	Transduction Laboratories
p16	P16 (F-12)	Mouse	IgG1	1/150	Santa Cruz Biotechnology
p57		Mouse	IgG1	1/150	Santa Cruz Biotechnology
Ki-67	MIB-1	Mouse	IgG1	1:50	Immunotech
HLA-G	W6/32	Mouse	lgG2a	1/100	Leinco Technologies

RESULTS

Most ectopic pregnancies occur in the ampulla. Figure 1A schematically illustrates uterine and fallopian implantation sites. Figure 1B shows the typical structure of an anchoring villous *in utero* (Fig. 1B1) and in a fallopian tube (Fig. 1B2). The fetal mesenchymal core is surrounded by a layer of cytotrophoblast covered by the syncytium, and the EVTs form cellular aggregates called TCCs. Using an anticytokeratin 7 antibody, which stains all trophoblast populations, trophoblastic cells were distinguished from non-epithelial maternal cells of the uterine decidua (Fig. 1B3) or the fallopian tube (Fig. 1B4). In pregnant tubes, trophoblast cells that had migrated into the fallopian tube tended to have the morphology of cohesive rounded cells (Figs 1B2,4 and 7B, D, F).

Absence of decidual cells in tubal implantation sites

To detect decidual cells in tubal pregnancies, we studied PRL expression in tubal implantation sites by means of RT-PCR. As shown in Fig. 2, PRL transcripts were observed in the secretory endometrium in normal pregnancies, and in the intrauterine decidua in tubal pregnancies. In contrast, no expression was detected in the tubal implantation sites or proliferative endometrium. As previously reported (27, 28), significant cross-reactivity of commercial anti-PRL antibodies with hPL precluded their use for immunohistochemical studies of placental tissue. IGFBP-1, another marker of decidual cells, was not expressed in the tubal implantation sites (Fig. 3C), but was clearly expressed in the intrauterine decidua of both normal (Fig. 3A) and tubal pregnancies (Fig. 3B). In the serial sections, vimentin staining (Fig. 3D) identified maternal mesenchymal cells, and cytokeratin or hPL staining confirmed the presence of invasive EVTs in tubal implantation sites (Fig. 3E and F).

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Pattern of trophoblast differentiation markers in uterine and tubal pregnancies (Table 3)

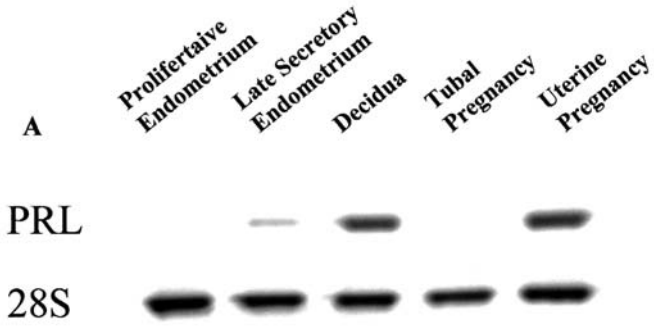
Integrin expression pattern

Figure 4A shows the intense and polarized localization of the α6 integrin subunit at the basal pole of villous trophoblasts. The α6 staining was no longer polarized and gradually weakened as trophoblast distance from the anchoring site increased, disappearing in the deepest layers. No differences in α6 integrin subunit distribution were found between the uterine and tubal pregnancies.

Uterine and tubal implantation sites displayed a similar α5β1 integrin distribution, which was exactly opposite to the α6 integrin subunit distribution, with positive staining of distal trophoblasts only (Fig. 4B).

Positive staining for the α1 integrin subunit was observed in the mesenchymal core of the fetal villi in the tubal and intrauterine pregnancies (Fig.4C). At the implantation site of the intrauterine and ectopic pregnancies, the αl integrin subunit was absent from the proximal part of the cell column. This αl integrin subunit was again expressed by invasive trophoblast cells in the distal part of the column (Fig.4C). The α1-expressing EVTs in distal columns were distinguished from maternal cells by cytokeratin 7 staining of serial sections (data not shown). This pattern is identical to that observed in intrauterine pregnancies, as previously reported (8, 29).

Fig. 2: *Analysis of prolactin (PRL) and 28S mRNA RT-PCR products amplified from proliferative endometrium (PE, m =10), secretory endometrium (SE, m = 10) and uterine decidua from patients with tubal pregnancy (decidua, m = 6), uterine pregnancy (UP, m = 5) and tubal pregnancy (TP, m = 6).* (A) Representative electrophoresis of prolactin (PRL) and 28S RT-PCR products showing the absence of PRL transcripts in proliferative endometrium and tubal pregnancy (TP). (B) PRL mRNA levels estimated from the density of the electrophoresis band. Values are mean ± SEM and represent the PRL to 28S band density ratio. Prolactin transcripts, used as a marker of decidualization, are restricted to late secretory endometrium and to the endometrium of patients with tubal pregnancy (decidua) or intrauterine pregnancy (UP).



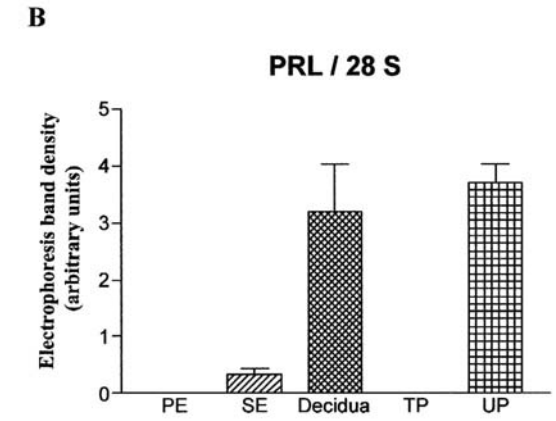


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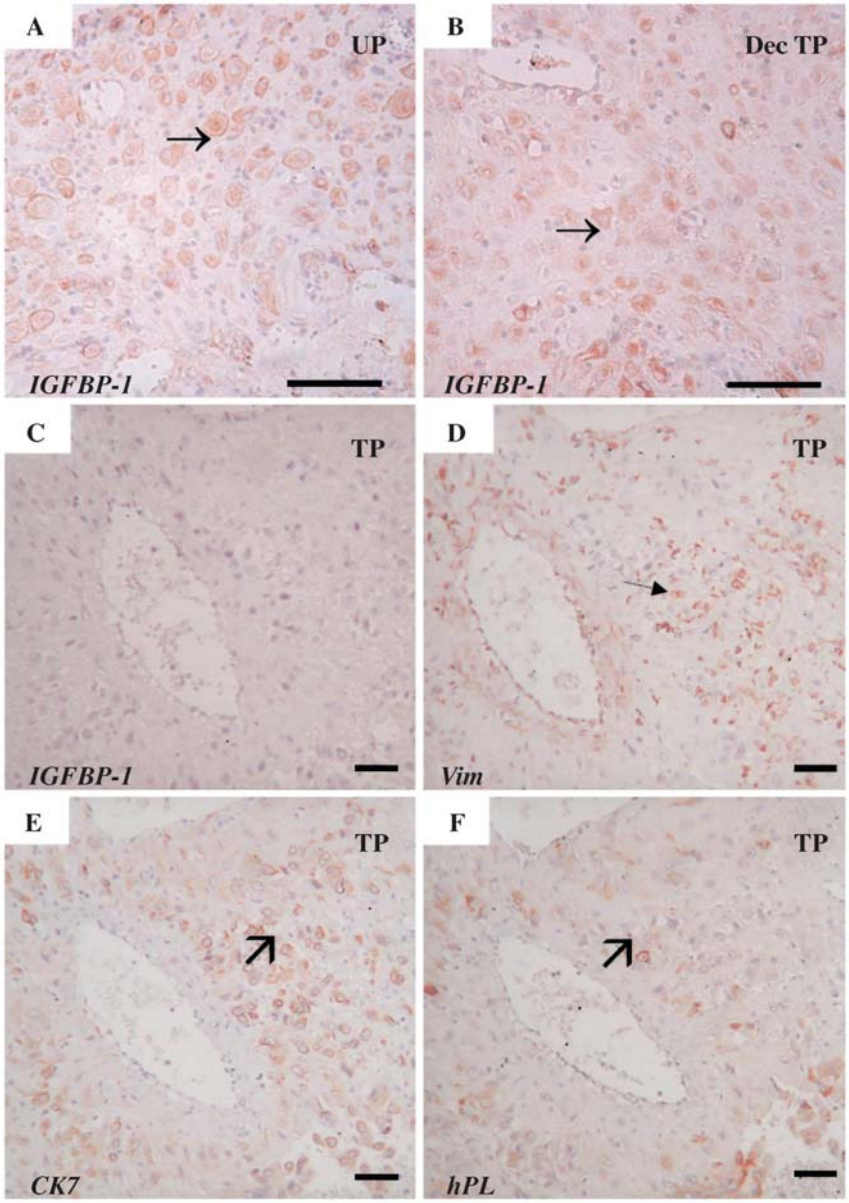
Fig. 3: *Immunoperoxidase staining for IGFBP-1, used as a marker of decidual differentiation.*
(A) IGFBP-1 detection in decidualized endometrial stromal cells (→) of an intrauterine pregnancy (UP).
(B) Endometrial stromal cells (→) in a representative case of tubal pregnancy (TP) also express IGFBP-1. Staining for IGFBP-1 (C), vimentin (D), CK7 (E) and hPL (F) was performed on serial sections from a representative tubal implantation site. Arrowheads show invasive extravillous trophoblasts (EVTs) reacting with anti-CK7 (E) and antihPL (F) antibodies. Vimentin-positive cells correspond to stromal cells and vascular cells. Scale bar = 50 μm.



E-cadherin and connexins

In both uterine and tubal implantation sites, E-cadherin was detected in villous trophoblasts and in proximal EVTs, but was minimally expressed in distal EVTs (Fig. 5A).

In both uterine and tubal implantation sites, Cx-40 staining was prominent in the proximal cell column, where cells were still in close contact with each other (Fig. 5B). Cx-40 was absent from distal and dispersed EVTs. Cx-43 was restricted to the villous trophoblasts of endometrial and tubal pregnancies. The pattern of expression was again identical in the two situations (data not shown).

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Other differentiation markers

We then examined the tubal implantation sites for the distribution of several other proteins that are differentially expressed during EVT differentiation in intrauterine placentation.

Cell cycle markers

Anti-Ki 67 (Fig. 6A), an S-phase marker, stained the villous cyto-trophoblast and EVTs closely apposed to fetal mesenchyme in the proximal part of the TCC, as reported in normal pregnancy (7, 30).

The cyclin-dependent kinase inhibitors pl6 and p57, which are markers of the G1 phase and G1-S phase of the cell cycle, respectively, were immunolocalized in the tubal pregnancies. Trophoblasts in cell columns were non-reactive, but deeply invasive EVTs were positive for these markers (data not shown). This pattern of expression is consistent with other recent data (7).

EGF-R and c-erb B2

In normal pregnancy, EGF-R is reported to be expressed in proximal but not in distal EVTs, whereas c-erb B2 displays the opposite pattern (31, 32). As shown in Fig.6B and C, EGF-R and c-erb B2 staining of the tubal pregnancy's EVTs showed reciprocal expression patterns similar to the intrauterine situation.

HLA-G

Only distal EVTs of the cell column reacted with anti-HLA-G antibody in the tubal implantation sites, with a pattern similar to that previously reported in uterine pregnancy (Fig. 6D) (33, 34).

Table 3: *First-trimester staining patterns of trophoblastic differentiation markers*

	Syncytium		Cytotrophoblasts					
			Villous		Extra-villous			
					Proliferative		Invasive	
	TP	UP	TP	UP	TP	UP	TP	UP
Cytokeratin 7	+	+	+	+	+	+	+	+
Integrins								
α6	0	0	+	+	+	+	0	0
α5β1	0	0	0	0	0	0	+	+
α1	0	0	0	0	0	0	+	+
Cell-cell adhesion molecules								
E-Cadherin	0	0	+	+	+	+	0	0
Connexin 40	0	0	0	0	+	+	0	0
Connexin 43	0	0	+	+	0	0	0	0
Proto-oncogenes								
EGF-R	+	+	+	+	0	0	0	0
c-erbB2	+	+	0	0	0	0	+	+
Cell cycle proteins								
Ki-67	0	0	+	+	+	+	0	0
p57	0	0	0	0	0	0	+	+
pl6	0	0	0	0	0	0	+	+
Others								
HLA-G	0	0	0	0	0	0	+	+
hPL	+	+	0	0	0	0	+	+

+: tubal pregnancy (TP) and uterine pregnancy (UP) mean, respectively, positivity in implantation sites from TPs and UPs. 0: negative staining.

HPL

In the tubal and uterine pregnancies, hPL staining predominated in the syncytiotrophoblast and EVTs in the distal column (Figs. 7A and B). Only a small percentage of EVT cells (cytokeratin-positive), (Fig. 7C and D) were positive for hPL. This pattern is identical to that described in uterine pregnancies by Kurman (35) and in ectopic pregnancies described by Earl and Chemnitz (36, 37).

Morphometry

We compared the length of EVT columns by measuring the distance separating the fetal mesenchymal core from the maternal mesenchyme. Mesenchymal cells were identified by vimentin staining (Fig. 7E and F). The trophoblastic nature of the cell column was confirmed by cytokeratin 7 reactivity (Fig. 7C and D). Mean length of the EVT columns was significantly higher in the tubal pregnancies (n = 100, 540 μm ± 33) than in the intrauterine pregnancies (n = 100, 170 μm ± 8.6, P< 0.0001) (Fig.7G). In the tubal pregnancies, the distal zone of the TCC was particularly enlarged (Fig. 7B, D and F). This zone was minimal or lacking in the intrauterine pregnancies (Fig.7A, C, E).

DISCUSSION

The decidual epithelial, together with stromal and inflammatory cells, secretes various molecules (prolactin, IGFBP-1, IL-1, IL-6, TNF-α, EGF, LIF, TGF-β, etc.) and matrix metalloproteinases or inhibitors (TIMPs) that regulate trophoblast differentiation, proliferation and invasion (11, 17, 21, 38).

Transfer of mouse blastocysts to ectopic non-decidualized soft tissues resulted in more extensive trophoblast invasion than observed in the uterus (39-41). When transplanted subcutaneously, trophoblasts also invaded blood vessels, leading to the formation of hemorrhagic nodules (18). The trophoblast overgrowth that occurred in the absence of decidua in IL-11R-knockout mice also supports the hypothesis that the decidua plays a role in restricting trophoblast invasion (42). These data therefore suggest that decidual tissue acts as a barrier to excessive trophoblast invasion.

In vitro studies also support decidual control of placentation, through feto-maternal paracrine interactions (22, 43, 44). For example, IGF-1 stimulates (while TGF-β inhibits) cytotrophoblast detachment from the distal column and migration in streams across the maternal extracellular matrix (45, 46).

To further examine the role of the decidua in the control of trophoblast invasion, we compared the distribution of trophoblast differentiation markers in tubal and intrauterine pregnancies.

As it was still controversial whether or not tubal implantation sites undergo decidual differentiation (2, 24, 26, 47-51), we examined the distribution of two key specific markers of decidual cells (PRL and IGFBP-1) with RT-PCR or immunohistochemistry (Figs 2 and 3). In contrast to the uterine sites, the tubal implantation sites did not contain PRL transcripts or IGFBP-1-positive cells, strongly supporting the absence of decidua formation. This is in agreement with our previous observation (25) of large numbers of uterine natural killer (uNK) cells in uterine decidua and their absence in tubal implantation sites.

In the proximal part of the anchoring villi, EVTs adhere to the fetal basement membrane through α6β4 integrin and actively proliferate, as revealed by Ki-67 staining (30, 52). These cohesive cells are linked by intercellular adhesion and junction molecules (E-cadherin and connexin 40) (53-55). They express the specific EGF-R receptor (31, 32). When differentiating towards an invasive phenotype, EVTs stop proliferating (Ki-67, pl6, p57), lose intercellular adhesion molecules (cadherin-E), switch to different junctional proteins (connexins 32, 40 and 43) and integrins (α6β4, α5β1, α1β1), and express distinct surface receptors (EGF-R, c-erb-B2, HLA-G) and specific hormones (hPL). This process is known as 'the phenotype switch' (6, 8, 9). Our data shows that all these markers had precisely the same expression pattern regardless of the implantation site (Table 3). This clearly indicates that the EVT phenotype switch is fully maintained in tubal pregnancies and does not require a differentiated decidua. The switch thus appears to be essentially insensitive to the maternal environment, as previously suggested by *in vitro* studies: expiant culture of placental villi or isolated EVTs on a type I collagen matrix or Matrigel is sufficient to induce this phenotype switch (5, 56, 57).

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Fig. 4: *Expression of α6, α5β1 and α1 integrins in uterine (UP) and tubal (TP) anchoring villi and cell columns at the fetal-maternal interface in first-trimester pregnancies.* Dual immunostaining for integrins (red): α6 subunit (panel A), α5β1 (panel B) and αl subunit (panel C); and for nuclear staining with DAPI (blue) in uterine (UP) and tubal pregnancy (TP). Single stainings (integrin or DAPI) are shown with a merge of the two images. α6 labeling is restricted to villous cytotrophoblast cells and to the proximal EVT of the trophoblastic cell column (TCC). α5 is present in the distal EVT of the TCC. **α1** is located in the villous mesenchyme and in the distal EVT of the TCC. No differences in the distribution of αl, α5βl, and α6 integrins were seen between uterine (UP) and tubal pregnancies (TP). Arrowheads show the first layers of the EVT and → the distal part of the column. VM = villous mesenchyme and EVT = extravillous trophoblast of the cell column. Scale bar = 100 µm.

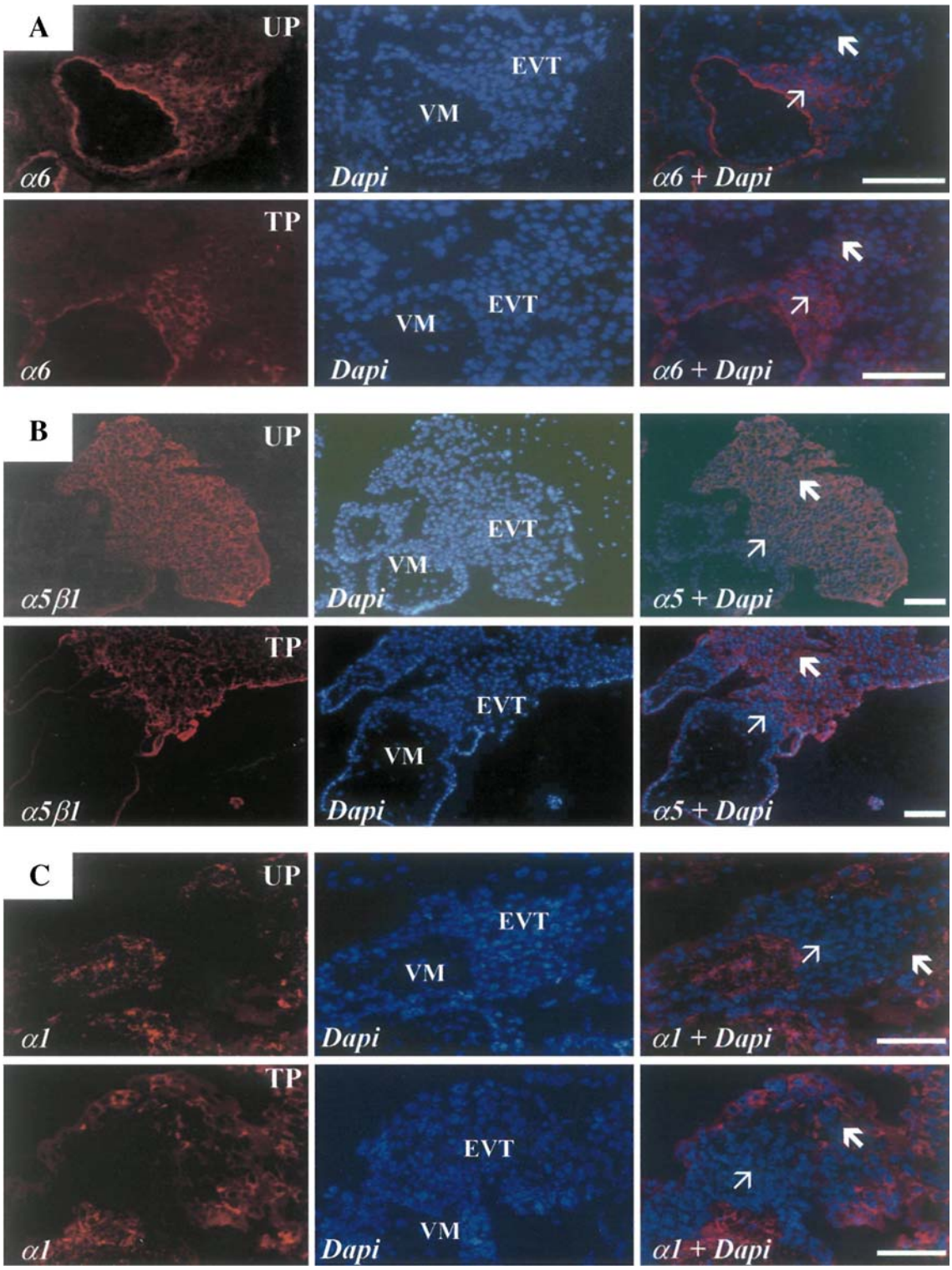

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Fig. 5: *Expression of E-cadherin and connexin 40 in first-trimester uterine and tubal anchoring villi and cell columns at the fetal-maternal interface.* Dual immunostaining for E-cadherin in panel A (red) and for nuclear staining with DAPI (blue). Single staining (integrin or dapi) are shown together with merged images. E-cadherin is expressed by villous cytotrophoblasts and the first layers (Arrowheads) of the proximal part of the TCC in both uterine (UP) and tubal pregnancies (TP). Immunostaining for connexin 40 (panel B) in UP and TP, and nuclear staining with DAPI (blue). Connexin 40 is expressed by the EVT of the TCC. Arrowheads show the first layers of the EVT and → the distal part of the column. VM = villous mesenchyme, EVT = extravillous trophoblast of the cell column. Scale bar = 100 µm.

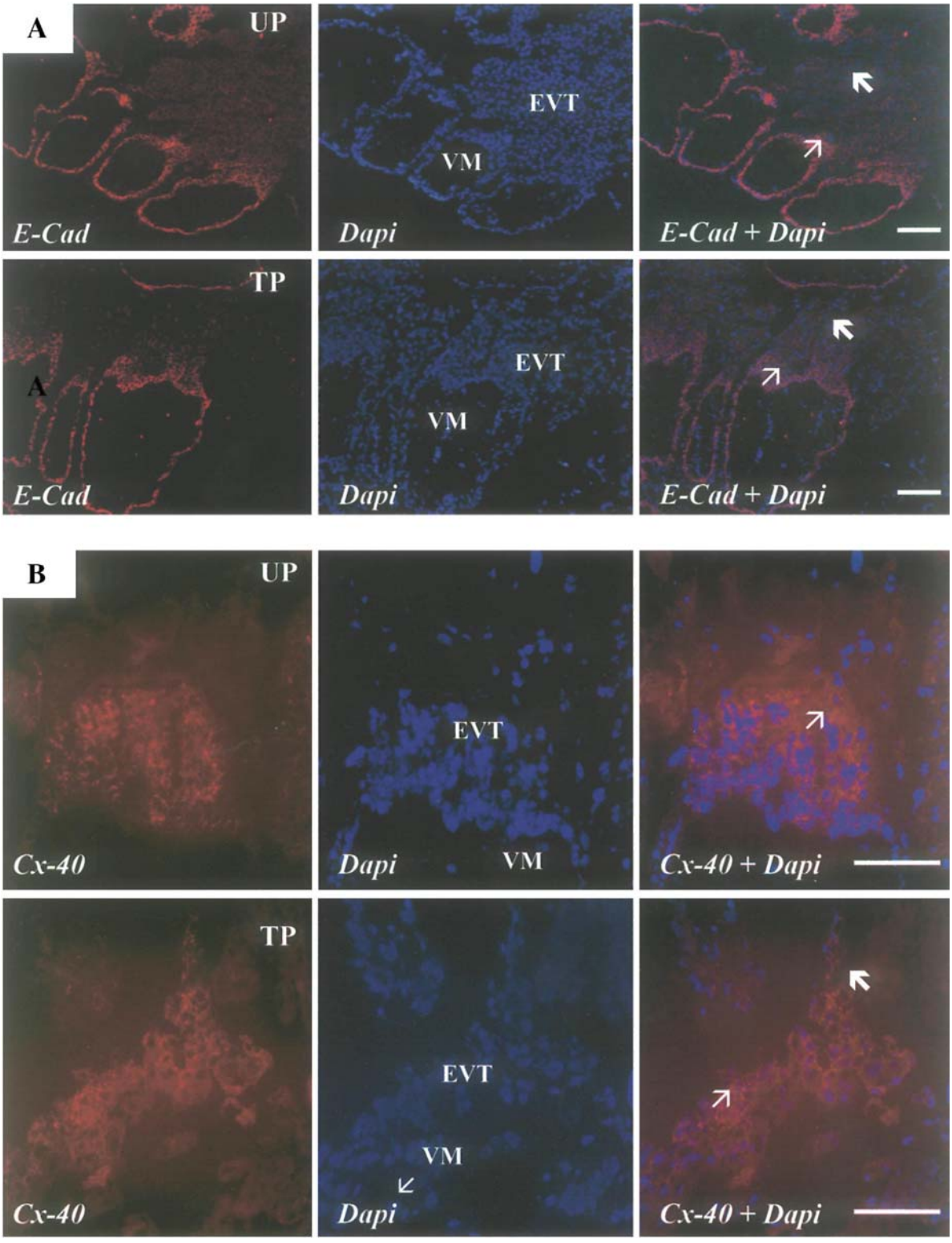

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Fig. 6: *Immunostaining for Ki-67, EGF-R, HLA-G and c-erb B2 in tubal pregnancy (TP).* Arrowheads show the first layers of the extravillous trophoblast (EVT) and → the distal part of the column. VM = villous mesenchyme, EVT = extravillous trophoblast of the cell column. Scale bar = 100 µm.

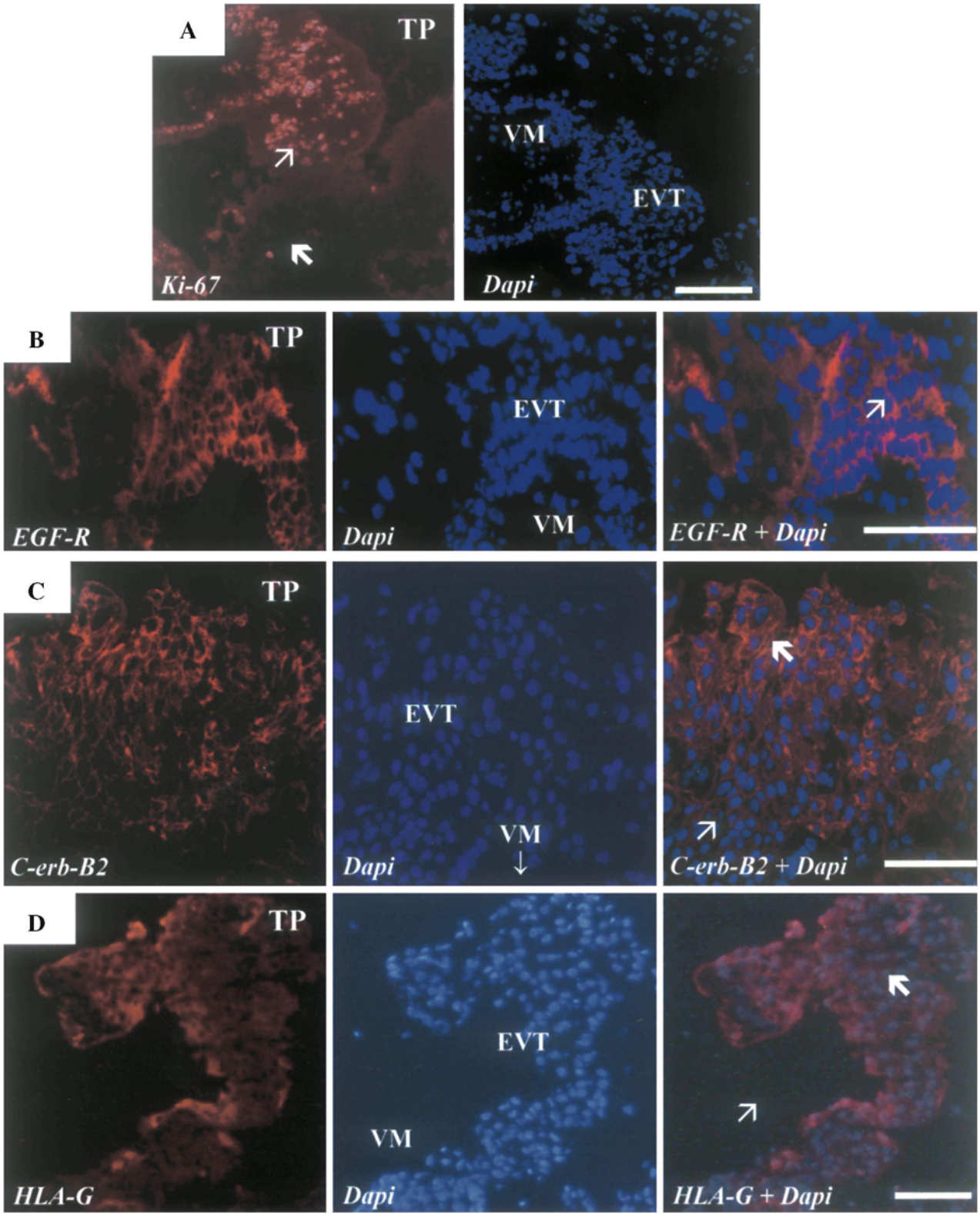

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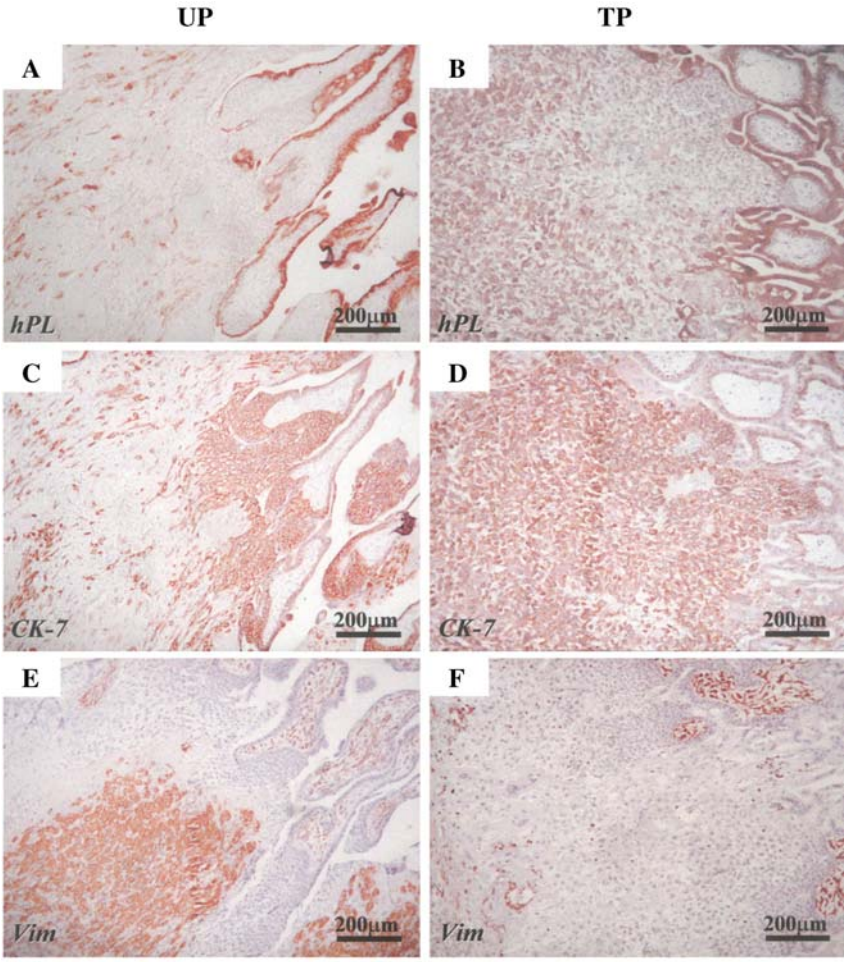
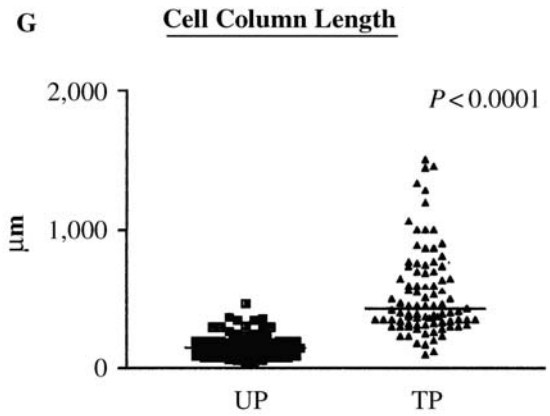
Fig. 7: *(A,B) hPL immunostaining identifies the syncytium and invasive extravillous trophoblasts (EVTs) in tubal (TPs) and uterine (UPs) pregnancies. (C,D)* Cytokeratin 7 staining identifies all villous and extravillous trophoblasts and permits precise measurement of the length of the column. (E,F) Vimentin staining identifies mesenchymal cells in fetal villi and in the tubal wall. Scale bar = 200 μm. (G) Comparison of the length of trophoblast cell columns (n = 100) in UPs and TPs (P< 0.0001).





Interestingly, the TCC was considerably longer in the tubal than uterine pregnancies (Fig. 7G). Despite the down-regulation of E-cadherin and the α6β4 integrin and the up-regulation of the α5βl and αlβl integrins in the distal part of the column, the EVTs remained cohesive in the absence of decidua (Figs. 7B and C). This points to a need for a maternal signal, presumably of decidual origin, to generate a motile phenotype. Identification of factors that control trophoblast invasion, paracrine regulation of invasion effectors (eg. inhibition of MMPs by TIMPs) or extra-cellular matrix components remains a major challenge for reproductive biologists.

Taken together, our data indicate that while the EVT switch is not triggered by decidua, decidual cells are likely to support EVT dispersion into the maternal endometrium.

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