The tumor suppressor gastrokine-1 is expressed in placenta and contributes to the regulation of trophoblast migration

F.B. Fahlbusch [a,*], M. Ruebner [b], H. Huebner [b], G. Volkert [a], C. Zuern [a], F. Thiel [b], M. Koch [b], C. Menendez-Castro [a], D.L. Wachter [c], A. Hartner [a], W. Rascher [a]

[a] Department of Pediatrics and Adolescent Medicine, University of Erlangen-Nürnberg, Loschgestrasse 15, 91054 Erlangen, Germany
[b] Department of Gynecology and Obstetrics, University of Erlangen-Nürnberg, Erlangen, Germany
[c] Department of Pathology, University of Erlangen-Nürnberg, Erlangen, Germany

1. Introduction

The orchestrated invasion of trophoblast cells into the uterine wall is the key process of hemochorial placentation in humans [1]. Its coordination involves feto-maternal cross-talk of multiple cytokines, enzymes and hormones [2–5]. Dysregulation of trophoblast invasion into the maternal stroma is found in pregnancy complications such as intra-uterine growth restriction and preeclampsia [5–7], as well as in gestational trophoblastic disease (reviewed by Ref. [8]). The penetrative nature of healthy trophoblast cells at the decidual level shows strong similarity to the metastasis of tumor cells seen in invasive carcinomas. Hence trophoblasts are often termed pseudo-malignant, as their cellular mechanisms for invasion of decidual stroma are likely related to those of malignant cells, except that in trophoblasts these mechanisms remain highly regulated [9]. Thus, understanding the molecular processes behind trophoblast cell invasion might translate into new treatment strategies of invasive carcinomas and metastasis.

Recently Gastrokine-1 (GKN1) has been identified as a novel functional tumor-suppressor gene in invasive gastric cancer (GC)

* Corresponding author. Tel.: +49 91318533118.
E-mail addresses: fabian.fahlbusch@uk-erlangen.de (F.B. Fahlbusch), Matthias.Ruebner@uk-erlangen.de (M. Ruebner), Hanna.Huebner@uk-erlangen.de (H. Huebner), Gudrun.Volkert@uk-erlangen.de (G. Volkert), tina_zuern99@hotmail.com (C. Zuern), Falk.Thiel@uk-erlangen.de (F. Thiel), Martin.Koch@uk-erlangen.de (M. Koch), Carlos.Menendez-Castro@uk-erlangen.de (C. Menendez-Castro), David.Wachter@uk-erlangen.de (D.L. Wachter), Andrea.Hartner@uk-erlangen.de (A. Hartner), Wolfgang.Rascher@uk-erlangen.de (W. Rascher).

[10,11]. Loss of GKN1 is indicative of a poor prognosis of intestinal-type GC [12] and is linked to a reduced cell–cell adhesion of tumor cells [13]. The transfection of GKN1 negative GC cells (MKN-28) with GKN1 reduced the formation of colonies [14] and induced Fas-pathway mediated apoptosis [15]. Moreover, GKN1 treatment of GBGC-823 GC cells induced cellular senescence via ERK stimulation [16], while in the AGS GC cell line GKN1 inhibited epithelial-to-mesenchymal transition by inactivating the PI3K/AKT pathway [17].

Physiologically, GKN1 is synthesized and secreted by mucosal epithelial cells of the gastric antrum [18]. Its role for gastric epithelial barrier function and restitution following epithelial injury is well recognized. As a secreted peptide [16], GKN1 is thought to exert mito- and motogenic repair functions as component of the gastric mucus layer [18,19] in part via interaction with members of the trefoil factor family (TFF) [20,21]. GKN1 protects the intestinal mucosal barrier by acting on cell–cell connections and by stabilizing perijunctional actin [22].

Previous studies [10,19] are supportive of GKN1 specificity for gastric type epithelia only. However, gene array analyses in placental tissues point to considerable expression levels of GKN1 in the placenta (Gene Atlas expression data, [23]). We used a stepwise approach to clarify the location and function of human GKN1 in healthy placenta, as well as in gestational trophoblastic disease: First we determined its localization using immunohistochemistry. Secondly, we confirmed its expression in different primary placental cell types in comparison to the invasive choriocarcinoma cell lines JAr, JEG-3 and ACH-3P. We hypothesized that − analogue to its expression pattern in the stomach − GKN1 expression is specific for non-invasive epithelial placental cell subtypes. We further hypothesized that GKN1 expression would be substantially more abundant in these cells, than in choriocarcinoma cell lines, further supporting tumor suppressive properties. To study functional aspects of placental GKN1 expression in vitro, we used time-lapse analysis of JEG-3 migration, an established model for trophoblast invasion [24]. We hypothesized that GKN1 reduces the motility of the JEG-3 choriocarcinoma cell line, possibly via AKT-mediated induction of E-cadherin.

2. Materials and methods

2.1. Placental tissue

Fresh samples of healthy human placenta at term ($n = 5$) were excised immediately after placental delivery, fixed in formaldehyde and embedded in paraffin. Tissue sections of 5 choriocarcinomas, 2 hydatidiform moles (1 complete/1 incomplete), 1 placental site trophoblastic tumor (PSTT) and 1 healthy antrum (positive control) were kindly provided by the Departments of Obstetrics & Gynecology and Pathology, University of Erlangen-Nürnberg, Germany. The latter also kindly provided placental sections ($n = 4$) of first trimester abortions with proven absence of chromosomal aberration.

2.2. Ethics

The study was approved by the Ethics Committee of the Medical Faculty of the University of Erlangen-Nürnberg (#2625-02/28/02). Written consent was given by every patient. Use of anonymized paraffin-embedded tissue samples was approved by the Ethics Committee 01/18/05.

2.3. Immunohistochemistry

Following de-paraffinization and rehydration, sections (1.5 μm) of placental tissue and antrum were subjected to heat-induced antigen retrieval via steam pressure cooking (20 and 12 min, respectively; 10 psi, 116 °C) in 1X TRS (#S2375, Dako GmbH, Hamburg, Germany). After washing with TBS, sections were analyzed using the ZytoChemPlus (AP) Polymer Kit (AP-S-008RED, ZYTOMED Systems GmbH, Berlin, Germany) according to the manufacturer's guidelines. Primary antibodies used are listed in Table 1. Biotinylated secondary antibodies (horse anti-mouse IgG #BA-2001, goat anti-rabbit IgG #BA-1000) were from Linaris (Dossenheim, Germany) and were used at a dilution of 1:500. Hematoxylin served as counterstain. In addition GKN1 immunoreactivity in placental sections was examined by an alternative approach using antigen retrieval with a microwave oven followed by peroxidase-DAB (3,3′-diaminobenzidine) staining (see Supplementary methods S1).

Table 1
Antibodies used for immunohistochemistry and Western blot analysis.

Antibody	Source	Dilution
Anti-human GKN1	Abcam, Cambridge, UK, (ab57623)	1:1000 (ZytoChem-Plus Kit); 1:100 (DAB-Peroxidase) (IHC)
Anti-human TFF1	Sigma Aldrich, Steinheim, Germany (#HPA003425)	1:1000 (IHC)
Anti-human TFF2	R&D Systems, Wiesbaden-Nordenstadt, Germany (# 366508)	1:1000 (IHC)
Anti-human E-cadherin	Abcam, Cambridge, UK, (ab1416)	1:250 (IHC); 1:1000 (WB)
Anti-human GAPDH-HRP	Santa Cruz Biotechnology, Heidelberg, Germany	1:1000 (WB)
Anti-human Pan-AKT	Cell Signaling Technology (New England Biolabs), Frankfurt am Main, Germany	1:1000 (WB)
Anti-human Phospho^{Ser473}-AKT	Cell Signaling Technology (New England Biolabs), Frankfurt am Main, Germany	1:2000 (WB)

IHC, immunohistochemistry; WB, Western blot analysis.

2.4. Fractionation and cultivation of primary placental cells

Human placentas were obtained from uncomplicated term pregnancies after elective caesarean section in accordance with the established guidelines of the local ethics committee. We isolated decidual stroma cells (DSC), extravillous trophoblast cells (EVT) and villous cytotrophoblasts (VT) from three healthy placentas at term. The procedures were carried out under sterile conditions. Protocols for immunomagnetic bead separation of VT and EVT isolation have been described in detail elsewhere [25,26]. Isolation of DSC from placenta via selective adhesion was carried out adapting the protocols of Frank et al. [27] and Lockwood et al. [28] (see Supplementary methods S1).

Table 2
Primer pairs and probes.

Gene	Type	Sequence (5′-3′)
GKN1	Forward Primer	GGAAAAGAAGCTTCAGGGTAAGG
GKN1	Reverse Primer	CATCGACTTTGTTTGGGTTGAC
GKN1	Probe	FAM-AGGACCACCTCCCAAGGGCCTG-TAMRA
TFF1	Forward Primer	GCCCAGACAGAGACGTGTACAG
TFF1	Reverse Primer	CGAACGGTGTCGTCGAAAC
TFF2	Forward Primer	TGTTTTGACAATGGATGCTGTTT
TFF2	Reverse Primer	CTGATCCGACTCTTGCTTTGG
r18S	Forward Primer	CGGCTACCACATCCAAGGAA
r18S	Reverse Primer	CCTGTATTGTTATTTTTCGTCACTACCT
E-cad	Forward Primer	GCCTCAGAAGACAGAAGAGAGACT
E-cad	Reverse Primer	GATCTGAACCAGGTTTTTAGGAAA
MMP-2	Forward Primer	ATGACAGCTGCACCACTGAG
MMP-2	Reverse Primer	ATTTGTTGCCCAGGAAAGTG
MMP-9	Forward Primer	TTGACAGCGACAAGAAGTGG
MMP-9	Reverse Primer	GCCATTCACGTCGTCCTTAT
TIMP-1	Forward Primer	AATTCCGACCTCGTCATCAG
TIMP-1	Reverse Primer	TGCAGTTTTCCAGCAATGAG
TIMP-2	Forward Primer	TGATCCACACACGTTGGTCT
TIMP-2	Reverse Primer	TTTGAGTTGCTTGCAGGATG
uPA	Forward Primer	AGGCCATTCTCTTCCTTGGT
uPA	Reverse Primer	TGACTGGAATTGTGAGCTGG
uPA	Probe	TCACCACCAAAATGCTGTGT

GKN1, gastrokine-1; TFF, trefoil factor; E-cad, E-cadherin; MMP, matrix metalloprotease; TIMP, tissue inhibitor of metalloproteases; uPA, urokinase.

2.5. Cell lines and cell culture

The ACH-3P cell-line [29] was a kind gift of U. Hiden, Dept. of Obstetrics and Gynecology, Medical University of Graz. Jar and JEG-3 choriocarcinoma cell lines were purchased from DSMZ (Braunschweig, Germany) and cultured in phenol-red free DMEM:F12 (Gibco-BRL) supplemented with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma–Aldrich). Cells were cultured under normoxic conditions in T-75cm^2 flasks at 37 °C under a humidified atmosphere containing 95% air, 5% CO_2. Every two passages ACH-3P cells were cultured in selection medium containing azaserine (5.7 μM) and hypoxanthine (100 μM) [29].

2.6. JEG-3 migration analysis via time-lapse imaging

Before plating JEG-3 cells on Ibidi μDish (35 mm, high, ibiTreat) culture-inserts (ibidi GmbH, Martinsried, Germany) they were counted via FACS as described in detail previously [30]. 5×10^4 JEG-3 cells were added into each of the two reservoirs to 100 μl phenol red-free DMEM:F12 (Gibco-BRL) with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin, 1% non-essential amino acids and incubated at 37 °C in a humidified atmosphere with 5% CO_2. Reaching confluency (12 h), the insert was removed and 2 ml of fresh media was added. After 1 h 5 ml of fresh media containing 0.1 μg/ml recombinant human GKN1 (#H00056287-P01, Abnova, Taipei City, Taiwan), or vehicle control (GKN1 solution buffer, 50 mM Tris–HCl, 10 mM reduced Glutathione, pH = 8.0) were added. After 4 h cells reached appropriate attachment and cell migration was captured by time-lapse phase-contrast imaging at 15 min intervals using the Axiovert 200 MAT inverse microscope (Carl Zeiss MicroImaging, Göttingen, Germany) equipped with CO_2- and temperature module S attached to the incubator PM-S_1 (Carl Zeiss). Data acquisition and analysis was carried out with the dedicated Axiovision software (v4.8.2, Carl Zeiss). Speed of JEG-3 migration was measured in μM/min at ten fixed horizontal grid-lines spanning between the two seam outlines, each 60 μM vertically apart. Furthermore, the seam area (nuclei free pseudopodic cytoplasma reaching into the preformed gap) was determined. Results shown are representative for at least three independent experiments.

2.7. Determination of Jeg-3 cell proliferation

For continuous assessment of the rate of cell proliferation, cell divisions – visible as bright spots during time-lapse phase-contrast imaging (see above) – were counted at the respective time points, as described by Huh et al. [31]. For verification, a 5-bromo-2′-deoxy-uridine (BrdU) incorporation assay into cellular DNA (#1299964, Roche) was performed, as previously described [32]. Cells were counterstained with hematoxylin. Nuclei with a positive staining for BrdU were counted along both sides of the former gap (2 cm from the seam on each side).

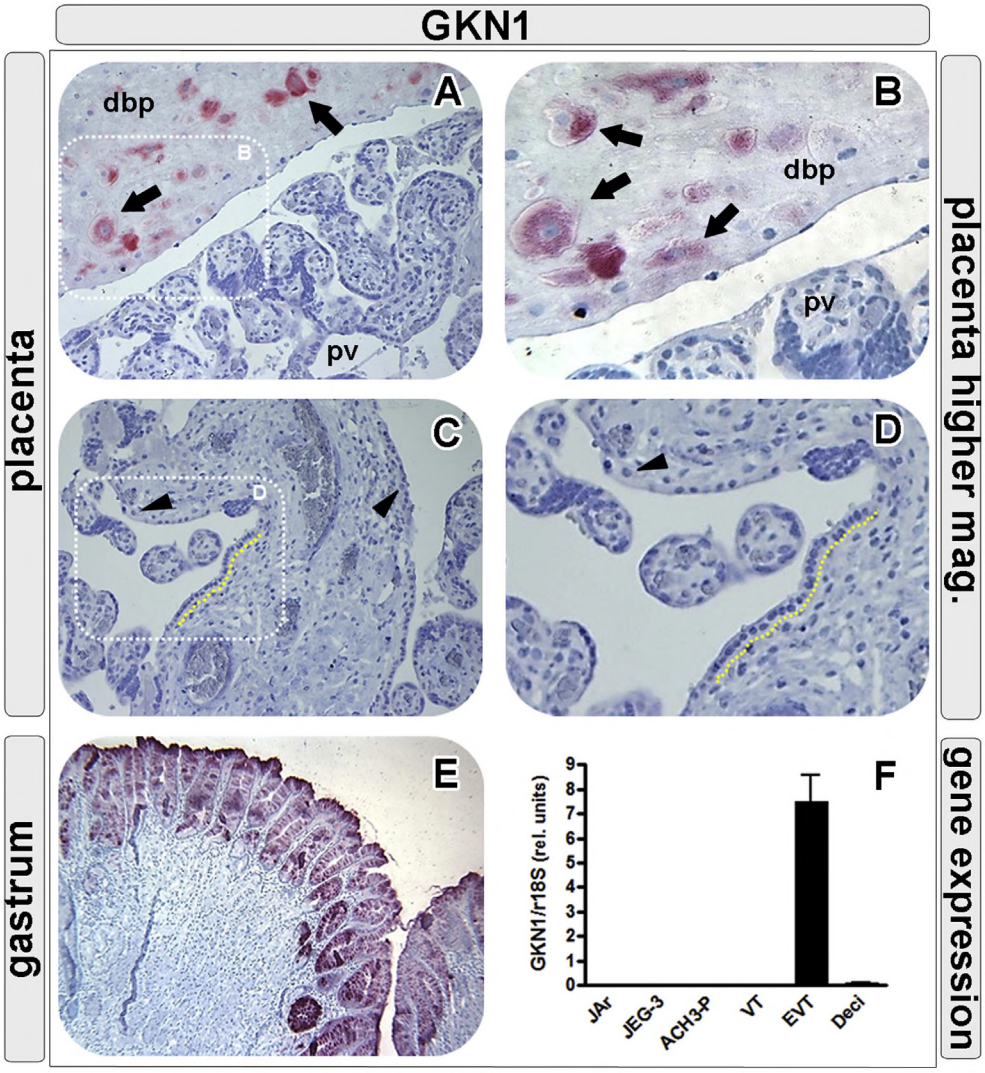

Fig. 1. Localization of gastrokine-1 (GKN1) in placental tissue at term (A–D), trophoblast cell lines and isolated primary placental cells (F). A: Decidual basal plate (dbp) at a magnification of 200× with adjacent placental villous tree (pv). Extravillous trophoblasts (EVT, dark arrows) are located in the dbp and stain positive for GKN1 (red). B: Magnification (400×) of the delineated area in A with focus on EVTs (dark arrows). C: Placental villous tree without dbp. Syncytiotrophoblast (SCT, dotted yellow line) and cytotrophoblasts (dark arrowheads) show no GKN1-positivity. D: Magnification (400×) of the delineated area in C with focus on the SCT. E: Gastric antrum (gastrum) served as the positive control for GKN1 specificity. GKN1 is expressed in surface mucous cells. F: Expression (mRNA) of GKN1 in immunomagnetic bead separated placental cells (VT = villous cytotrophoblast, EVT = extravillous trophoblast, Deci = decidual stroma cells) and the placental cell lines JAr, JEG-3 and ACH3-P.

2.8. Gene expression analysis of primary placental cells and cell lines

Isolation of mRNA and real-time PCR were performed as previously described elsewhere [33]. Primers and probes used for detection of GKN1, TFF1 and 2, E-cadherin, matrix are described in Table 2.

2.9. Gene expression analysis of stimulated Jeg-3 cells

For gene expression analysis of E-cadherin, MMPs, TIMPs and uPA, 25×10^3 cells/0.5 ml medium (DMEM:F12, 10% FCS) were seeded into a well of a 8 chamber slide. After adherence for 3 h cells were serum starved (DMEM:F12, 0.1% FCS) for 48 h and stimulated with 0.1 μg/ml GKN1 (Abnova) for 6 h, 12 h and 24 h. Isolation of mRNA and real-time PCR were performed as previously described elsewhere [33]. Primers and probes used are summarized in Table 2.

2.10. Western blot analysis of protein lysates from Jeg-3 cells

For protein expression analysis 10^5 cells/3 ml medium (DMEM:F12, 10% FCS) were seeded into a 3 cm petri dish. After adherence for 3 h cells were serum starved (DMEM:F12, 0.1% FCS) for 48 h and stimulated with 0.1 μg/ml GKN1 (Abnova) for 12 h. Protein concentration of cell lysates was determined using a protein assay kit (Pierce, Rockford, IL). Protein samples containing 30 μg total protein were denatured by boiling for 5 min and separated on a 12.5/7.5% agarose-gradient gel. After electrophoresis, the gels were electro-blotted (semidry) onto nitrocellulose membranes using a CAPS transfer buffer, blocked with 5% non-fat milk for 1 h at room temperature and incubated with the primary antibody overnight. Primary antibodies are listed in Table 1. Immunoreactivity was visualized with horseradish peroxidase-conjugated anti-rabbit IgG as a secondary antibody at a dilution of 1:1000 (Cell Signaling) for 1 h at room temperature, using the ECL system according to the manufacturer's instructions (Amersham, Braunschweig, Germany). Digital quantification of the blots was performed with my Image Analysis Software (v1.0, 2012, Thermo Fisher Scientific Inc.).

2.11. Statistical analysis

Results were expressed as mean ± standard error of the mean (SEM). Statistical analyses were performed using GraphPad Prism Version 4.0c (GraphPad Software, San Diego, CA, USA). Groups were compared using nonparametric Mann–Whitney U testing and Students *t*-test. A *p*-value of <0.05 was considered statistically significant.

3. Results

3.1. Expression and localization of placental GKN1, TFF-1 and -2

GKN1 protein expression was detected in the decidual basal plate of normal human placenta at term (Fig. 1A and B). Its localization was restricted to extravillous trophoblast cells (EVT). In contrast, villous cytotrophoblasts (VT) and the syncytiotrophoblast of the placental villous tree were completely negative for GKN1 (Fig. 1C and D). These data were confirmed by mRNA expression analyses in immunomagnetic bead separated primary placental cells from healthy human placenta at term (Fig. 1F). A high level of GKN1 mRNA expression was detected in HLA-G positive EVT only. In decidual stroma cells (DSC) GKN1 expression was at detection limits, while absent in VT and in the choriocarcinoma cell lines JEG-3, JAr and ACH3-P (Fig. 1F). In contrast to our findings in third trimester placentae, samples of first trimester placentae did not show GKN1 immunoreactivity in EVT (Supplementary Fig. 1A).

In accordance with the finding that GKN1 is not expressed in choriocarcinoma cell lines (Fig. 1F), no GKN1 immunoreactivity was detected in samples of choriocarcinoma (Fig. 2C). Similarly, no GKN1 was found in hydatidiform mole (Fig. 2B). Of the gestational trophoblastic diseases investigated in this study, only placental site trophoblastic tumor revealed some trophoblastic expression of GKN1 (Fig. 2D).

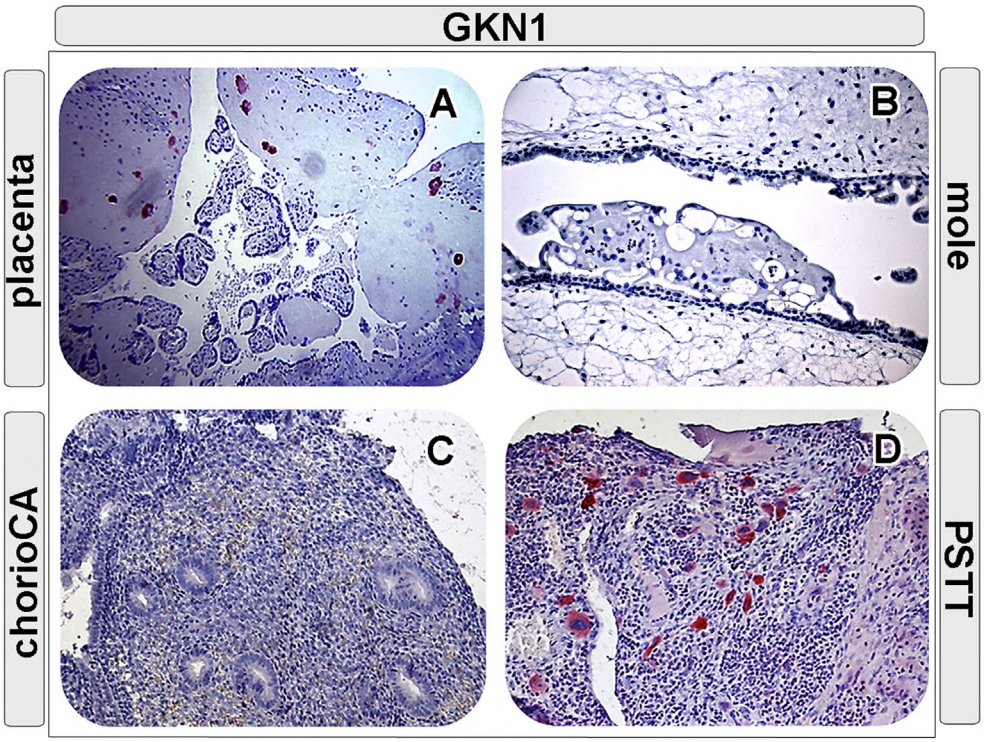

Fig. 2. Immunohistochemical detection of gastrokine-1 (GKN1) expression in healthy placenta at term (A, 200×) vs. the gestational trophoblastic neoplasias partial hydatidiform mole (mole) (B, 400×), choriocarcinoma (chorioCA) (C, 400×) and placental site trophoblast tumor (PSTT) (D, 400×). A: GKN1 is specifically expressed in extravillous trophoblasts within the decidual basal plate (red stain). B: No GKN1 expression is found in complete (not shown) and partial hydatidiform mole. Picture shows the typically enlarged, hydropic villi and central cavitation. In the center of the picture a small fraction of decidual basal plate with EVT is visible. C: No GKN1 expression is found in choriocarcinoma. Picture shows the typical morphologic pattern of choriocarcinoma with variable amounts of neoplastic mononucleate trophoblast alternating with syncytiotrophoblast. D: Placental site trophoblast tumor (PSTT) is composed of sheets of cells consistent with intermediate trophoblasts that invade and replace the myometrium. We found some strong GKN1 positivity (red stain) among cytoplasma rich trophoblasts with extravillous trophoblast morphology, possibly EVT giant cells.

The trefoil factors 1 and 2 (TFF1, TFF2) co-localize with GKN1 in the normal gastric antrum (Fig. 3A and B). TFFs can potentially bind to GKN1 to functionally activate it. Therefore, TFF1 and TFF2 expression was studied in placental tissue as well. We neither observed mRNA expression of TFF1 nor of TFF2 in primary placental cells and choriocarcinoma cell lines (data not shown). Moreover, TFF1 and TFF2 immunoreactivity was completely absent from human placenta at term (Fig. 3C and D). No co-localization of both factors with GKN1 was detected.

3.2. Functional analysis of GKN1 in placental cells

GKN1 treatment significantly reduced the migration of JEG-3 choriocarcinoma cell line after 7.5 h of observation (Fig. 4A and B, $p < 0.05$). The reduction remained significant until 12.5 h. Following GKN1 treatment, JEG-3 cells underwent morphologic changes. We observed a reduction of pseudopodic cytoplasma extensions, so that the total area of cytoplasmatic migrational seam was reduced throughout the entire observational period (Fig. 4C, D and F, $p < 0.002$). The proliferation rate of GKN1 treated JEG-3, as determined via the appearance of mitosis during time-lapse imaging and via BrdU at confluency (Fig. 4E) was not significantly different from the vehicle treated control.

We found that GKN1 co-localized with E-cadherin in EVT of the decidual basal plate (Fig. 5A). GKN1 significantly induced E-cadherin mRNA ($p < 0.05$) and protein ($p < 0.03$) expression in the choriocarcinoma cell line JEG-3 (Fig. 5B). Interestingly, the induction of E-cadherin coincided with the reduction of migration. Furthermore, GKN1 significantly ($p < 0.01$) inhibited Akt phosphorylation at Ser473, with constant protein levels of GAPDH and total Akt (Fig. 5B).

Moreover, we determined the levels of metalloprotease (MMP) −2 and −9, tissue inhibitor of metalloproteases (TIMP) −1 and −2, as well as urokinase (uPA) following GKN1 treatment as markers of an invasive cell phenotype. While MMP-2 and uPA expression levels were below detection limits (data not shown), GKN1 significantly ($p < 0.01$) reduced MMP-9 expression in JEG-3 after 12 h (Table 3). The levels of TIMP-1 and -2 mRNA remained unchanged (Table 3).

4. Discussion

In this study we demonstrated that GKN1 is specifically expressed in term extravillous trophoblasts (EVTs) at the decidual basal plate, while it was undetectable in the syncytiotrophoblast, in VT and DSC, as determined by IHC and expression analysis following isolation of functional placental cell subtypes. We were not able to detect placental expression of TFF1 or 2, factors which co-locate and putatively interact with GKN1 in the stomach [34]. In choriocarcinoma and hydatidiform mole immunohistochemical staining for GKN1 was absent, while present in placental site trophoblastic tumor. The commonly used choriocarcinoma cell lines (JAr, ACH-3P, JEG-3) showed no GKN1 expression. Thus, we presented evidence for placental expression of GKN1 predominantly in EVTs and showed that JEG-3 cells reduced their migratory activity after GKN1 treatment, likely via AKT-mediated induction of E-cadherin expression. To our knowledge this study is the first report on placental GKN1 expression and GKN1 localization to placental EVTs. In an early study on the tissue specific distribution of GKN1 by Northern blot analyses, Oien et al. [10] found GKN1 expression restricted to gastrum, while no GKN1 was found in the human placenta. The discrepancy of these results to the findings of our study might be related to differences in the placental sampling technique, because only the relatively small portion of EVTs specifically express GKN1, while most of the placental mass, especially the villous tree, is GKN1 negative.

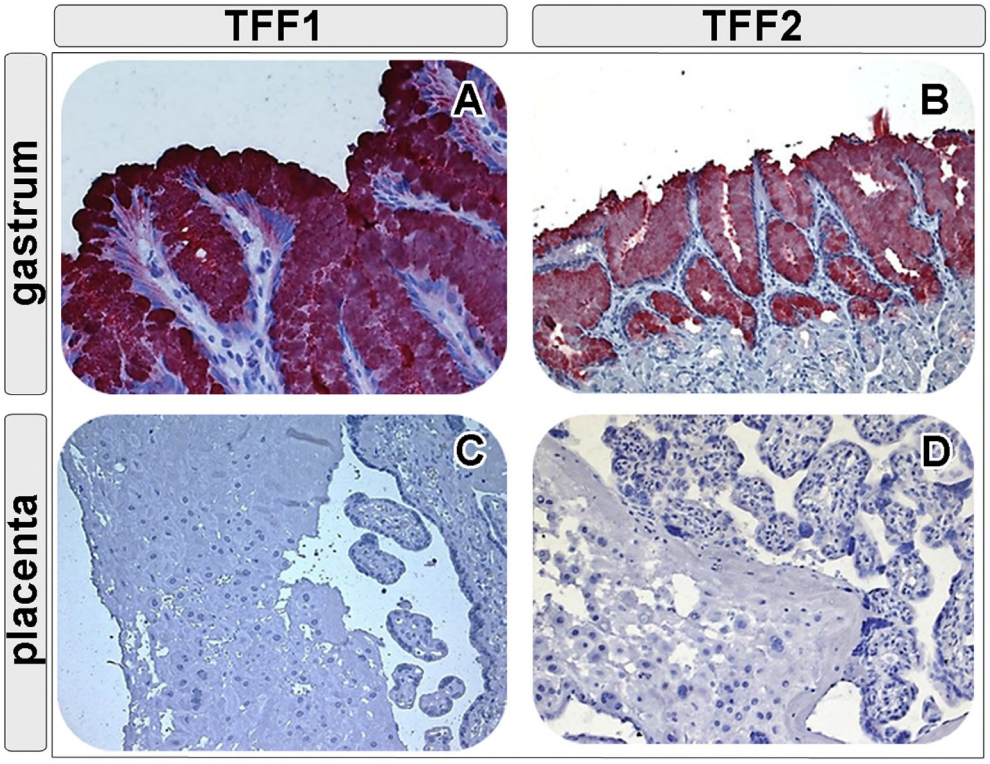

Fig. 3. Expression of trefoil factor 1 and 2 (TFF1, TFF2) in healthy placenta at term and in gastric antrum (gastrum; positive control). While TFF1 and TFF2 staining reveals strong positivity in the mucosa of the gastrum (A and B), no positive staining is found in placental tissue, neither at the level of the decidual basal plate nor in the placental villous tree (C and D). Magnification 200×.

EVTs exert important functions in placental development: After detachment from the proliferative zone of the proximal column of the placental anchoring villi EVTs invade into the maternal decidua where they control maternal spiral artery blood flow (endovascular trophoblasts) and modulate the maternal immune response (interstitial trophoblast) [35]. The process by which coherently attached trophoblasts change their morphology to a migratory phenotype is thought to closely resemble epithelial-to-mesenchymal transition [36,37], a process that is well recognized in malignant tumors [38]. Unlike carcinomas, the invasive capacity of trophoblasts is well-orchestrated and limited in duration and depth, preventing potential malignancy [36]. Consequently, placental biology is considered pseudo-malignant [9,39,40]. Dysregulation of trophoblast migratory activity is associated with trophoblast-associated gestational diseases: Shallow trophoblast invasion can lead to preeclampsia or intrauterine growth restriction, while trophoblasts with an increased invasive potential are predisposing for placenta increta/percreta, or the development of trophoblast tumors, such as the aggressively growing and highly metastatic choriocarcinoma [6,7,41]. This is in keeping with our observations of a lack of GKN1 expression in trophoblast-associated gestational diseases with an increased invasive potential like choriocarcinoma or mole, except for placental site trophoblastic tumor, where cytoplasma rich trophoblasts with extravillous trophoblast morphology (possibly EVT giant cells) stained positive for GKN1. This generally benign tumor is characterized by a lower invasive potential than choriocarcinomas. This finding might underscore the role of GKN1 as tumor-suppressor, as postulated for gastric cancer (GC) [17]. EVTs investigated in this study were obtained from third trimester placentas. During the course of gestation EVTs switch their phenotype from mesenchymal/migratory (first trimester) to epithelial/sessile (term) to convey e.g. anchoring functions in the decidua. Analysing placenta at term, we hypothesized that GKN1 might be involved in inhibition of EVT migration and might mediate firm adhesion. Our findings of a reduced migratory activity, reduced Akt activation and induced of E-cadherin expression in GKN1 treated JEG-3 cells are in keeping with findings obtained in the gut: Studies on GKN1 function in GC had previously shown that GKN1 treatment inhibited epithelial-to-mesenchymal transition in gastric carcinogenesis by inactivation of the PI3K/Akt-pathway [17] with a subsequent induction of E-cadherin expression. Akt signaling is known to reduce E-cadherin expression in eptithelial cells [42]. Thus, Akt mediates metastatic function in human cancer cells [43]. Loss of E-cadherin is a hallmark of metastatic carcinoma [44] and E-cadherin expression is lost commonly in diffuse, poorly differentiated GC [45].

In the placenta, PI3K/Akt is an important developmental pathway [46] that positively affects migration of human trophoblasts

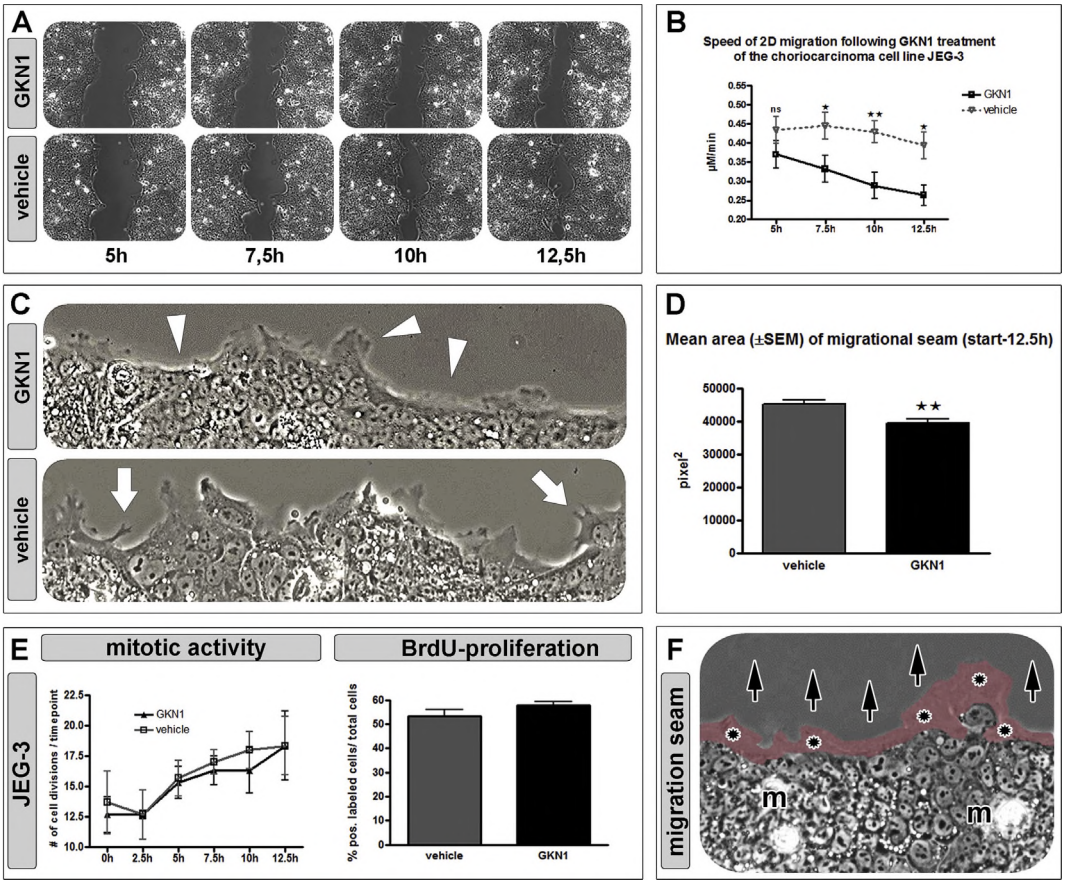

Fig. 4. Time-lapse imaging of JEG-3 migration (A and B) and changes in JEG-3 morphology (C, D and F) following GKN1 treatment ($\star = p < 0.05$, $\star\star = p < 0.02$). Analysis of JEG-3 proliferation rate via time-lapse live cell imaging and BrdU assay (E). A: Representative live images of vehicle and GKN1 treated JEG-3 cells at the respective time points (5 h–12.5 h). B: Migrational speed (µM/min) of the JEG-3 cell line at the respective time points: After 7.5 h GKN1 treatment significantly reduced JEG-3 migration. C: Morphological changes induced by GKN1 in JEG-3 cells. JEG-3 cell migration is based on pseudopodic extension of cytoplasma into the preformed gap (F: cytoplasmatic seam, indicated in red color and asterisks), direction of migration (arrows), m indicates bright cells undergoing mitosis). While vehicle treated cells retain a pseudopodic cytoplasmatic structure during migration (C; white arrows), GKN1-treated cells (C; white arrowheads) show a significant reduction in the number of pseudopodia, resulting in a significantly reduced area of migrational seam (D). E: GKN1 treatment did not significantly alter JEG-3 proliferation in comparison to vehicle, as determined by phase contrast visualization of mitosis (see F; m) and by BrdU staining.

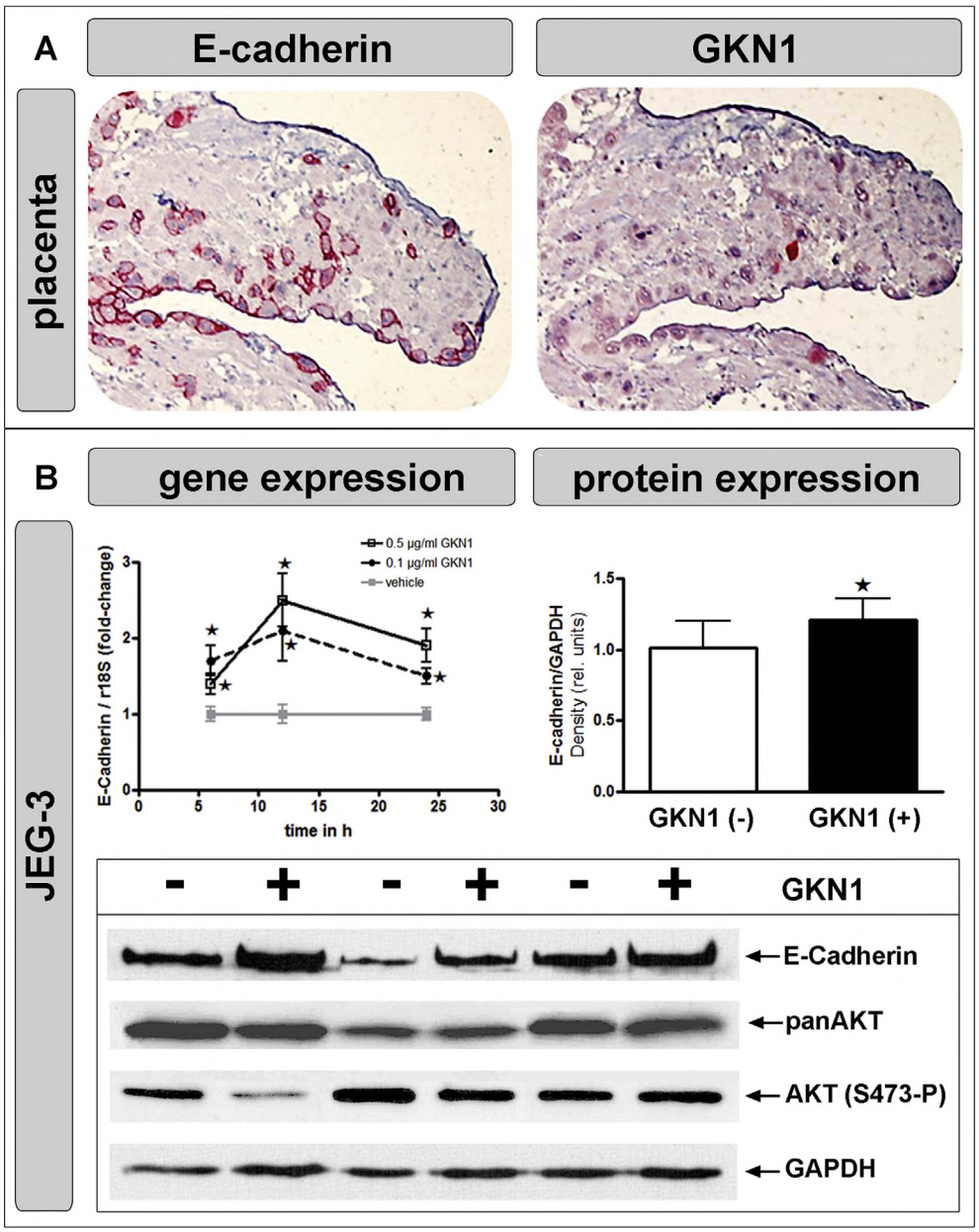

Fig. 5. E-cadherin expression in placental tissue (A, magnification 200×) and in the trophoblast cell line JEG-3 (B). A: Beside its expression in the syncytiotrophoblast of healthy third trimester placentas (not shown here), E-cadherin is expressed in extravillous trophoblasts. Depicted is the decidual basal plate of consecutive sections containing extravillous trophoblasts with staining for E-cadherin (left) and gastrokine 1 (GKN1, right) indicating co-localization. B: GKN1 treatment (0.1 and 0.5 µg/ml) of the JEG-3 cell line results in a significant induction of E-cadherin mRNA (left) and protein expression (right), with a concomitant reduction of AKT Ser473 phosphorylation ($p < 0.01$, bottom).

[47] and the extravillous trophoblast derived HTR-8/SVneo cell line [48]. The adherens junction protein E-cadherin is transiently down-regulated in EVTs of the first and second trimester [49], allowing trophoblast invasion.

Our results on GKN1 function in the trophoblast cell line JEG-3 closely resemble effects of GKN1 on the AGS GC cell line [17]: In our study GKN1 treatment altered the mobility of JEG-3 cells by inducing E-cadherin, reducing the formation of cytoplasmatic pseudo-podia and subsequently leading to a reduced migration. GKN1 was previously shown to reduce the proliferation rate of certain cancer cell lines, however, did not affect the proliferation of the HeLa cell line [16]. We did not observe changes in proliferation rates of JEG-3 in response to GKN1. This is supportive of a GKN1-mediated reduction of migration in JEG-3, which is not secondary to a reduced proliferation rate. The JEG-3 cell line was chosen for our experiments, because of its EVT-like characteristics [40] and because of its migratory activity [50].

Although we only examined two-dimensional migration, our finding that GKN1 reduces matrix metalloproteinase-9 (MMP-9, gelatinase B) expression in JEG-3 cells might point to a regulatory

Table 3
Expression of MMP-9, TIMP-1 and -2 following GKN1-treatment (12 h).

Gene name	Fold-induction	*p*-value
TIMP-1	0.88 ± 0.17	0.5
TIMP-2	1.18 ± 0.26	0.5
MMP-9	0.60 ± 0.13	0.01

role of GKN1 in three-dimensional tissue invasion. MMPs catalyze the destruction of decidual matrix [51]. Especially MMP-9 is highly expressed by first trimester EVT and is rate-limiting for its invasion [2,52]. Reduced expression of MMP-9 in EVT is associated with preeclampsia, which is characterized by insufficient trophoblast invasion [53]. Interestingly, MMP-9 knockout mice show an impairment of reproduction [54].

We and others [55] did not find placental expression of GKN1 activating TFF1 and TFF2. Thus, either trefoil factors are not essential for GKN1 action on EVTs, or alternatively, TFF1 or TFF2 might originate from other sources: It is known that the concentration of circulating maternal trefoil factors strongly increases during pregnancy [56]. TFF-1,-2 and-3 expression was found in the mucus of endocervix and endometrium [57]. It seems intriguing to hypothesize that there might be an interaction of fetal GKN1 and maternal trefoil factors on the level of the EVT, as discussed for gastric [34] epithelium. Such cross-talk could e.g. modify the resistance of GKN1 to protease degradation or affect the stabilization of cell–cell contacts of EVTs [13,58]. This might be of physiological relevance for EVT invasion and placental function, as in the second half of pregnancy a reduction of EVT invasion is thought to be tightly related to cell–cell linkage and transformation of multiple EVT into multinucleated giant cells [59,60]. We did not observe relevant GKN1 expression in first trimester EVT, which still display an invasive phenotype. This might strengthen our hypothesis that GKN1 becomes of functional relevance in late pregnancy, possibly inhibiting EVT invasion. However, our findings in first trimester placental tissue are limited by the fact that these samples were from abortions and by the limited number of samples. Although these tissues did not show overt histological abnormalities, we cannot completely rule out minor placental pathologies which might influence GKN1 expression. Nevertheless, our data suggest an important role for GKN1 for the plasticity of placental cells during pregnancy. Knowledge on placental GKN1 function might not only aid understanding of reproductive biology but further help to improve the understanding of its role in (gastric) cancer.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

The authors thank I. Allabauer, K. Schmied and R. Wachtveitl for their technical assistance. We also thank the research staff of the Departments of Gynecology & Obstetrics and Pathology at the University of Erlangen-Nürnberg for their collaboration. This work was supported by the Johannes & Frieda Marohn Stiftung (Fahl/2008), Medical Department, University of Erlangen-Nürnberg, Germany.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.placenta.2013.08.005.

References

[1] Elliot MG, Crespi BJ. Placental invasiveness mediates the evolution of hybrid inviability in mammals. Am Nat 2006;168(1):114–20.
[2] Bischof P, Meisser A, Campana A. Control of MMP-9 expression at the maternal-fetal interface. J Reprod Immunol 2002;55(1–2):3–10.
[3] Dey SK, Lim H, Das SK, Reese J, Paria BC, Daikoku T, et al. Molecular cues to implantation. Endocr Rev 2004;25(3):341–73.
[4] Karmakar S, Dhar R, Das C. Inhibition of cytotrophoblastic (JEG-3) cell invasion by interleukin 12 involves an interferon gamma-mediated pathway. J Biol Chem 2004;279(53):55297–307.
[5] Eastabrook G, Hu Y, von Dadelszen P. The role of decidual natural killer cells in normal placentation and in the pathogenesis of preeclampsia. J Obstet Gynaecol Can 2008;30(6):467–76.
[6] Huppertz B. Placental origins of preeclampsia: challenging the current hypothesis. Hypertension 2008;51(4):970–5.
[7] Seckl MJ, Sebire NJ, Berkowitz RS. Gestational trophoblastic disease. Lancet 376(9742):717–29.
[8] Lurain JR. Gestational trophoblastic disease I: epidemiology, pathology, clinical presentation and diagnosis of gestational trophoblastic disease, and management of hydatidiform mole. Am J Obstet Gynecol 2010;203(6):531–9.
[9] Soundararajan R, Rao AJ. Trophoblast 'pseudo-tumorigenesis': significance and contributory factors. Reprod Biol Endocrinol 2004;2:15.
[10] Oien KA, McGregor F, Butler S, Ferrier RK, Downie I, Bryce S, et al. Gastrokine 1 is abundantly and specifically expressed in superficial gastric epithelium, down-regulated in gastric carcinoma, and shows high evolutionary conservation. J Pathol 2004;203(3):789–97.
[11] Yoshikawa Y, Mukai H, Hino F, Asada K, Kato I. Isolation of two novel genes, down-regulated in gastric cancer. Jpn J Cancer Res 2000;91(5):459–63.
[12] Moss SF, Lee JW, Sabo E, Rubin AK, Rommel J, Westley BR, et al. Decreased expression of gastrokine 1 and the trefoil factor interacting protein TFIZ1/GKN2 in gastric cancer: influence of tumor histology and relationship to prognosis. Clin Cancer Res 2008;14(13):4161–7.
[13] Hnia K, Notarnicola C, de Santa Barbara P, Hugon G, Rivier F, Laoudj-Chenivesse D, et al. Biochemical properties of gastrokine-1 purified from chicken gizzard smooth muscle. PLoS One 2008;3(12):e3854.
[14] Shiozaki K, Nakamori S, Tsujie M, Okami J, Yamamoto H, Nagano H, et al. Human stomach-specific gene, CA11, is down-regulated in gastric cancer. Int J Oncol 2001;19(4):701–7.
[15] Rippa E, La Monica G, Allocca R, Romano MF, De Palma M, Arcari P. Overexpression of gastrokine 1 in gastric cancer cells induces fas-mediated apoptosis. J Cell Physiol.
[16] Xing R, Li W, Cui J, Zhang J, Kang B, Wang Y, et al. Gastrokine 1 induces senescence through p16/Rb pathway activation in gastric cancer cells. Gut 2012;61(1):43–52.
[17] Yoon JH, Kang YH, Choi YJ, Park IS, Nam SW, Lee JY, et al. Gastrokine 1 functions as a tumor suppressor by inhibition of epithelial-mesenchymal transition in gastric cancers. J Cancer Res Clin Oncol 2011;137(11):1697–704.
[18] Toback FG, Walsh-Reitz MM, Musch MW, Chang EB, Del Valle J, Ren H, et al. Peptide fragments of AMP-18, a novel secreted gastric antrum mucosal protein, are mitogenic and motogenic. Am J Physiol Gastrointest Liver Physiol 2003;285(2):G344–53.
[19] Martin TE, Powell CT, Wang Z, Bhattacharyya S, Walsh-Reitz MM, Agarwal K, et al. A novel mitogenic protein that is highly expressed in cells of the gastric antrum mucosa. Am J Physiol Gastrointest Liver Physiol 2003;285(2):G332–43.
[20] Westley BR, Griffin SM, May FE. Interaction between TFF1, a gastric tumor suppressor trefoil protein, and TFIZ1, a brichos domain-containing protein with homology to SP-C. Biochemistry 2005;44(22):7967–75.
[21] Newton JL, Allen A, Westley BR, May FE. The human trefoil peptide, TFF1, is present in different molecular forms that are intimately associated with mucus in normal stomach. Gut 2000;46(3):312–20.
[22] Walsh-Reitz MM, Huang EF, Musch MW, Chang EB, Martin TE, Kartha S, et al. AMP-18 protects barrier function of colonic epithelial cells: role of tight junction proteins. Am J Physiol Gastrointest Liver Physiol 2005;289(1):G163–71.
[23] Su AI, Wiltshire T, Batalov S, Lapp H, Ching KA, Block D, et al. A gene atlas of the mouse and human protein-encoding transcriptomes. Proc Natl Acad Sci U S A 2004;101(16):6062–7.
[24] Zygmunt M, Hahn D, Munstedt K, Bischof P, Lang U. Invasion of cytotrophoblastic JEG-3 cells is stimulated by hCG in vitro. Placenta 1998;19(8):587–93.
[25] Ruebner M, Strissel PL, Langbein M, Fahlbusch F, Wachter DL, Faschingbauer F, et al. Impaired cell fusion and differentiation in placentae from patients with intrauterine growth restriction correlate with reduced levels of HERV envelope genes. J Mol Med 88(11):1143–56.
[26] Fahlbusch FB, Dawood Y, Hartner A, Menendez-Castro C, Nogel SC, Tzschoppe A, et al. Cullin 7 and Fbxw 8 expression in trophoblastic cells is regulated via oxygen tension: implications for intrauterine growth restriction? J Matern-Fetal & Neonat Med: Off J Euro Assoc Perinatal Med, Feder Asia Oceania Perin Soc, Int Soc Perinatal Obstet 2012;25(11):2209–15.
[27] Frank GR, Brar AK, Cedars MI, Handwerger S. Prostaglandin E2 enhances human endometrial stromal cell differentiation. Endocrinology 1994;134(1):258–63.
[28] Lockwood CJ, Yen CF, Basar M, Kayisli UA, Martel M, Buhimschi I, et al. Preeclampsia-related inflammatory cytokines regulate interleukin-6 expression in human decidual cells. Am J Pathol 2008;172(6):1571–9.
[29] Hiden U, Wadsack C, Prutsch N, Gauster M, Weiss U, Frank HG, et al. The first trimester human trophoblast cell line ACH-3P: a novel tool to study autocrine/paracrine regulatory loops of human trophoblast subpopulations–TNF-alpha stimulates MMP15 expression. BMC Dev Biol 2007;7:137.
[30] Meissner U, Spranger R, Lehner M, Allabauer I, Rascher W, Dotsch J. Hypoxia-induced leptin production in human trophoblasts does not protect from apoptosis. Eur J Endocrinol 2005;153(3):455–61.

[31] Huh S, Ker DF, Bise R, Chen M, Kanade T. Automated mitosis detection of stem cell populations in phase-contrast microscopy images. IEEE Trans Med Imag 2011;30(3):586–96.
[32] Marek I, Volkert G, Jahn A, Fahlbusch F, Zurn C, Ozcan Z, et al. Lack of alpha8 integrin leads to morphological changes in renal mesangial cells, but not in vascular smooth muscle cells. BMC Cell Biol 11:102.
[33] Marek I, Volkert G, Jahn A, Fahlbusch F, Zurn C, Ozcan Z, et al. Lack of alpha8 integrin leads to morphological changes in renal mesangial cells, but not in vascular smooth muscle cells. BMC Cell Biol 2010;11:102.
[34] Menheniott TR, Kurklu B, Giraud AS. Gastrokines: stomach-specific proteins with putative homeostatic and tumor suppressor roles. Am J Physiol Gastrointest Liver Physiol 2013;304(2):G109–21.
[35] Vicovac L, Aplin JD. Epithelial-mesenchymal transition during trophoblast differentiation. Acta Anat 1996;156(3):202–16.
[36] Kokkinos MI, Murthi P, Wafai R, Thompson EW, Newgreen DF. Cadherins in the human placenta–epithelial-mesenchymal transition (EMT) and placental development. Placenta 2010;31(9):747–55.
[37] Floridon C, Nielsen O, Holund B, Sunde L, Westergaard JG, Thomsen SG, et al. Localization of E-cadherin in villous, extravillous and vascular trophoblasts during intrauterine, ectopic and molar pregnancy. Mol Hum Reprod 2000;6(10):943–50.
[38] Larue L, Bellacosa A. Epithelial-mesenchymal transition in development and cancer: role of phosphatidylinositol 3' kinase/AKT pathways. Oncogene 2005;24(50):7443–54.
[39] Ferretti C, Bruni L, Dangles-Marie V, Pecking AP, Bellet D. Molecular circuits shared by placental and cancer cells, and their implications in the proliferative, invasive and migratory capacities of trophoblasts. Hum Reprod Update 2007;13(2):121–41.
[40] Novakovic B, Rakyan V, Ng HK, Manuelpillai U, Dewi C, Wong NC, et al. Specific tumour-associated methylation in normal human term placenta and first-trimester cytotrophoblasts. Mol Hum Reprod 2008;14(9):547–54.
[41] Kaufmann P, Black S, Huppertz B. Endovascular trophoblast invasion: implications for the pathogenesis of intrauterine growth retardation and preeclampsia. Biol Reprod 2003;69(1):1–7.
[42] Grille S, Bellacosa A, Larue L. Regulation of E-cadherin by the Akt protein kinase B. J de la Societe de biologie 2004;198(4):375–8.
[43] Vivanco I, Sawyers CL. The phosphatidylinositol 3-kinase AKT pathway in human cancer. Nat Rev Cancer 2002;2(7):489–501.
[44] Cavallaro U, Christofori G. Multitasking in tumor progression: signaling functions of cell adhesion molecules. Ann N Y Acad Sci 2004;1014:58–66.
[45] Tahara E. Genetic pathways of two types of gastric cancer, vol. 157. IARC Scientific Publications; 2004. p. 327–49.
[46] Forbes K, Westwood M. Maternal growth factor regulation of human placental development and fetal growth. J Endocrinol 2010;207(1):1–16.
[47] Knofler M. Critical growth factors and signalling pathways controlling human trophoblast invasion. Int J Dev Biol 2010;54(2–3):269–80.
[48] Qiu Q, Yang M, Tsang BK, Gruslin A. Both mitogen-activated protein kinase and phosphatidylinositol 3-kinase signalling are required in epidermal growth factor-induced human trophoblast migration. Mol Hum Reprod 2004;10(9):677–84.
[49] Zhou Y, Fisher SJ, Janatpour M, Genbacev O, Dejana E, Wheelock M, et al. Human cytotrophoblasts adopt a vascular phenotype as they differentiate. A strategy for successful endovascular invasion? J Clin Invest 1997;99(9):2139–51.
[50] Fu J, Lv X, Lin H, Wu L, Wang R, Zhou Z, et al. Ubiquitin ligase cullin 7 induces epithelial-mesenchymal transition in human choriocarcinoma cells. J Biol Chem 2010;285(14):10870–9.
[51] Roth I, Fisher SJ. IL-10 is an autocrine inhibitor of human placental cytotrophoblast MMP-9 production and invasion. Dev Biol 1999;205(1):194–204.
[52] Campbell S, Rowe J, Jackson CJ, Gallery ED. In vitro migration of cytotrophoblasts through a decidual endothelial cell monolayer: the role of matrix metalloproteinases. Placenta 2003;24(4):306–15.
[53] Kolben M, Lopens A, Blaser J, Ulm K, Schmitt M, Schneider KT, et al. Proteases and their inhibitors are indicative in gestational disease. Eur J Obst, Gynecol Reprod Biol 1996;68(1–2):59–65.
[54] Dubois B, Arnold B, Opdenakker G. Gelatinase B deficiency impairs reproduction. J Clin Invest 2000;106(5):627–8.
[55] Samson MH, Poulsen SS, Obeid R, Herrmann W, Nexo E. Trefoil factor family peptides in the human foetus and at birth. Eur J Clin Invest 2011;41(7):785–92.
[56] Samson MH, Vestergaard EM, Milman N, Poulsen SS, Nexo E. Circulating serum trefoil factors increase dramatically during pregnancy. Scand J Clin Lab Invest 2008;68(5):369–74.
[57] Wiede A, Hinz M, Canzler E, Franke K, Quednow C, Hoffmann W. Synthesis and localization of the mucin-associated TFF-peptides in the human uterus. Cell Tissue Res 2001;303(1):109–15.
[58] Taupin D, Podolsky DK. Trefoil factors: initiators of mucosal healing. Nat Rev Mol Cell Biol 2003;4(9):721–32.
[59] al-Lamki RS, Skepper JN, Burton GJ. Are human placental bed giant cells merely aggregates of small mononuclear trophoblast cells? An ultrastructural and immunocytochemical study. Hum Reprod 1999;14(2):496–504.
[60] Huppertz B, Hemmings D, Renaud SJ, Bulmer JN, Dash P, Chamley LW. Extravillous trophoblast apoptosis–a workshop report. Placenta 2005;26(Suppl. A):S46–8.