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

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ABSTRACT

Introduction: Gastrokine-1 (GKN1) is a secreted auto-/paracrine protein, described to be expressed in the gastric mucosa. In gastric cancers GKN1 expression is commonly down-regulated. While current research focusses on the exploration of tumor-suppressive properties of GKN1 with regard to its potential clinical use in the treatment of gastroenterologic tumor disease, nothing is known about GKN1 expression and function in other organ systems. We investigated GKN1 expression in placental tissue and cells.
Materials and methods: GKN1 was localized using immunohistochemistry in first and third trimester placental tissue, hydatidiform moles and various gestational trophoblastic neoplasias. We determined the expression of GKN1 in immunomagnetic bead-separated term placental cells and in choriocarcinoma cell lines. The role of GKN1 for JEG-3 migration was studied using live cell imaging. E-cadherin, MMP-2 and -9, TIMP-1 and -2, as well as urokinase (uPA) expression levels were determined.
Results: GKN1 is expressed in healthy third trimester placentas. Its expression is specifically limited to the extravillous trophoblast (EVT). GKN1 expression is significantly reduced in choriocarcinoma cell lines and gestational trophoblastic neoplasias. GKN1 attenuates the migration of JEG-3 choriocarcinoma cells in vitro, possibly via AKT-mediated induction of E-cadherin. GKN1 treatment reduced MMP-9 expression in JEG-3.
Discussion: Besides its role in gastric physiology our results clearly indicate regulatory functions of GKN1 in the EVT at the feto-maternal interface during pregnancy. Based on our findings in the JEG-3 choriocarcinoma cell line, an auto-/paracrine role of GKN1 for EVT motility and villous anchorage at the basal plate is conceivable. Thus, the tumor suppressor GKN1 is expressed in placental EVT and might contribute to the regulation of EVT migration/invasion.

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)

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[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.