Trophoblast expression dynamics of the tumor suppressor gene gastrokine 2

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Abstract Gastrokines (GKNs) were originally described as stomach-specific tumor suppressor genes. Recently, we identified GKN1 in extravillous trophoblasts (EVT) of human placenta. GKN1 treatment reduced the migration of the trophoblast cell line JEG-3. GKN2 is known to inhibit the proliferation, migration and invasion of gastric cancer cells and may interact with GKN1. Recently, GKN2 was detected in the placental yolk sac of mice. We therefore aimed to further characterize placental GKN2 expression. By immunohistochemistry, healthy first-trimester placenta showed ubiquitous staining for GKN2 at its early gestational stage. At later gestational stages, a more differentiated expression pattern in EVT and villous cytotrophoblasts became evident. In healthy third-trimester placenta, only EVT retained strong GKN2 immunoreactivity. In contrast, HELLP placentas showed a tendency of increased levels of GKN2 expression with a more prominent GKN2 staining in their syncytiotrophoblast. Choriocarcinoma cell lines did not express GKN2. Besides its trophoblastic expression, we found human GKN2 in fibrotic villi, in amniotic membrane and umbilical cord. GKN2 co-localized with smooth muscle actin in villous myofibroblasts and with HLA-G and GKN1 in EVT. In the rodent placenta, GKN2 was specifically located in the spongiotrophoblast layer. Thus, the gestational age-dependent and compartment-specific expression pattern of GKN2 points to a role for placental development. The syncytial expression of GKN2 in HELLP placentas might represent a reduced state of functional differentiation of the syncytiotrophoblast. Moreover, the specific GKN2 expression in the rodent spongiotrophoblast layer (equivalent to human EVT) might suggest an important role in EVT physiology.

Keywords GKN2 · HELLP · IUGR · PE · Placenta · HLA-G · SMA

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Introduction

Gastrokines (GKNs) are known tumor suppressor genes in the stomach (Dai et al. 2014; Kim et al. 2014; Mao et al. 2012a; Rippa et al. 2011; Yan et al. 2011; Yoon et al. 2011). Our understanding of their roles in other organ systems like the mammary gland (Sjoblom et al. 2006) and the placenta (Antas et al. 2014; Fahlbusch et al. 2013) is just evolving. Currently, the gastrokine family consists of the orthologs GKN1, GKN2 and GKN3. All members of the GKN family share an evolutionary conserved BRICHOS domain, which has tumor-suppressive functions by regulation of epigenetic modification (Hedlund et al. 2009; Yoon et al. 2013).

So far, most data exist on the GKN1 ortholog: GKN1 expression was reduced or absent in gastric cancer (GC) and the respective cell lines. Restoration of GKN1 significantly reduced the proliferation and invasiveness of GC cells. GKN1-transfected and recombinant GKN1-treated GC cells further showed a reduction in epithelial-to-mesenchymal transition (EMT), concomitant with decreased levels of reactive oxygen species and expression of phosphatidylinositol 3-kinase (PI3K)/Akt pathway proteins (Mao et al. 2012a; Rippa et al. 2011; Yan et al. 2011; Yoon et al. 2011). Helicobacter pylori infection and long-term nonsteroidal anti-inflammatory drug administration down-regulated GKN1 expression in the gastric mucosa (Mao et al. 2012b). Recently, Yoon et al. showed that the GKN1–miR-185–DNMT1 axis suppressed gastric carcinogenesis through regulation of epigenetic alterations and of the cell cycle (Yoon et al. 2013). Interestingly, an E104T mutation in the BRICHOS region of GKN1 was identified in breast cancer (Sjoblom et al. 2006).

Compared with GKN1, the role of GKN2 in the stomach is just beginning to be uncovered (Dai et al. 2014). GKN2 expression is found in gastric foveolar cells (Otto et al. 2006). Helicobacter pylori-induced gastritis (Resnick et al. 2006) and GC (Du et al. 2003) are associated with a reduced GKN2 expression. Dai et al. found that GKN2 expression was significantly down-regulated or absent in GC cell lines (Dai et al. 2014), gastric intestinal metaplasia and tumor tissues. Overexpression of GKN2 suppressed the proliferation, migration and invasion of GC cells in part via cell cycle arrest (Dai et al. 2014). This effect was also visible in GKN2-treated nude mice xenograft models, along with a reduction in cyclin D1, cyclin E1 and matrix metalloproteinase 9 (MMP9) expression (Dai et al. 2014). Recently, interactions of GKN2 with GKN1 were found to play a regulatory role in the maintenance of gastric epithelial homeostasis (Kim et al. 2014).

The concept that gastrokines might have physiologic roles outside the stomach, especially in the placenta, is novel. We were able to identify GKN1 expression and regulatory functions in the human placenta (Fahlbusch et al. 2013). GKN1 was expressed in extravillous trophoblasts (EVT) of healthy third-trimester placentas and was significantly reduced in choriocarcinoma cell lines and gestational trophoblastic neoplasias. GKN1 showed tumor-suppressive properties by attenuating the migration of JEG-3 choriocarcinoma cells in vitro. This effect was associated with an AKT-mediated induction of E-cadherin. Moreover, GKN1-treated JEG-3 cells showed reduced MMP9 expression (Fahlbusch et al. 2013). Based on these findings, we hypothesized an auto-/paracrine role of GKN1 for EVT motility and villous anchorage at the basal plate. Additionally, GKN1 protein was found in smooth muscle cells of chicken gizzard, where it associates with filamentous (F)-actin and smooth muscle actin (SMA) highlighting its role in cell–cell contact, in actin network stabilization and in membrane anchorage (Hnia et al. 2008). Recently, GKN2 expression was found to be transiently induced in the maturing mouse yolk sac and co-localized with epithelial cell adhesion molecule (EpCAM) (Antas et al. 2014). The GKN2 transcript was also found in the mouse placenta (Antas et al. 2014). Based on these findings, we studied the localization and expression of GKN2 in the human and the rodent placenta. We were especially interested in gestational age-dependent GKN2 expression pattern in first- versus third-trimester human placentae. We further examined GKN2 expression in the human placental pathologies preeclampsia (PE), intra-uterine growth restriction (IUGR) and the HELLP syndrome (characterized by hemolysis, elevated liver enzymes and low platelet count). Based on its described role in the stomach (i.e., as a tumor suppressor (Dai et al. 2014) and as a regulator of mucosal integrity (Kim et al. 2014; Kouznetsova et al. 2007; Mao et al. 2012b)) and with respect to our previous findings for GKN1 (regulation of trophoblast migration (Fahlbusch et al. 2013)), we hypothesized that GKN2 is expressed in human placenta and might contribute to the pathophysiology of the above placental syndromes, which are accompanied in part by shallow placental invasion (Huppertz 2008) and reduced syncytial integrity (Ruebner et al. 2010, 2013).

Materials and methods

Human placental tissue

Fresh samples of human placenta were excised immediately after placental delivery, fixed in formaldehyde and embedded in paraffin. We analyzed tissue sections of healthy placentas ($n = 5$, gestational age (GA) 31–35 weeks) and of pregnancies complicated by IUGR (GA 31–37 weeks), PE (GA 34–36 weeks) and HELLP (GA 30–35 weeks) ($n = 5$, each). Moreover, placental sections of first-trimester abortions ($n = 4$, GA 8–12 weeks) were examined. Healthy human antrum served as positive control.

In a separate set of experiments, fresh samples of human appropriate for gestational age (AGA) ($n = 25$, GA 36.6 ± 0.6 weeks), IUGR ($n = 18$, GA 36.6 ± 0.9 weeks), PE ($n = 18$, GA 36.4 ± 0.9 weeks) and HELLP ($n = 8$, GA 34.4 ± 1.4 weeks) placentae were collected for qPCR gene expression analysis (GA values shown as mean ± SEM). The samples were obtained in a standardized sterile procedure immediately after placental delivery. A 2 × 2 cm segment, total depth ranging from basal plate to chorionic membrane, was excised at the central region of the placenta using a scalpel blade. Macroscopic calcifications were avoided. Amniotic membranes and basal plate were

removed. Samples were immediately snap-frozen in liquid nitrogen and stored at −80 °C until further processing.

The diagnosis of IUGR, PE and HELLP was based on general accepted criteria, outlined previously (Langbein et al. 2008). AGA placentas came from otherwise healthy newborns with cervical insufficiency or premature rupture of membranes (PROM) of the mother. IUGR, PE and HELLP patients included in this study did not suffer from other clinical diseases, such as cancer or diabetes. Cases of PE with combined IUGR or HELLP syndrome and vice versa were excluded.

Ethics

The study was approved by the Ethics Committee of the Medical Faculty of the University of Erlangen-Nürnberg (#2625-02/28/02 and #2180-05/05/04). Written consent was given by every patient. The use of anonymized paraffin-embedded tissue samples was approved by the Ethics Committee on January 18, 2005.

Animal procedures

All animal procedures were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and were approved by the local government authorities (Regierung von Mittelfranken, approval numbers AZ # 54-2531.31-12/06 and AZ # 54-2532.1-17/08) after evaluation of the local government's review board for animal research ethics. All surgery was performed under isoflurane or sodium pentobarbital anesthesia, and all efforts were made to minimize suffering. Pregnant female Wistar rats were obtained from Charles River (Sulzfeld, Germany) and were housed in a room maintained at 22 ± 2 °C, exposed to a 12-h dark/light cycle. The animals were allowed unlimited access to standard chow and tap water. Rat placentae were obtained following maternal killing via bleeding in deep anesthesia at d19 of pregnancy. 129/Sv mice of our own breeding were housed in a room maintained at 22 ± 2 °C, exposed to a 12-h dark/light cycle and with free access to standard chow (#1320, Altromin, Lage, Germany) and tap water. Sections of mouse placentae were obtained at d17.5 of pregnancy. All rodent placentae were fixed in methyl Carnoy or in paraformaldehyde solution for embedding in paraffin.

Cell lines and cell culture

JaR, JEG-3 and BeWo choriocarcinoma cell lines were purchased from ATCC (Wesel, Germany). BeWo cells were cultured in DMEM (high glucose) media (Sigma-Aldrich, Taufkirchen, Germany) supplemented with 10 % fetal calf serum (FCS) and 100 U/ml penicillin and 100 µg/ml streptomycin (1 % P/S, Sigma-Aldrich). JaR cells were grown in RPMI 1640 media (Sigma-Aldrich) supplemented with 10 % FCS, 2 mM L-glutamine, 10 mM HEPES, 4.5 g/l D-glucose, 0.1 mM nonessential amino acids and 1 % P/S. JEG-3 cells were cultured in DMEM/F12 (Sigma-Aldrich) media with 10 % FCS and 1 % P/S. Cells were cultured in 75 cm^2 T flasks at 37 °C under a humidified atmosphere containing 95 % air, 5 % CO_2.

Western blot analysis

For protein expression analysis, proteins were isolated from three frozen placental tissues with histologic signs of increased syncytial GKN2 expression in our immunostains (see supplementary figure S1 g–i) using a microdismembranator and re-suspended in RIPA buffer with 1 mM Na_3VO_4, 1.5 mM NaF and protein inhibitor cocktail (1 mM phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml of each pepstatin, leupeptin and chymostatin). Fifty micrograms of each lysate was resolved on a 12.5 % acrylamide-SDS gel and transferred to a nitrocellulose membrane using Tris–glycin buffer. Blocking was performed with 3 % nonfat milk. The membrane was incubated with a polyclonal rabbit anti-GKN2 primary antibody (USCN Life Science Inc., Houston, Tx, USA) at a dilution of 1:200 or with horseradish peroxidase-labeled GAPDH (Santa Cruz Biotechnology Inc., Heidelberg, Germany) at a dilution of 1:1000 at 4 °C overnight. Immunoreactivity was visualized with horseradish peroxidase-conjugated goat anti-rabbit antibody (Cell Signaling Technology Inc., Leiden, the Netherlands) at a dilution of 1:1000, using the ECL system according to the manufacturer's instructions (Amersham, Braunschweig, Germany). Protein bands were detected with GeneGnome (Syngene, Cambridge, UK). Digital quantification of the blots was performed with ImageJ Software (v1.48q, NIH, USA).

Gene expression analysis

For gene expression analysis, cultured cells were lysed and RNA was purified using the peqGold TriFast reagent (Peqlab, Erlangen, Germany). Fifty to hundred micrograms from snap-frozen placental tissues was minced using a Mikro-Dismembranator (Braun Biotech, Sartorius AG, Goettingen, Germany). Total RNA of placental tissues was purified by TriFast reagent (Peqlab). RNA of cell lines and tissues was pre-treated with DNase I (Sigma-Aldrich) before cDNA synthesis with the High-Capacity cDNA Reverse Transcription kit (Life Technologies, Darmstadt, Germany) in a thermal cycler (ABI2720) for 2 h at 37 °C. Quantification of GKN2 mRNA expression was achieved by quantitative real-time PCR analysis using the SYBR

Select MasterMix (Life Technologies); 10 nM forward and reverse primers were used to amplify 40 ng of cDNA. The expression was normalized using 18srRNA as a reference gene.

Primers were designed using Primer 3 software (Untergasser et al. 2012). The following primers were used (Eurofins Genomics, Ebersberg, Germany): GKN2 forward 5′-TAACATCATCAGCCCAAGCA-3′, reverse 5′-TGGATGCAATGTAGCCATGT-3′ and 18srRNA forward 5′-GCAATTATTCCCCATGAACG-3′, reverse 5′-GGCCTCACTAAACCATCCAA-3′.

Immunohistochemistry

Following de-paraffinization and rehydration, 2-µm sections of fixed placental tissue and antrum were cut with a Leitz microtome (Leica Instruments, Wetzlar, Germany). Unmasking was performed by cooking in target retrieval solution (TRS, Dako, Hamburg, Germany). Subsequently, endogenous peroxidase activity was blocked with 3 % H_2O_2 in methanol for 20 min at room temperature. Sections were layered with polyclonal rabbit anti-GKN2 primary antibody (USCN Life Science Inc.) at a dilution of 1:50 in 1 % BSA/TBS and incubated overnight at 4 °C. Biotinylated secondary antibodies came from Linaris (Dossenheim, Germany) and were used at a dilution of 1:500. 3,3′-Diaminobenzidine (DAB) staining was performed with a peroxidase detection method, as described previously (Hartner et al. 2001), using a DAB Peroxidase (HRP) Substrate Kit (SK-4100, Vector Laboratories Inc., Burlingham, CA, USA). Hematoxylin served as counterstain. For negative control samples, we used equimolar concentrations of pre-immune rabbit IgG. Sections were analyzed using a Leitz Aristoplan microscope (Leica Microsystems, Wetzlar, Germany). For immunofluorescence (IF) double-stainings, primary antibodies were applied simultaneously overnight. GKN2 antibody dilution and unmasking were performed as described above. Double-stainings with α-smooth muscle actin (SMA), histocompatibility antigen class I, G (MHC I, HLA-G) and GKN1 were performed: Monoclonal primary mouse anti-human SMA antibody came from Dako (#MO851, Dako, Clone 1A4) and was used at a dilution of 1:100 (1 % BSA/TBS). HLA-G primary antibody was from Thermo Scientific (#MA1-19454, Clone 4H84, Pierce Antibody Products, Rockford, IL, USA) and used at a dilution of 1:200. For GKN1 staining, unmasking was performed with cooking in TRS. The mouse monoclonal anti-GKN1 antibody came from Abcam (#ab 57623, Abcam, Cambridge, UK) and was used at a dilution of 1:100. After washing, slides were incubated with the respective secondary fluorescent antibodies for 2 h (Alexa Fluor 555 (red), 488 (green) and DAPI, all from Life Technologies) and embedded in Mowiol (Sigma-Aldrich). IF sections were analyzed using a Nikon Eclipse 80i microscope (Nikon GmbH, Duesseldorf, Germany). The specificity of GKN2 staining in placental tissue was confirmed by control stainings of gastric tissue (human and rodent), as described previously by others (Menheniott et al. 2013). Human and rodent negative control stains are shown in supplementary Fig. S2 and S3, respectively.

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 two-sided unpaired *t* test. A p value of <0.05 was considered statistically significant.

Results

Localization of GKN2 in human first- and third-trimester placental tissue

As shown in Fig. 1, GKN2 was strongly expressed in both first- (Fig. 1a–f) and third-trimester (Fig. 1g–l) human placental compartments: In the first trimester, prominent GKN2 immunoreactivity was seen in villous trophoblasts (VT) lining the basal syncytiotrophoblast (SCT), which itself also showed faint-positive GKN2 staining (Fig. 1a). Moreover, we observed strong positive staining for GKN2 in basal plate extravillous trophoblasts (EVT) (Fig. 1d). This localization was seen with both DAB-IHC (Fig. 1a, d) and IF (Fig. 1b, c, e, f) staining techniques. In the first trimester, the GKN2 staining in the EVT was hard to discriminate from decidual stroma cells and villous fibroblasts, which were also GKN2 positive (Fig. 1d). This was especially evident at earlier stages (villi not yet detectable) of first-trimester placental development (Fig. 1c, f), where positive GKN2 staining was found almost ubiquitously. However, using double-staining with HLA-G antibodies specific to EVT, a clear HLA-G/GKN2 co-localization in EVT was determined (Fig. 1e, f).

In the third trimester (Fig. 1g–l), the placental GKN2 staining profile changed. SCT and VT showed no GKN2 immunoreactivity (Fig. 1g, h, i, k). While the EVT retained its positive staining for GKN2 (Fig. 1j, k, l), the staining pattern differed from the first-trimester EVT (Fig. 1d). The third-trimester EVT showed a characteristic cap-like GKN2 staining which faced toward the fetal side (Fig. 1l), while no such orientation was found in the first trimester (Fig. 1e). HLA-G double-staining (Fig. 1k, l) clearly indicated GKN2 localization in the basal plate EVT, with a few GKN2-positive HLA-G-negative decidual stroma cells in between.

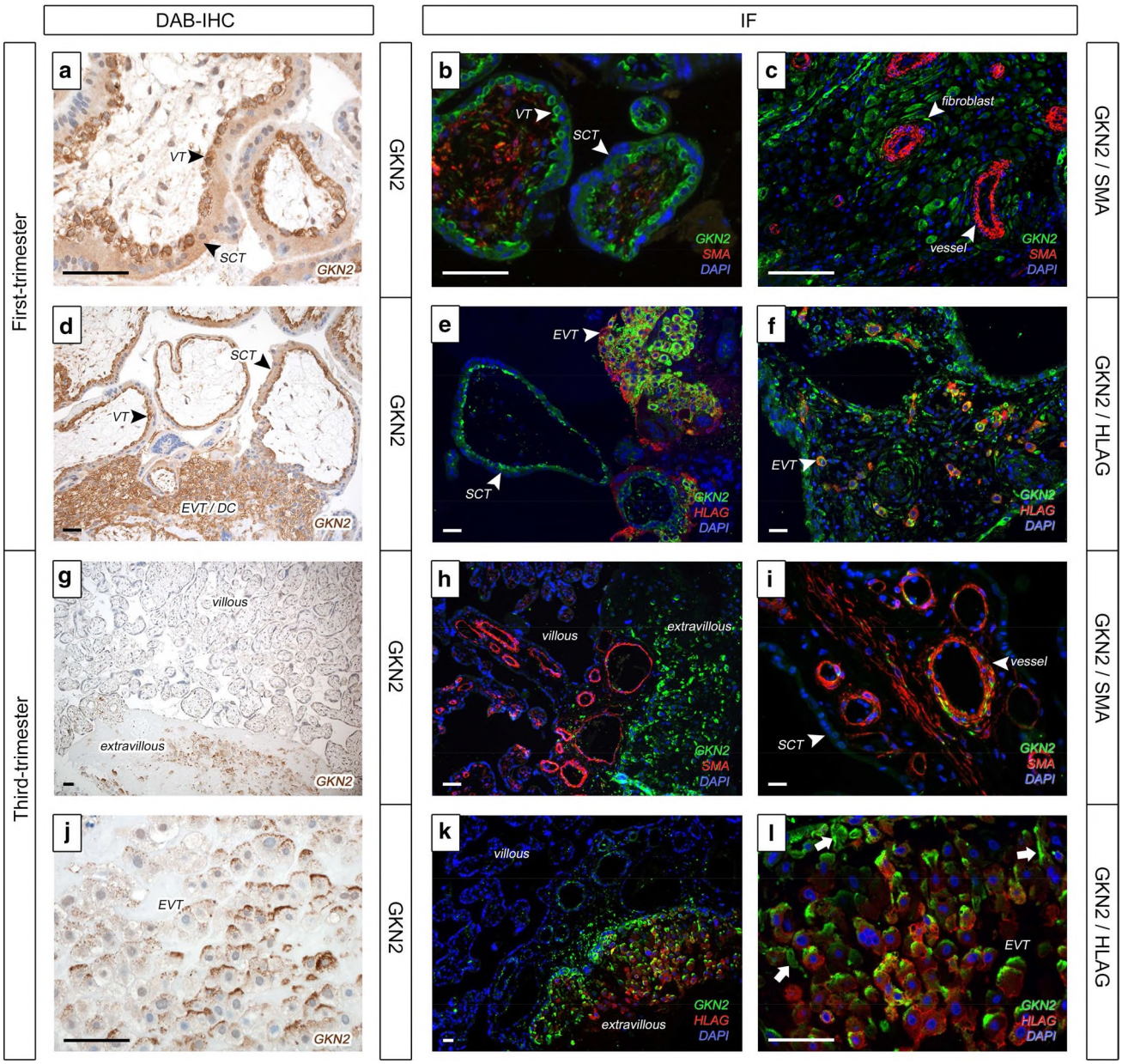

Fig. 1 Localization of gastrokine 2 (GKN2) in human first-trimester (**a**–**f**) and term placental tissues (**g**–**l**). Paraffin-embedded placental tissues were stained by immunohistochemistry (DAB-IHC; **a**, **d**, **g**, **j**) or immunofluorescence (IF; **b**, **c**, **e**, **f**, **h**, **i**, **k**, **l**) with anti-GKN2 (*brown* and *green*, respectively), anti-smooth muscle actin (SMA, *red*) or anti-HLA-G (*red*) antibody. Nuclei were stained by hematoxylin or DAPI (*blue*). In first-trimester placental sections villous (VT) and extravillous trophoblast (EVT), as well as decidual stroma cells (DC) and villous fibroblasts were GKN2 positive. The syncytiotrophoblast (SCT) showed a slightly positive GKN2 staining. EVT and fibroblasts of third-trimester placental sections were GKN2 positive as well, whereas there was no GKN2 expression in the SCT. **b**, **c** and **h**, **i** represent double-stainings of GKN2 (*green*) and SMA (*red*). **e**, **f** and **k**, **l** show co-localization of GKN2 (*green*) and HLA-G (*red*). **l**) *White arrows* (⇒) mark decidual stroma cells. *Yellow* immunofluorescence indicates overlap of *red* and *green* staining. The *bar* equals 100 µm

Strong GKN2 immunoreactivity was found in the EVT in proximity to anchoring villi attached to the basal plate (Fig. 1h, k). Double-staining further showed SMA/GKN2 co-localization in contractile elements of inner villous vessels (Fig. 1i). GKN2 co-localized with SMA in umbilical cord myofibroblasts (supplementary Fig. S4 a–c) and with HLA-G (supplementary Fig. S4 d–f) and GKN1 (supplementary Fig. S4 g–i) in EVT. GKN2 was mostly observed in a speckled cytoplasmic distribution pattern, which might indicate its localization in secretory granules (supplementary Fig. S4 a, c, i), as previously described for the stomach for both GKN2 (Otto et al. 2006) and GKN1 (Chen et al. 2011).

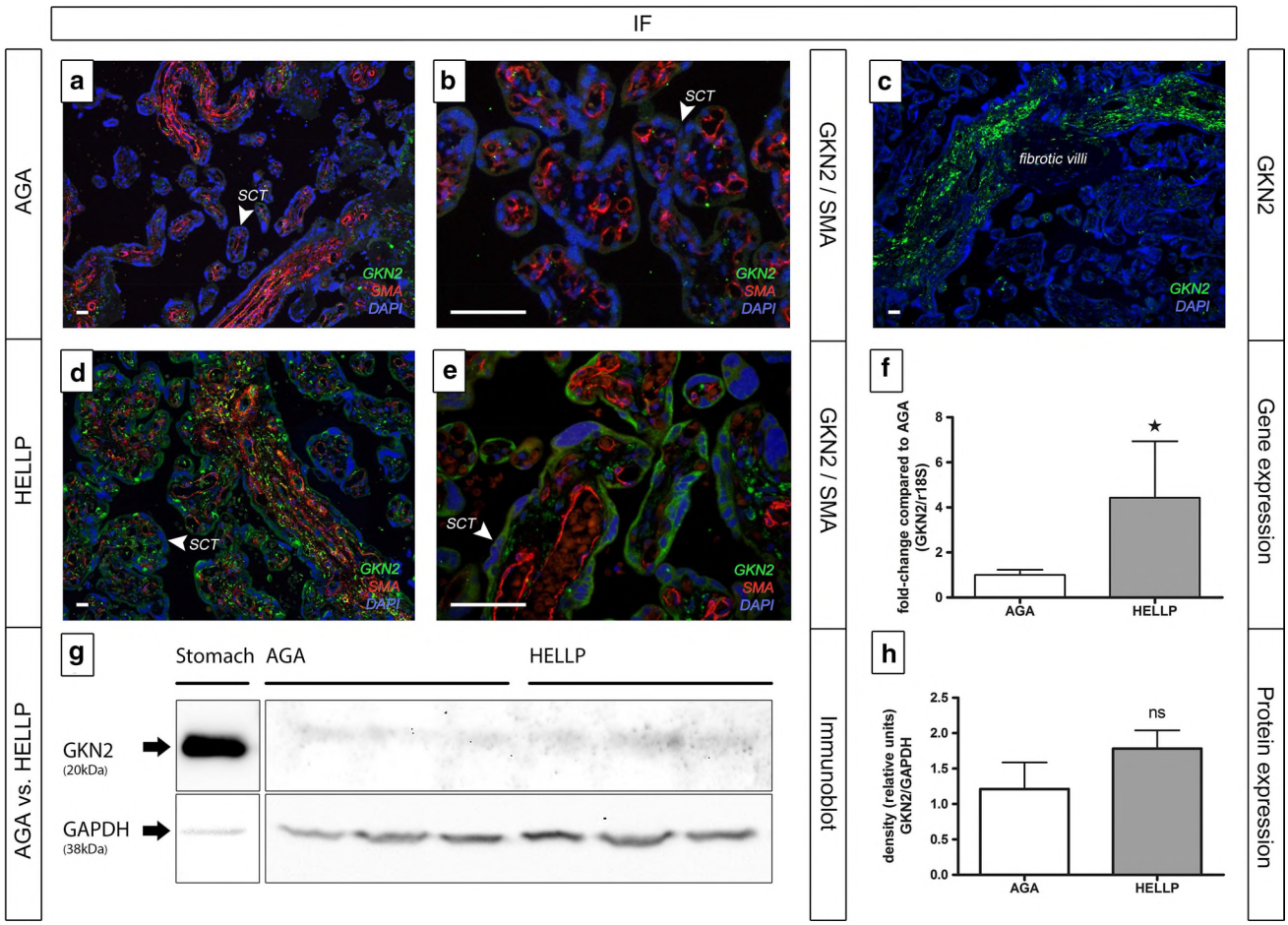

Fig. 2 GKN2 protein and mRNA expression in human appropriate for gestational age (AGA) and HELLP placentas. Third-trimester placental sections were stained with anti-GKN2 (*green*) and anti-SMA (*red*) antibodies, and GKN2 expression detected by immunofluorescence (IF; **a**–**e**) shows GKN2-positive fibrotic villi of an AGA placenta. The *bar* equals 100 µm. **f** gene expression of GKN2 in placentas of pregnancies complicated by HELLP syndrome ($n = 8$) was measured by quantitative real-time PCR and normalized to r18S (house keeping gene). Results (mean ± SEM) represent fold-change expression compared to AGA ($^*p < 0.05$). **g**, **h** Western blot analysis of GKN2 protein expression in AGA versus HELLP placentas ($n = 3$ each) normalized to GAPDH protein expression. Human stomach served as positive control. Results given as mean ± SEM

Expression of GKN2 in human placental pathologies

GKN2 expression levels of choriocarcinoma cell lines JEG-3, BeWO and JaR were below detection limits (data not shown). We did not find a significant induction of GKN2 mRNA expression in placental tissue (villous samples) of pregnancies complicated by IUGR or PE (data not shown). However, a significantly increased GKN2 expression was detected in placental samples of HELLP syndrome patients (Fig. 2f). Supportive of this finding, immunohistochemical analysis revealed increased positive GKN2 staining of the syncytiotrophoblast in three of five analyzed HELLP placentas (supplementary Fig. S1 g–i and Fig. 2e), as compared to healthy AGA placentas with GKN2-negative SCT (supplementary Fig. 1a–e, Fig. 2a, b). To quantify placental GKN2, western blot analysis (AGA vs. HELLP, $n = 3$) was performed (Fig. 2g) using samples of HELLP placentas with strong syncytial GKN2 expression (supplementary Fig. S1 g–i). Compared to the stomach (positive control, Fig. 2 g), GKN2 protein expression was low in the placenta. The increase in GKN2 in total HELLP placentas was small and did not reach statistical significance (Fig. 2h), which is probably due to the fact that we examined total placental tissue only, not isolated syncytium. Thus, samples might also contain GKN2-positive villous fibroblasts (Faye-Petersen et al. 2006) (Fig. 2c, d) or EVT which easily override syncytial GKN2 protein expression.

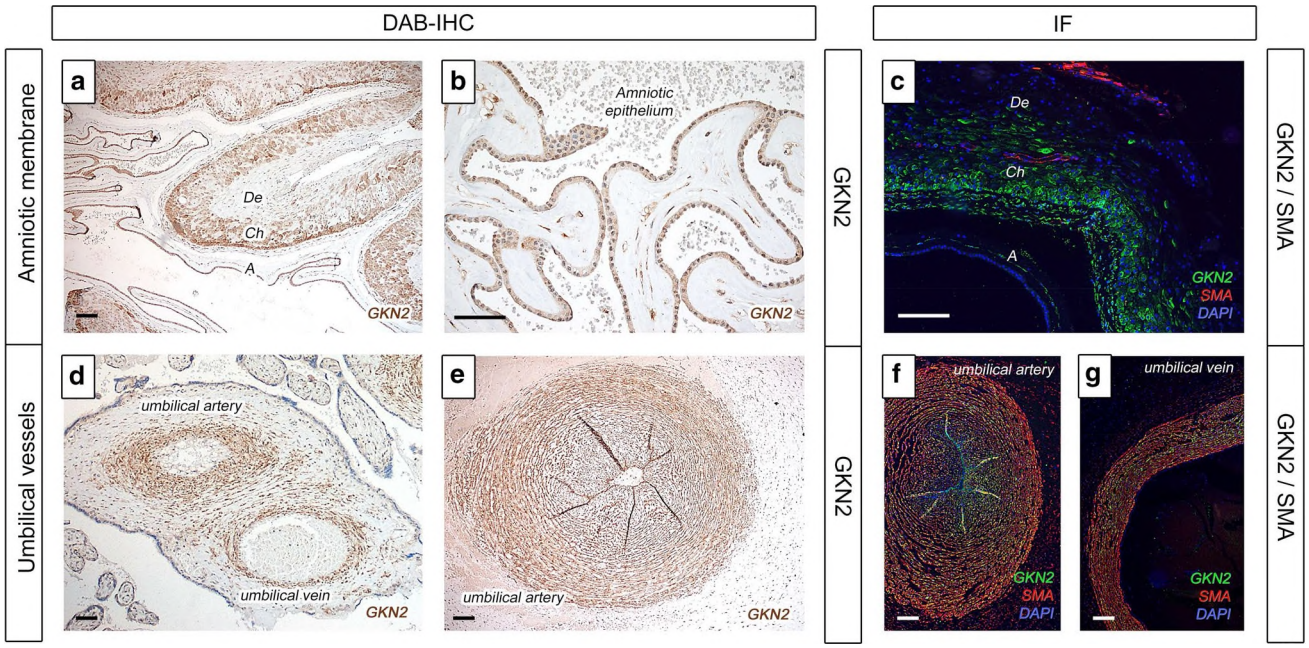

Fig. 3 GKN2 localization in human amniotic membrane and umbilical vessels of third-trimester placentas. Placental sections were stained by immunohistochemistry (DAB-IHC; **a**, **b**, **d**, **e**) or immunofluorescence (IF; **c**, **f**–**g**). The amniotic membrane consists of the amnion (A) and chorion layer (Ch), with the decidua (De) in close proximity to the Ch. The Ch, as well as the epithelium of the A shows positive staining for GKN2 (**a**–**c**). **d**–**g** umbilical vessels show a strong immunoreactivity for GKN2 in smooth muscle cells and surrounding myofibroblasts. **f**, **g** Represent an IF double-staining of GKN2 (*green*) and SMA (*red*). *Yellow* immunofluorescence indicates overlap of red and green staining. The *bar* equals 100 µm

Localization of GKN2 in human amniotic membrane and umbilical cord

As shown in Fig. 3, we found positive GKN2 staining in the chorion layer of the amniotic membrane (Fig. 3a, c) and in amniotic epithelium (Fig. 3a, b), comparable to findings of our own group (data not shown) and others in the mouse (Antas et al. 2014). In the umbilical cord, we determined GKN2 expression in SMA-positive myofibroblasts surrounding the umbilical arteries (Fig. 3d–f) and vein (Fig. 3d, g), as well as in SMA-positive smooth muscle cells of the vessel wall itself (Fig. 3f, g).

Localization of GKN2 in rodent placental tissue

As shown in Fig. 4, we examined the localization of GKN2 in rat (Fig. 4a–c) and mouse (Fig. 4d–f) placental tissue. In the rat, GKN2 was exclusively located in the glycogen cells of the spongiotrophoblast layer (Fig. 4a–c), while the labyrinth layer (SMA positive, Fig. 4b, c) and the giant cells (GiC) (Fig. 4a, c) showed no positive staining for GKN2. In the mouse, the complete spongiotrophoblast layer (i.e., spongiotrophoblast and glycogen cells) stained positive for GKN2 (Fig. 4d–f). Moreover, we were able to verify findings by Antas et al. (2014) showing positive GKN2 staining in the endodermal epithelium of the mouse yolk sac (data not shown).

Discussion

In our study, we demonstrate a differential placental GKN2 expression pattern throughout gestation. In first-trimester trophoblasts, GKN2 was commonly expressed in VT, SCT and EVT, whereas in the third trimester, only EVT retained their strong immunoreactivity for GKN2. Additionally, GKN2 was detected in some SMA-positive smooth muscle cells of villous vessels and in the surrounding myofibroblasts, as well as in some villous fibroblasts throughout gestation. Placentas from patients suffering from HELLP syndrome somewhat showed higher levels of GKN2 mRNA expression, possibly due to a sustained syncytial expression. The rodent placenta showed a specific expression of GKN2 in the spongiotrophoblast layer, which corresponds to the human EVT.

Similar to GKN1 (Oien et al. 2004), GKN2 was originally considered to be exclusively expressed in the stomach epithelium (Menheniott et al. 2013). Our previous work (Fahlbusch et al. 2013) showed that GKN1 is found in human placental EVT, and Antas et al. were recently able to detect GKN2 expression in the yolk sac of mice (Antas et al. 2014). So far, GKN2 expression has not been studied in human or rodent placenta. Comparing the GKN2 expression pattern of healthy placenta to our recently published findings on its ortholog

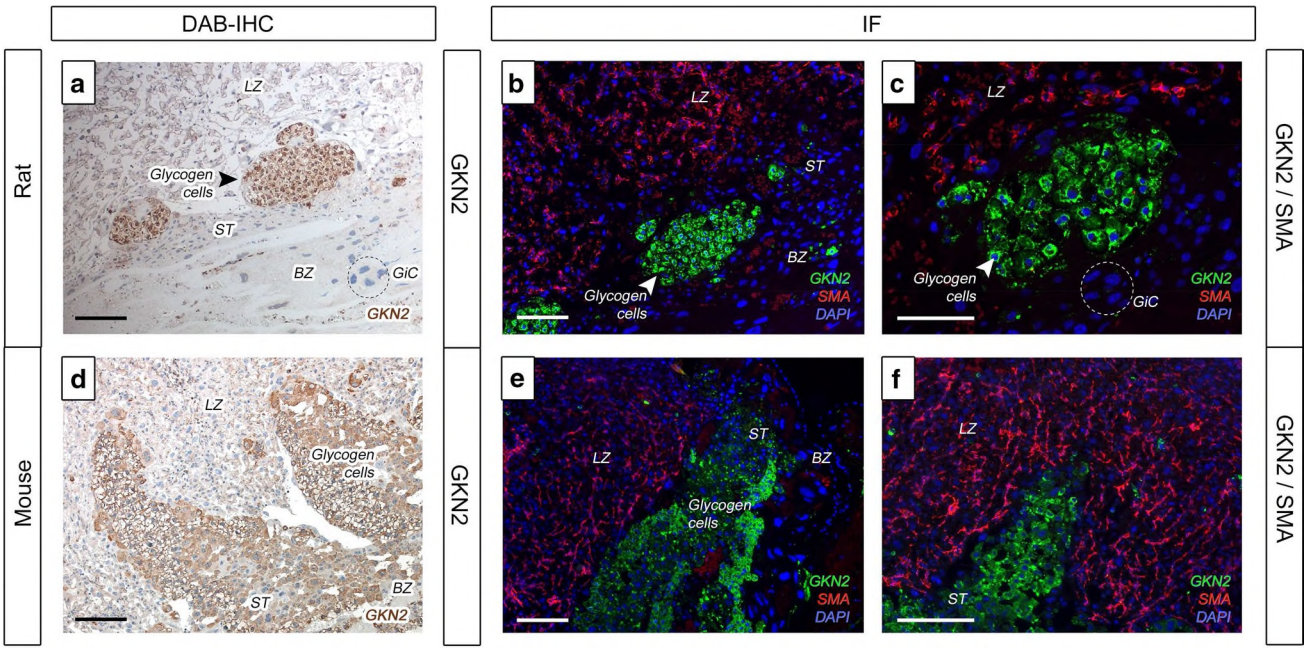

Fig. 4 GKN2 localization in rodent placental tissues. Sections of rat (**a**–**c**) and mouse (**d**–**f**) placentas were stained by immunohistochemistry (DAB-IHC; **a**, **d**) and immunofluorescence (IF; **b**, **c**, **e**, **f**). Rat and mouse placental sections show the labyrinth zone (LZ), the spongiotrophoblast layer (ST) and the basal zone (BZ). Glycogen cells are GKN2 positive. GKN2-negative giant cells (GiC) are indicated by a *dashed circle*. **b**, **c** and **e**, **f** represent immunofluorescence double-stainings of GKN2 (*green)* and SMA (*red*). Nuclei are stained *blue* by hematoxylin (**a**, **d**) or DAPI (**b**, **c**, **e**, **f**). The *bar* equals 100 µm

GKN1 (Fahlbusch et al. 2013), the following similarity can be noted: Using double-immunofluorescence, both GKN1 and GKN2 were expressed in the basal plate EVT (HLA-G positive). While GKN1 showed a homogenous staining pattern, GKN2 immunoreactivity seemed to be directed toward the villous tree resulting in a cap-shaped staining. The GKN2 staining pattern in the third trimester seems to have developmentally evolved from a placental state in the first trimester where all three trophoblast compartments show positive staining for GKN2. This finding might be of pathophysiologic relevance, as we found a tendency of increased GKN2 expression in placentas from pregnancies complicated by the HELLP syndrome. These HELLP placentas partly retained their syncytial immunoreactivity for GKN2, while in healthy AGA placentas the SCT was GKN2 negative. Hence, a disturbance of regular trophoblast development could be hypothesized. Interestingly, the concept of gestational pathogenesis via trophoblast maldifferentiation has been previously assumed to be involved in the development of human placental pathologies (Huppertz 2008). We have recently shown that aberrations in the syncytial cell fusion process are closely associated with these placental syndromes (Ruebner et al. 2013). These conditions are characterized by a failure of EVT to invade the decidua and to subsequently transform maternal spiral arteries, or the altered release of trophoblastic factors, respectively. In IUGR and PE, in contrast to HELLP, we did not observe a significant change in syncytial GKN2 expression. This finding is in line with our previous work indicating a more pronounced epigenetic impact on syncytial gene expression in HELLP placentas compared to other pathologies (Ruebner et al. 2013).

Our observation that GKNs are expressed in smooth muscle cells is shared by others (Hnia et al. 2008). In humans, we detected a strong co-localization of GKN2 with SMA, arguing for an expression of GKN2 in smooth muscle cells and myofibroblasts, too. GKN1 was described as a potential actin filament locker (Hnia et al. 2008). As both GKNs contain the same BRICHOS domain, a similar role for GKN2 might be conceivable. The finding that GKN2 was specifically expressed in the VT lining the basal SCT in the first trimester suggests an additional role of GKN2 in cell–cell adhesion as discussed for GKN1 in the chicken, where the BRICHOS domain stabilized actin fiber assembly (Hnia et al. 2008). In particular, in first-trimester VT lining the basal syncytiotrophoblast, only GKN2 immunoreactivity is found. This could argue for a regulatory role of GKN2 for VT cell differentiation, possibly via cell cycle regulation (Dai et al. 2014) or for trophoblast cell–cell contact, as seen for GKN1 in the colon (Chen et al. 2012; Walsh-Reitz et al. 2005).

The finding that GKN1 and GKN2 co-localized in the EVT might suggest an interaction of these two factors, in analogy to the co-localization in the stomach. Both of these proteins exert tumor-suppressive cellular effects on their own. GKN1 and GKN2 inhibit migration, proliferation and invasion (Dai et al. 2014; Fahlbusch et al. 2013; Yoon et al. 2011). This is in line with our finding that the invasive choriocarcinoma cell lines JEG-3, JaR and BeWo lack GKN2 expression, similar to gastric cancer cell lines, gastric intestinal metaplasia and tumor tissues (Dai et al. 2014). Moreover, individual effects of GKN2 in the placenta seem likely, as it co-localizes with GKN1 only in certain basal plate EVT and because it is present in other placental cell types without a concomitant expression of GKN1 (such as smooth muscle cells, fibroblasts, VT, SCT).

Functional findings from recent studies performed with gastric cancer cell lines indicate that GKN2 has a role in gastric mucosal homeostasis by regulating GKN1 activities (Kim et al. 2014). Surprisingly, GKN1 lost its ability to decrease cell proliferation in the presence of GKN2, although GKN2 itself is known to suppress proliferation (Dai et al. 2014). GKN1 increased GKN2 expression through inactivation of the NF-κB pathway (Kim et al. 2014). In the placenta, no functional data exist at the level of the EVT so far. Regarding the polarized expression pattern of GKN2 in EVT, it might be conceivable that GKN2 has a regulatory role in directed migration or invasion of EVT. Expression of proteins in a polarized fashion is known from urokinase receptors which are located at the leading edge of migrating EVT facing the maternal side (Multhaupt et al. 1994). The polarized expression of these receptors by invasive EVT correlated with the extent and the directionality of their migration. Hence, the polarized EVT staining pattern found for GKN2 facing the fetal side might argue for anti-migratory cell–matrix interactions facilitating anchorage in the third-trimester basal plate. Concomitantly, no such polarized pattern was found in invasive first-trimester EVT.

In rodents, we found specific GKN2 positivity in the spongiotrophoblast layer of the placenta. The functional role of the rodent placental compartments—i.e., the chorionic plate (embryonic side), the labyrinth and the trophoblast giant cell layer (facing the maternal decidua)—has been extensively studied (Cross et al. 2003; Georgiades et al. 2002; Simmons and Cross 2005). However, the function of the spongiotrophoblast layer (also called junctional zone) remains greatly elusive. It is known that the spongiotrophoblast integrity is essential for fetal viability by supporting the developing villous structure of the labyrinth (feto-maternal exchange surface) (Guillemot et al. 1994; Tanaka et al. 1997). Among the trophoblast giant cells, the spongiotrophoblast expresses several important auto- and paracrine factors, such as tissue remodelling factors (e.g., MMP/uPA), angiogenic factors (such as VEGF) and placental lactogens (reviewed by (Bouillot et al. 2006)). During rodent placental development (Rossant and Cross 2001), two types of cytotrophoblasts evolve from the spongiotrophoblast layer: the spongiotrophoblasts and the glycogen cells (Adamson et al. 2002; Rossant and Cross 2001). The latter are characterized as a specialized subtype of spongiotrophoblasts that migrate into the decidua. Based on this property, rodent glycogen cells are deemed to be the equivalent of human EVT (Cross et al. 2003; Georgiades et al. 2002; Simmons and Cross 2005). Analogous to rodent glycogen cells, distal human EVT are also rich in glycogen. Again, opposed to human EVT, little is known about the placental role of rodent glycogen cells (Bouillot et al. 2006; Georgiades et al. 2002; Tesser et al. 2010). Hence, in analogy to human GKN1, GKN2 could act as a negative regulator of spongiotrophoblast/glycogen cell migration (Dai et al. 2014; Fahlbusch et al. 2013).

We further demonstrate strong endodermal GKN2 staining in human amniotic membranes. This finding is in analogy to the study of Antas et al. who found strong GKN2 immunoreactivity in the rodent yolk sac endothelium (Antas et al. 2014). Based on its endodermal localization, it can be hypothesized that the expression of GKN2 in the stomach could be related to the embryonic formation of the primitive gut tube, which arises from the dorsal part of the yolk sac endoderm and is incorporated into the body during embryogenesis (Carlson 2004). The concentrated GKN2 staining in trophoblasts lining the basement membrane of the chorion might further suggest an anchorage function of GKN2 in these cells, as suggested for GKN1 in the chicken (Hnia et al. 2008).

Taken together, we found GKN2 expression in placenta of both humans and rodents. Its differential localization was gestational age dependent, and we determined some up-regulation of GKN2 in placentas from pregnancies complicated by the HELLP syndrome. This might point to a function of GKN2 in the differentiation of healthy and diseased placenta. Due to the localization of GKN2, we speculate that GKN2 may regulate trophoblast anchorage and migration; however, the exact role of GKN2 remains to be determined.

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Conflict of interest The authors declare that they have no conflict of interest.

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