REVIEW

CCN1 (CYR61) and CCN3 (NOV) signaling drives human trophoblast cells into senescence and stimulates migration properties

Friederike Kipkeewa, Manuela Kirschb, Diana Kleinc, Manuela Wuellingd, Elke Winterhagera, and Alexandra Gellhausa,b

aDepartment of Molecular Biology, University of Duisburg-Essen, Essen, Germany; bDepartment of Gynecology and Obstetrics, University of Duisburg-Essen, Essen, Germany; cInstitute of Cell Biology, University of Duisburg-Essen, Essen, Germany; dDepartment of Developmental Biology, University of Duisburg-Essen, Essen, Germany

ABSTRACT
During placental development, continuous invasion of trophoblasts into the maternal compartment depends on the support of proliferating extravillous trophoblasts (EVTs). Unlike tumor cells, EVTs escape from the cell cycle before invasion into the decidua and spiral arteries. This study focused on the regulation properties of glycosylated and non-glycosylated matricellular CCN1 and CCN3, primarily for proliferation control in the benign SGHPL-5 trophoblast cell line, which originates from the first-trimester placenta. Treating SGHPL-5 trophoblast cells with the glycosylated forms of recombinant CCN1 and CCN3 decreased cell proliferation by bringing about G0/G1 cell cycle arrest, which was accompanied by the upregulation of activated Notch-1 and its target gene p21. Interestingly, both CCN proteins increased senescence-associated β-galactosidase activity and the expression of the senescence marker p16. The migration capability of SGHPL-5 cells was mostly enhanced in response to CCN1 and CCN3, by the activation of FAK and Akt kinase but not by the activation of ERK1/2. In summary, both CCN proteins play a key role in regulating trophoblast cell differentiation by inducing senescence and enhancing migration properties. Reduced levels of CCN1 and CCN3, as found in early-onset preeclampsia, could contribute to a shift from invasive to proliferative EVTs and may explain their shallow invasion properties in this disease.

KEYWORDS
CCN1; CCN3; migration; placenta; senescence; trophoblast

Introduction
In mammalian species, the formation of a functional placenta is essential for normal fetal growth and development. Appropriate placentalization in humans relies on the ability of extravillous cytotrophoblasts (EVTs) to proliferate and then to invade the maternal tissue. Diploid EVT’s located in the proximal cell column continuously proliferate to provide a constant supply of invading EVT’s during the first trimester of pregnancy.1-4 To establish a connection between the placenta and the maternal vasculature, terminal differentiated trophoblast giant cells, characterized by endoreduplication up to 1000N of DNA,5 invade and transform the maternal vessels, and this transformation in turn guarantees nutrition and oxygen support to the placenta and the fetus.6,7

Before differentiating into the invasive pathway, the proliferative trophoblast cells of the cell column escape from the cell cycle when they come into contact with the maternal decidua. To prevent tumor-like behavior, such as that occurring in choriocarcinoma, the proliferation and invasion properties are temporally and spatially separated in EVTs. In humans, these two processes are tightly controlled by a plethora of multiple and complex signaling factors, such as growth factors, hormones, and chemokines.8-11 Preeclampsia, a complication in pregnancy, is known to coincide with an insufficient invasion of trophoblast cells into the decidua and the maternal spiral arteries. Such placentas lack sufficient maternal vascular remodeling, and this characteristic, combined with a restricted supply of oxygen and nutrition for the embryo, may result in intrauterine growth restriction. Therefore, deciphering this defined regulation process is important for understanding the pathogenesis of preeclampsia.

Previous studies have shown that the matricellular CCN protein family members CCN1 (CYR61) and CCN3 (NOV) play an important role in these regulatory processes.12-15 CCN proteins are known to regulate pivotal cellular processes, such as differentiation,
proliferation, migration, and angiogenesis.\textsuperscript{16,17} Downstream signaling events are mediated by integrins, bone morphogenetic proteins (BMPs), vascular endothelial growth factor (VEGF), Wnt proteins, and Notch.\textsuperscript{18}

CCN1 and CCN3 proteins occur in a secreted glycosylated form (g-CCN1 and g-CCN3) or in an intracellular non-glycosylated form (ng-CCN1 and ng-CCN3).\textsuperscript{19,20} As shown in earlier studies, these two types of proteins function differently in regulating trophoblast proliferation and migration.\textsuperscript{14,21} In the human placenta, CCN1 and CCN3 are expressed in endothelial cells of placental vessels, stromal cells, and interstitial EVT giant cells, and their expression levels increase during pregnancy.\textsuperscript{22} In the placentas of women with early-onset preeclampsia, CCN1 and CCN3 protein levels are significantly lower than in gestation-matched control placentas.\textsuperscript{23} We have already demonstrated that in the malignant trophoblast cell line Jeg3, a model of invasive EVT, CCN3 reduces cell proliferation and enhances migration properties.\textsuperscript{12-14} These studies showed that CCN3 acts by inducing the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK), phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt), and Notch/p21 pathways, mediating these separate functions for proliferation and migration/invasion in Jeg3 cells.\textsuperscript{14}

In the study reported here, we investigated the proliferation control of CCN1 and CCN3 in benign SGHPL-5 trophoblast cells, which are more similar to the in vivo situation than previous models. We confirmed that the proliferation of the SGHPL-5 cell line is reduced by CCN1 and CCN3, whereas the migration is mostly enhanced by these proteins. We found that the CCN1 and CCN3 proteins induce senescence of the trophoblast cells, which is accompanied by cell cycle arrest at G0/G1. Simultaneously, CCN1 and CCN3 seem to promote migration capability by activating focal adhesion kinase (FAK) and Akt kinase (protein kinase B), a finding suggesting that the CCNs play a regulatory role in controlling proliferation and stopping differentiation, inducing senescence and the onset of migration in EVTs.

Materials and methods

Cell culture and treatment of SGHPL-5 trophoblast cells

The cytotrophoblast cell line SGHPL-5 (kindly provided by G. Whitley, Division of Basic Medical Sciences, St George’s University of London, UK) was routinely cultivated in Ham’s F10 nutrient mixture (Biochrom AG, Berlin, Germany) supplemented with 10% fetal calf serum (FCS; Biochrom AG), 2 mM L-glutamine, and 1% penicillin/streptomycin (10,000 U/ml, 100x; Live Technologies, Carlsbad, CA, USA). Cells were seeded as specified in the following sections and allowed to attach for 24 h in normal culture medium. Synchronization in cell cycle phase distribution was achieved by serum starvation for another 24 h.

Cells were treated with 1 μg/ml recombinant human glycosylated CCN1 and CCN3 (g-rhCCN1, g-rhCCN3) from mouse myeloma cells (R&D Systems, Minneapolis, MN, USA); with 1 μg/ml non-glycosylated CCN1 and CCN3 (ng-rhCCN1, ng-rhCCN3) from E. coli (Peprotech, Hamburg, Germany); or with 1 μg/ml solvent control (0.1% bovine serum albumin [BSA] in phosphate-buffered saline [PBS]).

In vitro proliferation assay

Cells were seeded at a density of $5 \times 10^3$ cells per well in 12-well plates in triplicate. After 24 h of serum starvation, the cells were treated with 5% FCS and 1 μg/ml g-rhCCN1, ng-rhCCN1, g-rhCCN3, ng-rhCCN3, or PBS/0.1% BSA as a solvent control. An electronic cell counter (CASY-I; Schärfe Systems, Reutlingen, Germany) was used to count the cells 24 h and 48 h after plating, as previously described.\textsuperscript{15,24}

Analysis of cell cycle distribution

Cells were seeded at a density of $7 \times 10^5$ cells per well in 25-cm$^2$ cell culture flasks. After 24 h of serum starvation, cells were treated with 5% FCS and 1 μg/ml g-rhCCN1, ng-rhCCN1, g-rhCCN3, ng-rhCCN3, or PBS/0.1% BSA as a solvent control for 0 h, 4 h, or 24 h. Bromodeoxyuridine (BrdU) was added to the culture for the last two hours of the incubation period. Cells were then fixed and stained for newly synthesized DNA as marked by incorporated BrdU using a specific fluorescein isothiocyanate (FITC)-conjugated anti-BrdU antibody as well as total DNA by 7-amino-actinomycin D (7-AAD) according to the manufacturer’s protocol (FITC BrdU Flow Kit; BD Pharmingen, San Jose, CA, USA). Two-color flow cytometric analysis was used to detect cells actively synthesizing DNA (FL-1; FACSCalibur; Becton Dickinson, Heidelberg, Germany) and total DNA (FL-3). Positions in the G0/G1, S, and G2/M phases of the cell cycle were quantified with a classical DNA profile (FL-3; histogram plot of DNA content against cell numbers).

Annexin V apoptosis assay

Cells were seeded at a density of $9 \times 10^3$ cells per well in 6-well plates. After 24 h of serum starvation, the cells were treated with 1 μg/ml g-rhCCN1, g-rhCCN3, or...
PBS/0.1% BSA as a solvent control for 24 h. Annexin V apoptosis assays were performed as described by Koch et al. using flow cytometry (FACSCalibur, Becton Dickinson) in combination with FITC-coupled annexin and propidium iodide (PI; BD Pharmingen).

**Senescence-associated β-galactosidase staining**

SGHPL-5 cells were seeded in 6-well plates (3 × 10^5 cells per well), and experiments were performed with 1 μg/ml rhCCN1, rhCCN3, or PBS/0.1% BSA as a solvent control for 24 h or 48 h. Cells were washed with PBS and were then fixed for 15 min in 0.2% glutaraldehyde in PBS. After two washes with PBS, fixed cells were incubated in freshly prepared senescence-associated β-galactosidase (SA-β-Gal) staining solution (1 mg/ml X-Gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM MgCl_2 in PBS at pH 6.0) for 24 h at 37°C. At least three random fields were digitally photographed with a phase-contrast microscope (10× magnification). The numbers of total cells and of positive blue-stained cells were counted and depicted as SA-β-gal–positive cells per 100 cells.

**Analysis of migration**

Wound healing migration assays for analyzing horizontal migration properties were performed with co-culture inserts (ibidi GmbH, Martinsried, Germany). We tal migration properties were performed with co-culture Wound healing migration assays for analyzing horizon-

**RNA isolation and quantitative reverse-transcriptase polymerase chain reaction**

Total RNA was isolated from cells with the E.Z.N.A RNA extraction kit (Omega-Biotek, Norcross, GA, USA) and was reversely transcribed as previously described. Gene expression was quantified with the quantitative PCR Master Mix and SYBR green (Applied Biosystems, Darmstadt, Germany) and an ABI Prism 7300 sequence detector (Applied Biosystems). PCR reactions were carried out in triplicate with a final volume of 20 μl, with 1 μl (40 ng) cDNA, 1x reaction buffer containing SYBR green, and 10 pmol sense and anti-sense primers (for sequences, see Table 1). PCR was performed for 10 min

**Table 1. Sequences and National Center for Biotechnology Information (NCBI) accession numbers for polymerase chain reaction primers.**

| Gene       | NCBI accession number | Primer sequences (5’ → 3’) | Product length (bp) |
|------------|-----------------------|----------------------------|---------------------|
| β-actin    | NM_001101 sense: ACC AAC TGG GAC GAC ATG GAG AAA A | anti-sense: TAC GGC CAG AGG CTT ACA GGG ATA | 213 |
| p15INK4B   | NM_078487 sense: TGC TAG GAT GCG CAA ATC CC | anti-sense: AGG GTG TGG TGG AAT TGC GA | 199 |
| p16INK4A   | NM_000774 sense: CAT GGA GCC TTC GCG TGA C | anti-sense: GCC CTC CCG TAA CTA TT | 120 |
| p27Kip1    | NM_004064.3 sense: CAG CTT GCG CGA GTT CTA CT | anti-sense: AAG ATG TGG CTT CAG GTT GC | 236 |
| MMP-2      | NM_004350.2 sense: ATG ACA GCT GCA CTA CTT AG | anti-sense: ATT TGT TGC CCA GGA AAG TT | 174 |
| MMP-9      | NM_004994.2 sense: TGG ACA CCA ACA AAG AGT GG | anti-sense: GCC ATT CAC GTG TTC CTT AT | 179 |
| INT-4S     | NM_002205 sense: CTA CAA TGA TGT GGC CT CG | anti-sense: GGA TAT CCA TGG CCA TGC AG | 198 |
| INT-4L     | NM_002211.3 sense: TGG CTT TGC ATT ACT GCT GA | anti-sense: GGG TGG CCG ATT TGG CAT TC | 104 |

bp, base pairs; MMP, matrix metalloproteinase; INT, integrin.
SGHPL-5 cells were seeded onto sterile glass coverslips and fixed with ice-cold methanol for 10 min. Blocking was achieved with 1% BSA in PBS for 10 min at room temperature (RT). Cells were incubated with rabbit anti–Ki-67 antibody (Abcam, Cambridge, UK; ab66155, 1:100) for 1.5 h and with secondary Cy3-conjugated donkey anti-rabbit antibody (Abcam, Cambridge, UK; ab66155, 1:100) for 45 min at RT. Cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole hydrochloride (DAPI; 0.1 μg/ml) for 15 min at RT. Mowiol (Sigma Aldrich, Munich, Germany) was used as a mounting medium. Microscopy was carried out with a confocal laser-scanning microscope (Leica TCS SP5; Leica, Wetzlar, Germany).

**Immunofluorescence and microscopy**

SGHPL-5 cells were seeded onto sterile glass coverslips and fixed with ice-cold methanol for 10 min. Blocking was achieved with 1% BSA in PBS for 10 min at room temperature (RT). Cells were incubated with rabbit anti–Ki-67 antibody (Abcam, Cambridge, UK; ab66155, 1:100) for 1.5 h and with secondary Cy3-conjugated donkey anti-rabbit antibody (Dianova, Munich, Germany) for 45 min at RT. Cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole hydrochloride (DAPI; 0.1 μg/ml) for 15 min at RT. Mowiol (Sigma Aldrich, Munich, Germany) was used as a mounting medium. Microscopy was carried out with a confocal laser-scanning microscope (Leica TCS SP5; Leica, Wetzlar, Germany).

**Protein preparation and western blotting**

Cells were harvested in NETN lysis buffer (175 mM Tris-Base, pH 8; 100 mM NaCl; 1 mM EDTA, 0.4% Igepal CA-630) supplemented with ethylenediaminetetraacetic acid (EDTA)-free complete protease inhibitors and PhosSTOP phosphatase inhibitor cocktail (Roche, Penzberg, Germany) and homogenized by 5 passes through a 20G needle syringe. Cell lysates were centrifuged for 10 min at 13,000 rpm at RT, and cell debris were removed. Next, 30 to 50 μg of total protein was separated on a 12% polyacrylamide gel and transferred to a nitrocellulose membrane (Amersham Bioscences, Piscataway, NJ, USA). The following primary antibodies were used: polyclonal rabbit anti–β-actin (#A2066; Sigma Aldrich), polyclonal rabbit anti-p42/44 (#9154), polyclonal rabbit anti-phospho p42/44 (#4370), polyclonal rabbit anti-Akt (#4691), polyclonal rabbit anti–phospho-Akt (#3787), mouse anti-human cyclin D1 (#sc-8396; Santa Cruz Biotechnology, Dallas, TX, USA), mouse anti-human p21Waf1/Cip1 (#S2946), rabbit anti-human cleaved Notch 1 (#2421) (all from Cell Signaling Technologies, Danvers, MA, USA); mouse anti-human p16 (F-9) (sc-55600), monoclonal mouse α-Tubulin (#sc-8035) (both from Santa Cruz Biotechnology), and mouse anti-human glyceraldehyde 3-phosphate dehydrogenase (GAPDH; #5G4 Mab 6C5; HyTest, Turku, Finland). Secondary horseradish peroxidase (HRP)-conjugated antibodies were purchased from Santa Cruz Biotechnology.

Protein expression was analyzed as described by Yang et al. Detection was accomplished on X-ray films with Supersignal West Dura Extended Duration Substrate (Thermo Scientific Pierce, Rockford, IL, USA) according to the manufacturer’s protocol (Kodak, Stuttgart, Germany). For normalization of protein expression, rabbit anti–β-actin, rabbit anti-GAPDH, or mouse anti-α-Tubulin was used.

**Statistical analysis**

Statistical analysis of densitometric data from Western blot analyses and of the qRT-PCR results was performed with GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA). Statistical significance was determined with PASW Statistics 18 (IBM, Duesseldorf, Germany) using the Mann-Whitney U test for nonparametric independent two-group comparisons. For comparing CCN-treated samples with untreated controls, statistical significance was set at the level of P ≤ 0.05.

**Results**

**CCN1 and CCN3 decrease proliferation and induce G0/G1 cell cycle arrest in SGHPL-5 trophoblast cells**

As previously reported, both g-CCN3 and ng-CCN3 recombinant proteins, decrease proliferation of the malignant trophoblast cell line Jeg3. Because of the tumor characteristics of Jeg3 cells concerning proliferation control we investigated the influence of recombinant human CCN1 and CCN3 proteins on proliferation control in benign SGHPL-5 cells. SGHPL-5 cells endogenously express ng-CCN1 but not CCN3 protein (data not shown). When g-rhCCN1, ng-rhCCN3 or g-rhCCN3 was added to the cell culture medium, the numbers of SGHPL-5 trophoblast cells were significantly lower within 48 h than in control cultures (Fig. 1A), and g-rhCCN1 and ng-rhCCN3 the most effective proteins. The expression of the proliferation marker Ki-67 was clearly lower after treatment with both glycosylation forms of CCN1 and CCN3 than in control cells, as determined by immunocytochemistry (Fig. S1)

To identify the reasons for the reduction in cell numbers after the addition of CCNs, we investigated apoptosis and cell cycle arrest. Analysis of apoptosis with the Annexin V assay showed a significant increase in Annexin V staining after treatment with g-rhCCN1 but not with g-rhCCN3 (Fig. 1B). Antibodies against CCN1 alone or rh-CCN1 pre-incubated with anti-CCN1 did not increase Annexin V staining. Other markers of apoptosis, such as caspase-3
and p53, however, were not altered after the addition of either of the rhCCN proteins (data not shown). Moreover, we did not find that CCN1 and CCN3 induced any increase in the numbers of polyploid cells (n > 4) in SGHPL-5 cells as a marker of endoreduplication (Fig. 1C).

Cell cycle analysis of BrdU-labeled cells by fluorescence-activated cell sorting (FACS) showed that the number of cells arrested in the G0/G1 phase after treatment with ng-rhCCN1 (52.85% ± 0.55%), g-rhCCN1 (52.45% ± 2.69%), ng-rhCCN3 (53.06% ± 2.57%), or g-rhCCN3 (51.49% ± 2.17%) was significantly higher than that of control cells (40.05% ± 1.46%) or vehicle control (42.74% ± 0.86%) (Fig. 2). Accordingly, the fraction of cells in G2/M phase was significantly lower after the addition of ng-rhCCN1
The Notch-1 receptor is known to be expressed in cytotrophoblast cells of the human placenta\textsuperscript{26} and to regulate the cyclin/CDK inhibitor p21 as a downstream target of Notch-1 activation.\textsuperscript{27} Our recent studies using Jeg3 malignant trophoblast cells also showed a link between the CCN-induced Notch-1 signaling pathway and the decrease in cell proliferation.\textsuperscript{14}

Treating SGHPL-5 cells with g-rhCCN1, ng-rhCCN1, or g-rhCCN3 significantly enhanced the cleavage of the Notch-1 receptor (Fig. 3A). After 2 h, the expression of p21 protein was significantly upregulated by both glycosylation states of CCN1, whereas stimulation with glycosylated or non-glycosylated CCN3 enhanced p21 protein expression only slightly but not significantly (Fig. 3B). Interestingly, expression of cyclin D1, a positive regulator for the transition from the G1 to the S phase,\textsuperscript{28} was slightly but not significantly upregulated after the addition of both glycosylation states of CCN1 and CCN3 (data not shown).

**CCN1 and CCN3 induce cellular senescence via upregulation of the senescence marker β-galactosidase and p16 expression**

Since it is known that a G0/G1 cell cycle arrest via upregulation of the p21 pathway upon CCN1 is associated with cellular senescence,\textsuperscript{29} we performed senescence-associated β-galactosidase staining of CCN1- and CCN3-treated SGHPL-5 trophoblast cells. The number of cells with SA-β-Gal staining was significantly higher after 48 h of treatment with g-rhCCN1 and both glycosylation forms of CCN3 than in control cultures (Fig. 4A-B). Expression of the cyclin-dependent kinase inhibitors p15\textsuperscript{INK4B}, p27\textsuperscript{Kip1}, and p57\textsuperscript{Kip2}, as well as p16\textsuperscript{INK4A}, on mRNA level did not differ significantly between treated and untreated SGHPL-5 cells (Fig. S2). However, the increase in SA-β-Gal staining upon CCNs was preceded by a significant upregulation of the cell cycle regulator and senescence marker protein p16 upon 2 h treatment with g- and ng-CCN1 and g-CCN3.
Interestingly, p16 protein levels decreased upon g-CCN1 after 48 hours maybe due to an increase in turnover rate.

**CCN1 and CCN3 increase the migration of SGHPL-5 trophoblast cells via phosphorylation of FAK and Akt kinases**

In our recent studies using the choriocarcinoma cell line Jeg3, we found increased migration of Jeg3 cells after treatment with ng-CCN3 but not with g-CCN3. This increased migration was mediated by the activation of Akt and MAP kinases.13,14

We investigated the cell migration behavior of SGHPL-5 cells after treatment with g-rhCCN1, ng-rhCCN1, g-rhCCN3 or ng-rhCCN3 by using two separate assays to analyze horizontal and vertical migration properties. Horizontal migration of SGHPL-5 cells was performed as a wound healing assay using co-culture chambers and was mostly enhanced by the glycosylated forms of both CCN1 and CCN3 and by non-glycosylated CCN1 (Fig. 5A and 5C). In uncoated transwell migration assays analyzing vertical migration, we observed an increase in migration, but not significantly after treatment with ng-rhCCN1 or ng-rhCCN3, and a significantly decreased migration after treatment with g-rhCCN3 as well as no obvious change in migration upon g-CCN1 (Fig. 5B and 5D).

Figure 3. CCN1 and CCN3 activate Notch-1/p21 signaling in SGHPL-5 cells. (A) Exemplary Western blots of the cleaved Notch-1 receptor and (B) the expression of its target gene p21 expression in SGHPL-5 cells after treatment with CCN1 or CCN3 for 2 h or 8 h are shown. Levels of protein expression are normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or β-actin. Relative expression values of protein quantification are shown as mean expression levels normalized to GAPDH or β-actin above the Western blots. Columns represent the means of three independent measurements; error bars represent SEM. * P ≤ 0.05 versus control. Cleavage of the Notch-1 receptor and, thus, activation of the Notch pathway is enhanced in cells treated with CCN1 or CCN3 than in control cells. The activated Notch pathway in turn upregulates the expression of the cell cycle regulator p21. * P ≤ 0.05.
that the phosphorylation of FAK is significantly affected only by g-CCN3 and is slightly increased by ng-CCN3 after 8 h of CCN3 treatment (Fig. 7A). Both glycosylation forms of CCN1 and the glycosylated form of CCN3 significantly promote the phosphorylation of Akt after 2 h and 8 h of CCN treatment (Fig. 7B). The phosphorylation of ERK1/2 was not changed by treatment with CCN1 or CCN3 (Fig. 7C).

Figure 4. Senescence associated β-Gal staining in SGHPL-5 cells is enhanced by treatment with CCN1 and CCN3. (A) Analysis of cellular senescence in SGHPL-5 cells after treatment with CCN1 and CCN3 for 48 h as determined by SA-β-gal staining (shown as blue staining). Scale bar, 50 μM. (B) Numbers of SA-β-gal positive cells per 100 cells were determined by microscopic observation. The results showed that the occurrence of senescent cells after 48 h of treatment with glycosylated recombinant human CCN1 (g-rhCCN1), g-rhCCN3, or non-glycosylated (ng)-rhCCN3 treatment was significantly higher than that in untreated control cells (ctrl). (C) Exemplary Western blot of protein expression of the cell cycle regulator and senescence marker p16INK4A in SGHPL-5 cells after treatment with CCN1 or CCN3 for 2, 24, or 48 h. Levels of protein expression are normalized to α-tubulin. Densitometric analysis of relative expression values of protein quantification is shown as mean expression levels normalized to α-tubulin (N = 3) above the Western blot. Columns represent the means of three independent measurements; error bars represent SEM. * P < 0.05 vs. control. The expression of p16 is upregulated after 2 h of treatment with CCN1 and CCN3. *P ≤ 0.05.
Figure 5. CCN1 and CCN3 change the migration properties of SGHPL-5 trophoblast cells. (A) Exemplary phase micrographs of wound healing horizontal migration assays using ibidi co-culture chambers. SGHPL-5 cells were treated with glycosylated or non-glycosylated CCNs for 24 h. Untreated cells were used as controls (controls and vehicle controls). Each micrograph is representative of three independent experiments. Glycosylated and non-glycosylated CCNs stimulated the horizontal migration of SGHPL-5 cells; the most pronounced effect was achieved with glycosylated CCNs compared to controls. Scale bar, 500 μm. (B) Exemplary micrographs of migration assays using transwell chambers are shown. The cells were treated with glycosylated or non-glycosylated CCNs for 6 h. Five random fields per condition were photographed at 20× magnification. Interestingly, non-glycosylated CCN1 (ng-CCN1) and CCN3 (ng-CCN3) slightly induced migration, whereas glycosylated (g)-CCN3 significantly reduced the migration capability of SGHPL-5 cells. Scale bar, 50 μm. (C) Quantification of horizontal migration assays. Bars represent mean values of three independent experiments; error bars indicate SD. Horizontal migration was significantly enhanced after stimulation with glycosylated CCNs and non-glycosylated CCN1 compared to controls. N = 3 *P ≤ 0.05. (D) Quantification of vertical migration assays using transwell chambers. Bars represent mean values of three separate (glycosylated and non-glycosylated) experiments performed in duplicate; error bars indicate SD. Mean value of untreated cultures (control) was arbitrarily set at 100%. Vertical migration of SGHPL-5 cells was moderately higher after treatment with non-glycosylated (ng)-CCN3 and with ng-CCN1, not changed upon g-CCN1, but it was significantly reduced by treatment with g-CCN3. N = 3. *P ≤ 0.05.
CCN proteins are known to mediate the regulation of cell migration and invasion through diverse integrin receptors. Using Jeg3 trophoblast cells we confirmed that integrin α5β1 is the receptor for CCN3-promoted trophoblast migration. Both subunits of the integrin α5β1 are expressed by SGHPL-5 trophoblast cells (Fig. S3). Whether integrin α5β1 mediates the increased migration behavior of SGHPL-5 like the Jeg3 cells must be elucidated by future experiments.

The schematic overview (Fig. 8) summarizes the identified signaling pathways of both CCN proteins in SGHPL-5 cells. CCN1 and CCN3 decrease proliferation by inducing cell cycle arrest and bringing about senescence; they also activate Notch/p21 signaling and simultaneously increase migration by activating FAK and Akt, probably via integrins α5β1, which are expressed in SGHPL-5 cells.

**Discussion**

Cell cycle exit and subsequent differentiation into the invading cell type of trophoblast cells are central processes of placentation and are coordinated by an exact interplay between proliferation, differentiation, and invasion capabilities. This study focused on the molecular regulatory mechanisms, such as proliferation and migration, that are mediated by both glycosylation forms of the matricellular proteins CCN1 and CCN3. We have previously shown that these proteins control the proliferation process in Jeg3 cells as a model of EVTs. EVTs detach from the cell column and differentiate into the invasive phenotype; they then deeply invade the maternal decidua and maternal spiral arteries.

Previous studies showed that CCN1 and CCN3 are expressed at high levels in the human placenta during pregnancy, with expression in interstitial EVT cells, in endothelial cells of vessels, and in stromal cells. The levels of both CCN proteins are consistently high in the sera of non-pregnant and pregnant women. However, lower levels of CCN1 and CCN3 were detected in the sera of pregnant women with early-onset preeclampsia, a disease that is associated with insufficient trophoblast invasion. These findings indicate that CCNs are involved in the regulation of cell biological events at the feto-maternal interface.

More detailed analyses in previous studies showed that CCN3-mediated migration was induced by integrin α5β1 as the receptor and activator of Akt kinase, whereas Notch-1 and p21 are involved in antiproliferative capabilities of CCN3. In the present study we focused mainly on...
the role of CCN proteins in proliferation control, using the benign cytotrophoblast cell line SGHPL-5 as a model system for the \textit{in vivo} situation.

**CCN1 and CCN3 decrease proliferation of SGHPL-5 cells by inducing a G0/G1 cell cycle arrest, and then differentiate into a cellular senescent state**

During trophoblast differentiation, some of the cytotrophoblasts (CTBs) underlining the syncytiotrophoblast layer maintain their undifferentiated phenotype throughout pregnancy, thereby providing a reservoir of placental stem cells. The remainder of the CTBs differentiate into two subpopulations of trophoblast cells: syncytiotrophoblast cells (STBs) and invasive extravillous interstitial cytrophoblasts (EVTs).\textsuperscript{3} Until now, little has been known about the interplay of cell cycle regulators, and it has been impossible to determine whether trophoblast cells proliferate or exit from the cell cycle to allow further differentiation.\textsuperscript{35,36} The results of previous experiments

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure7.png}
\caption{Analysis of activation of focal adhesion kinase (FAK), Akt, and extracellular signal-related kinase (ERK) in SGHPL-5 trophoblast cells after treatment with CCN1 or CCN3. Densoitometric analysis of the expression of phosphorylated FAK (A), Akt (B), and ERK1/2 (C) compared to total expression of FAK, Akt, and ERK in SGHPL-5 cells after treatment with CCN1 or CCN3 for 0 or 8 h. Columns represent the means of three independent measurements; error bars represent SEM. *P ≤ 0.05 vs. controls. An exemplary Western blot is shown below the graphs. Phosphorylation of FAK is significantly enhanced only by the glycosylated form of CCN3. (B) Phosphorylation of Akt is significantly increased by both glycosylation forms of CCN1 and by glycosylated recombinant human CCN3 (g-rhCCN3). (C) Phosphorylation of ERK1/2 in SGHPL-5 trophoblast cells is not affected by CCN1 or CCN3. *P ≤ 0.05.}
\end{figure}
using the choriocarcinoma cell line Jeg3 suggested that CCN3 causes an imbalance between the proliferation and migration of human trophoblast cells. In the present study we found that both glycosylation forms of CCN1 and CCN3 proteins reduce the numbers of benign SGHPL-5 trophoblast cells, whereas in Jeg3 cells only CCN3 seems to regulate proliferation. Comparing the effect of CCNs on migration properties in both cell lines showed that Jeg3 trophoblast cells and SGHPL-5 cells are mostly stimulated by non-glycosylated CCN1 and CCN3. Thus, the regulation properties of CCNs on proliferation differ between the malignant and the benign trophoblast cell lines, and migration seems to be similarly regulated.

The reduced number of SGHPL-5 cells after treatment with CCN1 and CCN3 is based on cell cycle control and not on apoptosis. The analysis of cell cycle phase distribution found that reduced proliferation after treatment with CCN1 or CCN3 is associated with a G0/G1 cell cycle arrest characterized by an increased number of cells in the G0/G1 phase. The proportion of cells in the G2/M phase was significantly reduced by both glycosylation forms of CCN1 and CCN3. Arrest of or exit from the cell cycle is a precondition for a cell to pass into postmitotic states, such as quiescence, senescence, or terminal differentiation.

Studies of murine trophoblast giant cells have shown that terminal differentiation is marked by endoreduplication. However we did not detect an increase in the number of polyploid SGHPL-5 cells after treatment with either CCN. Instead, our results clearly showed that both CCN proteins induced cellular senescence in SGHPL-5 cells, as demonstrated by an increased expression of SA-β-gal, and the increased expression of p16, both are well-established markers of cellular senescence. Meanwhile, separate signaling pathways are mediated by CCNs and lead to alterations in proliferation and migration properties.

It is known that the Notch-1 receptor is expressed in CTBs of the human placenta and that this receptor regulates the cyclin/CDK inhibitor p21. In small cell lung cancer cells, Notch-1 signaling induces a p21-mediated cell cycle arrest. Furthermore, Notch signaling plays an important role in the regulation of proliferation in the placental cell column and of trophoblast invasion and differentiation of EVTs. Inhibiting the Notch signaling pathway in primary EVTs and SGHPL-5 cells enhanced...
proliferation in the placental cell column, invasion capability, and expression of EVT markers, as shown by Haider et al.\(^1\) In the present study we found that Notch-1 expression is associated with the proliferative capability of CTB cell column progenitor cells, which is highest during the first trimester of pregnancy. This finding strongly corroborates our findings that CCN1 and CCN3 activate Notch-1 signaling and thereby reduce proliferation of the cytotrophoblast cell line SGHPL-5, as reported by Haider et al.\(^1\)

CCN proteins are known to act via Notch-1 in other systems, such as myoblasts.\(^42\)\(^-\)\(^45\) Our recent studies showed that Notch/p21 signaling also seems to mediate the proliferation-reducing activity of CCN3 in malignant Jeg3 cells.\(^14\) In the present study we found that CCN1 and CCN3 cause a G0/G1 cell cycle arrest and induce cellular senescence in SGHPL-5 trophoblast cells; this effect is presumably mediated by activation of the Notch-1 receptor after upregulation of p21.

Normally, cellular senescence is a characteristic feature of aging. It protects against tumourigenesis by limiting the proliferation of potentially detrimental cells and restricts tissue damage.\(^46\) Recent studies by Krizhanovsky and colleagues\(^46\) clearly showed that, in the placenta, the fusion of CTBs to STBs induces cellular senescence and that this action may be necessary for proper STB function during embryonic development.\(^45\) The same finding has been reported in studies of mouse placentas. Zhang et al.\(^48\) showed that throughout gestational days 14.5 to 18.5 the labyrinthine trophoblast cells strongly express SA-\(\beta\)-gal, p53, and p21 and therefore induce cellular senescence. The induction of senescence by CCN1 has already been described in other cell types, such as fibroblasts,\(^49\) hepatic myofibroblasts,\(^50\) cell lines of non-small-cell lung carcinoma,\(^29\) and aging muscle cells.\(^51\)

**CCN1 and CCN3 induce the migration properties of SGHPL-5 cells by FAK and Akt signaling**

In addition to the inhibition of proliferation, the non-glycosylated forms of CCN1 and CCN3 in particular tend to enhance the vertical migration properties of SGHPL-5 trophoblast cells, whereas for horizontal migration properties the glycosylated CCNs exert the strongest effect. Interestingly, the application of glycosylated CCN3 results in less vertical migration of SGHPL-5 cells. So far we had no proven explanation for these separate effects of the various CCN forms on migration directions. However, it is already known that glycosylation controls diverse protein functions such as migration and invasion properties of the extravillous trophoblast.\(^52\) Thus, here the different glycosylated CCN proteins may differ in the modulation of focal adhesion structures.

The results in horizontal migration between the different CCN isoforms regarding its glycosylation may be explained by the fact that the glycosylated CCN proteins are the secreted isoforms which could act from outside on migration behavior of the cells in a paracrine manner and may therefore more efficiently increase migration compared to the non-glycosylated intracellular form of the CCNs which is located intracellularly and could only act in an autocrine manner.

Future investigations will focus on other potential signaling pathways that differ between the glycosylation forms of the CCN proteins. FAK is involved in integrin-mediated signal transduction pathways of the extracellular matrix and plays an important role in the regulation of cell proliferation, migration, and invasion.\(^53\) It is known that the activation of Akt and ERK1/2 is related to cell migration and the activation of FAK.\(^54\)\(^-\)\(^57\) and that these kinases are involved in trophoblast migration and invasion (reviewed by Chakraborty et al.\(^8\)). In SGHPL-5 cells, phosphorylation and thereby activation of FAK occurs only after treatment with glycosylated CCN3. The phosphorylation status of FAK does not change after treatment with CCN1. The phosphorylation and activation of Akt are induced by both CCN1 and CCN3. This finding has been verified for CCN3 in renal carcinoma cells.\(^58\)

Haslinger et al.\(^59\) verified the increase in SGHPL-5 migration after the application of epidermal growth factor by Akt signaling, in particular the Akt 1 and Akt 3 isoforms. In Jeg3 cells, epidermal growth factor–like domain 7 promotes migration and invasion by activating the MAPK, PI3K, and Notch pathways.\(^60\) In contrast here, phosphorylation and activation of ERK1/2 do not seem to play a role in CCN1/3-mediated regulation of migration in SGHPL-5 cells, because the phosphorylation status remains unchanged after treatment with both CCN proteins.

An important aspect of cell migration and invasion is the FAK/Akt-mediated enhanced expression and activity of MMP-2 and MMP-9.\(^31\)\(^-\)\(^33\) SGHPL-5 cells treated with g-CCN1 or g-CCN3 exhibit significantly higher mRNA expression of MMP-9. If the protein level or activity of MMP-9 is also increased upon g-CCNs is unknown up to now and has to be investigated in future experiments. However, we assume that the involvement of CCN1 and CCN3 in invasion is obvious, and the activation of these signaling cascades and the resulting changes in cell physiology seem to depend on the inducing factor and the trophoblast cell line.

Thus, in a receptor-dependent manner, CCN1 and CCN3 support the inhibition of trophoblast proliferation and promote the migration of invasive trophoblasts into the maternal decidua. This conclusion is easily
transferable to the placenta in vivo, because the Notch-1 receptor is expressed proximally in the placent al cell column, whereas the integrin α5β1 receptor is expressed distally in the invading EVTs, thereby providing a spatially distributed spectrum of action (Fig. 8).

Taken together, the results of this study show that CCN1 and CCN3 are key regulatory proteins of the EVTs that control proliferation and invasion. They could support the cell cycle exit of trophoblast cells located at the proximal column and simultaneously enhance the migration properties of the invasive trophoblasts detaching from the column. Thus, we assume that both CCN proteins regulate the switch of EVTs from the proliferating to the non-proliferating senescence phenotype but not endoreduplication. We further assume that the reduced levels of CCN1 and CCN3 observed in early-onset preeclampsia could lead to the increased proliferation and thereby the reduced invasion capability of EVT cells. Our findings regarding the coordinated multifunctional properties of both CCN proteins in the human placenta and their defined signaling cascades may inspire efforts aimed at correcting impaired pathways in reproductive diseases by interfering with the CCN molecules.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgments

We would like to thank Guy S. Whitley, London, for providing the SGHPL-5 cell line. The authors thank Claudine Kühn, Gabriele Sehn, Ursula Schmücker, Kathrin Kauschke, and Dagmar Thyssen for their excellent technical assistance. We are grateful to Dr. Florence Witte for English editing and critical reading of the manuscript.

Funding

This study was funded by the German Research Foundation (DFG) with the contract numbers WI 774/22-2 to Elke Winterhager/Alexandra Gellhaus and GE 2223/2-1 to Alexandra Gellhaus.

References

[1] Pijnenborg R, Dixon G, Robertson WB, Brosens I. Trophoblastic invasion of human decidua from 8 to 18 weeks of pregnancy. Placenta 1980; 1(1):3-19; PMID:7443635; http://dx.doi.org/10.1016/S0143-4004(80)80012-9
[2] Lyall F. Mechanisms regulating cytotrophoblast invasion in normal pregnancy and pre-eclampsia. Aust N Z J Obstet Gynaecol 2006; 46(4):266-73; PMID:16866784; http://dx.doi.org/10.1111/j.1479-828X.2006.00589.x
[3] Handwerger S. New insights into the regulation of human cytotrophoblast cell differentiation. Mol Cell Endocrinol 2010; 323(1):94-104; PMID:20036312; http://dx.doi.org/10.1016/j.mce.2009.12.015
[4] Ji L, Brkić J, Liu M, Fu G, Peng C, Wang YL. Placental trophoblast cell differentiation: physiological regulation and pathological relevance to preeclampsia. Mol Aspects Med 2013; 34(5):981-1023; PMID:23276825; http://dx.doi.org/10.1016/j.mam.2012.12.008
[5] Nakayama H, Scott IC, Cross JC. The transition to endoreduplication in trophoblast giant cells is regulated by the mSNA zinc finger transcription factor. Dev Biol 1998; 199(1):150-63; PMID:9676199; http://dx.doi.org/10.1006/dbio.1998.8914
[6] Zhou Y, Genbacev O, Damsky CH, Fisher SJ. Oxygen regulates human cytotrophoblast differentiation and invasion: implications for endovascular invasion in normal pregnancy and in pre-eclampsia. J Reprod Immunol 1998; 39(1-2):197-213; PMID:9786462; http://dx.doi.org/10.1016/S0165-0378(98)00022-9
[7] McMaster MT, Zhou Y, Fisher SJ. Abnormal placentation and the syndrome of preeclampsia. Semin Nephrol 2004; 24(6):540-7; PMID:15529288; http://dx.doi.org/10.1016/j.semnephrol.2004.07.002
[8] Chakraborty C, Gleeson LM, McKinnon T, Lala PK. Regulation of human trophoblast migration and invasiveness. Can J Physiol Pharmacol 2002; 80(2):116-24; PMID:11934254; http://dx.doi.org/10.1139/y02-016
[9] Knöller M. Critical growth factors and signalling pathways controlling human trophoblast invasion. Int J Dev Biol 2010; 54(2-3):269-80; PMID:19876833; http://dx.doi.org/10.1087/ijdb.2008.082769mk
[10] Knöller M, Pollheimer J. IFPA Award in Placentology lecture: molecular regulation of human trophoblast invasion. Placenta 2012; 33:S55-62; PMID:22019198; http://dx.doi.org/10.1016/j.placenta.2011.09.019
[11] Soncin F, Natale D, Parast MM. Signaling pathways in mouse and human trophoblast differentiation: a comparative review. Cell Mol Life Sci 2015; 72(7):1291-302; PMID:25430479; http://dx.doi.org/10.1007/s00018-014-1794-x
[12] Wolf N, Yang W, Dunk CE, Gashaw I, Ly C, Schmidt M, Winterhager E, Gellhaus A. Regulation of the matricellular proteins CYR61 (CCN1) and NOV (CCN3) by hypoxia-inducible factor-1α and transforming-growth-factor-β3 in the human trophoblast. Endocrinology 2010; 151(6):2835-45; PMID:20237132; http://dx.doi.org/10.1210/en.2009-1195
[13] Yang W, Wagener J, Wolf N, Schmidt M, Kimming R, Winterhager E, Gellhaus A. Impact of CCN3 (NOV) glycosylation on migration/invasion properties and cell growth of the choriocarcinoma cell line Jeg3. Hum Reprod 2011; 26(10):2850-60; PMID:21784733; http://dx.doi.org/10.1093/humrep/der239
[14] Wagener J, Yang W, Kauschke K, Winterhager E, Gellhaus A. CCN3 regulates proliferation and migration properties in Jeg3 trophoblast cells via ERK1/2, Akt and Notch signalling. Mol Hum Reprod 2013; 19(4):237-49; http://dx.doi.org/10.1093/molehr/gas061
[15] Winterhager E, Gellhaus A. The role of the CCN family of proteins in female reproduction. Cell Mol Life Sci 2014; 71(12):2299-311; PMID:24448904; http://dx.doi.org/10.1007/s00018-014-1556-9
[16] Leask A, Abraham DJ. All in the CCN family: essential matricellular signaling modulators emerge from the bunker. J Cell Sci 2006; 119(Pt 23):4803-10; PMID:17130294; http://dx.doi.org/10.1242/jcs.03270

[17] Kubota S, Takigawa M. The CCN family acting throughout the body: recent research developments. Biol Mol Concepts 2013; 4(5):477-94; PMID:25436754; http://dx.doi.org/10.1515/bmc-2013-0018

[18] Zuo GW, Kohls CD, He BC, Chen L, Zhang W, Shi Q, Zhang BQ, Kang Q, Luo J, Luo X, et al. The CCN proteins: important signaling mediators in stem cell differentiation and tumourigenesis. Histol Histopathol 2010; 25(6):795-806; PMID:20376786

[19] Chevalier G, Yeger H, Martinerie C, Laurent M, Alami J, Zuo GW, Kohls CD, He BC, Chen L, Zhang W, Shi Q, Kubota S, Takigawa M. The CCN family acting through...

[20] Gaspari I, Stiller S, Böing C, Kimmig R, Winterhager E. Premembranal regulation of the pro-angiogenic factor CYR61 in human endometrium. Endocrinology 2008; 149(5):2261-9; PMID:18202125; http://dx.doi.org/10.1210/en.2007-1568

[21] Yang GP, Lau LF. Cyr61, product of a growth factor-inducible immediate early gene, is associated with the extracellular matrix and the cell surface. Cell Growth Diff 1991; 2(7):351-7; PMID:1782153

[22] Gellhaus A, Schmidt M, Dunk C, Lye SJ, Kimmig R, Winterhager E. Decreased expression of the angiogenic regulators CYR61 (CCN1) and NOV (CCN3) in human placenta is associated with pre-eclampsia. Mol Hum Reprod 2006; 12(6):389-99; PMID:16675545; http://dx.doi.org/10.1093/molehr/gal044

[23] Gellhaus A, Schmidt M, Dunk C, Lye SJ, Winterhager E. The circulating proangiogenic factors CYR61 (CCN1) and NOV (CCN3) are significantly decreased in placenta and sera of preeclamptic patients. Reprod Sci 2006; 12(6):389-99; PMID:16675545; http://dx.doi.org/10.1093/molehr/gal044

[24] Gellhaus A, Dong X, Propson S, Maass K, Klein-Hitpass L, Kibschull M, Traub O, Willecke K, Perbal B, Lye SJ, et al. Connexin43 interacts with NOV: a possible mechanism for negative regulation of cell growth in choriocarcinoma cells. J Biol Chem 2004; 279(35):36931-42; PMID:15181016; http://dx.doi.org/10.1074/jbc.M404073200

[25] Koch Y, van Furden B, Kaiser S, Klein D, Kibschull M, Schorle H, Carpteineto A, Gellhaus A, Winterhager E. Connexin 31 (GJB3) deficiency in mouse trophoblast stem cells alters giant cell differentiation and leads to loss of oxygen sensing. Biol Reprod 2012; 87(2):37; PMID:22623621; http://dx.doi.org/10.1093/biolreprod.111.098079

[26] De Falco M, Cobelli L, Giraldi D, Mastroiacomo A, Perna A, Colucurci N, Miele L, De Luca A. Expression and distribution of notch protein members in human placenta throughout pregnancy. Placenta 2007; 28(2-3):118-26; PMID:17185135; http://dx.doi.org/10.1016/j.placenta.2006.03.010

[27] Devgan V, Mammucari C, Millar SE, Brisken C, Dotto GP. p21WAF1/Cip1 is a negative transcriptional regulator of Wnt4 expression downstream of Notch1 activation. Genes Dev 2005; 19(12):1485-95; PMID:15964998; http://dx.doi.org/10.1101/gad.341405

[28] Alt JR, Gladden AB, Diehl JA. p21 (Cip1) promotes cyclin D1 nuclear accumulation via direct inhibition of nuclear export. J Biol Chem 2002; 277(10):8517-23; PMID:11751903; http://dx.doi.org/10.1074/jbc.M108867200

[29] Jim Leu SJ, Sung JS, Chen MY, Chen CW, Cheng JY, Wang TY, Wang J. The matricellular protein CCN1 suppresses lung cancer cell growth by inducing senescence via the p53/p21 pathway. J Cell Biochem 2013; 114(9):2082-93; PMID:23553737; http://dx.doi.org/10.1002/jcb.24557

[30] Chen CC, Lau LF. Functions and mechanisms of action of CCN matrix metalloproteinases. Int J Biochem Cell Biol 2009; 41:771-83; PMID:18775791; http://dx.doi.org/10.1016/j.biocel.2008.07.025

[31] Chen JS, Huang XH, Wang Q, Chen XL, Fu XH, Tan HX, Zhang LJ, Li W, Bi J. FAK is involved in invasion and metastasis of hepatocellular carcinoma. Clin Exp Metastasis 2010; 27:71-82; PMID:20180147; http://dx.doi.org/10.1007/s10585-010-9306-3

[32] Chung TW, Lee YC, Kim CH. Hepatitis B viral HBx induces matrix metalloproteinase-9 gene expression through activation of ERK and Pdlns-3/akt pathways: involvement of invasive potential. FASEB J 2004; 18:1123-5; PMID:15132991; http://dx.doi.org/10.1096/fj.04-2126com

[33] Park CM, Park MJ, Kwak HJ, Lee HC, Kim MS, Lee SH, Park IC, Rhee CH, Hong SI. Ionizing radiation enhances matrix metalloproteinase-2 secretion and invasion of glioma cells through Src/epidermal growth factor receptor-mediated p38/Akt and phosphatidylinositol 3-kinase/Akt signaling pathways. Cancer Res 2006; 66:8511-9; PMID:16951163; http://dx.doi.org/10.1158/0008-5472.CAN-05-4340

[34] Carter AM, Enders AC, Pijnenborg R. The role of invasive trophoblast in implantation and placentation of primate. Philos Trans R Soc Lond B Biol Sci 2015; 370(1663):20140070; PMID:25602074; http://dx.doi.org/10.1098/rstb.2014.0070

[35] Genbacev O, McMaster MT, Fisher SJ. A repertoire of cell cycle regulators whose expression is coordinated with cell cycle progression in endoreduplication in rodent trophoblast cells. Am J Pathol 2000; 157(4):1337-51; PMID:11021837; http://dx.doi.org/10.1016/S0002-9440(10)64648-2

[36] Korgun ET, Celik-Ozenci C, Acar N, Cayli S, Desoye G, Demir R. Location of cell cycle regulators cyclin B1, cyclin D1 nuclear accumulation via direct inhibition of nuclear export. J Biol Chem 2002; 277(10):8517-23; PMID:11751903; http://dx.doi.org/10.1074/jbc.M108867200

[37] MacAuley A, Cross JC, Werb Z. Reprogramming the cell cycle for endoreduplication in rodent trophoblast cells. Mol Biol Cell 1998; 9(4):795-807; PMID:9529378; http://dx.doi.org/10.1091/mbc.9.4.795

[38] Collado M, Serrano M. The power and the promise of onco-gene-induced senescence markers. Nat Rev Cancer 2006; 6(6):472-6; PMID:16723993; http://dx.doi.org/10.1038/nrc1884

[39] Suriwannpong V, Borges MW, Ravi RK, Arnold DR, Nelkin BD, Baylin SB, Ball DW. Notch signaling induces cell
cycle arrest in small cell lung cancer cells. Cancer Res 2001; 61(7):3200-5; PMID:11306509

[41] Haider S, Meinhardt G, Velicky P, Otti GR, Whitley G, Fiala C, Pollheimer J, Knöfler M. Notch signaling plays a critical role in motility and differentiation of human first-trimester cytotrophoblasts. Endocrinology 2014; 155 (1):263-74; PMID:24189144; http://dx.doi.org/10.1210/en.2013-1455

[42] Sakamoto K, Yamaguchi S, Ando R, Miyawaki A, Kasasawa Y, Takagi M, Li CI, Perbal B, Katsube K. The nephroblastoma overexpressed gene (NOV/ccn3) protein associates with Notch1 extracellular domain and inhibits myoblast differentiation via Notch signalling pathway. J Biol Chem 2002; 277:29399-405; PMID:12050162; http://dx.doi.org/10.1074/jbc.M203772200

[43] Katsube K, Sakamoto K, Tamamura Y, Yamaguchi A. Role of CCN, a vertebrate specific gene family, in development. Dev Growth Differ 2009; 51(1):55-67; PMID:19128405; http://dx.doi.org/10.1111/j.1440-169X.2009.01077.x

[44] Haque I, De A, Majumder M, Mehta S, McGregor D, Kim KH, Chen CC, Monzon RI, Lau LF. Matricellular Jun JI, Lau LF. The matricellular protein CCN1 induces fibroblast senescence and restricts fibrosis in cutaneous wound healing. Nat Cell Biol 2010; 12(7):676-85; PMID:20526329; http://dx.doi.org/10.1038/ncb2070

[45] Kim KH, Chen CC, Monzon RI, Lau LF. Matricellular protein CCN1 promotes regeneration of liver fibrosis through induction of cellular senescence in hepatic myofibroblasts. Mol Cell Biol 2013; 33(10):2078-90; PMID:23508104; http://dx.doi.org/10.1128/MCB.00049-13

[46] Du J, Klein JD, Hassounah F, Zhang J, Zhang C, Wang XH. Aging increases CCN1 expression leading to muscle senescence. Am J Physiol Cell Physiol 2014; 306(1):C28-36.52; PMID:24196529; http://dx.doi.org/10.1152/ajpcell.00066.2013

[47] Liao WC, Liu CH, Chen CH, Hsu WM, Liao YY, Chang HM, Lan CT, Huang MC, Shyu MK. β1,4-Galactosyltransferase III suppresses extravillous trophoblast invasion through modifying β1-integrin glycosylation. Placenta. 2015; 36(4):357-64; PMID:25659296; http://dx.doi.org/10.1016/j.placenta.2015.01.008

[48] Schaller MD. Cellular functions of FAK kinases: insight into molecular mechanisms and novel functions. J Cell Sci 2010; 123(Pt 7):1007-13; PMID:20332118; http://dx.doi.org/10.1242/jcs.045112

[49] King WG, Mattaliano MD, Chan TO, Tsichlis PN, Brugge JS. Phosphatidylinositol 3-kinase and Rho family GTPases in Vav3-induced cell transformation, cell motility, and morphological changes. J Biol Chem 2002; 277(46):38569-79; PMID:12050162; http://dx.doi.org/10.1074/jbc.M203727200

[50] Goldman-Wohl D, Yagel S. United we stand not divided: the fetal tissues and organs of mice. Clin Exp Pharmacol Physiol 2014; 41(12):965-75; PMID:25345622; http://dx.doi.org/10.1111/1440-1681.12328

[51] King WG, Mattaliano MD, Chan TO, Tsichlis PN, Brugge JS. Phosphatidylinositol 3-kinase and Rho family GTPases in Vav3-induced cell transformation, cell motility, and morphological changes. J Biol Chem 2002; 277(20):17638-48; PMID:11884391; http://dx.doi.org/10.1074/jbc.M111575200

[52] Ren K, Jin H, Bian C, He H, Liu X, Zhang S, Wang Y, Shao RG. MR-1 modulates proliferation and migration of human hepatoma HepG2 cells through myosin light chains-2 (MLC2)/focal adhesion kinase (FAK)/Akt signal pathway. Mol Biol Cell 1997; 8(8):4406-18; PMID:9234699; http://dx.doi.org/10.1128/MCB.17.8.4406

[53] Haslinger P, Haider S, Sonderegger S, Otti GR, Whitley G, Knöfler M. AKT isoforms 1 and 3 downstream target in migration of colorectal cancer cells. Curr Cancer Drug Targets 2009; 9(1):26-36; PMID:18948272; http://dx.doi.org/10.2174/156800909785794599

[54] Turecková J, Vojtechová M, Krausová M, Sloncová E, Korinek V. Focal adhesion kinase functions as an akt downstream target in migration of colorectal cancer cells. Transl Oncol 2009; 2(4):281-90; http://dx.doi.org/10.1593/tlo.09160

[55] Liu S, Han L, Wang X, Liu Z, Ding S, Lu J, Bi D, Mei Y, Niu Z. Nephroblastoma overexpressed gene (NOV) enhances RCC cell motility through upregulation of ICAM-1 and COX-2 expression via Akt pathway. Int J Clin Exp Pathol 2015; 8(10):7851-9; PMID:26545622; http://dx.doi.org/10.36049/ijcep.055809

[56] Haslinger P, Haider S, Sonderegger S, Otten JV, Pollheimer J, Whitley G, Knöfler M. AKT isoforms 1 and 3 regulate basal and epithelial growth factor-stimulated SGHPL-5 trophoblast cell migration in humans. Biol Reprod 2013; 88(3):54; PMID:23303682; http://dx.doi.org/10.1095/bioreprod.112.104778

[57] Massimi M, Vecchione L, Piccirilli D, Spitalieri P, Amati F, Salvi S, Ferrazzani S, Stuhlmann H, Campagnolo L. Epidermal growth factor-like domain 7 promotes migration and invasion of human trophoblasts through activation of MAPK, PI3K and NOTCH signaling pathways. Mol Hum Reprod 2015; 21(5):435-51; PMID:25667199; http://dx.doi.org/10.1093/molehr/gav006