Impact of CCN3 (NOV) glycosylation on migration/invasion properties and cell growth of the choriocarcinoma cell line Jeg3

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BACKGROUND: Recently we have shown that the matricellular CCN3 protein expressed in invasive extravillous trophoblast cells (EVTs) is decreased in early-onset pre-eclampsia and is regulated by oxygen tension. Pathogenesis of pre-eclampsia relies on a shallow invasion of EVT into the spiral arteries, which leads to hypoxia accompanied by uteroplacental insufficiency. Here we investigated the function of glycosylated and non-glycosylated CCN3 protein on cell growth as well as migration and invasion properties of the malignant trophoblast cell line Jeg3 which is a widely used model for the invasive trophoblast.

METHODS AND RESULTS: Stable transfection of Jeg3 choriocarcinoma cells with full length CCN3 resulted in high expression of secreted glycosylated and cellular non-glycosylated CCN3. These cells revealed significantly reduced growth in cell numbers combined with a significantly increased migratory and invasive capacity. Matrix metalloprotease (MMP)-2 and MMP-9 activities were enhanced dependent on CCN3 expression, which could be confirmed by CCN3 knockdown studies. Using recombinant glycosylated and non-glycosylated CCN3, we revealed that CCN3 decreased growth in Jeg3 cell numbers independent of its glycosylation status, whereas only non-glycosylated CCN3 was able to enhance migration and invasion properties.

CONCLUSIONS: The present results suggest that CCN3 protein regulates the decrease in Jeg3 cell numbers independent of its glycosylation status, whereas migratory and invasive properties are influenced only by non-glycosylated CCN3. An impaired balance in the expression of glycosylated and non-glycosylated CCN3 could contribute to the shallow invasion of EVT observed in pre-eclampsia.

Key words: CCN3 (NOV) / trophoblast / pre-eclampsia / invasion / glycosylation

Introduction

Pre-eclampsia (PE) is a pregnancy-specific disease characterized by development of hypertension and proteinuria after 20 weeks of gestation. It affects about 2–8% of all pregnancies and it is a major contributor of maternal mortality worldwide (Steegers et al., 2010). This disease originates in the placenta, probably due to an inadequate cytotrophoblast invasion and vascular remodeling. As a consequence, the high-resistance, non-dilated vessels cannot carry sufficient blood supply to meet the demand of the fetus in the second and especially third trimester of pregnancy (Wang et al., 2009). This reduced placental perfusion ends with widespread maternal endothelial dysfunction associated with changed expression of numerous placenta-derived growth and angiogenic factors, such as soluble vascular endothelial growth factor receptor 1 (sFlt-1), which cause the maternal symptoms described above (Young et al., 2010).

Within this concert of factors leading to pre-eclampsia, we could show that CCN proteins seemed to play an important role (Gellhaus et al., 2006, 2007; Wolf et al., 2010). CCN3 (NOV, nephroblastoma overexpressed) belongs to the CCN family, which constitutes multifunctional secreted proteins that act as matrix cellular regulators (Dhar and Ray, 2010; Zuo et al., 2010). A 55-kDa secreted CCN3 protein was observed in the culture supernatant of some mammalian derived cell lines such as human glioblastoma G59 cells and human...
adrenocortical NCI-H295R cells. In G59 cells, the expression of an additional 48-kDa form could be shown in the cytosolic compartment (Lazar et al., 2007). Inasmuch as two putative N-glycosylation sites were present at position 97 (NQTG) and 280 (NCTS) in the human CCN3 polypeptide sequence, the different CCN3 forms observed are the result of posttranslational glycosylations (Chevalier et al., 1998). A very recent study revealed that recombinant rat CCN3 as well as human CCN2 (CTGF) derived from mammalian cell lines are N-glycosylated (Bohr et al., 2010).

The CCN proteins can integrate and modulate the signals of integrins, bone morphogenic proteins (BMPs), vascular endothelial growth factor, Wnt proteins and Notch (Zuo et al., 2010). CCN3 plays a basic role in osteogenesis, chondrogenesis and angiogenesis. Recently it has been shown that CCN3 knockout mice exhibit inhibited vascular smooth muscle cell migration and proliferation indicating the involvement of CCN3 in both, proliferation and migration processes (Shimoyama et al., 2010). The association between CCN3 expression and tumor cell metastasis is still under debate. A comprehensive study on Ewing’s sarcoma patients showed that high CCN3 expression was associated with a higher risk of metastasis (Benini et al., 2005; Vallacchi et al., 2008) indicating again that CCN3 is associated with cell migration and invasion.

In recent years trophoblast cell research has underlined the striking similarities between the proliferative, migratory and invasive properties of placental cells and cancer cells. In our earlier studies we revealed that the CCN3 protein is expressed in the human placenta in the invasive interstitial extravillous trophoblast (EVT) giant cells (Gellhaus et al., 2006). As it has been shown for cancer cells, we assume that impaired trophoblast invasion might be associated with deregulated CCN3 expression because the CCN molecules are able to react to oxidative stress, a characteristic for the placental environment in pre-eclampsia (Wolf et al., 2010). Further evidence for the role of CCN3 in trophoblast invasion and migration is significantly reduced expression levels in women with pre-eclampsia (Gellhaus et al., 2006, 2007).

Here we wanted to investigate if the secreted glycosylated and cellular non-glycosylated form of the CCN3 protein fulfill different functions in migration and invasion or growth properties using a malignant trophoblast cell line as a model for the invasive trophoblast.

Materials and Methods

Cell culture and stable transfection

The human Jeg3 choriocarcinoma cell line was obtained from American Type Tissue Collection (ATCC number: HTB-36™, Manassas, VA). Cells were routinely cultured in minimum essential media (MEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Biochrome, Berlin, Germany), 1.2 mM L-glutamine (Invitrogen) and maintained in a humidified 5% CO2 atmosphere at 37°C.

Stable transfectants expressing CCN3 were obtained from Jeg3 cells by using Fugene6 transfect reagent (Roche Applied Science, Mannheim, Germany). CCN3 expression vector was constructed by inserting the entire coding region of the human CCN3 cDNA (Genbank Accession NM_002514) into a mammalian expression vector pcMV-NOVH (S) (Chevalier et al., 1998), which contains a G418 resistance gene. Transfection with pcMV-NOVH (S) or empty vector was performed according to the manufacturer’s protocol and cells were selected in medium containing 500 µg/ml G418 sulfate (Biochrome) for 2–3 weeks. The colonized cells were isolated, amplified and used for subsequent experiments.

siRNA knockdown of CCN3 expression

Small interfering RNA (siRNA) oligonucleotides against CCN3 and control siRNA were synthesized by Dharmacon Research, Inc. (Lafayette, CO, USA) using their custom SMARTpool and siCONTROL non-targeting siRNA pool, respectively. For knockdown of CCN3, CCN3-expressing cells (Jeg3/CCN3) were transfected with siLentFect lipid reagent (Bio-Rad, Hercules, CA, USA) with a final concentration of 10 nM siRNA. After 24 h incubation, the cells were harvested for further analysis. The knockdown of CCN3 expression was confirmed by real-time RT–PCR and Western blot.

RNA extraction and quantitative RT–PCR

Total RNA was isolated using E.Z.N.A. RNA extraction kit (Omega-biotech, Norcross, GA, USA) and reverse transcribed as previously described (Gellhaus et al., 2004). Gene expression was quantified using the qPCR Master Mix for SYBR green (Applied Biosystems, Darmstadt, Germany) and the GeneAmp 5700 sequence detection system (Applied Biosystems, Darmstadt, Germany) with primer sets indicated in Table I. For a quantitative measurement β-actin was used as an internal control. The PCR reactions were carried out in triplicate in a final volume of 20 µl with 1 µl (40 ng) cDNA, 1 × reaction buffer containing SYBR green and 10 pmol sense and antisense primers. PCR was performed for 10 min at 95°C followed by 40 cycles of 10 s denaturation at 95°C and 1 min annealing at 60°C. Specificity of the amplification products was confirmed by melting curve analysis. The PCR fragments were also visualized on 2% ethidium bromide-stained agarose gels. Ten-fold series dilution of purified PCR products starting at 1 pg to 0.1 fg were used as standards, providing a relative quantification of the unknown samples. The quantity of cDNA in each sample was normalized to the β-actin content.

Western blot

Protein extracts were prepared from cells by homogenization with modified RIPA lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1% NP-40, 0.25% Na-deoxycholate, 1 mM EDTA, 0.1% SDS) supplemented with EDTA-free complete protease inhibitors (Roche, Penzberg, Germany). Protein content was determined using the Roche photometric assay (Roht, Karlsruhe, Germany). Protein samples (cell lysate: 30 µg; recombinant CCN3 proteins: 100 ng) were separated on a 10% polyacrylamide gel and electrophoretically transferred to nitrocellulose membrane (Amer sham Biosciences, Piscataway, NJ, USA). For the analysis of protein expression, membranes were blocked with 5% non-fat dried milk in Tris-buffered saline (TBS) with 0.03% Tween-20 and then incubated with polyclonal goat anti-human CCN3 (1: 500; R&D Systems, Wiesbaden, Germany). Primary antibody binding was detected using the following secondary antibodies: anti-goat IgG and anti-mouse IgG antibody conjugated to horseradish peroxidase (1:10000; Pierce, Rockford, IL, USA). Detection was achieved with the ECL chemiluminescence kit (Pierce) according to the protocol using X-ray films (Kodak, Stuttgart, Germany). Blots were stripped in a buffer containing 62.5 mM Tris–HCl, pH 6.8, 2% SDS, 100 µM β-mercaptoethanol at 55°C and reprobed with mouse anti-human GAPDH antibody (1:1000, Chemicon, Hampshire, UK) for normalization of protein expression.

Heparin-binding assay

Confluent Jeg3 cells and Jeg3/CCN3 cells were cultured for 24 or 48 h in serum-free media. After removing the cell debris, conditioned media were incubated overnight at 4°C with 200 µl of heparin-agarose beads.
and Jeg3/CCN3 cells (5\*10^5 diluted Matrigel (BD Biosciences)). To examine cell invasion, parental Jeg3 size for estimation of cell migration and filters precoated with 1:5

Control cells were treated with DMSO only. Described above. For treatment of Jeg3/CCN3 cells with Brefeldin A (Peprotech, NJ, USA), respectively, or solvent 0.1% BSA–PBS in triplicate.

For treatment of Jeg3 cells with the conditioned medium of Jeg3/CCN3 cells, cells were collected and electrophoresed in 10% polyacrylamide gel containing 0.1 mg/ml gelatin (Sigma, St. Louis, MO) under non-reducing conditions. The loading was normalized by measuring the total cell numbers. After electrophoresis the gel was rinsed twice in the renaturation buffer (2.5% Triton-X 100), incubated in developing buffer (50 mM Tris–HCl, 5 mM CaCl_2, at pH 8.8) at 37°C for 24 h, stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 (Serva, Heidelberg, Germany) for 4 h and destained in methanol/acetatic acid/water (4:5:4.5). Proteolytic MMP activity was identified as a clear band on a blue background. The gel was scanned and a semi-quantification was performed by ‘quantity one’ software (Bio-Rad, California, USA). MMP activity in transfected or pretreated Jeg3 cells was expressed in relation to the parental cells.

Table I Characteristics of primers used for quantitative PCR.

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<th>Sequence</th>
<th>PCR product (bp)</th>
<th>GenBank accession number</th>
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<td>251</td>
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For treatment of Jeg3 cells with the conditioned medium of Jeg3/CCN3 cells, cells were plated at a density of 1 × 10^4 cells/well in 12-well plates in triplicate and cumulative cell numbers were counted every day after plating (24, 48 and 72 h) using a multichannel electronic cell counter (Casy1; Schaerfe System, Reutlingen, Germany) as previously described (Gellhaus et al., 2004).

For treatment of Jeg3/CCN3 cells with Brefeldin A (Peprotech, NJ, USA) and non-glycosylated CCN3 protein from Escherichia coli (Peprotech, NJ, USA), respectively, or solvent 0.1% BSA–PBS in triplicate. Twenty-four hours later cumulative cell numbers were counted as described above. For treatment of Jeg3/CCN3 cells with Brefeldin A (BFA, Biozol, Eching, Germany), cells were cultivated for 24 h with BFA dissolved in DMSO (1 ×) supplemented with cell culture medium. Control cells were treated with DMSO only.

Migration and invasion assay

Assays were performed in BD FalconTM cell culture inserts (BD Biosciences, Massachusetts, USA) with uncoated porous filters (8 μm pore size) for estimation of cell migration and filters precoated with 1:5 diluted Matrigel (BD Biosciences) to examine cell invasion. Parental Jeg3 and Jeg3/CCN3 cells (5 × 10^5) in 500 μl MEM were seeded into the upper chambers of the inserts and 10% FCS in MEM was added to the lower chamber. The cells were allowed to migrate or invade for 24 h in a humidified tissue culture incubator at 37°C, 5% CO2 atmosphere. Cells at the lower side of the inserts were incubated with Calcein-AM (Invitrogen) for 1 h, detached from the insert membrane with dissociation buffer (Biozol, Eching, Germany) and their fluorescence was quantified by using a fluorescence microplate reader (FLUOstar Omega) with excitation at 480 nm and emission at 520 nm. Serial dilution of cells starting at 250–4000 cells was used as standards, providing a relative quantification of the cells numbers.

Jeg3 cells (5 × 10^5) which had been pretreated with 1 μg/ml recombinant CCN3 proteins (glycosylated CCN3 and non-glycosylated CCN3 protein, respectively (see above)) in MEM for 24 h were trypsinized and seeded into the upper chambers of the inserts. Ten percent of FCS was added to the lower chamber and cells were allowed to migrate or invade for 24 h in the presence of 1 μg/ml recombinant CCN3 in both sides of the chamber. As a control 0.1% BSA/PBS was added to the culture medium. For quantification of migrated/invaded cells Calcein-AM staining was used as described above.

Gelatin zymography

The biological activity of MMP-2 and MMP-9 was evaluated by gelatin zymography. Conditioned culture media (without FCS) of Jeg3 and Jeg3/CCN3 cells were collected and electrophoresed in 10% polyacrylamide gel containing 0.1 mg/ml gelatin (Sigma, St. Louis, MO) under non-reducing conditions. The loading was normalized by measuring the total cell numbers. After electrophoresis the gel was rinsed twice in the renaturing buffer (2.5% Triton-X 100), incubated in developing buffer (50 mM Tris–HCl, 5 mM CaCl_2, at pH 8.8) at 37°C for 24 h, stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 (Serva, Heidelberg, Germany) for 4 h and destained in methanol/acetatic acid/water (4:5:4.5). Proteolytic MMP activity was identified as a clear band on a blue background. The gel was scanned and a semi-quantification was performed by ‘quantity one’ software (Bio-Rad, California, USA). MMP activity in transfected or pretreated Jeg3 cells was expressed in relation to the parental cells.

Endoglycosidase digestion

For analysis of CCN3 glycosylation pattern 50 μg of cell lysate, 50 μg of methanol precipitated conditioned medium or 100 ng of recombinant CCN3 proteins were digested with endoglycosidase Hf (Endo Hf) or peptide N-glycosidase F (PNGase F) (New England BioLabs, Inc., Frankfurt, Germany), respectively. PNGase F removes all types of N-linked carbohydrates, whereas Endo Hf removes only high mannose and some hybrid types of N-linked carbohydrates. Proteins were denatured with 2 μl 10 × glycoprotein denaturing buffer at 95°C for 10 min. Following addition of 2 μl 10% Nonidet P-40 and 2 μl Endo Hf
or PNGase F reaction buffer, the reaction mix was incubated at 37°C for 1 h with 1000 units PNGase F or 2000 units Endo Hf, respectively. As a negative control, protein samples were incubated with buffers alone without addition of enzymes. Subsequent western blots were prepared as described above.

**Statistical analysis**

All values were expressed as mean ± SEM of three individual experiments. Statistic assays were performed using Mann–Whitney U-test and a P value of ≤0.05 was considered to be statistically significant.

**Results**

**Glycosylation of transfected CCN3 in Jeg3 trophoblast cells**

Since parental Jeg3 cells express very low level of CCN3 protein (Gellhaus et al., 2004) we transfected CCN3 to analyse its function in the human malignant trophoblast. CCN3 mRNA (Fig. 1A) and protein expression (Fig. 1B) were highly increased in two CCN3-transfected Jeg3 cell clones (#1, #3) compared with the parental Jeg3 and vector control cells. The two clones differed in their expression levels with Jeg3/CCN3 #1 revealing a higher amount and Jeg3/CCN3 #3 showing a lower amount of CCN3 transcript as well as protein. Both Jeg3/CCN3 cell clones displayed a 55-kDa secreted CCN3 protein in the conditioned medium and a 48-kDa cytosolic CCN3 protein (Fig. 1B). To verify that the difference in molecular weight is due to different glycosylation of the CCN3 protein an endoglycosidase digestion was performed (Fig. 1C, D). Digestion of the secreted 55-kDa CCN3 protein in the conditioned medium of Jeg3/CCN3 cells using PNGase F resulted in a reduction of molecular weight, whereas treatment with Endo Hf had no effect (Fig. 1C). Enzymatic digestion of the cytosolic 48-kDa CCN3 did not result in a decrease in molecular weight (Fig. 1D).

PNGase F removes all types of N-linked carbohydrates, whereas Endo Hf removes only high mannose and some hybrid types of N-linked carbohydrates, these data suggest that the secreted CCN3 has N-linked oligosaccharide chains of the complex type.

**CCN3 decreased Jeg3 trophoblast cell numbers independent from its glycosylation status**

Jeg3/CCN3 cells revealed a significantly smaller increase in cell numbers (P < 0.05) compared with controls from day 1 onwards after plating (Fig. 2A) which confirmed previous findings (Gellhaus et al., 2004).

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**Figure 1** CCN3 expression and glycosylation pattern in Jeg3 CCN3 transfectants. (A). Quantitative PCR of CCN3 in Jeg3/CCN3-transfected cell clones #1 and #3, in vector control cells as well as in parental Jeg3 cells. mRNA expression was normalized to β-actin (*P < 0.05). (B). Western blot analysis of CCN3 expression. CCN3 was identified in CCN3-transfected clones #1 and #3 in both the cell lysate (48-kDa, non-glycosylated) and the medium (55-kDa, glycosylated) GAPDH (39-kDa) was used as the loading control. (C) Glycosylation pattern of secreted CCN3 and (D) intracellular CCN3 of Jeg3/CCN3 cells after endoglycosidase digestion with PNGase F and Endo Hf.
Since it is known that glycosylated CCN3 is secreted from the Jeg3/CCN3 cells as shown by immunoblotting (Fig. 1B), we investigated cell numbers of parental Jeg3 cells treated with conditioned medium of Jeg3/CCN3 cell clones containing the glycosylated CCN3 protein. After 24 h of cultivation in Jeg3/CCN3 conditioned medium, Jeg3 cell number was significantly decreased about 20% (Fig. 2B). To further analyse if the secreted glycosylated CCN3 is responsible for the reduced cell numbers, we used Brefeldin A (BFA), a substance known to block secretion of proteins (Misumi et al., 1986). Treatment with BFA for 24 h resulted in nearly complete absence (#1) or strong decrease (#3) of glycosylated CCN3 in the conditioned medium (Fig. 2C). Because of the toxicity of BFA (Shao et al., 1996) we only investigated cell numbers within the first 24 h. As shown in Fig. 2D, BFA treatment led to a slight reduced cell number of Jeg3 parental and vector control cells probably due to the toxic effect of BFA. However, the significant reduced cell numbers of Jeg3/CCN3 cell clones was abrogated after BFA treatment (Fig. 2D), which again confirmed that the secreted glycosylated CCN3 protein decrease Jeg3 growth properties.

Since changes in the glycosylation profile of proteins can lead to alterations in activity and function (Kukuruzinska and Lennon, 1998; Arnold et al., 2007) we wanted to know whether the glycosylation of CCN3 mediates this function. Therefore, we used recombinant N-glycosylated CCN3 (55-kDa) from mouse melanoma cells, as confirmed by deglycosylation with PNGase F, and non-glycosylated CCN3 protein (36.2-kDa) produced in E. coli (Fig. 3A). Treatment with both, glycosylated and non-glycosylated CCN3, resulted in a significant reduction in Jeg3 cell numbers after 3 days (Fig. 3B). However, compared with the CCN3 transfectants, which exhibit both forms of the CCN3 protein, the effect was less prominent (compare Fig. 2A and 3B). To give evidence for a specific action of CCN3, we incubated Jeg3 cells with the recombinant glycosylated CCN3 together with a CCN3 blocking antibody. As shown in Fig. 3C, the reduced growth caused by the glycosylated recombinant CCN3 protein was abrogated by blocking the CCN3 protein.

Thus, the CCN3 protein has the ability to decrease the growth in cell numbers of Jeg3 cells independent of its glycosylation status.

**CCN3 enhanced cell migration and invasion of Jeg3 trophoblast cells**

We further investigated the migratory and invasive capability of CCN3-transfected Jeg3 cells expressing both glycosylated and non-glycosylated CCN3 protein by using transwell assays without matrigel (migration) or with matrigel-coated filters (invasion).

Migration and invasion were significantly increased in Jeg3/CCN3 cells after 24 h compared with controls (Fig. 4A). In addition,
transcription levels of MMP-2 and MMP-9 were significantly elevated in the CCN3 transfectants (Fig. 4B, C). Gelatin zymography assays revealed elevated MMP-2 and to a lesser extent MMP-9 activity in the Jeg3/CCN3-conditioned medium (Fig. 4D).

Next, we knocked down the CCN3 expression in the transfectants by siRNA. As shown in Fig. 5A both cellular CCN3 and secreted CCN3 protein were almost completely abolished after CCN3 siRNA treatment (80–90% efficiency). CCN3 silencing significantly reduced the amount of both migrating and invading cells (P < 0.05) (Fig. 5B). In addition, CCN3 knockdown resulted in a down-regulation of MMP-2 and MMP-9 mRNA levels (Fig. 5C) and a significant decrease of MMP-2 and MMP-9 activity (Fig. 5D).

To determine whether the secreted glycosylated CCN3 is responsible for the enhanced migration properties in Jeg3/CCN3 cells, we used again BFA treatment to block the secretion of CCN3. BFA treatment did not significantly attenuate the enhanced migration of Jeg3/CCN3 cells (Fig. 5E). Thus, we assume that the secreted glycosylated CCN3 protein was not responsible for the enhanced migratory properties of Jeg3 cells.

Enhanced migration and invasion properties of Jeg3 cells upon CCN3 was dependent on its glycosylation

Since we recently showed that the migratory and invasive properties of Jeg3 cells were influenced by treatment with recombinant non-glycosylated CCN3 (Wolf et al., 2010), we re-examined the migration and invasion capacity of the Jeg3 parental cells using both the glycosylated and non-glycosylated form of recombinant CCN3 protein. We could confirm the results by Wolf et al. (2010) with enhanced migration and invasion properties of the Jeg3 cells using the non-glycosylated CCN3 (data not shown).

Using glycosylated CCN3, we observed no obvious effect on migration and invasion properties of Jeg3 cells (Fig. 6A). In addition, MMP2-mRNA expression in Jeg3 cells was significantly increased 48 h after addition of non-glycosylated CCN3 (Fig. 6B). In contrast, treatment with glycosylated CCN3 protein resulted in unchanged levels of MMP-2 expression. Both recombinant CCN3 forms did not significantly up-regulate MMP-9 activity (data not shown).
Figure 4 CCN3 enhanced Jeg3 cell migration and invasion and elevated MMP activity. (A) CCN3-transfected Jeg3 cell clones exhibited a significantly higher migration and invasion rate compared with control cells. (B) MMP-2 and (C) MMP-9 mRNA levels were measured and normalized to β-actin. (D) MMP activity in conditioned media of Jeg3 cells and Jeg3/CCN3 cells was analyzed by gelatin zymography: MMP-9 (Gelatinase B, 92-kDa) and MMP-2 (Gelatinase A, 72-kDa) bands. Above: densitometric analysis of MMPs activity. CCN3 overexpression significantly enhanced MMP-2 and MMP-9 activities in conditioned media compared with controls. *P < 0.05.
Figure 5 Enhanced migration and invasion and MMP activation were clearly dependent on CCN3. Jeg3/CCN3 cells were treated with siCCN3 or siControl. (A) Immunoblots revealed reduced CCN3 proteins in both cell lysates and conditioned media under CCN3 siRNA treatment. (B) Inhibition of CCN3 expression reduced Jeg3 cell migration and invasion. (C) MMP-2 and MMP-9 mRNA levels and (D) MMP-2 and MMP-9 activities were decreased after siCCN3 RNA treatment. (E) The number of migrated CCN3 cells was not significantly changed after 24 h BFA treatment compared with untreated vector control cells. *P < 0.05.
and may act in the CCN3 transfectants in a synergistic way.

of its glycosylation status is able to reduce growth in Jeg3 cell numbers

migration and invasion. Only the non-glycosylated form is able
glycosylated CCN3, have different properties with respect to
that the two forms of CCN3 protein, glycosylated and non-
and -9 transcription and activity. Moreover, we could give evidence
results in a smaller increase in cell numbers, but increased migration
of glycoproteins, such as CCN3 are associated with many
physiological and pathological processes, including migration and
differentiation, cell proliferation and tumor invasion. N-glycoproteins
are highly regulated during growth and differentiation, are expressed
in a fairly specific manner on tumor cells and are important for metas-
tasis (reviewed by Kuruzinska and Lennon, 1998; Miyoshi et al., 2010).
Recently it has been shown for the trophoblast that hyperglycosylation
of human choriongonadotrophin hCG is a marker of early human tro-
phoblast invasion (Guibourdenche et al., 2010), which points to an
important role of glycosylation for trophoblast function.

The role of CCN3 in regulating cell proliferation has been controver-
sially discussed, with the full-length form originally described as being
anti-proliferative, whereas the amino-truncated nuclear form was
shown to stimulate proliferation and act as an oncogene (Planque and
Perbal, 2003). This study and our previous results demonstrated that
Jeg3 cells transfected with CCN3 grew at a slower rate than the parental
Jeg3 cells that express only low levels of CCN3 (Gellhaus et al., 2004).
This is corroborated by investigations of Benini et al. (2005) and Laurent
et al. (2003), which showed that transfection of CCN3 into glioblastoma
and Ewing’s sarcoma cells resulted in reduced proliferative activity both
in vitro and in vivo. Jeg3 cells—though carcinoma cells—are well-
established models for the invasive trophoblast because this cell line
expresses cytokeratine 7, hCG, HLA-G and is recommended for regu-
lation studies on trophoblast invasion-related genes (Hannan et al.,
2010). However, their possibility for unlimited growth in contrast to
primary trophoblast cells is probably related to their tumor origin. But
with these characteristics, this cell line gave the opportunity to discrimi-
nate between effects on cell growth versus migration/invasion proper-
ties. Transfection of full-length CCN3 protein into Jeg3 trophoblast
cells, which lead to an overexpression of both forms of CCN3 resulted
in less growth in cell numbers. In addition cell growth in the presence of
both recombinant CCN3 proteins, glycosylated or non-glycosylated
CCN3, reduced growth rates pointing to the fact that not the glyco-
sylation status but the protein itself is important for regulating cell growth. In
comparison to the CCN3 transfectants expressing both CCN3 forms,
the effect of each recombinant CCN3 protein is less strong. This
phenomenon could be due to a synergistic effect of both CCN3
forms in the transfectants or by the fact that the CCN3-transfected
cells constitutively express CCN3 at a high level. CCN3 transfectants
showed a significant increase of their migration and invasion properties.
These findings could be confirmed by other studies in glioblastoma or
Ewing’s sarcoma cells overexpressing CCN3 (Laurent et al., 2003;
Benni et al., 2005).

Our experiments revealed an activation of MMP-2 and MMP-9 in
Jeg3 cells upon CCN3. Since Jeg3 cells serve as a model for the inva-
sive trophoblast, we would surmise a similar role for CCN3 in EVTs.
This idea is supported by recent studies on placental explant cultures
from first-trimester placentas (Wolf et al., 2010). In this study we
revealed an increased invasion of EVTs under low oxygen (3% O2),
which was accompanied by an enhanced expression of CCN3.
In pre-eclamptic placental tissues MMP-2 and/or MMP-9 proteo-
lytic activity of trophoblasts were found to be defective (Shokry
et al., 2009), which is hypothesized to induce shallow invasion of
the trophoblast cells. A role for both MMPs in trophoblast invasion
has been proved in rats where inhibition of MMP activity could
indeed impair trophoblast invasion with subsequent changes in vascu-
lar remodeling and placental perfusion (Verlohren et al., 2010).
However, CCN3 action seems to be cell specific since in Ewing’s

To summarize, our results showed that CCN3 overexpression
results in a smaller increase in cell numbers, but increased migration
and invasion of Jeg3 cells, which is correlated with increased MMP-2
and -9 transcription and activity. Moreover, we could give evidence
that the two forms of CCN3 protein, glycosylated and non-
glycosylated CCN3, have different properties with respect to
migration and invasion. Only the non-glycosylated form is able
to trigger these processes in Jeg3 cells. The CCN3 protein independent
of its glycosylation status is able to reduce growth in Jeg3 cell numbers
and may act in the CCN3 transfectants in a synergistic way.

Discussion

In the present study, we revealed that the CCN3 protein independent
of its glycosylation status is involved in choriocarcinoma cell growth.
However, migration and invasion properties of this trophoblast cell
line as well as activation of MMP-2 and MMP-9 are dependent on
the non-glycosylated but not on the glycosylated form of CCN3.
Thus, the two CCN3 forms in Jeg3 cells, a 48-kDa, non-glycosylated
CCN3 protein in the cell lysate and a 55-kDa secreted, glycosylated
CCN3 protein, serve different roles in malignant trophoblast cell
physiology. It is well known that changes in the glycosylation profile
of glycoproteins, such as CCN3 are associated with many

Figure 6 Change in migration and invasion properties of Jeg3 cells
upon CCN3 was dependent on its glycosylation. (A) Migration
and invasion of Jeg3 cells were not significantly changed upon treatment
with glycosylated recombinant CCN3. (B) MMP-2 mRNA expression
level in Jeg3 cells was significantly elevated after 48 h treatment with
non-glycosylated CCN3 proteins but not with glycosylated CCN3.
*p < 0.05.
sarcocoma high CCN3 levels could increase cell migration and invasion independent of promoting MMP-2 and MMP-9 activities (Benini et al., 2005). To our surprise the glycosylated secreted form of CCN3 was not able to change migration and invasion properties in contrast to the non-glycosylated intracellular CCN3 protein as we have already shown previously (Wolf et al., 2010).

It remains questionable how the non-glycosylated form of CCN3 which up to now has been only found intracellularly but not in the supernatant of the CCN3 transfectants can mediate enhanced migration and invasion. Further investigations are needed to unravel if the balance of intracellular non-glycosylated and secreted glycosylated CCN3 protein could contribute to coordinated trophoblast migration and invasion behavior.

The question arises in which signal cascade CCN3 is integrated to serve its different functions. Although CCN proteins may play important roles in fine-tuning major signaling pathways, such as Wnt, BMP and Notch, the precise function and mechanism of action of these proteins remain undefined (Zuo et al., 2010). It is known that CCN3 activities are mediated through interactions with integrin receptors as an outside–inside signaling. It can be speculated that this signaling cascade is triggered by the secreted glycosylated CCN3 from outside. Huang et al. (2011) could show that CCN3 enhances migration of human osteosarcoma cells through avβ5 integrin and integrin-linked kinase (ILK) and AP-1 pathways. Moreover, the integrin α5β1-dependent signaling pathway was reported to be implicated in MMP-2 and MMP-9 regulation of trophoblast cell migration and invasion (Jovanovic et al., 2010), but it remains elusive if this pathway is triggered by the intracellular CCN3, which would require an inside–outside signaling pathway.

In summary, our results demonstrated that high CCN3 expression results in reduced growth capacities and increased migration and invasion of Jeg3 cells, which is correlated with increased MMP-2 and -9 activities. Deregulated CCN3 expression might result in aberrant MMP-2 and -9 activities, which could be one reason for the shallow invasion and immature remodeling of the uterine vessels observed in pre-eclampsia. We could give evidence that change in glycosylation of CCN3 results in different physiological functions: independent of its glycosylation CCN3 reduces cell numbers, whereas non-glycosylated cellular CCN3 seems to be responsible for the enhanced migration/invasion properties of Jeg3 cells. Further investigations have to clarify whether the two CCN3 proteins activate different signaling cascades to achieve their functions.

**Authors’ roles**

W.Y., J.W., N.W.: participated in study design, acquisition of data, analysis of data, in manuscript drafting and critical discussion. M.S., R.K.: involved in study design, manuscript drafting and critical discussion. E.W., A.G.: participated in study design, acquisition and interpretation of data, manuscript drafting and critical discussion.

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