Decreased expression of the angiogenic regulators CYR61 (CCN1) and NOV (CCN3) in human placenta is associated with pre-eclampsia

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The pregnancy disorder pre-eclampsia (PE) is thought to be caused in part by shallow invasion of the extravillous trophoblast (EVT) leading to uteroplacental insufficiency and hypoxia. Here, we focused on the expressions of cysteine-rich 61 (CYR61, CCN1) and nephroblastoma overexpressed (NOV, CCN3), members of the CCN family of angiogenic regulators, in human placenta during normal pregnancy compared with pre-eclamptic and HELLP placentae using quantitative RT–PCR, western blotting and immunocytochemistry. During normal pregnancy, both proteins showed increasing expression levels and were strongly coexpressed in endothelial cells of vessels, stromal cells and interstitial EVT giant cells. However, NOV showed an earlier onset of expression in villous endothelial cells during gestation compared with CYR61, which may signify distinct roles of these proteins in placental angiogenesis. In early-onset pre-eclamptic placentae, both CYR61 and NOV were expressed at a significantly lower level compared with normal matched controls. This decrease of CYR61 and NOV in pre-eclamptic placentae is not associated with a decrease of the endothelial marker CD34 or vimentin. No obvious changes in the localization of CYR61 and NOV in pre-eclamptic placentae were detected but a change in the intracellular distribution in trophoblast giant cells. Our data point to a potential role of both molecules in the pathogenesis of early-onset PE.

Key words: angiogenesis/CCN/CYR61/NOV/placenta/pre-eclampsia

Introduction

Pre-eclampsia (PE), a multisystemic disorder affecting about 5–10% of pregnancies towards the end of the second trimester of gestation, is one of the leading causes of pregnancy-related maternal and fetal morbidity and mortality (Redman and Sargent, 2005; Sibai et al., 2005). In the most severe cases, this disease is often complicated by intrauterine growth restriction and premature birth.

Clinically, this disease is characterized by hypertension and proteinuria (Pridjian and Puschett, 2002). Currently, it is discussed whether the HELLP syndrome, an acronym for hemolysis, elevated liver enzymes and low platelet counts, represents a severe variant of PE or another type of disease (Sibai, 2004). At present, women at risk of this disease are identified on the basis of epidemiological and clinical risk factors, but the diagnostic criteria remain unclear. To date, the only effective treatment of PE is the delivery of the fetus and placenta.

Although the pathophysiology of PE is still unknown, the placenta is considered to play a key role in this disease (Myatt, 2002). This is mostly due to the finding that PE occurs even in the absence of a fetus as in molar pregnancy. The haemochorial placentation in humans involves a complex interaction of the trophoblast and the uterus. Extravillous trophoblast (EVT) cells migrate through the uterine stroma and erode local spiral arteries to gain access to the maternal blood supply. Normally, the invasive trophoblast remodels the maternal vessels by replacing the vascular smooth muscle and endothelial cells and converting them to vessels with low resistance and therefore high blood flow capacity (Fisher, 2004).

One of the most favoured hypotheses is that PE is generated by shallow invasion of the EVT followed by an incomplete remodelling of the maternal vascular structures which leads to uteroplacental insufficiency and fetal growth retardation which in turn can influence placental angiogenesis and development (Zhou et al., 1993; Fisher, 2004). The nature of the limited invasion is presently unknown, although it could result from defective EVT differentiation.

Several molecular mechanisms are known to affect trophoblast differentiation, such as specific transcriptional regulators, genes involved in the invasion process as well as adhesion molecules and integrins which play a key role in cell migration (Cross, 2000; Chakraborty et al., 2002). Defects in any of these processes could result in shallow invasion and immature remodelling of the uterine vessels observed in pre-eclamptic placenta. However, the abundance of genes that have been implicated in the pathogenesis of PE indicates that this disease may not be explained by a simple single mechanism.

Recent lines of evidence suggest that failed trophoblast invasion is linked to the maternal vascular pathology through the abnormal placentation production of vasculogenic/angiogenic factors such as vascular endothelial growth factor (VEGF) (Fisher, 2004; Mayhew et al., 2004a). Zhou et al. demonstrated that the expressions of VEGF-A and VEGF receptor-1 are down-regulated in cytotrophoblasts of pre-eclamptic...
placenta tissues and that the release of the soluble VEGF receptor-1 (sFlt-1), an antagonist of VEGF and placental growth factor (PIGF), is increased (Zhou et al., 2002). However, there are some discrepancies about the regulation of VEGF in the literature (Mayhew et al., 2004a; Sgambati et al., 2004). Several investigations have demonstrated that other growth factors and their receptors such as PIGF and insulin-like growth factor I (IGF-I) are also dysregulated in serum or placental tissues of women with PE (Sane et al., 2004; Lam et al., 2005). It is speculated that an imbalance in the production of angiogenic/growth factors at the maternal–fetal interface and their release into the maternal circulation could lead to the clinical signs of this pregnancy disorder such as hypertension and proteinuria.

Because angiogenesis and/or migration of trophoblasts are affected in this disorder, we have focused on molecules that are discussed as key players in these processes. We have evaluated the expressions of cysteine-rich 61 (CYR61, CCN1) and nephroblastoma overexpressed (NOV, CCN3), members of the CCN family, in the human placenta during normal pregnancy and in the placentae of pathological pregnancies. The CCN family of proteins consists of six members, CYR61 (CCN1), connective tissue growth factor (CTGF, CCN2), NOV (CCN3) and Wnt-induced secreted proteins (WISP1–3, CCN4–6), which are matricellular proteins involved in the regulation of various cellular processes such as adhesion, migration, proliferation and differentiation (Perbal, 2001, 2004; Bleau et al., 2005; Rachal and Brigstock, 2005). CYR61 and NOV have been shown to promote proangiogenic activities in endothelial cells in vivo through integrin receptors and are highly expressed in vascular structures during embryogenesis (Babic et al., 1998; Ellis et al., 2000, 2003; Leu et al., 2002; Lin et al., 2003; Chen et al., 2004). Moreover, these proteins are found to be expressed in the human placenta (Kolesnikova and Lau, 1998). The significance of CYR61 for developmental processes is strengthened by the observation that targeted disruption of the CYR61 gene in mice results in embryonic lethality because of impaired allantoic vessel bifurcation in the placenta and compromised vessel integrity in embryonic arteries (Mo et al., 2002).

In the light of the results from mice lacking CYR61, the aim of this study was to investigate the expressions of CYR61 and NOV in the human placenta during normal pregnancy and in the placentae complicated by early and late PE (ePE and IPE) and HELLP syndrome to address the question whether these molecules are dysregulated in these pregnancy-associated diseases. Here, we demonstrate that CYR61 and NOV show increasing levels in human placenta during pregnancy with strong expression in endothelial cells of vessels, in stromal cells and in interstitial EVT giant cells. Furthermore, we found a decreased level of these angiogenic regulators in early pre-eclamptic placental tissues compared with gestationally matched normal placentae. Our data suggest that the angiogenic factors CYR61 and NOV may be involved in the pathogenesis of PE through their potential effect on placental development and function.

**Materials and methods**

**Patients and placental collection**

Placental tissue was obtained from the Department of Gynecology and Obstetrics of the University Hospital Essen, the Department of Obstetrics and Gynecology, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, and the Morgentaler Clinic, Toronto. Written consent was received from women before surgery. The respective ethics committee approved consent forms and protocols to use the tissue. Placental tissue was obtained at the time of vaginal delivery. Caesarean section or as abortion material (6–18 weeks’ placenta tissues). Thirty-three pregnant women were recruited for the study of gene expression during normal pregnancy classified into the following four groups: first trimester (6–13 weeks, n = 7), second trimester (14–26 weeks, n = 6), preterm (27–36 weeks, n = 11) and term (37–41 weeks, n = 9). For the study of pre-eclamptic and HELLP placental tissues versus matched control normotensive pregnancies without any signs of PE or HELLP, the following groups were analysed: pregnancies complicated by early-onset PE and delivered before 34 weeks (25–33 weeks, n = 11), IPE and delivered after 34 weeks (34–39 weeks, n = 8) (ePE and IPE groups are defined according to Zhong et al., 2005) and HELLP syndrome (23–30 weeks, n = 6). For the clinical details of the patients, see Tables I and II. Because the gestational age was different between the ePE, HELLP and IPE groups, their respective gestational age-matched groups were used as controls (control 1: 23–33 weeks, n = 12 and control 2: 34–39 weeks, n = 9). For the control group, women with chronic hypertension, renal disease, collagen vascular disease, any evidence of intra-partum infection or other pregnancy complications such as fetal anomalies or chromosomal abnormalities were excluded from this study.

PE was diagnosed according to international criteria (Roberts and Redman, 1993; Sibai et al., 2005). Generally, PE was defined as a blood pressure of at least 140/90 mmHg on two occasions at least 6 h apart occurring after 20 weeks of gestation in women known to be normotensive beforehand and detectable urinary protein (proteinuria) ≥[1+ (≥30 mg/dl) by dipstick]. The HELLP group was further characterized by the same characteristics as for ePE along with abnormal liver function with enhanced liver enzyme concentrations glutamate oxalacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) and thrombocytopenia.

Early pre-eclamptic and HELLP groups were further discriminated from the normal controls by the following findings: lower mean birth (only HELLP group) and placental weight, significantly higher mean maximal pressure

| Table 1. Clinical details of pregnant women from normal pregnancies of first trimester, second trimester, preterm and term |
|---------------------------------|-----------------|-----------------|-----------------|
|                                | 1st trimester   | 2nd trimester   | Preterm         |
| Maternal age (years)           | ND              | 25.3 ± 6.9      | 27.1 ± 5.2      |
| Gestational age (weeks)        | 9.1 ± 2.4       | 24.3 ± 2.3      | 32.4 ± 2.7      |
| Birth weight (g)               | 675.8 ± 228.7   | 1666.2 ± 422.4  | 3345 ± 458.8    |
| Placental weight (g)           | 447.5 ± 244     | 409.4 ± 138.9   | 539.2 ± 55.5    |
| Pregnancy BMI (kg/m²)          | ND              | 26.7 ± 3.1      | 26.3 ± 4.2      |
| SBP (mm Hg)                    | ND              | 114.2 ± 9.2     | 114.4 ± 12.4    |
| DBP (mm Hg)                    | ND              | 67.5 ± 5.2      | 66.9 ± 5.9      |
| Proteinuria*                   | 0               | 0.125 ± 0.4     | 0.125 ± 0.4     |
| GOT (U/l)                      | 24.3 ± 7.7      | 50 ± 9.7        | 22.4 ± 12.6     |
| GPT (U/l)                      | 22.3 ± 9        | 27.5 ± 39.1     | 14.8 ± 9.3      |
| Thrombocytes (1000/mcl)        | 253.7 ± 53.4    | 200.1 ± 124.6   | 209.1 ± 72.4    |
| Smokers (n)                    | 0               | 0               | 0               |

SBP, maximal systolic blood pressure; DBP, maximal diastolic blood pressure; GOT, glutamate oxalacetate transaminase; GPT, glutamate pyruvate transaminase; ND, not determined.

*1 = 30 mg/dl; 2 = 100 mg/dl; 3 = 300 mg/dl.
Clinical details of pregnant women with early and late PE and HELLP syndrome and the matched normal control groups (Control 1 and 2)

<table>
<thead>
<tr>
<th></th>
<th>Control 1</th>
<th>Early PE</th>
<th>HELLP</th>
<th>Control 2</th>
<th>Late PE</th>
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<tr>
<td>Maternal age (years)</td>
<td>26 ± 5.9</td>
<td>31.0 ± 5.2</td>
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<td>30.4 ± 5.3</td>
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<td>Gestational age (weeks)</td>
<td>28.3 ± 3.2</td>
<td>29.8 ± 2.5</td>
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<td>Birth weight (g)</td>
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<td>1141.1 ± 398</td>
<td>776.7 ± 334.5</td>
<td>2723.8 ± 761.2</td>
<td>3106.9 ± 1100.1</td>
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<td>Placental BMI (kg/m²)</td>
<td>366.4 ± 142.7</td>
<td>284.4 ± 87.2</td>
<td>259.3 ± 63.5</td>
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<td>Pregnancy BMI (kg/m²)</td>
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<td>SBP (mm Hg)</td>
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<td>108.5 ± 17.3</td>
<td>170.8 ± 29.1</td>
<td>114.4 ± 6.2</td>
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<td>DBP (mm Hg)</td>
<td>67 ± 5.4</td>
<td>107.7 ± 12.5</td>
<td>105.8 ± 14.6</td>
<td>70.6 ± 7.8</td>
<td>94.4 ± 5.6</td>
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<td>Proteinuria*</td>
<td>0.2 ± 0.4</td>
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<td>3†</td>
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<td>GOT (U/l)</td>
<td>51.7 ± 60.5</td>
<td>53.9 ± 68.1</td>
<td>116.7 ± 138.4</td>
<td>33.3 ± 25.4</td>
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<td>GPT (U/l)</td>
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<td>87.9 ± 149.2</td>
<td>140.2 ± 107.6</td>
<td>28.8 ± 111.9</td>
<td>15.1 ± 18.3</td>
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<td>Thrombocytes (1000/mcl)</td>
<td>240.9 ± 84.8</td>
<td>193.2 ± 115.1</td>
<td>81.8 ± 191</td>
<td>221.8 ± 111.9</td>
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<td>Smokers (n)</td>
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Values are shown as mean ± SD.

PE, pre-eclampsia; SBP, maximal systolic blood pressure; DBP, maximal diastolic blood pressure; GOT, glutamate oxalacetate transaminase; GPT, glutamate pyruvate transaminase; ND, not determined. Control 1 and Control 2 are gestational age-matched controls of normal pregnancies for early PE/HELLP and late PE, respectively.

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<th>Control 1</th>
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<th>Control 2</th>
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<td>(systolic and diastolic) and proteinuria (also applied to IPE, see Table II). Early pre-eclamptic patients exhibited a significantly higher diastolic blood pressure compared with late pre-eclamptic patients. The women with HELLP syndrome were characterized by a significantly higher level of the liver enzyme GPT and a significantly lower quantity of thrombocytes with respect to the controls. For RNA and protein isolation, only chorionic tissue from the central part of the placenta was collected, and contamination with maternal decidua and amniotic membranes was excluded by morphological observation. Tissue processing was equally performed in the participating clinical centres involved in this study. Tissues were frozen in liquid nitrogen and stored at –80°C until extraction of matched RNA and protein samples. For immunohistochemistry and histology, we removed the chorionic villi tissue together with parts of the decidua basal plate. The samples were embedded in OCT cryo-medium (Tissue-Tek, Sakura, Zoeterwoude, the Netherlands), frozen in liquid nitrogen and stored at –80°C.</td>
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**Standard RT–PCR**

Two micrograms of total placental RNA samples was digested with DNase I (Invitrogen, Karlsruhe, Germany). The reverse transcription and the following PCR reaction were performed as described previously (Gellhaus et al., 2004). The PCR was generated using primers specific for CYR61, NOV, CD34 and vimentin designed based on the published sequence generated by MWG Biotech (Ebersberg, Germany). The following primer sequences were used:

- **CYR61** (AF307860) 5’-primer, GTGACGGAGGATCATCAAGGACC, 3’-primer, ATTCTGCGCCTGTGAAGGGTGG; NOV (NM_002514) 5’-primer, CACCGCGGATAGGGAGGATA, 3’-primer, GGTTAGGGCCTCCAGTGA; CD34 (NM_001101) 5’-primer, CACCCCTGTCTCAACATG, 3’-primer, GCACTCAAGGTTCTCAGTCCAGT; and vimentin (NM_003830) 5’-primer, GAGAACTTCCGCTCGAAG, 3’-primer, TCCACAGCTCCTAGTGCAGGT. The PCR was performed for 34 cycles of 1 min denaturation at 94°C, 1 min annealing at 59°C and 0.5 min elongation at 72°C. The PCR amplification products were studied using a confocal laser-scanning microscope (LSM 510, Zeiss, Oberkochen, Germany).

**Quantitative real-time RT–PCR**

Two to four micrograms of total placental RNA samples was DNase-digested and reverse transcribed as described previously (Gellhaus et al., 2004). Gene expression of CYR61, NOV, CD34 and vimentin was quantitated using the qPCR Master Mix for SYBR green (Eurogentec, Seraing, Belgium) and the GeneAmp 5700 sequence Detection System (Applied Biosystems, Darmstadt, Germany). For a quantitative measurement, β-actin (NM_001101) was used as an internal control: 5’-primer, ACCAATGGGGCACTGAGAAGAA; and 3’-primer, TACGGCCAGAGGCGTAGGTTAG (213 bp PCR product). The PCR reactions were carried out in triplicate in a final volume of 25 μl with 2 μl (80 ng) cDNA, 1× reaction buffer containing SYBR green, 10 pmol sense and antisense primers CYR61, NOV, CD34, vimentin and β-actin (for primer sequences, see above). The specificity of the amplification products was confirmed by melting curve analysis and by agarose gel electrophoresis. The PCR fragments were visualized on 2% ethidium bromide-stained agarose gels. Ten-fold 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 PCR was performed for 10 min at 95°C followed by 45 cycles of 10 seconds denaturation at 95°C and 1 min annealing at 60°C. The quantity of cDNA in each sample was normalized to the β-actin cDNA.

**Immunofluorescence and microscopy**

Indirect immunofluorescence of 7 μm placental cryostat sections was performed as described previously (Winterhager et al., 1991). The following primary antibodies were used: rabbit polyclonal antibody against CYR61 (1: 75, Aviva antibody, San Diego, CA, USA), rabbit polyclonal antibody against NOV (1: 150) (Chevalier et al., 1998), monoclonal mouse anti-Ki67 (1: 100, Novocastra, Newcastle, UK), monoclonal mouse anti-human CD31 (platelet endothelial cell adhesion molecule-1 (PECAM-1), 1: 150, Dako, Hamburg, Germany) and monoclonal mouse anti-human cytokeratin 7 (1: 150, Dako). The following appropriate secondary antibodies were used: donkey anti-mouse Alexa Fluor® 488 (1: 300, MoBiTech, Geettingen, Germany) and Cy3-conjugated goat anti-rabbit IgG (1: 300, Dianova, Munich, Germany). After immunolabelling, the DNA-specific dye 4',6-diamidino-2-phenylindole (DAPI) hydrochloride (0.1 μg/ml; 15 min, 37°C) was used to counterstain the nuclei. The sections were mounted with Mowiol (Sigma, Munich, Germany) and were studied using a confocal laser-scanning microscope (LSM 510, Zeiss, Oberkochen, Germany).

**Western blot analysis**

Protein extracts were prepared from placental tissues 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 BCA protein assay (Perbio Science, Bonn, Germany).
Protein samples (30 μg) were separated on a 12% polyacrylamide gel for the analysis of CYR61 and NOV expressions and on a 10% polyacrylamide gel for analysing CD34 expression and electrophoretically transferred to polyvinylidene difluoride membrane (Amersham Biosciences, Piscataway, NJ, USA). Membranes were blocked with 5% non-fat dried milk in Tris-buffered saline (TBS) with 0.15% Tween-20 and incubated with the primary antibody. The following primary antibodies were used: rabbit polyclonal CYR61 antibody (1 : 25000; kindly provided by N. Schuetze, Wuerzburg, Germany; Schutze et al., 2005), goat anti-human NOV (1 : 1000; R&D Systems, Wiesbaden, Germany), mouse anti-human CD34 (1 : 150, Santa Cruz Biotechnology, Heidelberg, Germany) and mouse anti-human GAPDH antibody (1 : 1000, Chemicon, Hampshire, UK) for normalization of protein expression. Primary antibody binding was detected using the following secondary antibodies: anti-rabbit IgG, anti-goat IgG and anti-mouse IgG antibody conjugated to horseradish peroxidase (1 : 10000; Santa Cruz Biotechnologies). Detection was achieved with the ECL chemiluminescence kit (Amersham Biosciences) according to the protocol using X-ray films (Kodak, Stuttgart, Germany).

**Statistical analysis**

The gene expression data of the quantitative RT–PCR experiments and the western blot analysis as well as the clinical data of pregnant women were analysed for statistical significance by the Mann–Whitney test for non-parametric independent two-group comparisons with the program SPSS 10 for Windows (SPSS Inc, Chicago, IL, USA). Differences with a $P$ value $≤ 0.05$ were regarded as statistically significant.

**Results**

**CYR61 and NOV expressions in human placenta during normal pregnancy**

A total of 33 normal placentae from women were included into this analysis (Table I). The transcript levels of the angiogenic regulator genes CYR61 and NOV were analysed in the chorionic villi tissue of placenta from normal pregnancies in the four gestational groups: first trimester, second trimester, preterm and term by quantitative RT–PCR (Figure 1). Both genes revealed a significantly increasing expression level ($P ≤ 0.05$) in placenta tissues during pregnancy using $β$-actin as the normalizing housekeeping gene. Although the tendency in the temporal expression pattern of both molecules during pregnancy was similar, there were some differences (Figure 1). In detail, NOV mRNA levels increased between the first and second trimesters and remained constant throughout the early third trimester, but a significant increase was seen at term as compared with first trimester levels. CYR61 expression revealed two peaks with enhanced expression, one in the second trimester (14–26 weeks) and one at the end of pregnancy (37–41 weeks). During the course of pregnancy, NOV showed a lower up-regulation (four-fold) of mRNA expression compared with CYR61 (eight-fold). Highest increase of CYR61 mRNA (six-fold) in the placenta was observed between first and second trimesters of pregnancy, whereas NOV was only two-fold up-regulated. Interestingly, after 26 weeks of pregnancy, the CYR61 transcript level was significantly down-regulated compared with the NOV mRNA expression. The results were independent from the chosen housekeeping gene (GAPDH and Ubiquitin B) (data not shown).

To confirm the results of CYR61 and NOV transcript expressions in placental tissue throughout normal pregnancy on protein level, we performed western blot analysis of placentae for each gestational group (Fig. 2). We could verify the increase of CYR61 and NOV protein expressions during the course of gestation. The protein expression pattern of both genes in the four gestational groups correlated to their transcript levels (compare Figures 1 and 2).

**Figure 1.** CYR61 and NOV mRNA expressions in human placenta during normal pregnancy. (A, B) Quantitative RT–PCR analysis of CYR61 and NOV mRNA in the first trimester (7–13 weeks), second trimester (14–26 weeks), preterm (27–36 weeks) and term (37–41 weeks) placentae after normalization to $β$-actin. During the course of pregnancy, there was a significantly increasing level of CYR61 and NOV mRNA in the human placenta. CYR61 showed two peaks with enhanced expression in the second trimester and at term. (A) Representation of single data of each examined placenta. (B) Data represent means ± SD. *$P ≤ 0.05$; *$^{1,3}$, relative expression is significantly enhanced in reference to first trimester (1) or preterm (3) placental tissues.
Localization of CYR61 and NOV proteins in human placenta during normal pregnancy

Immunocytochemical localization of CYR61 and NOV expressions in the human placenta in different gestational stages was performed in combination with various marker molecules (Figure 3). Because it is well known that CYR61 and NOV expressions are often associated with proliferative activity (Babic et al., 1998; Lin et al., 2003; Perbal, 2004), Ki67 was used as a marker to test the association of CYR61 and NOV expressions between the proliferating extravillous cytotrophoblast cells of the cell column and the non-proliferating interstitial EVT giant cells. In first trimester human placentae, double immunostaining of CYR61 or NOV with Ki67 revealed that neither protein was detectable in proliferating cytotrophoblast cells of a cell column in early stages of placental development (Figure 3A and C). However, both proteins showed an intense punctuated cytoplasmic and weak nuclear expression in clusters of the non-proliferating EVT giant cells localized in the decidua (Figure 6E and G). CYR61 and NOV were not expressed in cytotrophoblast and syncytiotrophoblast cells in all stages of pregnancy (Figure 3A–D). Thus, the expression of CYR61 and NOV was restricted to non-proliferating EVT giant cells with an absence in cytotrophoblast and syncytiotrophoblast cells. However, intense immunostaining for CYR61 as well as NOV was found in mesenchymal and stromal cells of the placental villi during all stages of pregnancy (Figure 3A–D).

Moreover, both proteins were highly coexpressed by endothelial cells of vessels within the vascularized villi of second trimester placenta tissues and showed a further increase near term (Figure 3B and D). We confirmed the localization of CYR61 and NOV in endothelial cells using CD31 as an endothelial marker (Figure 3E–H). Interestingly, we found a different onset of expression between CYR61 and NOV in endothelial cells of placental vessels during gestation. Whereas NOV showed an expression in endothelial cells of vessels in sections of first trimester placentae (13 weeks) (Figure 3G, arrow), CYR61 was not detected in endothelial cells of early placentae (Figure 3E, arrow) but in stromal cells. With ongoing pregnancy, coexpression of both CCN proteins could be demonstrated in endothelial cells of all vessels in the chorionic villi from the second trimester onwards (Figure 3F and H; 40 weeks).

Although we could demonstrate that there was an increasing transcript level of CYR61 and NOV in human placenta from early to late pregnancy (see Figure 1), there was no obvious relation to an enhanced immunolabelling of both proteins in all compartments such as endothelial cells, stromal cells and EVT giant cells.

CYR61 and NOV expressions in pre-eclamptic and HELLP placenta

We investigated 25 pathological placentae for the expression levels of both genes, (i) 11 placentae from women with early-onset PE (25–33 weeks, ePE), (ii) 8 placentae from women with PE in late pregnancy (34–39 weeks, lPE) and (iii) 6 placentae from women with HELLP syndrome (23–30 weeks). The expression data of the pathological placentae were compared with control placentae of the corresponding gestational stages of women with normotensive pregnancies: Control 1 (23–33 weeks, Co 1, n = 12) for ePE and HELLP, and Control 2 (34–39 weeks, Co 2, n = 9) for IPE. For clinical parameters of the women with PE and HELLP, see Table II.

The transcript levels of CYR61 and NOV were significantly (CYR61: \(P = 0.0001\); NOV: \(P = 0.004\)) lower in early pre-eclamptic placentae compared with the respective control group analysed by quantitative RT–PCR (Figure 4). Although variation of the expression levels of both genes was high among individual placenta tissues as...
Normal pregnancy. CYR61 and NOV revealed no expression in proliferating cytotrophoblast cells (CT) in a cell column (CC) of first trimester placentae (A, C; 9 weeks) but strong expression in stromal cells (Str) throughout gestation. Both proteins were not expressed in the syncytiotrophoblast (ST) during pregnancy. An intense expression of both proteins was found in endothelial cells of vessels (V) especially in term placentae (B, D; 40 weeks). CYR61 and NOV were strongly coexpressed—we used vimentin, which is present in all cells of mesenchymal origin such as villous stromal cells but also in endothelial cells. However, even this marker for both mesenchymal and endothelial cells does not indicate any change in expression levels in pre-eclamptic and HELLP placentae compared with normal controls (Fig. 4D).

The significant down-regulation of CYR61 and NOV in placentae from women with early-onset PE was confirmed in early pre-eclamptic placentae using western blot analysis (Figure 5A and B). Neither the HELLP placentae nor the late pre-eclamptic placentae revealed a significant change in the CCN protein levels. Again CD34 showed a significant up-regulation in early pre-eclamptic and also in HELLP placentae compared with controls (Figure 5C). These results confirmed that the decreased level of CYR61 and NOV expressions in early pre-eclamptic placentae could not be associated with a decrease in marker gene expression of endothelial cells.

**Localization of CYR61 and NOV proteins in pre-eclamptic and HELLP placentae**

Although the transcript levels of CYR61 and NOV were reduced in early pre-eclamptic placentae, no obvious difference in staining intensity and expression pattern of both proteins compared with normal control placentae of matched stages could be observed (Fig. 6). As in healthy placentae, expression of both proteins was absent in cytotrophoblast cells and in the syncytiotrophoblast analysed by double immunolabelling with cytokeratin 7 (data not shown). In the pathological placenta tissues, CYR61 and NOV were strongly coexpressed in endothelial cells of vessels and in stromal cells of the chorionic villi as in normal placentae (Figure 6B and D).

However, a difference in the localization and distribution of both proteins in interstitial EVT giant cells of early pre-eclamptic and HELLP placentae was found (Figure 6E–H). Whereas the control placentae showed a strong punctuated cytoplasmic and very weak nuclear expression in vessels of early placenta tissues but not in the trophoblast (Dye et al., 2001; Huppertz, 2006), whereas the endothelial marker CD31 was also found to be expressed in subpopulations of trophoblast cells as well as on platelets, monocytes and lymphocytes and thus not suitable for western blot analysis (Couch et al., 1998; Dye et al., 2001). Real-time PCR analysis revealed a significant up-regulation of CD34 in early pre-eclamptic placentae compared with normal matched controls, suggesting that either the amount of endothelial cells or the CD34 expression level per cell was elevated (Figure 4C). The analysis of CD34 transcript expression in HELLP placentae revealed no significant changes.

To associate the expression of both CCN molecules with the amount of villous stromal cells—the second cell population where CYR61 and NOV were expressed—we used vimentin, which is present in all cells of mesenchymal origin such as villous stromal cells but also in endothelial cells. However, even this marker for both mesenchymal and endothelial cells does not indicate any change in expression levels in pre-eclamptic and HELLP placentae compared with normal controls (Fig. 4D).
expression of CYR61 and NOV in clusters of giant cells (Figure 6E and G, arrows), the giant cells of pre-eclamptic and HELLP placentae demonstrated a diffuse staining of both CCN proteins in the cytoplasm as well as in the nucleus (shown for early PE placentae, Figure 6F and H, arrows).

Discussion
This study shows that CYR61 and NOV, which represent multifunctional proteins of the CCN family (Perbal, 2004; Rachal and Brigstock, 2005), were strongly expressed in human placenta with increasing expression levels during the course of normal pregnancy. Both proteins were prominently coexpressed in endothelial cells of vessels, in villous stromal cells and in interstitial EVT giant cells which point to a possible involvement of both genes in angiogenic processes as well as migration properties of EVT cells in human placental development.

The increasing expression levels of CYR61 and NOV mRNA and protein during normal pregnancy may be explained by the increased density of villi during the development of the human placenta accompanied by an increasing amount of fetal capillaries and stromal cells (Castellucci et al., 1990; Benirschke and Kaufmann, 2000). The increase in expression in gestational human placenta is paralleled by investigation of the CYR61 expression in mouse placenta where CYR61 transcripts increased from mid (11.5 days post coitum) to late gestation (18.5 days post coitum) (O’Brien and Lau, 1992; Kireeva et al., 1997; Latinkic et al., 2001).

Interestingly, we found a different onset of expression between CYR61 and NOV in endothelial cells of placental vessels during gestation. In early first trimester placentae, only NOV was detected in the
developing chorionic vasculature. As it is known that NOV is expressed in endothelial cells, promotes cell adhesion in vascular endothelial cells and induces neovascularization in vivo (Ellis et al., 2000; Lin et al., 2003), we assume that NOV may play a role in placental vasculogenesis which is known to occur in the first trimester placenta (Kaufmann et al., 2004). In contrast to NOV, CYR61 was not found in endothelial cells of early first trimester placenta tissues but was found from the second trimester onwards. Analysis of the CYR61 knockout mouse, which suffered from embryonic death because of vascular defects in the placenta and embryo, demonstrated that CYR61 is not required for vasculogenesis but is essential for vessel bifurcation in ongoing vascular development (Mo et al., 2002). Kaufmann et al. reported that the strongest growth spurt in the villous tree development is observed between the first and second trimester with a tremendous increase in villous mass and branching-angiogenesis followed by a stage of villi differentiation post 26 weeks gestation characterized by non-branching angiogenesis (Kaufmann et al., 2004). This may account for our observed lack of CYR61 during the period of vasculogenesis in the first trimester placentae. The expression of CYR61 in the endothelial cells of placental vessels during later gestation seems to be required for the precise regulation and development of a branching capillary network that occurs in the second trimester. This proposed function of CYR61 in placental angiogenesis is in accordance with our results showing a peak in expression in the second trimester and a strong down-regulation at preterm. Thus, the increased expression of both CCN molecules is associated with the enhanced angiogenesis during normal placental development.

This study demonstrated a significant decrease in expression levels of CYR61 and NOV mRNAs and proteins in placentae of women suffering from early-onset PE, but not from lPE, compared with normotensive controls.
Figure 6. Localization of CYR61 and NOV proteins in pre-eclamptic placentae. Double immunolabelling of CYR61 and NOV with CD31 (A–D) and cytokeratin 7 (CK7) (E–H). Analysis of representative healthy control (24 weeks: A, C; 26 weeks: E, G) and early pre-eclamptic placenta sections (B, D; 26 weeks; F, H; 30 weeks). (A–D): green, CD31; (E–H): green, CK7; (A–H): red, CYR61 and NOV, respectively; (E–H): blue, DAPI staining; (A–H): yellow, coincident staining. The expression pattern of both proteins in pre-eclamptic placenta sections was unaltered compared with the control placenta sections: CYR61 and NOV were strongly expressed in the endothelium of vessels (V, arrows in B, D). No expression was found in the syncytiotrophoblast but strong expression in stromal cells and in interstitial extravillous trophoblast (EVT) giant cells (GC). But note the difference in the localization and distribution of both proteins in the giant cells in pre-eclamptic placentae compared with normal controls (E–H). Whereas the control placentae showed a strong punctuated cytoplasmic and weak nuclear expression of CYR61 and NOV in clusters of giant cells (E, G, arrows), the pre-eclamptic placentae demonstrated a diffuse staining in the cytoplasm as well as in the nucleus (F, H, arrows). Scale bar represents: (A–D), 80 μm; (E–H), 20 μm.

gestational-matched controls. Using CD34 and vimentin as endothelial and stromal markers, we could not attribute the down-regulation of CYR61 and NOV in early pre-eclamptic placentae to a decreased proportion of endothelial and stromal cells in the villous tissue. Our results showed that the transcript and protein levels of the vascular endothelial marker CD31 revealed no down-regulation but rather an increase in early pre-eclamptic placentae compared with normal controls, suggesting no decline in fetal endothelial cell proliferation in placental villi during angiogenesis in the pre-eclamptic placenta samples. Our results are further confirmed by data of other groups investigating placent al villous morphology in PE, who showed that this syndrome is not associated with impoverished growth of villi and fetal vasculature (Mayhew et al., 2004b; Egбор et al., 2005). Furthermore, Lyall et al. revealed no difference in the expression of the endothelial marker CD31 between pre-eclamptic and normotensive controls (Lyall et al., 1995). One can speculate that the elevated levels of CD34 in early pre-eclamptic placentae could be a compensatory mechanism resulting from increased pro-inflammatory endothelial cell activation (Lam et al., 2005; Redman and Sargent, 2005), a higher proportion of endothelial cells or an enhanced expression of CD34 per cell.

In contrast to the observed decrease in CYR61 and NOV protein levels by western blot analysis, our immunohistochemical analysis showed no clear difference in staining intensity between the endothelium and stromal tissues of normal and pre-eclamptic placenta, but we suggest that the quantitative real-time PCR and western blot techniques are the more appropriate analyses to investigate gene and protein expression levels. Our immunohistochemical findings are in line with the observation of Antebły et al., who reported a similar quantification problem because of variable immunostaining intensities, analysing the expression of growth factor receptor-protein bound 2 (GRB2) in pre-eclamptic placentae (Antebły et al., 2005).

As CYR61 and NOV are coexpressed in endothelial cells of pre-eclamptic placenta tissues and it is well known that they exhibit proangiogenic functions and mediate endothelial cell growth, migration, adhesion and survival, it is likely that these molecules act in an autocrine manner on the endothelium. However, because the amount of endothelial cells, and thereby vessels, are not decreased in the pre-eclamptic placentae, it remains speculative what the consequences of the reduction in CCN proteins are in relation to angiogenesis. It is also possible that endothelial and stromal derived CYR61 and NOV act in a paracrine manner on trophoblast cells. It is known that the CCN molecules regulate the production and/or activity of other angiogenic molecules, e.g. basic fibroblast growth factor (bFGF) and VEGF (Rachal and Brigstock, 2005), that in turn act on both trophoblast and endothelial cells during placental development (Dunk and Ahmed, 2001; Antebły et al., 2004). Therefore, it is possible that the decrease in CYR61 and NOV in PE may contribute to the reported decreases in growth factors such as VEGF, PIGF and IGF (Zhou et al., 2002; Sane et al., 2004; Lam et al., 2005) and thereby influences trophoblast differentiation. However, it remains unclear if the decrease of CYR61 and NOV is a cause or a consequence of PE.

Further evidence for a possible involvement of CYR61 and NOV in placental function was the finding of strong expression levels in the non-proliferating interstitial EVT giant cells. This expression is associated with findings in the mouse placenta, where CYR61 is localized in trophoblast giant cells (Mo et al., 2002). We suggest that the EVT giant cells may require both CCN proteins as they differentiate along the invasive pathway after escaping from the cell cycle. It is reported that NOV and CYR61 stimulate migration and invasion properties in Ewing’s sarcoma cells (Benini et al., 2005; Bleau et al., 2005). The expression of CYR61 and NOV also further supports the hypothesis of a vascular endothelial phenotype of the EVT cells suggested by the expression of vascular cell adhesion molecule-1 (VCAM-1), PECAM-1.
and VE-cadherin (Zhou et al., 1997b). In PE, the molecules representing the vascular phenotype failed to express properly (Zhou et al., 1993, 1997a). We did not evaluate whether the amount of CYR61 and NOV was reduced in EVT giant cells; however, we observed a difference in the localization and distribution of both proteins in interstitial EVT giant cells of pre-eclamptic placentae. Whereas the control placenta giant cells showed a strong punctuated cytoplasmic weak nuclear expression of CYR61 and NOV, the giant cells of pre-eclamptic and HELLP placentae demonstrated a diffuse staining of both CCN proteins in the cytoplasm and nucleus. We have previously demonstrated that NOV localizes predominantly in the nucleus of the invasive trophoblast cell line JEG3 and shown that NOV over-expressing JEG3 trophoblast cells revealed a reduced cell proliferation compared with parental cells (Gellhaus et al., 2004). Furthermore, it is also known that the function of CCN proteins seems to be dependent on their subcellular localization (Perbal, 2001, 2004). A hypothesis is that if NOV is accumulated in the nucleus, it leads to growth stimulation, whereas if NOV is secreted or remains at the cell membrane, it inhibits proliferation. Therefore, we suggest that the aberrant localization pattern of NOV and CYR61 is likely to negatively affect trophoblast invasion and remodelling of the spiral arteries, thereby compromising blood flow to the maternal-fetal interface in PE.

It is intriguing that both severe pre-eclamptic and HELLP placenta samples show aberrant giant cell expression of both proteins, yet only the pre-eclamptic group reveal decreased placental expression levels. We found no clear regulation of CYR61 and NOV in the placenta tissues from women with HELLP syndrome and late-onset of PE suggesting different aetiologies between early-onset pre-eclamptic placentae and the later maternal pathology of PE. It is hypothesised that HELLP is a different pregnancy-associated disease compared with PE because this syndrome exhibits different pathophysiology accompanied by other molecular mechanisms (Baxter and Weinstein, 2004). The diagnosis criteria for early-onset PE and HELLP are often miscellaneous and not well defined. As we found no significant changes in expression levels of the angiogenic molecules CYR61 and NOV in HELLP placentae, we assume that these proteins are not involved in the liver metabolism and haematoapoiesis disorders that are the main characteristics of HELLP.

In conclusion, our results showed that the expression of the CCN proteins CYR61 and NOV in human placenta increased during normal pregnancy. Because both proteins have a different onset in expression in endothelial cells of placental vessels during pregnancy, they may play different roles in angiogenesis during normal placental development. Whereas CYR61 seems to be more involved in branchangiogenesis in the placenta, NOV may play a role in early placental vasculogenesis.

Interestingly, CYR61 and NOV were significantly lower expressed in early pre-eclamptic placentae compared with normal matched controls accompanied by a change in intracellular distribution of both proteins in interstitial EVT cells which adds novel molecules to the orchestra of candidate genes that showed a dysregulated expression in PE and are associated with placental angiogenesis and EVT migration.

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