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CCM2 and CCM3 proteins contribute to vasculogenesis and angiogenesis in human placenta

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Summary. Placenta as an ideal model to study angiogenic mechanisms have been established in previous studies. There are two processes, vasculogenesis and angiogenesis, involved in blood vessel formation during placental development. Therefore, blood vessel formation is a crucial issue that might cause vascular malformations. One of the vascular malformations is cerebral cavernous malformation (CCM) in the central nervous system, consisting of endothelium-lined vascular channels without intervening normal brain parenchyma. Three CCM loci have been mapped as *Ccm1, Ccm2, Ccm3* genes in CCM. In order to investigate whether CCM proteins participate in blood vessel formation, we report here the expression patterns of CCM2 and CCM3 in developing and term human placenta by means of immunohistochemistry and Western blot analysis. CCM2 and CCM3 were obviously detected in the vascular endothelium during early pregnancy. Moreover, vascular endothelium of stem villi revealed a moderate immunostaining for CCM2 and, to a lesser extent, in the endothelium of mature intermediate villi in term placenta. Interestingly, CCM3 immunostaining was weakly localized in the endothelium of mature intermediate villi and showed lesser expression going toward stem villi in term placenta. The expression patterns of the proteins were clearly identified in the vascular endothelium of human placenta, suggesting that they might play roles during angiogenesis and vasculogenesis. Furthermore, with this study, CCM2 and CCM3 have been described for the first time in the human placenta.

Key words: CCM2, CCM3, Placenta, Angiogenesis, Vasculogenesis

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Introduction

The placenta is a multifaceted organ that plays a critical role in maintaining and protecting the developing fetus. Normal development and function of the placenta requires extensive vasculogenesis and subsequent angiogenesis, in both maternal and fetal tissues. Vasculogenesis is the formation of the primitive vascular network de novo from progenitor cells, and angiogenesis is identified as the extension of blood vessels from preexisting vascular structures (Demir et al., 1989, 2006; Geva et al., 2002; Charnock-Jones et al., 2004). Many factors, such as vascular endothelial growth factor (VEGF), angiopoietins (Angpt-1 and -2) and their receptors are involved in the molecular regulation of these diverse developmental steps. The spatial and temporal localization of these factors have been studied extensively in the placenta (Geva et al., 2002; Demir et al., 2006).

It is well known that genetic and angiogenic factors also play an important role in the pathogenesis of several vascular anomalies in human placenta (Breier, 2000; Reynolds and Redmer, 2001; Reynolds et al., 2002). Considerable advances have been made in recent years to identify the genetic and molecular determinants of a variety of vascular anomalies using molecular genetic approaches. So far, several genes have been defined in vascular anomalies, such as cerebral cavernous malformations (CCMs). CCMs are vascular malformations that can occur as a sporadic or a familial autosomal dominant disorder with incomplete penetrance and variable clinical expressions (Liquori et al., 2007). CCMs are also characterized by abnormally enlarged capillary cavities without intervening brain parenchyma (Clatterbuck et al., 2001) that can result in seizures, hemorrhage, recurrent headaches, and focal neurological deficits (Felbor et al., 2006). Up to now, three CCM loci have been identified; on chromosomes

7q21-22 (Ccm1; Online Mendelian Inheritance in Man [OMIM] 116860), 7p13-15 (Ccm2; OMIM 603284), and 3q25.2-27 (Ccm3; OMIM 603285), and 3 genes have been cloned, KRIT1 on Ccm1, MGC4607 on Ccm2, and PDCD10 on Ccm3 (Revencu and Vikkula, 2006).

The mechanisms involved in cerebral blood vessel angiogenesis during development are still poorly defined. Therefore, there is a lack in the understanding of the mechanisms that lead to CCMs. The identification of the CCM genes has broadened the understanding of the mechanisms underlying CCMs. Recent studies have indicated possible angiogenic functions of these genes (Plummer et al., 2005; Revencu and Vikkula, 2006). Guzeloglu-Kayisli et al. (2004) reported that CCM1 was expressed in endothelial cells of human placenta using a well-established in vivo model of angiogenesis by detailed histological examination. They pointed out that CCM1 was expressed during early angiogenesis by endothelial cells and may play a key role in vessel formation and/or development. However, there is still no knowledge about whether CCM2 and CCM3 would also have a role, like CCM1, in human placenta, as observed in the central nervous system.

We hypothesized that as indicated for CCM1 by Guzeloglu-Kayisli et al. CCM2 and CCM3 might also be involved in angiogenesis in both early and term human placenta (Guzeloglu-Kayisli et al., 2004). Therefore, our aim was to investigate the expression and distribution of CCM2 and CCM3 proteins in human placental tissues during development, and to determine whether there were any alterations in their expression by using immunohistochemistry and Western blot analysis. This is so far the first study which has attempted to determine CCM2 and CCM3 expression in human placental tissues.

Materials and methods

Tissue collection

A total of 14 samples of human placental tissues [5 samples of 22-28 days (fourth week pc.); 5 samples of 29-35 days (fifth week pc.); 4 samples of 36-42 days (sixth week pc.)] in the first trimester obtained after legal termination of pregnancy by curettage for medical or psychosocial reasons, which were unlikely to affect placental structure and function. Furthermore, a total of 6 term human placentas collected from clinically normal pregnancies terminated by vaginal deliveries were also studied. Tissues were classified as described previously (Demir et al., 1989) by assessing the embryonic developmental stages according to the Carnegie classification (O'rahilly, 1973). None of the normal pregnancies were receiving hormone treatment. The umbilical vessels of term placenta were dissected and clamped for the perfusion of each placental vascular bed with a phosphate buffered saline solution.

Tissues were supplied from the Department of Obstetrics and Gynecology, School of Medicine, Akdeniz University, Antalya, Turkey. Informed consent forms and protocols to use the tissues were approved by the Ethical Committee of School of Medicine, Akdeniz University, Antalya, Turkey.

Immediately after obtaining the tissues, placental samples were divided into two parts, one of which was embedded in paraffin for further immunohistochemical analysis, while the other one was preserved in liquid nitrogen for Western blot analysis.

CCM2 and CCM3 antibody production

A CCM3/PDCD10 (CCM3)-specific rabbit polyclonal antibody was synthesized and affinity-purified against the antigenic peptide KIPDEINDRVR FLQTIKD (Open Biosystems, Huntsville, AL) as previously described (Tanriover et al., 2008). The CCM2 antibody synthesis, characterization and validation have been previously described (Seker et al., 2006).

Immunohistochemistry

For CCM2 and CCM3 immunohistochemistry, paraffin sections were deparaffinized and blocked for endogenous peroxidase activity with methanol containing 3% H_2O_2 for 15 min and for nonspecific binding with universal blocking reagent (BioGenex, San Ramon, CA, USA) for 10 min at room temperature (Tanriover et al., 2005). Anti-rabbit CCM2 and CCM3 diluted in dilution buffer (1/250) were applied for 1h at room temperature in a humidified chamber. For negative controls the primary antibodies were replaced by normal rabbit IgG serum (Vector Lab. Burlingame, CA, USA) at the same concentration. After several washes in PBS, sections were incubated with biotinylated goat anti-rabbit IgG secondary antibody (1/400 dilution Vector Lab. Burlingame, CA, USA) for 30 min followed by LSAB streptavidin-peroxidase complex (Dako, Carpinteria, CA, USA) incubation for 30 min and were rinsed with PBS. Antibody complexes were visualized by incubation with diaminobenzidine (DAB) chromogen (BioGenex). Sections were counterstained with Mayer's hematoxylin (Dako), dehydrated, mounted and examined by a Zeiss-Axioplan (Oberkochen, Germany) microscope.

Analysis of staining intensities

The intensity of immunoreactivity was quantitatively evaluated by two observers blinded to the type and source of the tissues, in which positive stainings were grouped according to the following categories: 0 (no staining), + (weak but detectable), ++ (moderate or distinct), +++ (strong) and ++++ (very intense) (Sati et al., 2007). The results are presented in Table 1.

Double immunohistochemistry

Until the primary antibody step, the same protocol

was applied to the slides for double staining as described above. CCM2 and CCM3 antibodies were applied and then incubated with Alexa 488 donkey anti-rabbit secondary antibody (A21206; 1/300 dilution, Invitrogen, Eugene, Oregon, USA) for 1h at room temperature. After several washes in PBS, sections were incubated with a CD34 mouse monoclonal antibody (1/50 dilution, Santa Cruz Biotechnology Inc, Santa Cruz, Ca, USA) for 2h at room temperature. Following the washing steps in PBS, sections were incubated with Alexa 555 donkey anti-mouse secondary antibody (A31570; 1/300 dilution, Invitrogen) for 1h at room temperature. The sections were mounted with UltraCruz™ Mounting Medium (Santa Cruz Biotechnology Inc.) for fluorescence with DAPI (Santa Cruz Biotechnology Inc.) and examined under the Olympus BX61 fluorescence microscope.

Western blot analysis

Total protein from the tissues was extracted in a lysis buffer (10 mM Tris-HCL, 1 mM EDTA, 2.5% SDS, 1 mM phenyl methyl sulfonylfluoride, 1 µg/ml leupeptin) supplemented with Complete® protease inhibitor cocktail (Boehringer, Mannheim, Germany) (Tanriover et al., 2004). The protein concentration was determined using a standard BCA assay (Smith et al., 1985; Wiechelman et al., 1988) and 50 µg protein was applied per lane. Prior to electrophoresis, samples were heated for 5 min at 95°C and then subjected to SDS polyacrylamide gel electrophoresis under standard conditions. Afterwards, samples were transferred onto PVDF membranes (BioRad, Hercules, CA, USA) in a buffer containing 0.2 mol/l glycine, 25 mM Tris and 20% methanol, overnight. The membranes were blocked for 1 h with 5% nonfat dry milk (BioRad) in TBS-T to decrease nonspecific binding, then the membranes were incubated with rabbit polyclonal antibody against human CCM2 and CCM3 (both dilution 1/1000) in 5% nonfat dry milk in TBS-T for 2h. After several washes in TBS-T the membranes were incubated with horse peroxidase-labeled anti-rabbit IgG (dilution 1/10,000; Vector Laboratories) for 1 h. Immunolabelling was visualized using the chemiluminescence based SuperSignal CL HRP Substrate System (Pierce, Rockford, IL, USA) and the membranes were exposed to Hyperfilm (Amersham, Piscataway, NJ, USA). Afterwards, the membranes were stripped using Stripping solution (Pierce), and equal loading of proteins in each lane was confirmed by re-probing the membranes with mouse monoclonal anti-human ß-actin (Abcam, Cambridge, UK).

Results

Immunohistochemistry

Distribution of CCM2 in human placenta

First trimester. During early pregnancy both cytotrophoblast and syncytiotrophoblast cells were immunopositive with CCM2. The immunoreactivity of CCM2 in the syncytiotrophoblasts was higher than that of cytotrophoblasts (Fig. 1a). On the other hand, CCM2 revealed a moderate immunostaining in both the proximal and distal cytotrophoblastic cell columns. Moreover, CCM2 was clearly detected in the vascular endothelium of immature intermediate villi with a strong immunoreactivity (Fig. 1a), and to a lesser extent in the endothelium of stem villi. CCM2 immunoreactivity was moderately observed in stromal and Hofbauer cells (Fig. 1a). Interestingly, hematopoietic cells showed the strongest immunoreactivity for CCM2 (Fig. 1b). In addition, there was a moderate CCM2 immunolabelling localized in the cytoplasm of angiogenic cell cords with or without primitive lumen (Fig. 1b) (Table 1a).

Term placenta. Trophoblast cells revealed a strong immunoreactivity for CCM2 as also observed during early weeks of pregnancy. Moreover, CCM2 immunostaining was mainly observed in the stromal cells of term placenta (Fig. 1c) (Table 1b). There was a moderate CCM2 immunolabelling localized in the endothelium of vessels in stem villi (Table 1b), and a weak immunolabelling was also observed in vascular endothelium of mature intermediate and terminal villi of

Table 1. The quantitative analysis of CCM2 and CCM3 expressions during first trimester **(a)** and term **(b)** human placental tissues.

a)	1st trimester	CT	SN	CC	VE	SC	HbC	ACC	HC
	CCM2	++	+++	++	+++	++	++	++	++++
	CCM3	++	+++	+++	+++	+	+++	++	++++

b)	Term	CCM2			CCM3		
		TB	SC	VE	TB	SC	VE
	Mature intermediate	+++	+++	+	+++	++	+
	Terminal	+++	+++	+	+++	++	+
	Stem	+++	+++	++	+++	++	±

CT: cytotrophoblast; SN: syncytiotrophoblast; CC: cell columns; VE: vascular endothelium; SC: stromal cells; HbC: Hofbauer cells; ACC: angiogenic cell cords; HC: hematopoietic cells; TB: trophoblast.

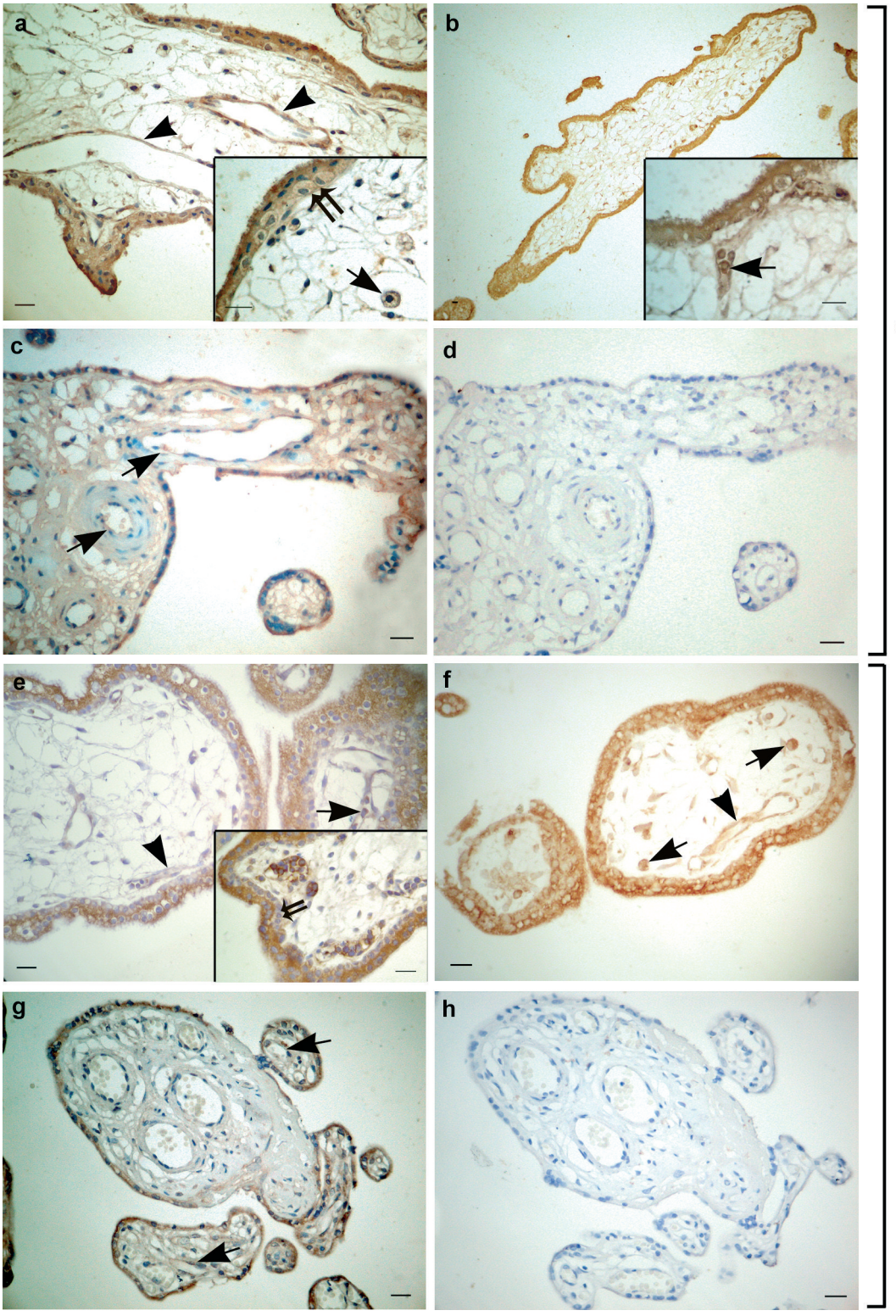

Fig. 1. Localization of CCM2 **(a, b, c, d)** and CCM3 **(e, f, g, h)** proteins in first trimester **(a, b, e, f)** and term human placenta **(c, d, g, h). a.** CCM2 was observed in the vascular endothelium of immature intermediate villi (arrowheads) and also detected in syncytiotrophoblasts, cytotrophoblasts (double arrow) and Hofbauer cells (inset, arrow). **b.** Low magnification image of immature intermediate villi in human placental tissue section was presented. Angiogenic cell cords with or without primitive lumen revealed moderate immunoreaction with CCM2, in addition to strong immunoreactivity in the hematopoietic cells (inset, arrow). **c.** Vascular endothelium in mature intermediate villi was weakly immunolabelled with CCM2 in term placenta (arrows). **d.** Negative-control section. Please note the absence of CCM2 immunostaining in the negative control staining. **e.** CCM3 immunoreactivity was observed in syncytiotrophoblasts, cytotrophoblasts (double arrow), Hofbauer, hematopoietic cells (arrow) and angiogenic cell cords (arrowhead) in immature intermediate villi in the first trimester. **f.** Vascular endothelium (arrowhead) and Hofbauer cells (arrows) were also immunopositive with CCM3. **g.** CCM3 expression in mature intermediate villi of human term placenta. Vascular endothelium showed weak immunoreactivity with CCM3 in mature intermediate villi (arrows). **h.** Negative-control section. Please note the absence of CCM3 immunostaining in term placenta. Scale bars: 50 μm.

term placenta (Fig. 1c) (Table 1b).

No immunoreactivity was observed on the slides where primary antibodies were replaced with normal rabbit IgG during early weeks of pregnancy or term placenta (Fig. 1d).

Distribution of CCM3 in human placenta

First trimester. Cytotrophoblast and syncytiotrophoblast cells revealed a similar staining pattern as shown for CCM2 immunolabelling (Fig. 1e). On the other hand, the proximal cytotrophoblastic cell columns revealed a strong immunoreactivity with CCM3 compared to the distal cell columns in the first trimester placenta. CCM3 immunolabelling was very intense in the immature intermediate villi where Hofbauer cells exhibited a strong immunoreactivity (Fig. 1f). The stromal cells of the chorionic villi revealed a weak to moderate staining. However, the hematopoietic cells exhibited a strong immunoreactivity (Fig. 1e) and angiogenic cell cords were moderately immunostained (Fig. 1e) (Table 1a).

Term placenta. Trophoblast and stromal cells exhibited a similar immunolabelling with CCM3 as observed for CCM2 in term placenta (Table 1b). The vascular endothelium showed a weak immunoreactivity with CCM3 in the mature intermediate villi (Fig. 1g) (Table 1b). Moreover, the reaction was also weak in the endothelium of terminal villi, while very weak immunoreaction was detected in the endothelium of vessels in stem villi of human term placenta (Table 1b).

Negative control immunostaining with normal rabbit IgG confirmed the specificity of CCM3 staining patterns in first trimester and term placenta (Fig. 1h).

Double staining with CD34

CD34/CCM2 and CD34/CCM3 double-labeling were used to confirm the endothelial staining of CCM2 and CCM3 on serial sections of first trimester and term placenta. CCM2 and CCM3 immunolabelling were detected in the vascular endothelium, which was also labeled by CD34 in first trimester and term placenta (Fig. 2a,b). CCM2 and CCM3 immunolabelings were also seen in trophoblast and stroma with Alexa 488 (green). CD34 was observed only in vascular endothelium with Alexa 555 (red).

Western Blot

CCM2 and CCM3 expressions were analyzed by Western blots of normal human placental tissues during early pregnancy (Fig. 3a) and term (Fig. 3b). The blots revealed clear bands for CCM2 and CCM3 corresponding to 47 kDa and 25kDa, respectively, in both first trimester and term placenta. Equivalent amounts of total proteins were loaded per lane as indicated by the immuno-expression of ß-actin (43kDa). According to the Western blot results, both CCM2 and CCM3 showed no significant alterations either in the first trimester placenta as pregnancy progressed or in term placentas.

Discussion

CCM genes have been shown to be involved in many biological processes, including angiogenesis and malformations, such as CCMs. It has been shown that Krit1, product of the Ccm1 gene, was expressed during

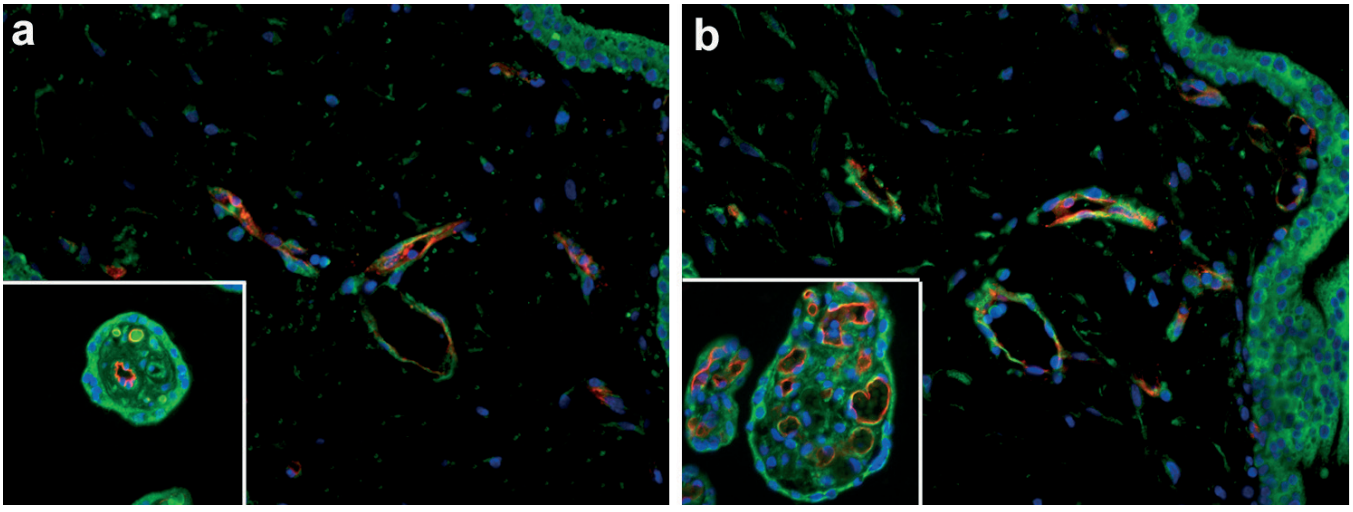

Fig. 2. Representative fluorescence photomicrographs of CD34/CCM2 and CD34/CCM3 double-staining in first trimester **(a, b)** and term placenta (insets). **a.** CCM2 immunostaining was detected in vascular endothelium in first trimester and term human placenta (inset). **b.** CCM3 immunoreactivity was observed in vascular endothelium, as well as CD34 in first trimester and term placenta (inset). Green: CCM2 or CCM3; blue: dapi; red: CD34. x 40

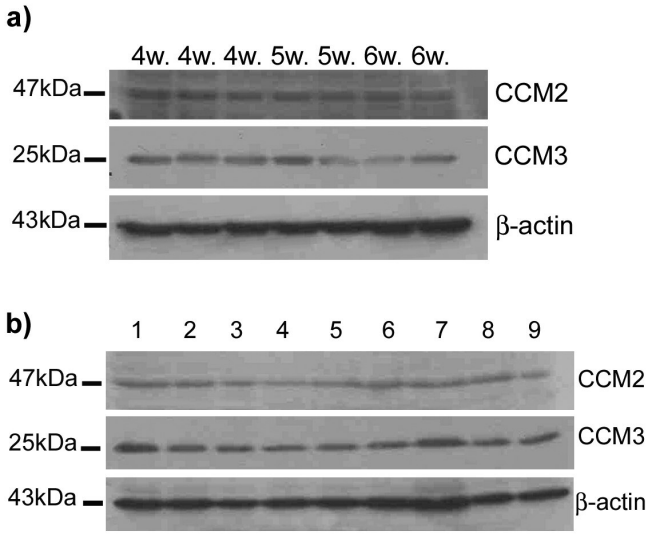

Fig. 3. a. Western blot analysis of CCM2 and CCM3 in the first trimester of human placenta. Bands were detected for CCM2 and CCM3, corresponding to 47kDa and 25kDa, respectively. **b.** Immunoblots of human term placenta extracts were detected as well. The immunoexpression of ß-actin (43kDa) was used to confirm equivalent amounts of total proteins loaded per lane.

early angiogenesis by placental endothelial cells, suggesting an important role in the vessel formation (Guzeloglu-Kayisli et al., 2004). Other CCM genes, Ccm2 and Ccm3, might also be associated with angiogenesis and vasculogenesis during placentation, similar to that shown for Ccm1. Since the vascular development and angiogenesis are closely linked with several genes and proteins, placenta is a useful model to investigate the functional roles of Ccm2 and Ccm3 genes. Based on this hypothesis, we aimed to investigate the presence of CCM2 and CCM3 proteins during human placentation by immunohistochemistry and Western blot analysis.

Both vasculogenesis and angiogenesis are regulated by the capacity of endothelial cells to adhere to each other and assemble into new vascular structures. CCM proteins are also required to regulate endothelial cell-cell association during formation of the cardiovascular system development (Kleaveland et al., 2009). A variety of receptors for extracellular matrix, cell-cell adhesion etc. are reported to play a pivotal role in these processes (Risau et al., 1988; Demir et al., 1989; Hanahan, 1997). So, it is possible that some new proteins, such as CCM2 and CCM3, which are involved in CCMs in the human brain, might also be involved in vascular system development in human placental tissues.

In this study we reported CCM2 and CCM3 proteins localized in the blood vessels of human placenta with a structural homogeneity, regardless of the developmental stage of the first trimester. Since both CCM2 and CCM3 are expressed in the endothelial cells both genes could possibly be related in endothelial cell functions in early pregnancy. Interestingly, CCM2 immunolabelling in the vascular endothelium was moderately observed in stem villi, whereas there was a weak immunoreaction in endothelium of mature intermediate and terminal villi at term. On the other hand, CCM3 expression in vascular endothelium decreased from the terminal villi towards the stem villi, where there was very weak immunoreactivity. So, that it is possible to speculate that at different villous segments of the human placental villous tree, which were identified according to their fetal vessel types and the stromal architecture, these two proteins might compensate each other. On the other hand, it seems they also act separately in stem villi vascular endothelium where abundant CCM2 expression was observed.

Differentiation of multipotential mesenchymal cells, pre-endothelial cell proliferation and migration, together with cell–cell connections are the most important subsequent steps for a successful vasculogenesis (Hanahan, 1997; Demir et al., 2006). Thereafter, the angiogenic cell cords and presumptive vessel lumens lined by the vessel structure are transformed into vessel tubes and then into mature vasculature through angiogenic remodeling (Demir et al., 1989, 2004; Suri et al., 1998). The important finding of this study was the observed strong expression of CCM2 and CCM3 in the presumptive endothelial cells in the vasculogenic areas and in endothelial cells of growing vessels, which send out capillary sprouts for new vessel development. From this point of view, our study also suggests that CCM2 and CCM3 may participate in vasculogenesis and vascular growth. Our results showing the expression of CCM2 and CCM3 in human placental tissues in the first trimester are consistent with previous report by Guzeloglu-Kayisli et al. where they have shown the expression of CCM1 in the placental endothelial cells, suggesting a possible role during early angiogenesis (Guzeloglu-Kayisli et al., 2004), but also a weak CCM2 and CCM3 expression in the vascular endothelium of mature intermediate and terminal villi in term placenta supporting the idea that this process might continue to take place in the later stages of pregnancy as well.

In addition to endothelial cells, our results showed that CCM2 and CCM3 proteins were also localized in the angiogenic cell cords with or without primitive lumen, Hofbauer cells and embryonic hematopoietic cells. Furthermore, like many potent paracrine angiogenic factors (Koch et al., 1992; Millauer et al., 1993) CCM2 and CCM3 might act as paracrine factors. It has been shown that Hofbauer cells, the macrophages of the placenta, express VEGF (Khan et al., 2000; Demir et al., 2004). Furthermore it was proposed that these VEGF-expressing Hofbauer cells interact with primitive vascular structures and therefore might play crucial roles in a paracrine matter in both vasculogenesis and angiogenesis in the human placenta (Demir and Erbengi, 1984; Demir et al., 2004; Seval et al., 2007). Consistently, in our study we have found that Hofbauer

cells express both CCM2 and CCM3, and thus, might have an influence on placental angiogenesis.

During early stages of placentation it is well know that the human placental villous tree is composed of two trophoblastic layers and a central extra-embryonic mesoderm. The relation between the mesenchymal core and the trophoblastic layers leads to the suggestion that they may influence each other's functions in a paracrine manner (Demir et al., 1995; Cervar et al., 1999). CCM2 and CCM3 were both localized in trophoblast cells. So, these two proteins might play an important role in coordination of trophoblast differentiation and migration, in addition to regulating trophoblast invasion.

In conclusion, our results showed that recently identified new critical genes, CCM2 and CCM3, are involved in vascular morphogenesis, and studies in the molecular pathways underlying vasculogenesis and angiogenesis will lead to a better understanding of several associated obstetrical complications. Further studies should aim to find other molecules interacting with CCM2 and CCM3, and to identify which signaling pathways are affected by these proteins. Moreover, these studies will definitely be useful to elucidate the cellular processes involved in pathogenesis of CCM in the central nervous system.

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