

# **Protocadherin 12 (VE-cadherin 2) is expressed in endothelial, trophoblast and mesangial cells**

Christine Rampon<sup>1</sup>, Marie-Hélène Prandini<sup>1</sup>, Stéphanie Bouillot<sup>1</sup>, Hervé Pointu<sup>2</sup>, Emmanuelle Tillet<sup>1</sup>,  
Ronald Frank<sup>3</sup>, Muriel Vernet<sup>2</sup> and Philippe Huber<sup>1</sup>.

<sup>1</sup>Laboratoire Développement et Vieillessement de l'Endothélium CEA-Inserm EMI-0219 and <sup>2</sup>Atelier de Transgène, Département de Réponse et Dynamique Cellulaires, CEA-Grenoble, 17 rue des Martyrs 38054 Grenoble, France.

<sup>3</sup>Department of Chemical Biology, German Research Centre for Biotechnology, D-38124 Braunschweig, Germany.

Author for correspondence: Philippe Huber, DRDC-DVE, 17 rue des Martyrs CEA-Grenoble 38 054 Grenoble. Tel: 33-438 78 41 18, Fax: 33-438 78 49 64, e-mail: phuber@cea.fr.

Short title: Protocadherin 12 tissue expression

## **ABSTRACT**

Protocadherin 12 protein (PCDH12, VE-cadherin 2) is a cell adhesion molecule which has been isolated from endothelial cells. Here, we have used Northern and Western blots, immunohistology and flow cytometry to examine the distribution of PCDH12 in mouse tissue. It is an N-glycosylated protein of 150 kDa mass. In the endothelium, PCDH12 immunoreactivity was variable and dependent upon the vascular bed. In both the embryo and embryonic stem cell differentiation system, signals were localized in vasculogenic rather than angiogenic endothelium. In addition, the protein was strongly expressed in a subset of invasive cells of the placenta, which were identified as glycogen-rich trophoblasts. In adult mice, strong PCDH12 signals were observed in mesangial cells of kidney glomeruli whereas expression was not detected in other types of perivascular cells. As opposed to most protocadherins, PCDH12 is not expressed in early embryonic (day 12.5) and adult brains. As a first approach to obtain insight into PCDH12 function, we produced transgenic mice deficient in PCDH12, which were viable and fertile. They did not display any obvious histomorphological defects. We conclude that PCDH12 has a unique expression pattern and that its deficiency does not lead to conspicuous abnormalities. Moreover, PCDH12 is the first specific marker for both glycogen-rich trophoblasts and mesangial cells.

Keywords: cadherin, endothelium, mouse, placenta, trophoblast cells, glomeruli, mesangial cells, knockout

## INTRODUCTION

Cadherins constitute a superfamily of transmembrane glycoproteins mediating calcium-dependent cell-cell adhesion [1]. These molecules are characterized by a variable number of cadherin ectodomains located in the extracellular part, a single transmembrane segment and a cytoplasmic domain. The cadherin superfamily includes several groups: the classical cadherins, such as E-, N- or VE-cadherin, the protocadherins, the desmocollins, the desmogleins, and other related proteins including Fat, T-cadherin and Ret [2]. Classical cadherins have been extensively studied. These molecules are involved in cell cohesion, tissue morphogenesis and cell behavior [3-6]. The cytoplasmic domain of classical cadherins interacts with catenins, allowing association with the cytoskeleton and cadherin/cytoskeleton association is required for strong cell-cell interaction.

Although recently discovered, protocadherins constitute the largest group of the superfamily [7, 8]. They were first identified a decade ago by a PCR strategy using primers amplifying the cadherin ectodomain [9]. Full length cDNA sequencing revealed that their cytoplasmic domains had no significant homology between each other and with those of classical cadherins. Hence, protocadherins do not possess catenin-binding sequences, and for those that have been tested, are not or only weakly associated with the cytoskeleton [8]. Recently, 52 novel protocadherins have been identified [10]. They are organized in three large clusters,  $\alpha$ ,  $\beta$ , and  $\gamma$ , on mouse chromosome 18. The vast majority of protocadherins, including those from the three clusters, are mostly or exclusively expressed in the central nervous system [9, 11, 12]. Their specific distribution in distinct neuronal populations may reflect their function in the establishment of neuronal circuits [8]. In contrast, protocadherin LKC, 12, 18 and  $\mu$  were not detected in the central nervous system, but in various visceral organs [13-18]. In general, protocadherins display weak homotypic adhesion properties [9]. It is thus likely that these molecules are not involved in tissue cohesion. Additionally, heterophilic interactions have been suggested for some of them, for example with matrix proteins, suggesting functions independent of cell-cell connection [7, 17, 19, 20]. Little is known about the *in vivo* biological activities of

protocadherins. The first cues on protocadherin properties suggest diverse roles in tissue organization, cell survival and proliferation [7, 8].

In this paper, we focused on vascular endothelial-cadherin 2, also called PCDH12 in the systematic protocadherin nomenclature [14, 15]. This molecule was first identified by RT-PCR in endothelioma cells using degenerated primers amplifying the cadherin ectodomain [14]. By Northern blot, PCDH12 was specifically detected in endothelioma cell lines and in highly vascularized organs. In transfected Chinese hamster ovary cells, PCDH12 was localized at intercellular junctions where it promoted calcium-dependent homophilic adhesion. PCDH12 did not co-immunoprecipitate with catenins, and was only weakly associated with the cytoskeleton. Furthermore, junctional localization of PCDH12 did not modify the paracellular permeability of a confluent monolayer, suggesting that this molecule is not involved in the control of endothelial permeability. Moreover, in contrast to VE-cadherin, PCDH12 did not modify cell migration and density-dependent cell growth.

In this paper, we showed that PCDH12 expression in the endothelium was variable and dependent upon the vascularization process or the vascular bed. Surprisingly, we noticed high expression levels in glycogen-rich trophoblasts and glomerular mesangial cells. Additionally, we produced PCDH12-deficient mice, which were viable, fertile and did not harbor obvious morphological defects.

## MATERIALS AND METHODS

### *RNA extraction and analysis*

RNA were extracted from cells or embryoid bodies with the Tri Reagent from Molecular Research Center. For RT-PCR analysis, RNA was reverse transcribed with the Superscript II from Invitrogen. PCR amplification was performed with PCDH12 specific oligonucleotides: 5'-GGCCCCAGAAACACATTCAGA-3' and 5'-TCTGTGGGGCCTCCTCCTTGT-3'. CD31, VE-cadherin and HPRT primers were previously described [21, 22]. Amplification products were visualized either by ethidium bromide staining (Fig. 6B) or after transfer onto nylon membrane (Oncor) and hybridization with internal oligonucleotides (Fig. 7B).

For Northern blot analysis, a Mouse Adult Tissue Blot containing 20 µg of RNA of various tissues was purchased from Seegene and successively hybridized with two <sup>32</sup>P-labeled probes: a PCR fragment corresponding to nucleotides 2,589-3,000 of mouse PCDH12 cDNA [14] and a 1.7 kb Eco RI-Sph I fragment from mouse VE-cadherin cDNA [23].

### *Gene targeting, ES cell manipulation and mouse breeding*

A PCDH12 genomic clone was isolated from a λ phage 129/Sv mouse genomic library (Stratagene). For the targeting vector, a 2 kb Sac I – Xmn I fragment located upstream of the initiation ATG was inserted upstream of a promoterless LacZ gene followed by a phosphoglycerate kinase-neomycine resistance gene. A 2.9 kb Xmn I – Nco I genomic fragment, located downstream of the initiation codon, was inserted in the targeting vector, downstream of the neomycine resistance unit. AT1 ES cells [24] were electroporated with the linearized vector using established procedures [22]. Clones were selected with G418 (250 µg/ml) and ganciclovir (2 µM). Resistant clones were screened by Southern blot analysis. DNA were digested with Hind III, and hybridized with a probe external to the recombination fragment (probe A, Fig. 2). Homologous recombination events were confirmed by digestion with Eco RI and hybridization with probe B (Fig. 2). Five clones out of 336 screened were

targeted at the *PCDH12* locus. Mouse chimeras derived from two clones with correct recombination event were crossed with CD1 mice (Charles River). Heterozygous mice were intercrossed to give homozygous mice. Animals from independent ES clones were not mated together.

### ***Wild type animals and tissue preparation***

All protocols in this study were conducted in strict accordance with the “*Ministère de l'Éducation Nationale, de la Recherche et de la Technologie*” Guidelines for the Care and Use of Laboratory Animals. Experiments were performed with tissues from CD1 mice (Charles River). The animals were allowed to acclimatize for a period of 1 week before experimental manipulation. For embryo and placenta production, mice were mated and the day on which a vaginal plug was found was designated 0.5. Pregnant females were killed by cervical dislocation and conceptuses were dissected in PBS. Isolation of glomeruli from kidneys was done by passing tissues through a series of steel sieves exactly as described [25]. For lung endothelial cell isolation, cells were dissociated and labeled with an endothelial-specific antibody directed against CD31 [26]. Briefly, tissues were minced with a scalpel and incubated with a solution of 0.5% collagenase-H (Roche) in a solution of Dulbecco's modified Eagle's medium supplemented with 20% fetal calf serum (both from Invitrogen) and 0.1% Dnase I (Sigma) at 37°C for 90 min. The cell suspension was filtered on a 40- $\mu$ m sieve to remove aggregates. Red cells were lysed in a solution of 0.14 M  $\text{NH}_4\text{Cl}$ , 17 mM Tris-HCl pH 7.6 for 2 min at room temperature. Cells were washed in PBS, labeled with anti-CD31 antibody and the CD31<sup>+</sup> population was isolated using MiniMACS separation columns (Miltenyi Biotec). The CD31<sup>-</sup> population was collected after two successive filtration on independent columns. RNA were prepared as described above.

### ***Cell culture and in vitro differentiation of ES cells***

H5V cells were grown as previously described [27]. ES cells were allowed to differentiate by LIF cytokine removal. ES cell differentiation was performed in semi-solid medium containing 50 ng/ml

VEGF (Peprotech) and 100 ng/ml bFGF (generous gift from Dr Andrew Baird, La Jolla, CA), as previously described [28]. Resulting embryoid bodies were harvested at indicated days. To analyze the angiogenic sprouts, 11 day-old-embryoid bodies were placed in type I-collagen with above cytokines and further cultured for 3 days. In-gel immunohistology was performed as described [28].

### ***Antibodies***

A rabbit antiserum was raised against a recombinant fragment spanning amino-acids 94 to 354 of the extracellular domain of PCDH12, as previously described [14]. The antiserum was affinity purified against peptides coupled to sepharose 4B. The bound antibodies were eluted using 10 volumes of 0.1 M glycine pH 2. Fractions were neutralized with 1 volume of Tris HCl 1M pH 8 and dialyzed against PBS. Before use, anti-PCDH12 antibodies were incubated for 1 h with an acetone extract of NIH 3T3 cells to reduce non-specific staining, as previously described [29]. Anti-PCDH12 antibodies were used at a concentration of 0.1 µg/ml for Western blot analysis and 5 µg/ml for immunohistology.

Rat anti-mouse CD31 (MEC13) was a gift from Dr A. Vecchi [26] and was used as undiluted hybridoma supernatant. Rabbit anti-human podocin (P35) was a gift from Dr C. Antignac [30] and was used at 1:1,000 dilution. Rabbit anti-mouse PDGF R $\beta$  was a gift from Dr W.B. Stallcup [31] and was used at a concentration of 2 µg/ml.

As secondary antibodies, the cyanine 3-conjugated antibodies, IgG or Fab fragments were from Jackson ImmunoResearch Laboratories and the alexa 488-conjugated antibodies were from Molecular Probes; all were used at recommended dilutions. The peroxidase-conjugated anti-rabbit antibody was from Biorad and used at 1:200 dilution.

### ***SPOT analysis***

Epitope mapping of the PCDH12 extracellular domain was performed as previously described [32]. The antigen sequence was divided into 15-mer amino acid sequences; each peptide overlapping with the next one by 12 amino acids. The overlapping peptides were synthesized spotwise as an array (spot

diameter of 2mm, distance of 4 mm, 25 spots a row) on a specially manufactured cellulose membrane (AC01; AIMS Scientific Products, GmbH, Braunschweig, Germany) as described by Frank and Overwin, 1996 [33] with an ABIMED ASP 222 automated SPOT robot. The peptides are linked to the membrane via a polyethylene glycol spacer through their carboxy terminal amino acid and are N-terminally acetylated. The peptide membrane was solvated in an ethanol bath, washed three times (10 min each) with 20 mM Tris-HCl pH 8, 140 mM NaCl, 0.1% Tween 20 (TBST), saturated with 10% fetal calf serum (Invitrogen) in TBST overnight at 4°C and incubated 1 h at room temperature with antiserum (dilution 1:5,000) in TBST. After three washes (10 min each) in PBST, the membrane was incubated 1 h with peroxidase-conjugated anti-rabbit antibody, washed as above, and revealed with the Chemiluminescent ECL kit (Perkin Elmer).

Peptides corresponding to each epitope were synthesized by Eurogentec and were coupled to CNBr-activated Sepharose beads (Amersham).

### ***Immunoblot analysis and N-glycosidase treatment***

Protein extracts were obtained by homogenization with a Polytron in a solution containing 20 mM Tris-HCl pH 7.2, 1 mM EDTA, 1 mM EGTA, 1mM Na<sub>3</sub>VO<sub>4</sub>, 10 mM β-glycerophosphate, 5 mM NaF, 1 mM dithiotreitol, 0.27 M sucrose, 4 μg/ml leupeptin, 1 % Triton, 0.5% NP40 and the antiprotease cocktail from Roche. Protein concentration was determined with a Micro-BCA kit (Pierce), using BSA as standard. Proteins were electrophoresed in denaturing polyacrylamide (6%) gels (SDS-PAGE) in reducing conditions, transferred onto Hybond ECL membrane (Amersham) and incubated with antibodies as described above. The molecular mass standard was from Biorad.

Five hundred μg of placenta and lung extracts were incubated with 10 units N-glycosidase F (Roche) overnight at 37°C in recommended condition assay.

### ***Histology***



For immunolocalization, tissues were snap-frozen in OCT compound and sectioned at 10- $\mu$ m in a cryomicrotome (Leica). Sections were permeabilized in paraformaldehyde 4%, Triton 0.5%-PBS for 3 min, fixed in 4% paraformaldehyde-PBS for 20 min, saturated with 2% BSA/PBS and incubated with primary and secondary antibodies, all at room temperature, using standard procedures. All experiments, except those with embryoid bodies, were performed in parallel on tissues from wild type and PCDH12<sup>-/-</sup> mice, as negative control. For co-immunolabeling with two antibodies from the same species (e.g., PCDH12/podocin and PCDH12/PDGF R $\beta$ ), Fab secondary antibodies were used, as previously described [34]. Briefly, slides were sequentially incubated with antibodies as follows: 1/ the anti-podocin or anti-PDGF R $\beta$  antibodies, 2/ the cyanine 3-conjugated anti-rabbit IgG Fab fragment, 3/ the anti-PCDH12 antibody and 4/ the alexa 488-conjugated anti-rabbit IgG antibody. For negative controls, either one or the other of the primary antibodies was omitted and no cross-reactivity was observed. Sections were incubated for 3 min in Hoechst 33258 (Sigma) for nuclear staining. Fluorescent slides were mounted in Fluorsave reagent (Calbiochem).

For immunoperoxidase labeling, sections were incubated after secondary antibody with diaminobenzidine (Dako), followed by nuclear staining with Harris hematoxylin (Sigma). Slides were mounted in Entellan (Merck).

For hematoxylin/eosin or periodic acid-Schiff (all from Sigma) staining, paraffin sections were prepared using standard procedures, from paraformaldehyde prefixed tissues. Slides were mounted in Entellan or Aquatex (Merck). Complete autopsies and histopathological analyses of mice were performed by the Institut Clinique de la Souris (Strasbourg).

Slides were observed under a fluorescent microscope (Axioplan, Zeiss) and pictures were made with a digital camera (Spot 2, Diagnostic Instruments). Alternatively, slides were observed with a confocal fluorescence microscope (Leica TCS SP2) and images were acquired for cyanine 3 (excitation 543 nm, collection 560-650 nm).

## RESULTS

### *PCDH12 mRNA expression profile in mouse tissues*

*PCDH12* transcripts were previously shown to be present in endothelioma cell lines and some highly vascularized organs, namely lung, heart and kidney [14, 15], suggesting that *PCDH12* could be expressed exclusively or primarily in endothelial cells. We first extended this study by Northern blot to other organs and compared *PCDH12* expression pattern to that of *VE-cadherin*, an endothelial specific marker (Fig. 1). Expression profiles were strikingly similar between *PCDH12* and *VE-cadherin*, reinforcing the previous hypothesis. The strongest *PCDH12* signals were in placenta, lung and heart. *PCDH12* mRNA size was calculated to be 6 kb, as opposed to 7 kb in the previous study, with no alternative transcript.

### *Production of PCDH12-deficient mice*

To gain insights into *PCDH12* biological activity in vivo, we generated *PCDH12*-deficient mice. These mice were produced using standard ES cell technology using positive-negative selection. Briefly, a 12 kb clone encompassing *PCDH12* gene 5'-end was isolated from a mouse genomic DNA library. The homologous recombination ("knockout strategy") was designed to remove 1.85 kb of *PCDH12* gene, including the initiation codon and 1,689 bp of N-terminal coding sequence (563 amino-acids) (Fig. 2A). A promoterless *LacZ* gene was inserted instead of this region ("knockin strategy"), however, this construct failed to produce any *LacZ* expression in the transgenic mice, probably because of a mislocalization of the reporter gene downstream of the promoter region. Alternatively, important regulatory regions may have been removed after recombination.

Homologous recombination events in ES clones were screened by Southern blot analysis using probes external to the recombination fragments (Fig. 2A, B). Analysis of clones on both 5'- and 3'-sides revealed that 1.5% of them were targeted at the *PCDH12* locus. Two independent clones were used to derive *PCDH12*-deficient mice which displayed identical characteristics. Homozygous mice were

viable and fertile, and appeared in a Mendelian ratio from heterozygote intercrosses (Table 1). Complete autopsies and histopathological analyses of adult (8-10 week old) homozygous animals (n = 4) and wild types (n = 2) were performed. Data did not reveal any morphological changes compared to wild types (not shown), indicating that PCDH12 deficiency did not lead to gross abnormalities in adult mice. A detailed phenotypic analysis of PCDH12-deficient mice will be described elsewhere. In this paper, these mice were used as negative control for analysis of PCDH12 tissue distribution.

### ***Characterization of PCDH12 in tissue extracts***

A rabbit antiserum was previously raised against an extracellular portion of PCDH12 (amino-acids 94-354) [14]. Although purified by affinity chromatography against the recombinant antigen, the antibody generated a high background in *in vivo* studies (not shown). To increase the specificity of this antibody, we purified the antiserum against peptides spanning the antibody epitopes. The recognized epitopes were mapped using peptide array analysis (SPOT analysis) (Fig. 3A). Four peptide clusters reacted with the antibody on SPOT membranes (peptides P1-P4). The pre-immune serum was not immunoreactive with these peptides (not shown). A library search showed that these peptides were highly specific to PCDH12. Epitope-purified antibodies were tested by Western blot using protein extracts from H5V cells, an endothelioma cell line, as well as placenta, for which the highest *PCDH12* mRNA levels were found. *PCDH12*<sup>-/-</sup> placenta extracts were used to control antibody specificity. P1 and P2-purified antibodies independently reacted with a 150 kDa protein in wild type but not in *PCDH12*-deficient extracts, demonstrating that this band corresponded to PCDH12 (Fig. 3B and not shown). P3- and P4-purified antibodies yielded weak signals and were discarded. PCDH12 could also be detected in uterus, ovary, spleen and total embryo at E12.5 (Fig. 4A). The PCDH12 protein sequence contains three putative N-glycosylation sites [14]. Extracts were treated with N-glycosidase and the molecular mass shifted to 130 kDa (Fig. 4B), corresponding to the theoretical molecular mass deduced from the amino-acid sequence. Interestingly, PCDH12 migration was consistently slightly reduced in H5V and placenta extracts compared to other organs (Fig. 4A, B), however, differences

were abolished after deglycosylation. Therefore, these features indicate that PCDH12 is a N-glycosylated protein but suggest that the N-glycosylation content is distinct in H5V cells and placenta compared to other tissues.

### ***PCDH12 expression in the endothelium***

P1 and P2-purified anti-PCDH12 antibodies were used for immunohistological localization of embryo and adult tissues. Immunoreactivity was tested in parallel on PCDH12-deficient tissue sections. P1 antibody yielded specific signals whereas P2 antibody also reacted non-specifically with cell nuclei (not shown). Therefore, P2 antibody was only used to confirm immunodetection with P1 antibody. In the embryo (E12.5), PCDH12 was detected in most visceral vessels, namely in the periphery of the duodenum (Fig. 5A-C), the liver (not shown) and the somites (Fig. 5D-F), as well as in the lung (Fig. 5G-I), as determined by colocalization with CD31, an endothelial-specific marker. PCDH12 was not detected in limb buds or in the brain (not shown), suggesting that PCDH12 was present in vasculogenic rather than angiogenic vessels. At E7.5, PCDH12 was detected in peripheric vessels of the deciduum (Fig. 5J-L), whereas expression was undetectable later on (not shown). At higher magnification (Fig. 5M-O), staining was not exclusively localized at cell junctions but rather diffuse at the cell surface, when compared to CD31 labeling (arrowheads).

To further examine expression in the two types of vessel formation, we used the embryonic stem (ES) cell differentiation system, which successively recapitulates the vasculogenesis and angiogenesis steps of vascular development. In the first step, embryoid body (EB) formation is accompanied by differentiation in the endothelial lineage. Endothelial cells eventually assemble into a plexus evoking the first events of vascular development in the visceral layer of the embryo. In a second step of culture, EB are embedded in collagen and angiogenic sprouts (AS) develop from the bodies into the gel [28, 35]. As illustrated in Fig. 6A, immunolabeling experiments showed a consistent staining in CD31+ cells of EB, whereas expression was absent or undetectable in angiogenic sprouts.

PCDH12 mRNA appearance during EB formation was followed by RT-PCR. PCDH12 was first detected at day 4, similar to VE-cadherin (Fig. 6B). These data indicate that the onset of both molecules is concomitant in the endothelial differentiation program.

In the adult, PCDH12 endothelial expression was diffuse, not associated to junctions and barely detectable by immunohistology. The highest expression levels were localized in the innermost vessels of the ovarian follicles, in the endometrium, the spleen and the choroid vessels of the eye (Fig. 7A and not shown). However, PCDH12 staining could not be detected in organs like the lung although high expression levels were detected in Western and Northern blots. To know in which cells of the lung PCDH12 was present, we dissociated lungs by collagenase treatment, performed a CD31 immunolabeling and sorted the cells into CD31<sup>+</sup> and CD31<sup>-</sup> populations, using a microbead technology. PCDH12 mRNA were only detected by RT-PCR in CD31<sup>+</sup> cells (Fig. 7B), indicating that PCDH12 was located in the endothelium and not other cell types.

### ***PCDH12 is a marker of glycogen-rich trophoblast cells***

From midgestation, the mouse placenta is composed of different layers (for review, see [36-38]), namely the chorionic plate, the labyrinth, the spongiotrophoblast layer (also called junctional zone) (Fig. 8A) and a discrete line of trophoblast giant cells (not visible at this magnification). The placenta is tightly associated with the maternal deciduum and the metrial triangle (Fig. 8A). These different layers show distinct vascular pattern, as illustrated by CD31 labeling (Fig. 8B); the spongiotrophoblast layer being almost devoid of vessels. PCDH12 immunohistology revealed a strong staining in a subset of cells in the spongiotrophoblast and the decidual layers in placentas of wild types (Fig. 8C) but not in PCDH12<sup>-/-</sup> mice (Fig. 8D). PCDH12<sup>+</sup> cells formed tightly associated islets in the spongiotrophoblast layer and the proximal deciduum (arrows), whereas they formed cords in the distal deciduum (arrowheads). In this tissue, PCDH12 was clearly localized at the cell junctions, as demonstrated by visualization in the XZ-axis in confocal microscopy (Fig. 8E). Immunoperoxidase detection of PCDH12 associated with hematoxylin counterstaining of placenta sections showed that the expressing

cells were highly vacuolated (Fig. 8F), as previously described for the glycogen-rich trophoblast cells [37, 38]. Glycogen labeling of placenta sections with the periodic acid-Schiff reaction confirmed that the vacuolated PCDH12<sup>+</sup> cells were morphologically identical to the glycogen-rich trophoblast cells (Fig. 8G).

### ***PCDH12 is a component of kidney glomeruli***

A strong PCDH12 staining was also present in adult kidney glomeruli (Fig. 9A). Control experiment with PCDH12<sup>-/-</sup> kidney sections did not show immunostaining (Fig. 9A). Three cell types are located in the glomeruli: endothelial cells, podocytes and mesangial cells that can be specifically identified by CD31, podocin and PDGF R $\beta$  labeling, respectively [26, 30, 39]. PCDH12 was only co-distributed with PDGF R $\beta$  (Fig. 9A), demonstrating that mesangial cells expressed PCDH12. Western blot analysis confirmed that PCDH12 was present in glomeruli, although undetectable in total kidney extracts (Fig. 9B). Interestingly, PCDH12 could not be detected in other types of perivascular cells (not shown).

## DISCUSSION

Approximately 80 protocadherins have been identified in the human genome and their genomic organization has been well characterized [8, 10]. However, general understanding of protocadherin function remains elusive and very few publications address the question of their biological activity. As a first approach to gain insights into PCDH12 *in vivo* activity, we studied its tissue distribution and produced PCDH12-deficient mice.

In this study, we generated an anti-PCDH12 antibody usable for *in vivo* investigation. As previously suspected, PCDH12 is expressed in endothelial cells. In the embryo, PCDH12 could be detected by immunohistology in the endothelium of visceral organs and somites. The labeling was diffuse and not particularly concentrated at the cell junctions, suggesting that PCDH12 was distributed on the entire cell surface. This finding contrasts with previous data on H5V cells and PCDH12-transfected CHO cells where PCDH12 was localized at intercellular junctions [14]. Interestingly, PCDH12 junctional localization in H5V and placenta is correlated with a differential glycosylation pattern. This feature is consistent with a role of the carbohydrate moiety in homotypic adhesion.

Several features suggest that PCDH12 expression is related to vasculogenesis rather than angiogenesis. First, staining could not be detected in brain or parietal vessels, which are produced by angiogenesis, as opposed to vasculogenesis which only occurs in visceral and somitic vessels [40, 41]. Second, this differential expression was confirmed in the ES-differentiation system, where PCDH12 was co-expressed with CD31 in intra-embryoid endothelial plexus and not in angiogenic sprouts. One possible explanation for this feature is that PCDH12 transcription is activated at the angioblast stage and is downregulated in mature endothelial cells. Therefore, PCDH12 may be used as a marker to distinguish vasculogenic and angiogenic vessels.

Except in some specific vessels (in the ovary, spleen, endometrium and eye choroid), PCDH12 could not be detected by immunohistology in adult vasculature although its presence was demonstrated by Northern and Western blots in a number of highly vascularized organs. The non-junctional localization

of PCDH12 likely explains this discrepancy. Similarly, N-cadherin, which is not concentrated at endothelial junctions, is readily detectable in endothelial cell extracts but is hardly visible in fixed cells or tissues [42] and our own observations], with some exceptions [43]. Therefore, PCDH12 may be only visible by immunohistology in cells strongly producing this molecule.

In contrast, glycogen-rich trophoblast cells harbored strong PCDH12 signals at their cell-cell junctions. Other trophoblast cell types of the placenta were not immunoreactive. As far as we know, PCDH12 is the first identified protein marker of this lineage. These cells invade the interstitium of the decidual layer where they remain independent from the maternal vascular system [37, 38]. Although the function of these cells is unknown, they seem to be specialized in glycogen synthesis and storage. They are also known to secrete IGF2, a protein hormone important for foeto-maternal interaction, and calcyclin, an inducer of placental lactogen hormone secretion by trophoblasts [44-46]. Therefore, PCDH12 may be involved in decidual invasion, glycogen metabolism or hormonal secretion. Further work is needed to evaluate these functions in PCDH12-deficient placentas.

Strong PCDH12 signals were localized in kidney glomeruli. Surprisingly, PCDH12 was codistributed with PDGF R $\beta$  and not with CD31, indicating that PCDH12 was located in mesangial instead of endothelial cells. The mesangial cells represent a specialized subtype of pericytes, which are required for glomerular capillary loop assembly and regulation of intraglomerular blood flow. PDGF R $\beta$ , as well as smooth muscle actin, are commonly used to mark pericytes in general and mesangial cells in particular. To our knowledge, PCDH12 is the first specific marker of mesangial cells. In addition, PCDH12 is the first member of the cadherin superfamily identified in this cell type.

The link between the three PCDH12-expressing cell types, namely endothelial, glycogen-rich trophoblast and mesangial cells, is not obvious. However in humans, endothelial, cytotrophoblast (a type of invasive trophoblast) and mesangial cells participate in pre-eclampsia pathogenesis, a common complication of pregnancy in humans, characterized by hypertension, glomerular lesions and defective trophoblast invasion [47]. Although pre-eclampsia has never been described in mice, PCDH12



expression pattern is intriguing in respect of the still unestablished connection between placental and renal dysfunctions in this pathology.

PCDH12-deficient mice were viable and close histological examination did not reveal morphological defects, suggesting that PCDH12 is not involved in tissue morphogenesis. Functional redundancy between members of the protocadherin family has been evoked for PCDH8 knockout [48]; however, there is no evidence of other protocadherins in endothelial cells, as previously shown by PCR amplification of cadherin domains using degenerated primers [14]. Defects induced by PCDH12 deficiency may be more subtle and function of organs like kidney and placenta should be carefully evaluated in physiological and pathological situations.

## **ACKNOWLEDGEMENTS**

We are grateful to Stéphane Saddier for library screening. We thank Susanne Daenicke for expert technical assistance in peptide array synthesis. This study was supported by the Ligue contre le Cancer and the Association de Recherche contre le Cancer.

## REFERENCES

- [1] B.M. Gumbiner, Regulation of cadherin adhesive activity, *J Cell Biol* 148 (2000) 399-404.
- [2] B.D. Angst, C. Marcozzi, A.I. Magee, The cadherin superfamily: diversity in form and function, *J Cell Sci* 114 (2001) 629-41.
- [3] B. Ranscht, Cadherins and catenins: interactions and functions in embryonic development, *Curr Opin Cell Biol* 6 (1994) 740-6.
- [4] L. Shapiro, D.R. Colman, The diversity of cadherins and implications for a synaptic adhesive code in the CNS, *Neuron* 23 (1999) 427-30.
- [5] C. Redies, Cadherins in the central nervous system, *Prog Neurobiol* 61 (2000) 611-48.
- [6] T. Yagi, M. Takeichi, Cadherin superfamily genes: functions, genomic organization, and neurologic diversity, *Genes Dev* 14 (2000) 1169-80.
- [7] S.T. Suzuki, Recent progress in protocadherin research, *Exp Cell Res* 261 (2000) 13-8.
- [8] M. Frank, R. Kemler, Protocadherins, *Curr Opin Cell Biol* 14 (2002) 557-62.
- [9] K. Sano, H. Tanihara, R.L. Heimark, S. Obata, M. Davidson, T. St John, S. Taketani, S. Suzuki, Protocadherins: a large family of cadherin-related molecules in central nervous system, *Embo J* 12 (1993) 2249-56.
- [10] Q. Wu, T. Maniatis, A striking organization of a large family of human neural cadherin-like cell adhesion genes, *Cell* 97 (1999) 779-90.
- [11] H. Sago, M. Kitagawa, S. Obata, N. Mori, S. Taketani, J.M. Rochelle, M.F. Seldin, M. Davidson, T. St John, S.T. Suzuki, Cloning, expression, and chromosomal localization of a novel cadherin-related protein, protocadherin-3, *Genomics* 29 (1995) 631-40.
- [12] K. Yoshida, K. Yoshitomo-Nakagawa, N. Seki, M. Sasaki, S. Sugano, Cloning, expression analysis, and chromosomal localization of BH-protocadherin (PCDH7), a novel member of the cadherin superfamily, *Genomics* 49 (1998) 458-61.

- [13] N. Okazaki, N. Takahashi, S. Kojima, Y. Masuho, H. Koga, Protocadherin LKC, a new candidate for a tumor suppressor of colon and liver cancers, its association with contact inhibition of cell proliferation, *Carcinogenesis* 23 (2002) 1139-48.
- [14] P. Telo, F. Breviario, P. Huber, C. Panzeri, E. Dejana, Identification of a novel cadherin (vascular endothelial cadherin-2) located at intercellular junctions in endothelial cells, *J Biol Chem* 273 (1998) 17565-72.
- [15] D. Ludwig, J. Lorenz, E. Dejana, P. Bohlen, D.J. Hicklin, L. Witte, B. Pytowski, cDNA cloning, chromosomal mapping, and expression analysis of human VE-Cadherin-2, *Mamm Genome* 11 (2000) 1030-3.
- [16] T. Wolverson, M. Lalande, Identification and characterization of three members of a novel subclass of protocadherins, *Genomics* 76 (2001) 66-72.
- [17] M. Goldberg, C. Peshkovsky, A. Shifteh, Q. Al-Awqati, mu-Protocadherin, a novel developmentally regulated protocadherin with mucin-like domains, *J Biol Chem* 275 (2000) 24622-9.
- [18] M. Goldberg, M. Wei, B. Tycko, I. Falikovich, D. Warburton, Identification and expression analysis of the human mu-protocadherin gene in fetal and adult kidneys, *Am J Physiol Renal Physiol* 283 (2002) F454-63.
- [19] K. Senzaki, M. Ogawa, T. Yagi, Proteins of the CNR family are multiple receptors for Reelin, *Cell* 99 (1999) 635-47.
- [20] G.R. Phillips, H. Tanaka, M. Frank, A. Elste, L. Fidler, D.L. Benson, D.R. Colman, Gamma-protocadherins are targeted to subsets of synapses and intracellular organelles in neurons, *J Neurosci* 23 (2003) 5096-104.
- [21] D. Vittet, M.H. Prandini, R. Berthier, A. Schweitzer, H. Martin-Sisteron, G. Uzan, E. Dejana, Embryonic stem cells differentiate in vitro to endothelial cells through successive maturation steps, *Blood* 88 (1996) 3424-31.

- [22] S. Gory-Fauré, M.H. Prandini, H. Pointu, V. Roullot, I. Pignot-Paintrand, M. Vernet, P. Huber, Role of vascular endothelial-cadherin in vascular morphogenesis, *Development* 126 (1999) 2093-2102.
- [23] G. Breier, F. Breviario, L. Caveda, R. Berthier, H. Schnurch, U. Gotsch, D. Vestweber, W. Risau, E. Dejana, Molecular cloning and expression of murine vascular endothelial- cadherin in early stage development of cardiovascular system, *Blood* 87 (1996) 630-41.
- [24] T. Buchou, M. Vernet, O. Blond, H.H. Jensen, H. Pointu, B.B. Olsen, C. Cochet, O.G. Issinger, B. Boldyreff, Disruption of the regulatory beta subunit of protein kinase CK2 in mice leads to a cell-autonomous defect and early embryonic lethality, *Mol Cell Biol* 23 (2003) 908-15.
- [25] P. Mundel, P. Gilbert, W. Kriz, Podocytes in glomerulus of rat kidney express a characteristic 44 KD protein, *J Histochem Cytochem* 39 (1991) 1047-56.
- [26] A. Vecchi, C. Garlanda, M.G. Lampugnani, M. Resnati, C. Matteucci, A. Stoppacciaro, H. Schnurch, W. Risau, L. Ruco, A. Mantovani, et al., Monoclonal antibodies specific for endothelial cells of mouse blood vessels. Their application in the identification of adult and embryonic endothelium, *Eur J Cell Biol* 63 (1994) 247-54.
- [27] C. Garlanda, C. Parravicini, M. Sironi, M. De Rossi, R. Wainstok de Calmanovici, F. Carozzi, F. Bussolino, F. Colotta, A. Mantovani, A. Vecchi, Progressive growth in immunodeficient mice and host cell recruitment by mouse endothelial cells transformed by polyoma middle-sized T antigen: implications for the pathogenesis of opportunistic vascular tumors, *Proc Natl Acad Sci U S A* 91 (1994) 7291-5.
- [28] O. Feraud, M.-H. Prandini, D. Vittet, Vasculogenesis and angiogenesis from in vitro differentiation of mouse embryonic stem cells, *Methods in Enzymology* (2003).
- [29] M.A. Nieto, K. Patel, D.G. Wilkinson, In situ hybridization analysis of chick embryos in whole mount and tissue sections, *Methods Cell Biol* 51 (1996) 219-35.
- [30] S. Roselli, O. Gribouval, N. Boute, M. Sich, F. Benessy, T. Attie, M.C. Gubler, C. Antignac, Podocin localizes in the kidney to the slit diaphragm area, *Am J Pathol* 160 (2002) 131-9.

- [31] U. Ozerdem, K.A. Grako, K. Dahlin-Huppe, E. Monosov, W.B. Stallcup, NG2 proteoglycan is expressed exclusively by mural cells during vascular morphogenesis, *Dev Dyn* 222 (2001) 218-27.
- [32] R. Frank, Spot-synthesis: An easy technique for the positionally addressable, parallel chemical synthesis on a membrane support., *Tetrahedron* 48 (1992) 9217-9232.
- [33] R. Frank, H. Overwin, SPOT synthesis. Epitope analysis with arrays of synthetic peptides prepared on cellulose membranes, *Methods Mol Biol* 66 (1996) 149-69.
- [34] A. Negoescu, F. Labat-Moleur, P. Lorimier, L. Lamarcq, C. Guillermet, E. Chambaz, E. Brambilla, F(ab) secondary antibodies: a general method for double immunolabeling with primary antisera from the same species. Efficiency control by chemiluminescence, *J Histochem Cytochem* 42 (1994) 433-7.
- [35] O. Feraud, Y. Cao, D. Vittet, Embryonic Stem Cell-Derived Embryoid Bodies Development in Collagen Gels Recapitulates Sprouting Angiogenesis, *Lab Invest* 81 (2001) 1669-1681.
- [36] J. Rossant, J.C. Cross, Placental development: lessons from mouse mutants, *Nat Rev Genet* 2 (2001) 538-48.
- [37] P. Georgiades, A.C. Ferguson-Smith, G.J. Burton, Comparative developmental anatomy of the murine and human definitive placentae, *Placenta* 23 (2002) 3-19.
- [38] J.C. Cross, D. Baczyk, N. Dobric, M. Hemberger, M. Hughes, D.G. Simmons, H. Yamamoto, J.C. Kingdom, Genes, development and evolution of the placenta, *Placenta* 24 (2003) 123-30.
- [39] P. Soriano, Abnormal kidney development and hematological disorders in PDGF beta-receptor mutant mice, *Genes Dev* 8 (1994) 1888-96.
- [40] W. Risau, I. Flamme, Vasculogenesis, *Annu Rev Cell Dev Biol* 11 (1995) 73-91.
- [41] W. Risau, Mechanisms of angiogenesis, *Nature* 386 (1997) 671-4.
- [42] D. Salomon, O. Ayalon, R. Patel-King, R.O. Hynes, B. Geiger, Extrajunctional distribution of N-cadherin in cultured human endothelial cells, *J Cell Sci* 102 (1992) 7-17.

- [43] H. Gerhardt, S. Liebner, C. Redies, H. Wolburg, N-cadherin expression in endothelial cells during early angiogenesis in the eye and brain of the chicken: relation to blood-retina and blood-brain barrier development, *Eur J Neurosci* 11 (1999) 1191-201.
- [44] R.W. Redline, C.L. Chernicky, H.Q. Tan, J. Ilan, Differential expression of insulin-like growth factor-II in specific regions of the late (post day 9.5) murine placenta, *Mol Reprod Dev* 36 (1993) 121-9.
- [45] P. Georgiades, M. Watkins, G.J. Burton, A.C. Ferguson-Smith, Roles for genomic imprinting and the zygotic genome in placental development, *Proc Natl Acad Sci U S A* 98 (2001) 4522-7.
- [46] R.L. Farnsworth, F. Talamantes, Calcyclin in the mouse decidua: expression and effects on placental lactogen secretion, *Biol Reprod* 59 (1998) 546-52.
- [47] P.K. Lala, C. Chakraborty, Factors regulating trophoblast migration and invasiveness: possible derangements contributing to pre-eclampsia and fetal injury, *Placenta* 24 (2003) 575-87.
- [48] A. Yamamoto, C. Kemp, D. Bachiller, D. Geissert, E.M. De Robertis, Mouse paraxial protocadherin is expressed in trunk mesoderm and is not essential for mouse development, *Genesis* 27 (2000) 49-57.

## FIGURE LEGENDS

### Figure 1: Northern blot showing tissue distribution of PCDH12.

Twenty  $\mu\text{g}$  of total RNA from indicated tissues (8 week-old female and E17.5 placenta) was electrophoresed under denaturing conditions and hybridized with a *PCDH12* specific probe (top). The same blot was rehybridized with a *VE-cadherin* probe (middle). The data showed similar expression patterns for *PCDH12* and *VE-cadherin*. Ethidium bromide stain of RNA showed equivalent loading in each lane (bottom).

### Figure 2: Disruption of the mouse PCDH12 locus by homologous recombination

(A) Gene-targeting strategy. Maps of the *PCDH12* wild type allele, targeting vector and the resulting *PCDH12*<sup>-</sup> allele after homologous recombination. The position of the initiation codon is indicated (ATG). A Sac I-Xmn I fragment (2 kb) was inserted upstream of a promoterless LacZ gene (NTR-LacZ) followed by a neomycine resistance transcription unit (pGKneo). A Xmn I-Nco I fragment (2.9 kb) was inserted downstream. A thymidine kinase transcription unit (TK) was located at the 3'-end for negative selection with ganciclovir. The restriction sites used for genotyping are indicated as well as the sizes of the generated fragments. Restriction sites are marked as follows: E, Eco RI; H, Hind III; N, Nco I; S, Sac I; X, Xmn I. (B) Identification of the ES *PCDH12*<sup>+/-</sup> clones by Southern blot. The correct targeting events were established by the appearance of a 3.2 kb band in Hind III digests with probe A, on the 5'-side, and by a 6 kb band in Eco RI digests with probe B, on the 3'-side.

### Figure 3: Immunopurification of anti-PCDH12 antibody and identification of PCDH12 in tissue extracts

(A) Peptide SPOT analysis of the epitopes recognized by polyclonal antibody directed against an extracellular portion of PCDH12 (R<sup>94</sup>-I<sup>354</sup>). The sequences of the four corresponding epitope peptides (P1-P4) are shown below. (B) Western blot analysis of 40  $\mu\text{g}$  of protein extracts from H5V cells and

placenta at E12.5, incubated with anti-PCDH12 antibody, purified by affinity chromatography with P1 peptide. Extracts from a *PCDH12*-deficient mouse were used as negative controls. A specific signal was detected at 150 kDa (arrowhead). Similar data were obtained with P2 purified antibody (not shown), whereas fractions purified with P3 and P4 resulted in poor signal levels (not shown).

**Figure 4: Immunoblot analysis of various tissue extracts**

(A) Protein extracts (40 µg) from adult (12-week-old) and embryonic (E12.5) tissues as well as embryoid bodies at day 5 (EB d5) were analyzed by SDS-PAGE and immunoblotted with P1 anti-PCDH12 antibody. Forty µg of protein extracts were used in each lane. H5V extracts were used as control. Migration of PCDH12 from H5V and placenta was consistently slightly reduced compared to lung or other organs (arrowheads). (B) N-glycosidase treatment of protein extracts shifted the molecular mass to 130 kDa. After deglycosylation, placenta and lung PCDH12 co-migrated at identical positions.

**Figure 5: Immunohistology showing endothelial expression of PCDH12 in embryo and deciduum**

Cryosections (10 µm) of E12.5 embryos (A-I) and E7.5 decidua (J-O) were incubated with P1 anti-PCDH12 (A, D, G, J, M) and anti-CD31 (B, E, H, K, N) antibodies. Merge images (C, F, I, L, O) showed a codistribution of both markers, indicating that PCDH12 is expressed in endothelial cells of duodenum (A-C), somites (D-F) and lung (G-I). In the deciduum at E7.5 (J-L), PCDH12 was only expressed in peripheric CD31+ cells. At higher magnification (M-O), one can see that PCDH12 immunoreactivity is not specifically localized at CD31-labeled cell junctions (arrowheads). Sections from *PCDH12*-deficient embryos incubated in parallel showed a similar punctuated pattern in the center of the duodenum (\*), indicating that this signal was unspecific (not shown).



**Figure 6: PCDH12 is codistributed with CD31 in ES-derived embryoid bodies**

(A) ES cells were allowed to differentiate into embryoid bodies in semi-solid medium supplemented with VEGF and bFGF for 11 days (EB). Immunolabeling of cryosections (10 µm) revealed that PCDH12 is expressed in most bodies and codistributed with CD31. Day 11 embryoid bodies were subsequently placed in type I collagen. In this secondary culture, angiogenic sprouts arise from embryoid bodies and develop in the gel. After 3 days, the gels were dried and incubated with PCDH12 and CD31 antibodies for whole-mount staining. In general, PCDH12 immunoreactivity was negative in the sprouts (AS). (B) RT-PCR analysis of PCDH12 expression during ES cell differentiation. RNA were prepared at day 3, 4, 5, 6 and 7 ; day 0 being the first day of differentiation. *PCDH12* mRNA was first detected at day 4, similar to VE-cadherin mRNA. *HPRT* amplification was used as positive control. cDNAs were amplified in the same PCR reaction.

**Figure 7: PCDH12 endothelial expression in adults**

(A) Immunolocalization of PCDH12 (left) and CD31 (middle) in cryosections. PCDH12 immunoreactivity was undetectable in most adult tissues (12 week-old). Specific signals were clearly visible in the innermost vessels of the ovarian follicles, in spleen vasculature and in choroid vessels in the eye. Nuclei staining (Hoechst) was performed in the eye to visualize the retina. (B) Cells from collagenase-treated lungs were labeled with an anti-CD31 antibody and sorted in CD31+ and CD31- populations using a micro-bead technology. RNA was prepared from these cells and RT-PCR was performed using *PCDH12*, *CD31* and *HPRT* primers. Amplification products were hybridized with specific internal probes. For PCDH12, a radioactive signal was detected only in the CD31+ cells, indicating that PCDH12 expression in lungs is restricted to endothelial cells. Abbreviations: ch, choroid; srl, cone and rod layer; f, follicle; ogl, outer granular layer; rpe, retinal pigment epithelium.

**Figure 8: Immunohistological localization of PCDH12 in E12.5 placenta**

(A) Hematoxylin/eosin staining of a E12.5 placenta associated with maternal deciduum showing the different layers. The white lines were added to indicate the limits between layers. Giant cells are located at the interface between spongiotrophoblast layer and deciduum (not shown). (B) CD31 immunolabeling (brown) of a placenta section. Nuclei were counterstained with hematoxylin. (C, D) PCDH12 (red) and CD31 (green) fluorescent immunolabeling of wild type (C) and *PCDH12*<sup>-/-</sup> (D) placenta sections. PCDH12<sup>+</sup> cells were located in the spongiotrophoblast layer, where they are assembled in islets (arrow), and in the deciduum, where they formed islets closed to the spongiotrophoblast layer (arrow) and cords in the distal part (arrowheads). (E) PCDH12 fluorescent labeling. Horizontal (XY) and vertical (XZ) images were obtained with a confocal laser-scanning microscope. (F) High magnification image of the spongiotrophoblast layer showing a PCDH12 staining (brown) in an islet of highly vacuolated cells. Nuclei were counterstained with hematoxylin. (G) High magnification image of glycogen-rich cells in the spongiotrophoblast layer stained with the periodic acid-Schiff (PAS) reaction and counterstained with hematoxylin. Abbreviations: cp, chorionic plate; d, deciduum; gi, giant cell; gt, glycogen rich trophoblast cells; l, labyrinth layer; mt, metrial triangle; s, spongiotrophoblast layer; sp spongiotrophoblast cells.

### **Figure 9: Characterization of PCDH12 expression in kidney glomeruli**

(A) Co-immunolabeling experiments of PCDH12 with either CD31 (endothelial), podocin (podocytic) or PDGF R $\beta$  (mesangial). The data only showed a codistribution with PDGF R $\beta$ , indicating that PCDH12 is expressed by mesangial cells. No PCDH12 signal could be detected in *PCDH12*<sup>-/-</sup> glomeruli. (B) Western blot analysis of protein extracts from isolated glomeruli or total kidney, incubated with an anti-PCDH12 antibody. H5V extracts were used as positive control. All tissues were from 8-10 week-old mice.

**Table 1: Genotype analysis of PCDH12<sup>+/-</sup> crosses**

Genotype*			Total number of animals
Wild type	PCDH12 <sup>+/-</sup>	PCDH12 <sup>-/-</sup>	
101 (25.6%)	195 (49.4%)	99 (25%)	395

\*Mice from two independent ES clones were genotyped at 3 weeks of age.