Endothelium-specific ablation of PDGFB leads to pericyte loss and glomerular, cardiac and placental abnormalities

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Summary

Platelet-derived growth factor-B (PDGFB) is necessary for normal cardiovascular development, but the relative importance of different cellular sources of PDGFB has not been established. Using Cre-lox techniques, we show here that genetic ablation of *Pdgfb* in endothelial cells leads to impaired recruitment of pericytes to blood vessels. The endothelium-restricted *Pdgfb* knockout mutants also developed organ defects including cardiac, placental and renal abnormalities. These defects were similar to those observed in *Pdgfb* null mice. However, in marked contrast to the embryonic lethality of *Pdgfb* null mutants, the endothelium-specific mutants survived into adulthood with persistent pathological changes, including brain microhemorrhages, focal astrogliosis, and kidney glomerulus abnormalities. This spectrum of pathological changes is reminiscent of diabetic microangiopathy, suggesting that the endothelium-restricted *Pdgfb* knockouts may serve as models for some of the pathogenic events of vascular complications to diabetes.

Key words: PDGFB, Endothelium, Cre, loxP, Pericytes, Microaneurysm

Introduction

In the process of vasculogenesis leading to the formation of primitive vascular plexuses and large vessels of vertebrate embryos, vascular smooth muscle cells (VSMCs) and pericytes (PCs) are induced de novo from surrounding undifferentiated mesenchyme and recruited to the proximity of the endothelium (Beck and D'Amore, 1997; Hungerford et al., 1996). Various in vitro experiments as well as genetic analyses in mice have implicated transforming growth factor β (TGFβ) and downstream signaling molecules in this process (Antonelli-Orlidge et al., 1989; Hirschi et al., 1998; Li et al., 1999; Oh et al., 2000; Sato and Rifkin, 1989; Yang et al., 1999). Later, in conjunction with the formation of new blood vessels through angiogenic mechanisms, VSMCs and PCs are recruited to the new vessels in a process involving proliferation and migration of the preexisting pool of VSMCs and PCs (Benjamin et al., 1998; Nicosia and Villaschi, 1995). Genetic analysis has implicated the receptor tyrosine kinase platelet-derived growth factor receptor β (PDGFRβ), its ligand PDGFB, and downstream signaling pathways in the longitudinal spreading of VSMCs and PCs along sprouting vessels (Enge et al., 2002; Hellström et al., 1999; Klinghoffer et al., 2001; Lindahl et al., 1997; Lindblom et al., 2003; Tallquist et al., 2003).

VSMCs and PCs express PDGFRβ, and therefore probably constitute a primary target for PDGFB in the embryo (Holmgren et al., 1991; Lindahl et al., 1997). However, the critical source of PDGFB in the process of VSMC/PC recruitment is not firmly established. Endothelial cells have been shown to express PDGFB in vitro (Jaye et al., 1985) and in vivo (Hellström et al., 1999; Lindahl et al., 1997), suggesting that PC recruitment is controlled by short-range PDGFB/PDGFRβ signaling between the endothelium and the PCs. However, several other cell types express PDGFB, for example megakaryocytes/platelets and monocytes/macrophages (Heldin and Westermark, 1999). These sources are implicated in inflammation and wound healing, but may contribute also to vascular development. PDGFB is also expressed by neurons (Sasahara et al., 1991). It is not known whether any of the extra-endothelial sources of PDGFB may have more long-range paracrine, or endocrine, effects on VSMC and PC growth and vascular recruitment.

Pdgfb and *Pdgfrb* null mutant mouse embryos die perinatally. Death is probably connected to microvascular dysfunction, manifested as widespread microvascular hemorrhage and generalized edema (Levéen et al., 1994; Soriano, 1994). The PC-deficient mutant microvessels of PDGFB- and PDGFRβ-deficient embryos show endothelial cell hyperplasia, hypervariable diameter, abundant microaneurysms, abnormal endothelial ultrastructure and signs of increased permeability (Hellström et al., 2001). In addition to these widespread signs of microvascular dysfunction, certain organs show rather specific defects, in particular the kidney, heart and placenta (Levéen et al., 1994; Ohlsson et al., 1999; Soriano, 1994). In kidneys, the glomeruli are malformed as a consequence of deficient recruitment of mesangial cells (Levéen et al., 1994; Soriano, 1994). Like PCs, mesangial cells

express PDGFRβ and may be attracted into the developing glomerular tuft by PDGFB released from endothelial cells (Lindahl et al., 1998). Other studies, however, have localized glomerular PDGFB expression to podocytes (Alpers et al., 1992) and to the mesangial cells themselves (Floege et al., 1992). Thus, the critical source of PDGFB in glomerulogenesis remains unclear.

To what extent the heart and placenta abnormalities of *Pdgfb* and *Pdgfrb* mutants are secondary to microvessel dysfunction is also not established. VSMC and PC recruitment is defective in both organs (Lindahl et al., 1997; Ohlsson et al., 1999), and hence these organ defects may be secondary to microvascular dysfunction; however, other functions for PDGFB/PDGFRβ have also been suggested at these sites. Cardiomyocytes express PDGFRβ transiently during early development (Soriano, 1994), and chimeric analysis has indicated that PDGFRβ may have a cell-autonomous role in these cells as well as in other muscle lineage cell types (Crosby et al., 1998). Placenta trophoblasts express PDGFB and PDGFRβ (Goustin et al., 1985), and *Pdgfb* or *Pdgfrb* null mice show a reduction in trophoblast numbers (Ohlsson et al., 1999), suggesting that the proliferation of these cells is under autocrine control.

In order to directly test the relative importance of various cellular sources of PDGFB in embryonic development, we have taken advantage of the Cre-lox system for conditional gene inactivation (Gu et al., 1994; Rajewsky et al., 1996). We generated mice carrying a *Pdgfb* allele flanked by loxP sites (Enge et al., 2002) and crossed these with transgenic mice that express Cre under the vascular endothelial specific Tie1 promoter (Gustafsson et al., 2001). The resulting offspring showed compromised PC recruitment to retinal blood vessels and developed a diabetic retinopathy-like condition (Enge et al., 2002). In the present study, we compare the endothelium-specific *Pdgfb* knockout with *Pdgfb* null mice in order to clarify the role of endothelial PDGFB expression during development. Interestingly, we found that endothelium-specific knockouts and complete *Pdgfb* null embryos developed a similar set of defects in glomeruli, microvessels, heart and placenta. However, in contrast to the null mutants, most endothelium-specific knockouts survived into adulthood with persistent microvascular pathology. Thus, these mice allowed us to unambiguously identify endothelial-derived PDGFB as source for PC recruitment and to address the role of PCs in the adult microvasculature.

Materials and methods

Endothelial cell-specific *Pdgfb* knockout mice

A detailed description of the generation of these mice has been published elsewhere (Enge et al., 2002). Briefly, a targeted mutation was introduced at the *Pdgfb* locus by homologous recombination in embryonic stem cells, ES-cells. In the mutant *Pdgfb* allele (PDGFBflox), the fourth exon, which codes for the major part of the PDGFB protein, was surrounded by loxP-sites (Fig. 1A). Intercrossing of *Pdgfb flox/+* mice, and crosses with $Pdgfb^{+/-}$ mice (Levéen et al., 1994) gave rise to *Pdgfb flox/flox* and *Pdgfb flox/–* mice with expected Mendelian frequencies. *Tie1-Cre* transgenic mice (Gustafsson et al., 2001) that express Cre under the mouse *Tie1* promoter (Korhonen et al., 1995) were mated with $Pdgfb^{+/-}$ mice to generate $Tie1Cre^{+}$ $Pdgfb^{+/-}$ offspring, which were subsequently crossed with *Pdgfb flox/flox* mice to create $Tie1Cre^{+}$ *Pdgfb lox/–* (lox/–) animals together with various controls: $Tie1Cre^{+}$, *Pdgfb lox/+* (lox/+); $Tie1Cre^{0}$, *Pdgfb flox/–* (flox/–); and $Tie1Cre^{0}$, *Pdgfb flox/+* (flox/+) (Fig. 1B). The mice were bred into the background of the *XlacZ4* reporter mice that express β-galactosidase in VSMCs and PCs (Tidhar et al., 2001). Mice in breeding were genotyped by PCR using the following primers:

BF, 5′-GGGTGGGACTTTGGTGTAGAGAAG-3′;
BB1, 5′-TTTGAAGCGTGCAGAATGCC-3′; and
BB2, 5′-GGAACGGATTTTGGAGGTAGTGTC-3′.

For genotyping, primers were combined with tail or toe DNA in Gittchier buffer [166 mM ammonium sulfate, 670 mM Tris (pH 8.8), 67mM $MgCl_2$, 50 mM β-mercaptoethanol, 67 μM EDTA, 0.1% Gelatine]. Thirty-two cycles of touch-down PCR were run, with each cycle being: 96°C for 30 seconds; 61-55°C for 30 seconds; and 65°C for 1 minute and 30 seconds. For the first seven cycles, the annealing temperature was decreased stepwise by 1°C; the PCR reaction ended with 25 cycles using an annealing temperature of 55°C. This generated diagnostic fragments of 265, 400 and 624 bp for the wild-type, flox and *Pdgfb* null alleles, respectively. The extent of recombination in vivo was controlled by PCR analysis of microvascular fragments as described (Enge et al., 2002).

Histological analysis

Tissues were fixed in 4% paraformaldehyde (PFA), paraffin embedded and sectioned at 4-5 μm. Sections were stained with Hematoxylin and Erythrosin (H/E) according to standard protocols, with α-smooth muscle actin (SMA) as previously described (Hellström et al., 1999), and with Periodic Acid Schiff (PAS), Hotchkiss McManus modified (Bio-optica 130802). Staining of whole tissues and sections for β-galactosidase were performed as described (Hogan et al., 1994), and sections were counterstained with Erythrosin. Images were captured using a Nikon Eclipse E1000 microscope. Vibratome brain sections (200 μm thick) were stained with isolectin (*Bandeiraea simplicifolia*, Sigma L-2140) and GFAP labeling was achieved using a polyclonal rabbit antibody (1:75, Dako Z 0334) as described (Enge et al., 2002). Confocal images were captured using a Leica TCS NT microscope system. All images were processed using Adobe Photoshop software.

Glomerulus morphometry

The maximum capillary diameter was measured in individual glomeruli on H/E-stained sections (as illustrated in Fig. 3). Mature glomeruli were selected at random from different kidney areas of embryonic day (E) 18.5 and postnatal day (P) 21 mice. In P21 mice, only glomeruli in the deep peripapillary region of the cortex were selected for analysis in order to compare glomeruli of a similar size and developmental age. The number of glomeruli analyzed ranged from 13 to 22 per animal at E18.5, and from 19 to 42 in the adult animals. Images were captured using a Nikon Eclipse E1000 microscope equipped with a Nikon Plan Fluor 60X lens. The Easy Image Measurement 2000 software was used to measure the actual capillary diameter, giving each animal its own range of values. *P* values were calculated using a *t*-test, two-sample unequal variance/two-tailed distribution. Graphs were produced using Microsoft Excel software.

Albuminuria measurements

For urine sampling, mice were placed in metabolic cages and urine collected during a 24-hour period (volume range 0.1-2 ml). Ten μl samples were diluted and assayed by ELISA using AlbuwellM kits (Exocell) according to the manufacturer's instructions. In total, 22 mice of two age groups were analyzed: 6 months and >1 year. Results were calculated as mg per 10g body weight per hour. *P* values were calculated using a *t*-test, two-sample unequal variance/two-tailed distribution. Graphs were produced using Microsoft Excel software.

PDGFB protein measurements

Glomeruli were isolated as described (Takemoto et al., 2002), and frozen and thawed three times in lysis buffer [60% Acetonitrile, 1%

Trifluoric Acid (TFA) 1% Tween]. PDGFB concentrations were then analyzed by homogenous proximity ligation as described (Fredriksson et al., 2002). Prior to analysis the samples were diluted in 100 mM Tris (pH 8.0). After dilution, 1 μl of each sample was analyzed in triplicate. The analyses were carried out on 22 mice belonging to two age groups: 6 months and >1 year. *P* values were calculated using a *t*-test, two-sample unequal variance/two-tailed distribution. Graphs were produced using Microsoft Excel software.

Results

Endothelium-specific *Pdgfb* ablation

Deletion of exon 4 in the *Pdgfb* gene results in a null allele (Levéen et al., 1994). To generate a conditionally inactivated *Pdgfb* allele, loxP sites were positioned on either side of exon 4 by homologous recombination in ES-cells. This resulted in a functionally intact *Pdgfb* flox allele (Enge et al., 2002) (Fig. 1A). Mice in which the Cre recombinase is expressed under the Tie1 promoter (Gustafsson et al., 2001) were bred onto a $Pdgfb^{+/-}$ background and then crossed with *Pdgfb flox/flox* mice to generate an endothelium-restricted inactivation of the *Pdgfb* gene (schematically illustrated in Fig. 1B). In litters of such crosses, the $Tie1Cre^{+}$, *Pdgfb lox/–* (lox/–) constitute the endothelium-restricted knockouts, whereas the $Tie1Cre^{0}$, *Pdgfb flox/+* (flox/+), $Tie1Cre^{0}$, *Pdgfb flox/–* (flox/–) and $Tie1Cre^{+}$, *Pdgfb lox/+* (lox/+) represent controls. In previous analyses, we found that the deletion was partial and inter-individually variable, but that most individuals showed >70% recombination in the endothelial population (Enge et al., 2002). Whereas the *Pdgfb* null mice invariably die at perinatal time points, lox/– mice were born at the expected Mendelian frequency, and most reached adulthood and were fertile.

Renal abnormalities in endothelium-specific *Pdgfb* mutants

Endothelial cells in the developing kidney glomerular tuft express Tie1 (Lindahl et al., 1998). To confirm that the Tie1 promoter directs Cre expression to the renal endothelium, we crossed *Pdgfb* mutants with the R26R reporter strain (Soriano, 1999) and identified Cre-activity using β-gal staining (Fig. 2). In the kidney, abundant β-gal staining was seen in the glomeruli, as well as in the tubular interstitium (Fig. 2A). At higher magnification, it was evident that the β-gal staining was confined to endothelial cells within the glomerular (Fig. 2B,C) and interstitial capillaries (Fig. 2D,E), and that most but not all endothelial cells were β-gal positive (Fig. 2E). Based on these observations, we conclude that the Tie1-Cre transgene is expressed in the vascular endothelial cell population in the kidney. The proportion of β-gal-positive versus negative endothelial cells in the kidney (>70%) is comparable to the ratio of recombined versus unrecombined *Pdgfb* flox alleles in brain capillaries reported previously (Enge et al., 2002), suggesting that the R26R and *Pdgfb* flox alleles recombine with similar efficiency in the presence of Cre. To confirm that recombination at the *Pdgfb* locus led to attenuated PDGFB protein levels in the glomerulus, we applied a sensitive and specific method based on PDGFB-binding aptamers and proximity-dependent DNA ligation (Fredriksson et al., 2002). Using this method, we could demonstrate a reduction in PDGFB protein levels in glomerular preparations from lox/– mice in comparison with controls (see below Fig. 4J,K).

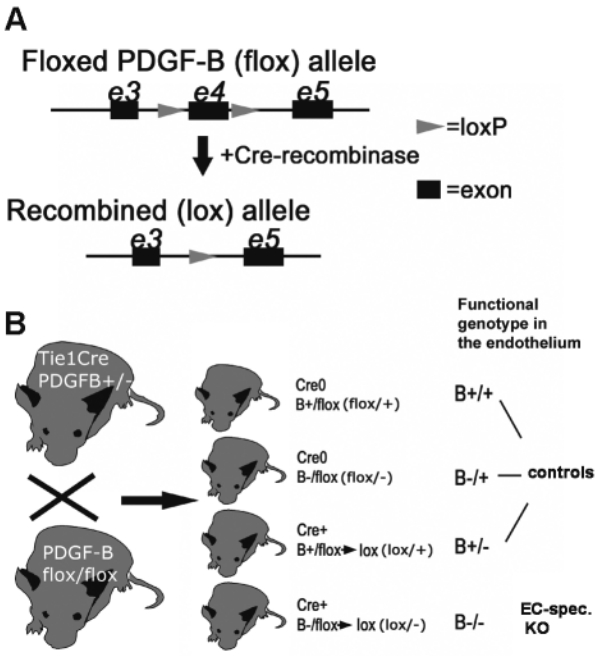

Fig. 1. Endothelium-specific *Pdgfb* knockout strategy. (A) Outline of the conditional (*flox*) and recombined (*lox*) alleles. (B) Breeding scheme used to generate endothelium-specific *Pdgfb* knockouts and various littermate controls. Abbreviations for genotypes used in subsequent figures are shown in parentheses.

We next compared the morphological appearance of glomeruli in wild type (+/+), lox/–, $Pdgfb^{-/-}$ (–/–), and control (flox/+, lox/+, flox/flox and flox/–) E18.5 embryos. $Pdgfb^{-/-}$ embryos lack mesangial cells, which result in typical ballooning of the capillary tuft, i.e. the tuft is replaced by a single expanded capillary loop (Fig. 3A,B). In lox/– embryos, ballooning was readily apparent, but variable in extent between individual glomeruli (Fig. 3D,F). Whereas some lox/– glomeruli were morphologically indistinguishable from –/– glomeruli (Fig. 3D), others showed an intermediate phenotype (Fig. 3F). Flox/flox, lox/+ and flox/– glomeruli were all indistinguishable from the wild type (Fig. 3C,E and data not shown). SMA staining confirmed the absence of mesangial cells in –/– glomeruli (data not shown), whereas lox/– glomeruli showed a variable picture, with some containing a small SMA-positive mesangial core (Fig. 3H) and others lacking it (Fig. 3I,J). The variability of the lox/– phenotype was also apparent from analysis of glomerular capillary diameters (Fig. 3K). Together, these analyses demonstrate that the glomerular phenotype of lox/– embryos is similar to the –/– phenotype, but slightly milder.

The postnatal renal phenotype was analyzed using H/E-stained paraffin sections of kidneys. Interestingly, the mesangial deficiency was largely corrected in 3-week-old animals (Fig. 4A,B, and data not shown). However, all lox/– animals showed glomerular dilation at this age in comparison with controls, both measured as an increased glomerulus diameter (Fig. 4A,B) and as an increased diameter of the individual capillary loops (Fig. 4C). By 6 months of age the picture was further normalized and no morphological signs of increased glomerular pathology were apparent in lox/– mice up to 21 months of age (Fig. 4D-G). Increased mesangial matrix

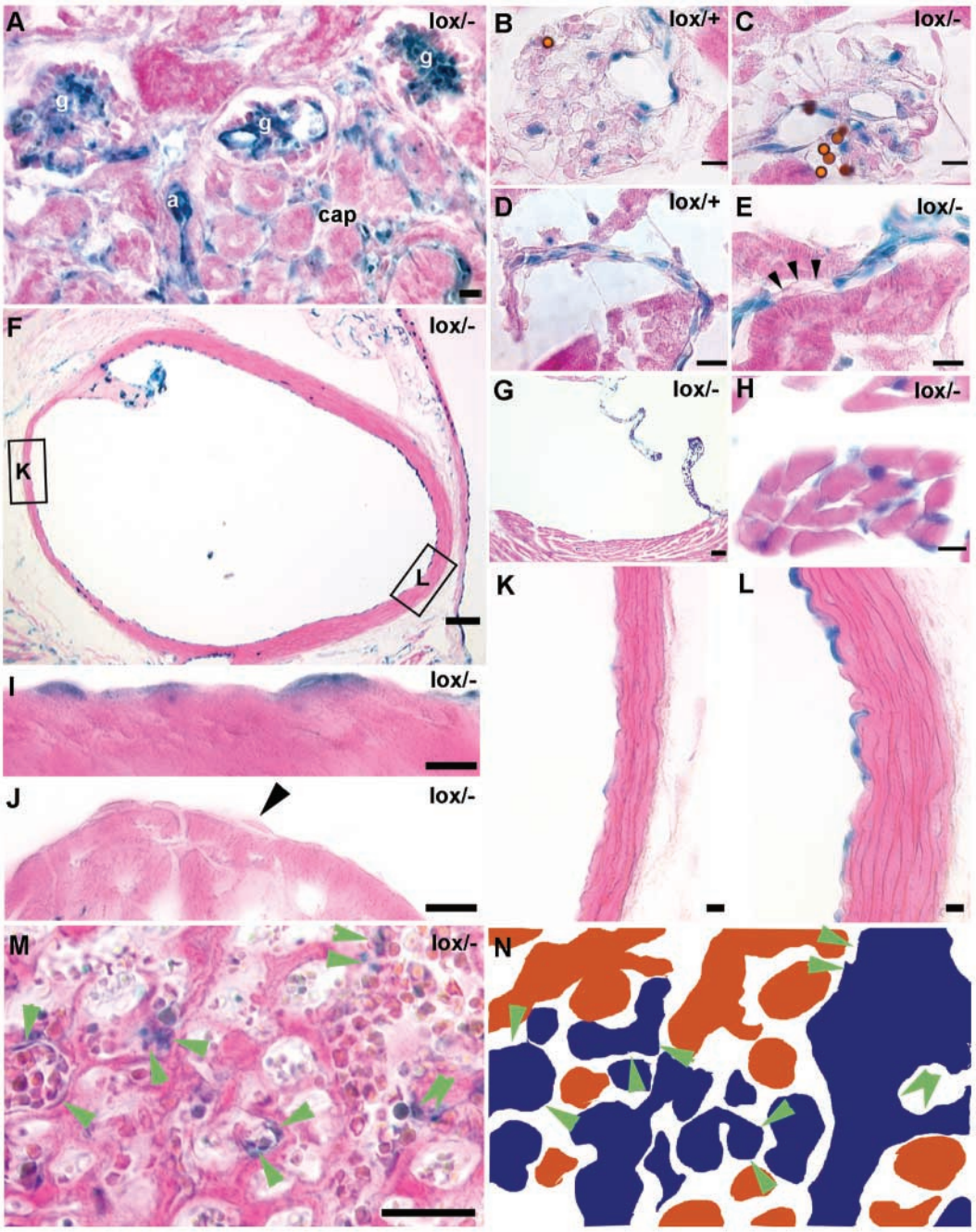

Fig. 2. Tie1-Cre-mediated *lacZ* expression in R26R mice. By crossing Tie1-Cre and R26R reporter mice, the expression and efficiency of the Cre recombinase could be studied. These crossings also contained the *Pdgfb* conditional and null alleles. Sections are stained for β-gal (blue) and are counterstained with Erythrosin (pink). (A) An overview section of the kidney cortex shows a *lacZ* expression pattern compatible with vascular expression. g, glomerulus; a, artery; cap, capillary. (B,C) Glomerular endothelial expression is evident at higher magnification. (D,E) Peritubular capillary endothelium is largely but not homogenously β-gal-positive (arrowheads). (F-L) In the heart and its large vascular connections, the endocardium, the cardiac valves and endothelium are β-gal positive, albeit not at 100% (arrow in J indicates an unstained endothelial cell). Deep in the myocardium, the capillaries are the only β-gal-positive structures (H). The endothelium of the large vessels, here illustrated by the aortic arch (F), show a chimeric Cre expression (K,L; boxed regions in F at higher magnification). (M) The placenta vessels that are lined by a β-gal-stained endothelium (green, arrowheads) could be classified as fetal by their content of large immature erythrocytes. (N) Diagrammatic representation of M, showing maternal sinuses (red) and fetal vessels (blue). Scale bars: 10 μm in A-E,H-L; 100 μm in F,G,M.

accumulation has been observed in mutants of PDGFRβ signaling (Klinghoffer et al., 2001), and in PDGFB retention (Lindblom et al., 2003) mutants, but was not seen in the PDGFB lox/– glomeruli stained by periodic acid-Schiff's (PAS) reagent (Fig. 4D-G). However, lox/– mice older than 12 months developed mild but significant increases in albumin content in the urine (Fig. 4I), suggesting that lox/– glomerular filtration become abnormal at older age. The glomerular

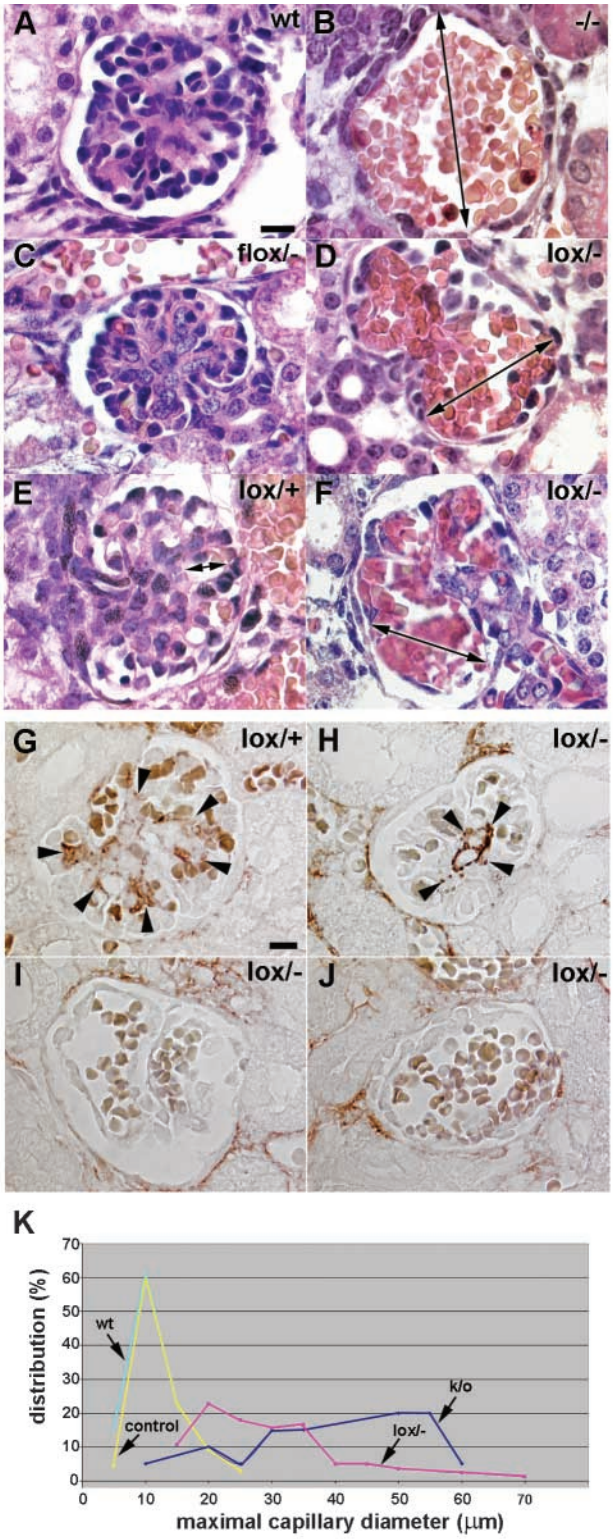

Fig. 3. Glomerular abnormalities in endothelium-specific *Pdgfb* knockouts. Hematoxylin/Erythrosin (H/E) staining (A-F) and α-smooth muscle actin immunostaining (G-J) of E18.5 kidney glomeruli of different genotypes. Normal glomeruli containing a mesangial cell core (outlined by arrowheads, G) were seen in wild-type (A) and control (C,E,G) kidneys, whereas the *Pdgfb*$^{-/-}$ (B) and some of the lox/– (D,J) glomeruli completely lacked mesangial cells. In these cases, the glomerular capillary tufts become replaced by single dilated capillary loops. Most lox/– glomeruli show a reduced mesangial core (outlined by arrowheads, H), leading to a reduction in tuft complexity and dilation of the remaining capillary loops (F,H). (K) Morphometric analysis of glomerular capillary dilation; the relative distribution of capillary diameters in glomeruli of the same genotype is shown. The capillary diameters of the lox/– animals differ significantly from those of all other groups (P<0.005). Note that the range of values, but not their distribution, is similar for lox/– and –/– (k/o). There is no difference between controls (sum of flox/+, flox/–, lox/+) and wild type (P>0.05). Measured diameters have been corrected to the closest 5 μm to yield an illustrative figure. Scale bar: 10 μm.

PDGFB protein content was lower in lox/– mice of both age groups although statistically significant differences were obtained only in mice older than 12 months (Fig. 4J,K).

Cardiac and placenta abnormalities in endothelium-specific *Pdgfb* mutants

Both cardiac and placenta abnormalities have been reported in *Pdgfb* and *Pdgfrb* mutant embryos at late gestation (Hellström et al., 1999; Levéen et al., 1994; Ohlsson et al., 1999; Soriano, 1994). The cardiac abnormalities include dilation, myocardial hypotrophy with thinning of the myocardial wall, myocardial hypertrabeculation and septal abnormalities (Hellström et al., 1999; Levéen et al., 1994; Soriano, 1994) (M. Hellström and M. Kalén, unpublished). When compared with *Pdgfb*$^{-/-}$, +/+ and various other controls (flox/+, flox/–, lox/+), we found that E18.5 lox/– embryos showed similar heart abnormalities as the same age –/– embryos (Fig. 5A-D). We also analyzed the heart morphology at three different postnatal ages (1 month, 6 months and >1 year). Like the kidney glomeruli, the heart phenotype normalized histologically with age. At 1 month of age, the myocardium of lox/– mice was already of normal thickness, and no statistically significant deviations were noticed at older ages (data not shown). To address the cause of the embryonic myocardial hypotrophy, we analyzed cardiac Cre expression in Tie1-Cre/R26R mice. Expression was evident in the endocardium (Fig. 2I), interstitial capillaries (Fig. 2H), valvular mesenchyme (Fig. 2F,G) and endothelium of the large arteries (Fig. 2F,L). A chimeric situation was evident, with areas of β-gal-positive as well as negative cells (Fig. 2J,K arrowheads). The *lacZ* expression sites in the postnatal heart were compatible with an early endocardial/endothelial expression, including the valvular mesenchyme, which is derived from the cardiac cushion. The cardiac cushion arises through mesenchymal transdifferentiation of endocardial cells, and has previously been shown to express *lacZ* in Tie1-Cre/R26R crosses (Gustafsson et al., 2001).

In addition, the placental abnormalities typical of *Pdgfb*$^{-/-}$ and *Pdgfrb*$^{-/-}$ embryos (Ohlsson et al., 1999) were reproduced in the lox/– embryos (Fig. 5E-J). These defects include dilation of both fetal and maternal vessels due to a reduction in the number of PCs and trophoblasts (Ohlsson et al., 1999). Both the cardiac and placental phenotypes in lox/– embryos showed some degree of interindividual variation (data not shown). Analysis of placental Cre expression in Tie1-Cre/R26R mice revealed a mosaic expression largely confined to endothelial cells of the fetal vessels within the labyrinthine layer of the

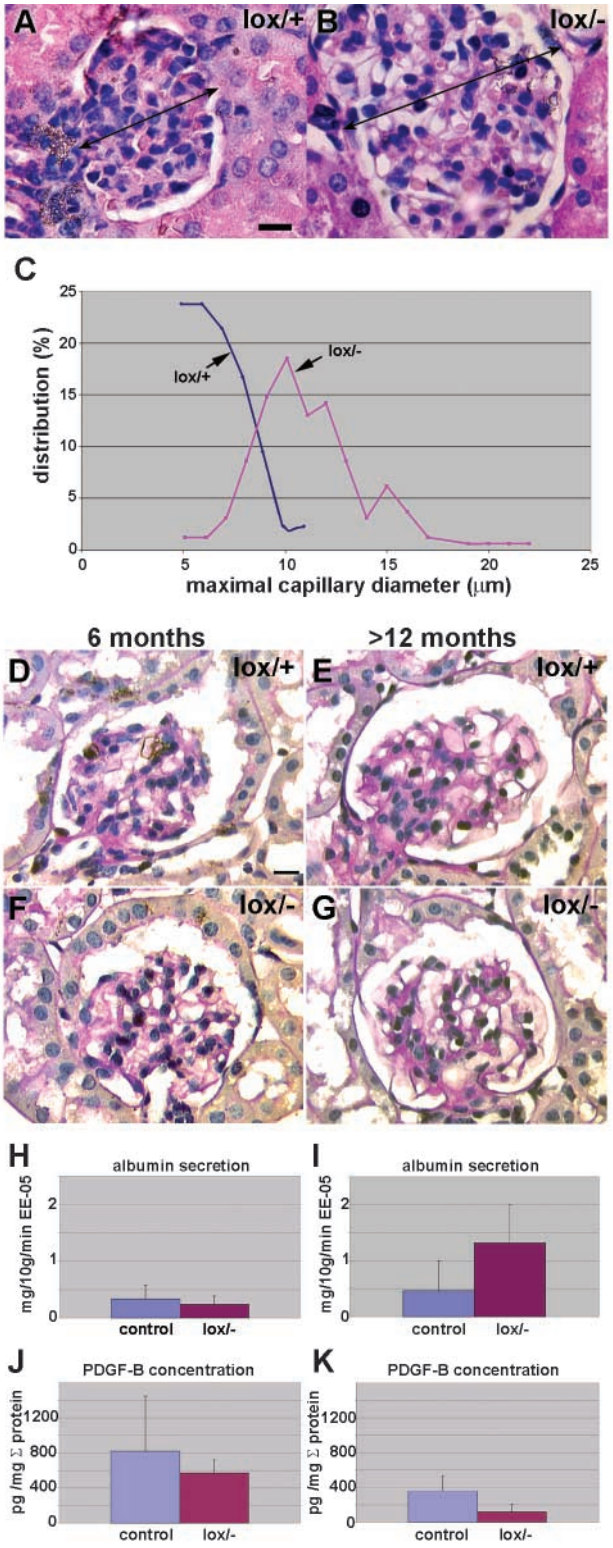

Fig. 4. Glomerular abnormalities in adult endothelium-specific *Pdgfb* knockouts. H/E staining of 3-week-old kidneys revealed an increased glomerulus diameter (A,B) and capillary loop diameter (C) in lox/– mice compared with controls. Capillary diameters of the lox/– animals differ significantly from those of the control group (P<0.005). The relative distribution of the maximal capillary diameter in the control and endothelium-specific *Pdgfb* knockout groups is shown. Measured diameters have been corrected to the closest 1 μm to yield an illustrative figure. PAS staining of adult kidneys did not reveal signs of pathology at the age of 6 months (D,F) or >1 year (E,G). Glomerular cell count and matrix volume were comparable in the various genotypes, Scale bar: 10 μm). (H,I) Urine albumin levels were increased in lox/– mice that were more than a year old (P<0.05), but not in 6-month-old lox/– mice (P>0.05). Glomerular PDGFB protein levels (J,K) were reduced in lox/– mice and the reduction was significant (P<0.005) in mice that were more than a year old. Bars represent levels (±s.d.) of albumin secretion (H,I) and PDGFB protein (J,K).

placenta (Fig. 2M,N). Occasional β-gal-positive leukocytes were seen in the placenta, as well as other vessels (data not shown), confirming that the Tie1 promoter also contributes some expression to the myeloid lineage (Gustafsson et al., 2001).

Endothelium-specific *Pdgfb* deletion leads to impaired PC recruitment to brain microvessels

To allow for visualization of PC recruitment to brain microvessels, we crossed the Tie1-*Cre/Pdgfb/flox* alleles onto the *XlacZ4* transgenic background in which *lacZ* expression is restricted to vascular smooth muscle cells and PCs from late gestation onwards (Abramsson et al., 2002; Klinghoffer et al., 2001; Tidhar et al., 2001). Whole-mount β-gal-staining of E15.5 brains allowed for quantification of PC density in various brain regions following dissection. Using this method, we observed a significant reduction in PC density in lox/– embryos. We compared the PC densities in the midbrain of control, $Pdgfb^{-/-}$ and lox/– embryos (Fig. 6). Vascularization of this region follows a stereotypical pattern: blood vessels enter the brain tissue perpendicular to the surface (radial branches) and ramify to form a capillary plexus in the subventricular zone. *XlacZ4*-positive PCs were found in association with the radial vessels, as well as with the vessels of the subventricular plexus, in both mutants and controls, however the densities differed markedly. The lox/– embryos showed intermediate densities between those of the control and –/– situations [70-90% reduction the two lox/– embryos shown (Fig. 6C,D,G,H,K,L), compared with >95% reduction in the $Pdgfb^{-/-}$ embryo (Fig. 6B,F,J)]. Both the lox/– and $Pdgfb^{-/-}$ mutants showed irregular capillary diameter, with focal distensions (microaneurysms) and hemorrhages (Fig. 6B-D,G,H). A noticeable variation in PC coverage between neighboring capillaries was seen in lox/– midbrain (Fig. 6K). Even in the lox/– embryo with a 90% overall reduction in *XlacZ4*-positive PCs, individual capillaries were found with seemingly normal PC coverage and distribution (Fig. 6L). This result would be expected from a chimeric situation in which most capillaries are composed of PDGFB-negative endothelial cells, whereas individual capillary segments may be composed of mostly unrecombined cells retaining PDGFB expression, which hence stimulate local recruitment of PCs.

Analysis of adult brain tissue by double staining for β-gal-positive PC nuclei and the endothelial marker PECAM1 showed that the PC deficiency of lox/– mice persisted into adulthood (Fig. 7A-D). In the striatum, capillary density was lower but, strikingly, numerous tortuous capillaries with increased diameter were seen (Fig. 7B, arrows). These

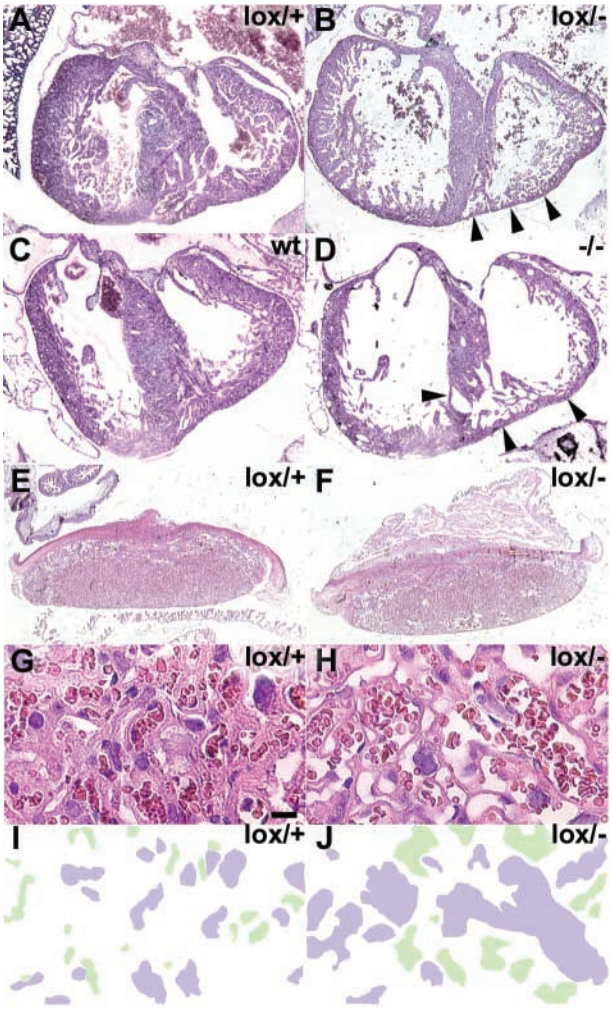

Fig. 5. Cardiac and placental abnormalities in endothelium-specific *Pdgfb* knockouts. H/E staining shows cardiac (A-D) and placental (E-H) abnormalities in E18.5 lox/– embryos similar to those seen in *Pdgfb*$^{-/-}$ embryos. Arrowheads (B,D) indicate the thin myocardium typical for –/– and lox/– animals. The placenta vessels in G and H that could be classified as either maternal or fetal by their erythrocyte content are outlined in I and J, respectively, to visualize their dilation in the lox/– animal (maternal sinuses are colored green, fetal vessels are colored purple). Scale bar in G: 10 μm.

abnormal capillary stretches lacked association of β-gal-positive PC nuclei, without exception (Fig. 7B). Capillaries in the striatum with associated PCs were straighter and more uniform in diameter (Fig. 7B, arrowheads). In the cerebellum, lox/– mice showed a substantial reduction of capillary density in the gray matter, and the presence of numerous enlarged capillaries (Fig. 7C,D).

Gross inspection of dissected brains of postnatal lox/– animals revealed scattered small hemorrhages deep in the cerebral parenchyma (Fig. 7E). Stained vibratome sections showed focal sites of upregulated expression of glial fibrillary acidic protein (GFAP; Fig. 7G,H, arrows) and increased density of microglial cells (Fig. 7H, arrowheads). These hallmarks of reactive gliosis that occur secondary to brain injury were seen at sites of bleeding (Fig. 7G,H), but not generally in lox/– brain tissue (Fig. 7F). Where analyzed, bleeding seemed to start from capillary branching points, sites that often harbor PCs in the normal situation.

Discussion

Endothelium-derived PDGFB acts in a paracrine fashion to recruit VSMCs/PCs

Previous work has demonstrated that PDGFB and PDGFRβ are necessary for the recruitment of PCs and VSMCs to developing microvessels. Whereas PDGFRβ is expressed by the PCs and VSMCs, the important source(s) of PDGFB has not been firmly established. Our current analysis identifies the endothelial cells as the critical source of PDGFB in PC and VSMC recruitment to blood vessels. In addition, our analysis shows that endothelial PDGFB is critical for proper heart and placenta development. As the PC and VSMC population in both these organs is PDGFRβ positive during development (Lindahl et al., 1997; Ohlsson et al., 1999), it is possible that both these phenotypes are secondary to microvessel dysfunction. However, our data do not exclude the possibility that endothelium-derived PDGFB has direct effects on, for example, cardiomyocytes and trophoblasts.

The importance of endothelium-derived PDGFB in PC, VSMC and mesangial recruitment suggests that PDGFB acts mainly through a short-range paracrine route. The inability of hematopoietic cell-derived PDGFB to compensate for the lack of endothelial PDGFB may seem surprising, given the presence of platelets and monocytes – two cell types with proven capacity to produce and release high levels of PDGFB – in developing microvessels. However, these cells probably release PDGFB mainly in conjunction with thrombus formation, blood clotting and inflammatory conditions (Heldin and Westermark, 1999). An important role for hematopoietic PDGFB in vascular turnover and homeostasis is also unlikely given the lack of vascular pathology in mouse radiation chimeras substituted with PDGFB-negative bone marrow (Kaminski et al., 2001). We have also failed to see any effects on vascular development by macrophage-specific knockout of PDGFB (M.E., unpublished). Likewise, neuronal PDGFB expression, which appears at detectable levels at late gestation, and increased postnatally (Sasahara et al., 1992; Sasahara et al., 1991), is not able to compensate for the loss of endothelial PDGFB in the CNS vasculature. A neuron-specific knockout of PDGFB also lacked detectable vascular as well as neuronal and glial phenotypic abnormalities (Enge et al., 2003). In summary, therefore, the only source of PDGFB with a clear developmental function detected so far is the vascular endothelium.

Role of endothelial PDGFB in glomerular development and postnatal function

Both genetic approaches and the use of pharmacological inhibitors have defined a crucial role for PDGFB and PDGFRβ in mesangial cell recruitment into developing glomeruli (Levéen et al., 1994; Lindahl et al., 1998; Sano et al., 2002; Soriano, 1994). Our present data identify the glomerular endothelium as a critical PDGFB source in this process. To our surprise, the severe reduction in mesangial cells observed at late embryonic age was largely corrected postnatally. By 3

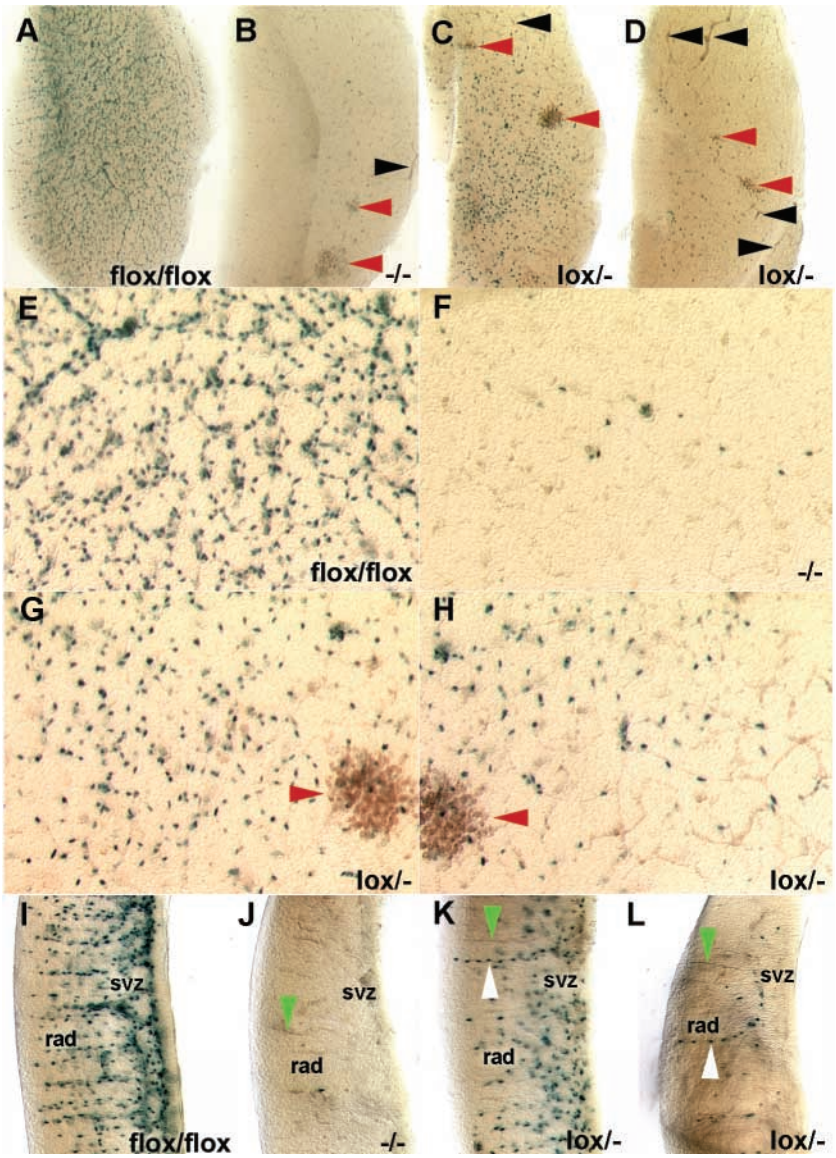

Fig. 6. Reduced PC recruitment to brain capillaries in endothelium-specific *Pdgfb* knockouts. Whole-mount β-gal staining of PCs in the midbrain. (A-D) Ventricular view of entire midbrain hemispheres; (E-H) Distribution of PCs (nuclear staining) in the subventricular zone (SVZ) plexus of the hemisphere. Black arrowheads indicate extensively dilated vessels; red arrowheads indicate focal hemorrhages. Note the dramatic PC deficiency in $Pdgfb^{-/-}$ tissue (B,F,J) and the intermediate PC densities in lox/– tissue (C,D,G,H,K,L). (I-L) Sections (~100 μm thick) of midbrain hemispheres, with the meningeal surface to the left and the ventricular surface to the right. The distribution of β-gal-positive PCs along the radial vessels (rad) is seen. In the lox/– embryos (K,L) many radial branches completely lack β-gal-positive PC coverage (green arrowheads), as in the –/– embryos (J), whereas other branches show normal coverage (white arrowheads).

weeks of postnatal age, the glomeruli appeared to have acquired a normal number of mesangial cells, although a mild increase in capillary diameter (and total glomerular diameter) persisted. The reason for this recovery is unclear. One possibility is that PDGFB regulates the rate of mesangial cell recruitment, but is not exclusively involved in the process. In such a scenario, PDGFB would be expected to cause a delay rather than an irreversible block in mesangial cell recruitment. Alternatively, other sources of PDGFB (such as macrophages or podocytes) could substitute for the loss of endothelial PDGFB, which could potentially be tested by generating combinations of, for exampl, endothelial, podocytic and hematopoietic knockouts. Such crosses are ongoing in our laboratory. The partial deletion of the *Pdgfb* gene could also provide an explanation, as a few remaining PDGFB-producing endothelial cells in each glomerulus might be enough for delayed recruitment. The recently discovered novel PDGFRβ agonists PDGFC and PDGFD (Bergsten et al., 2001; Gilbertson et al., 2001; LaRochelle et al., 2001; Li et al., 2000) also raise the possibility that other PDGF ligands could replace PDGFB as the PDGFRβ ligand in glomerulogenesis. So far we have been unable to detect PDGFD expression at the mRNA level in developing glomeruli. Although PDGFC is primarily a PDGFRα ligand, it might mediate PDGFRβ signaling through PDGFRβ/α heterodimers (Gilbertson et al., 2001). PDGFC is expressed by metanephric mesenchyme in the developing kidney (Ding et al., 2000; Li et al., 2000), and both PDGFRα and PDGFRβ are expressed by developing mesangial cells (Eitner et al., 2002; Floege et al., 1997; Lindahl et al., 1998; Matsumoto et al., 2002). Finally it is possible that factors other than PDGFs may play a role in mesangial cell recruitment.

The albuminuria observed in lox/– mice older than 12 months suggests that endothelium-derived PDGFB has a role in the maintenance of glomerulus function. PDGFB is upregulated in glomeruli in conjunction with injury, suggesting a protective role that may be operational also in the normal aging glomerulus. One would expect such a role to be mediated by the mesangial cells (as these are the only glomerular cells known to harbor PDGF receptors). However, the albuminuria implicates a problem with the podocytes, the endothelial cells or the intervening glomerular basement membrane. Therefore, the age-associated glomerular pathology in the lox/– mice probably reflects that mesangial cells influence the function of other glomerular cell types. Interestingly, the lox/– albuminuria was not accompanied by signs of morphological glomerular abnormalities. Glomerular extracellular matrix accumulation (glomerulosclerosis) has previously been reported in PDGFRβ signaling mutants, in which the intracellular domain was replaced with that of PDGFRα (Klinghoffer et al., 2001), and in PDGFB retention-motif knockouts, in which the C-terminal heparan sulphate proteoglycan-binding motif was deleted (Lindblom et al., 2003). In the latter case, albuminuria preceded glomerulosclerosis, which might suggest that glomerulosclerosis would develop also in the lox/– mice if they lived long enough.

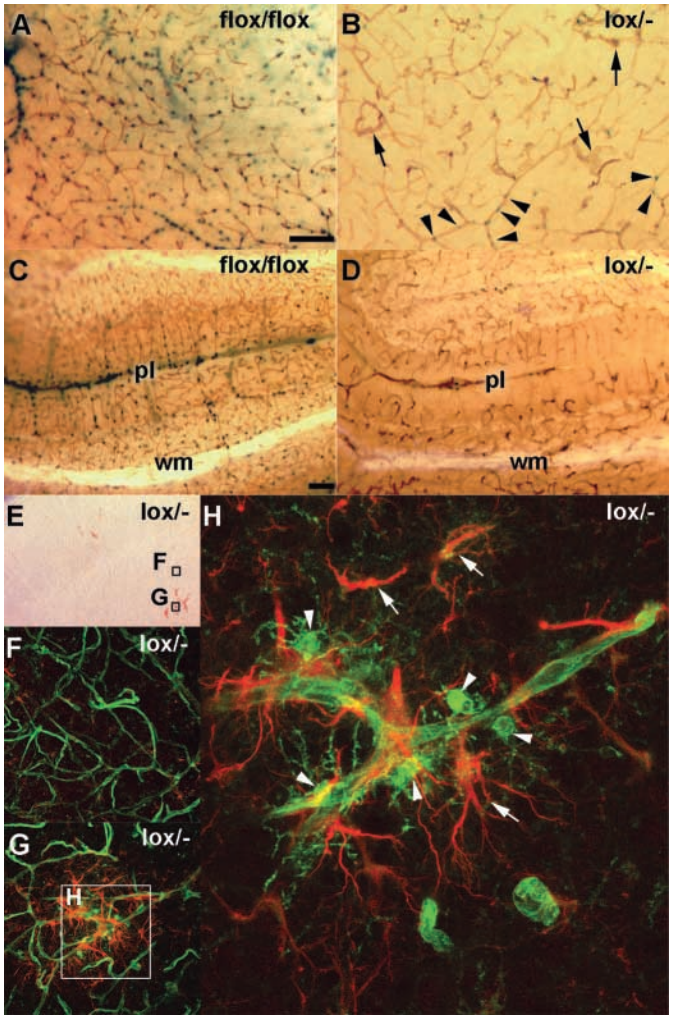

Fig. 7. Reduced microvessel density, increased microvessel diameter, microhemorrhage and focal astrogliosis in adult endothelium-specific *Pdgfb* knockouts. (A-D) Double staining for β-gal-positive PC nuclei (blue) and PECAM1 (brown) in the striatum (A,B) and cerebellum (C,D) of 3-week-old control (flox/flox; A,C) and lox/– (B,D) mice. Note the reduced number of PCs associated with the microvessels in the lox/– brain and the numerous pathological microvascular profiles seen in areas of complete PC loss (B, arrows). Remaining PCs in lox/– striatum correlate with more normal profiles, as judged by their even diameter and straight outline (B, arrowheads). A dramatic reduction of microvascular density is seen in lox/– cerebellum (D). Microhemorrhage, visible by macroscopic inspection in vibratome sections of P12 mice (E), was analyzed further by confocal microscopy using isolectin B4 coupled to FITC (green) and anti-GFAP antibodies coupled to rhodamin (red). The regions around the microhemorrhages (G,H; H represents the boxed region in G at higher magnification) showed strong GFAP expression in reactive astrocytes (arrows), as well as isolectin B4 staining of microglia (arrowheads). Regions in the lox/– brain without signs of microhemorrhage lacked signs of reactive gliosis (F). pl, pial surface; wm, white matter. Scale bars in A,C: 100 μm.

Why do *Pdgfb* null mice die and endothelium-specific *Pdgfb* knockout mice survive?

Considering the extensive PC loss, the prevalent microvascular bleedings, and the glomerular, cardiac and placental changes, it was a surprise to find that the majority of the PDGFB lox/– mice survive birth and reach reproductive age without overt problems, in sharp contrast to the null mutants that invariably die before or at birth. The likely explanation for this, supported by both genetic and morphological data, is that the endothelium-specific *Pdgfb* knockout is incomplete, i.e. all endothelial cells do not recombine their *Pdgfb flox* allele, resulting in a chimeric vasculature with regard to endothelial PDGFB production. Genetic analysis of freshly isolated microvascular fragments demonstrated that the ratio between *Pdgfb flox* and *lox* alleles in the endothelium varied between individual mice, but that most of them show more than 70% recombination, and some more than 90% (Enge et al., 2002). This corresponds well with the data obtained by crossing Tie1-Cre with ROSA26R mice, which resulted in Cre-activated *lacZ* expression in 50-90% of the endothelial cells depending on the site analyzed (Gustafsson et al., 2001) (this study). This correlates with the actual PC loss seen in *Pdgfb* lox/– brain microvessels, which is in the order of 50-90% in different individuals (Enge et al., 2002), compared with 95-98% in the complete knockout (this study). The existence of parallel midbrain radial vessels devoid of, or normally invested by, PCs in the endothelium-restricted knockouts also strongly suggests that the vascular bed is chimeric with regard to endothelial PDGFB expression and recruitment of PCs. It appears from these observations that mice can tolerate up to 90% PC loss without severe organ dysfunction, but die when the loss is more extensive. We have observed a small number of dead newborn lox/– pups, which may represent rare individuals in which the recombination efficiency led to higher than 90% PC loss (data not shown). Based on our previous experience with inter-individual variation in the Tie1-Cre-mediated recombination efficiency, we assume that the perinatal lethal cases represent situations in which recombination is near complete. Our data do not formally rule out the possibility that the perinatal death of *Pdgfb* null embryos reflects the loss of non-endothelial expression of PDGFB, but because selective knockout of any of the other major sources of PDGFB (neurons or hematopoietic cells) leads to viable mice without any obvious developmental or pathological defects, we consider this possibility unlikely.

Diabetic microangiopathy-like condition in endothelium-specific *Pdgfb* knockout mice

Although the lox/– mice survive, they show several pathological changes associated with microvascular dysfunction in the CNS, such as microaneurysm formation, microhemorrhage, and associated astro- and microgliosis. It is unclear whether these defects solely reflect the persistence of the developmental defects, or whether they also develop as a result of a need for continuous endothelial PDGFB expression in the postnatal animal. Irrespective of the exact cause of the microvascular defects in the adult mice, the *Pdgfb* lox/– mouse provides a model for microvascular changes that bears striking resemblance to diabetic microangiopathy (Cogan et al., 1961; Engerman, 1989; Kuwabara and Cogan, 1963). As the microvascular pathology of *Pdgfb* lox/– mice involves PCs and mesangial cells, which are both affected in diabetic microangiopathy (Frank, 1991; Wada et al., 2002), these mice

may provide a useful model for some of the later steps in this microvascular complication.

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