

Intussusceptive angiogenesis and expression of Tie receptors during porcine metanephric kidney development

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Summary. Intussusceptive angiogenesis (IA) is required for normal embryonic vascular development. The Tie family of receptors and their ligands, the angiopoietins, play an important role in the growth or regression of blood vessels which are important not only during development but also throughout an organism's life. The presence of IA was investigated in glomerular capillaries of the fetal porcine metanephros using Mercox II resin casts. The first signs of IA were observed in stage III glomeruli. Stage IV and V glomeruli showed numerous signs of aligned pillar formation and their successive merging to delineate the vascular entities. Furthermore, immunohistochemistry was used to determine the exact locations of the Tie receptors in the developing porcine metanephric kidneys. Tie1 and Tie2 were found in endothelial cells of all glomeruli. Strong expression of the receptors was found in podocytes of stage V glomeruli whereas a weaker expression was observed in the cuboidal epithelial cells of stage III and IV glomeruli. Remarkably, the receptors were also found in the parietal epithelium of Bowman's capsule. These findings indicate that there might be an association between Tie receptors and IA during porcine metanephric development and during glomerulogenesis in particular.

Key words: Pillar formation, Tie1 receptor, Tie2 receptor, Angiopoietin, Glomerulus

Introduction

After the formation of the embryonic vascular network by vasculogenesis (Risau, 1997), most of the new vessels in the developing organism and in the adult arise through angiogenesis. This process is responsible for the growth of the primary vascular plexus into a mature and efficient transport route for the blood (Papetti and Herman, 2002). The two main mechanisms of angiogenesis, sprouting and intussusceptive angiogenesis (IA), play a major role in the expansion of a capillary network but IA is also involved in its remodeling (Burri and Tarek, 1990). Specifically, the origin of the renal vasculature is controversial: the classical experiments that support its development via angiogenesis involve the transplantation of the avascular metanephros onto the quail chorioallantoic membrane (Sariola et al., 1983). On the other hand, experiments to support that the renal vasculature originates via vasculogenesis involved the use of *Tie-1/LacZ* transgenic mice to follow kidney endothelial cell development. Tie-1 receptor tyrosine kinase is expressed in endothelial precursor cells and cells expressing this marker were found in the avascular metanephros (Loughna et al., 1997). According to Makanya et al. (2005), the first step in metanephric microvascular growth involved angiogenic sprouting which formed the initial vascular plexuses of the renal lobules. The vascular growth model switched to intussusception which contributed to vascular amplification and remodeling. Ultimately, the maturation of the vasculature was achieved by intussusceptive pruning and branching remodeling.

IA is a type of new blood vessel formation in which a capillary is longitudinally split into two vascular

channels due to the formation and merging of intraluminal tissue pillars. The process of IA was first discovered on the basis of scanning electron microscopy (SEM) with vascular corrosion casts (Caduff et al., 1986). Three forms of IA are recognized, depending on the outcome or phenotype of these forms, i.e. intussusceptive microvascular growth, intussusceptive arborization and intussusceptive branching remodeling (Burri and Djonov, 2002). Despite this diversity, all forms of IA are characterized by the formation of intraluminal pillars, which form the hallmark of intussusception. Pillars in the initial stages are recognized as tiny shallow depressions on the surface of resin intravascular casts. Larger tissue pillars appear as deep broader holes on the casts and can be differentiated from tissue meshes purely by their sizes, with all holes $<2.5 \mu\text{m}$ in diameter being considered to represent tissue pillars (Makanya et al., 2009). According to the model that Burri and Djonov (2002) proposed, the pillar formation proceeds through 4 stages: I) endothelial cells of the opposite sides of the lumen migrate to each other and make contact. II) a central pillar is formed perforating the blood vessel. III) the pillar is encircled by pericytes and myofibroblasts that deposit extracellular matrix. IV) the pillars increase in size and fuse with each other splitting the capillary into two.

Angiopoietins and vascular endothelial growth factors (VEGFs) form two families of growth factors involved in angiogenesis (Carmeliet, 2000; Kässmeyer et al., 2009) and both are crucial for the development of renal vasculature (Wakelin et al., 2004; Woolf et al., 2009). Angiopoietin 1 (Angpt1) and angiopoietin 2 (Angpt2) both exert their functions through the tyrosine kinase receptor Tie2, which is mainly found on the cell membranes of vascular endothelial cells. Angpt1 is a blood vessel maturation factor, an anti-inflammatory agent and it promotes endothelial quiescence (Fukuhara et al., 2010). In addition to the maintenance of vascular quiescence, Angpt1 can also function as a potent angiogenic stimulator, both *in vivo* as well as *in vitro* (Fiedler and Augustin, 2006). Surprisingly, both effects of Angpt1 are induced through the activation of Tie2 receptor, which can initiate different molecular pathways depending on the presence or absence of endothelial cell-cell contacts (Fukuhara et al., 2010). Angpt2 binds Tie-2 without activating the receptor and it antagonizes Angpt1-induced Tie-2 phosphorylation (Maisonpierre et al., 1997). Depending on the presence or absence of VEGFA, Angpt2 induces angiogenesis or vascular degeneration, respectively (Lobov et al., 2002; Scharpfenecker et al., 2005). There are clear indications that the angiopoietins play an important role during intussusceptive angiogenesis (Augustin, 2001; Kurz et al., 2003). Targeted deletion of Tie2 expression in mice leads to deficient pillar formation (Patan, 1998). Angpt1 overexpressing mice show a vascular phenotype of enlarged vessels with abundant small invaginations that are reminiscent to intussusceptive angiogenesis (Burri

and Tarek, 1990; Thurston et al., 1999, 2005). In addition, overexpression of Angpt2 affects ongoing intussusceptive angiogenesis in the chick chorioallantoic membrane as it leads to the remodeling of a previously uniform capillary mesh into an arborized vascular tree (Winnik et al., 2009). Tie1 is a Tie2 homologue and an orphan receptor that does not serve as a high-affinity angiopoietin receptor but is required for normal embryonic vascular development (Partanen et al., 1992). Tie1-Tie2 interactions have been implicated in the regulation of Angpt1-induced Tie2 signal transduction, indicating ligand-independent functions of Tie1 (Saharinen et al., 2005; Seegar et al., 2010). Tie heterodimers are preferentially bound by Angpt2, thus rendering Tie2 unavailable for Angpt1 activation. In absence of Angpt2, Angpt1 can separate the heterodimers and activate Tie2 homodimers. In absence of Tie1, Angpt2 can activate Tie2 (Kim et al., 2006; Seegar et al., 2010).

The development of the nephron could be divided into five stages according to Friis (1980). Mesenchymal cells condense around branches of the ureter into a renal vesicle (stage I), which matures in an S-shaped body (stage II). The latter becomes invaded by endothelial cells, which assemble into a single glomerular capillary loop, which subsequently expands into a complex tuft of branched capillaries (glomerulus). Mesangial cells, which share a common origin with smooth muscle cells and pericytes, also infiltrate the glomerulus (Gomez and Norwood, 1999). Stage III glomerulus is spherical or oval with a diameter of less than $50 \mu\text{m}$ and has a distinct Bowman's space and a few capillary loops are present. Stage IV glomerulus has a round shape, a diameter of about $50 \mu\text{m}$ and it contains more capillary loops. In both stages a monolayer of cuboidal epithelial cells (presumptive podocytes) is arranged at the outer side of the glomerulus. Stage V glomerulus has a diameter of $100 \mu\text{m}$; it contains multiple capillaries and the podocytes and endothelial cells are flattened.

In previous studies on mouse metanephrogenesis, low levels of angiopoietins and Tie2 transcripts were present from the inception of the metanephros; there were relatively low levels of Angpt1 and Tie2 mRNA and proteins expressed around E12.5, when interstitial capillaries populate the organ; protein levels of Angpt1, Angpt2 and Tie2 were upregulated by E14.5 and E16.5, when the first layers of vascularized glomeruli are forming (Kolatsi-Joannou et al., 2001). Later, Tie2 was expressed by capillaries in the nephrogenic cortex, glomerular tufts, and vasa recta. Angpt1 mRNA was found to localize to condensing renal mesenchymal cells, proximal tubules and glomeruli in addition to maturing tubules of the outer medulla. Angpt2 transcripts were more spatially restricted, being detected only in differentiating outer medullary tubules and the vasa recta bundle area (Yuan et al., 1999). Previous investigations by our research group regarding the role of angiopoietins in the porcine developing metanephros revealed that

Angpt1 is predominantly present in the stage V glomeruli which are located in the juxtamedullary region of the metanephric cortex, while Angpt2 is observed in the stage III and IV glomeruli that are located in the outer cortex as well as in stage V glomeruli (De Spiegelaere et al., 2011). In order to gain more insight into the molecular mechanism of IA, the present study focuses on the localization of the angiotensin receptors Tie1 and Tie2 in the porcine glomeruli using immunohistochemical staining. Additionally, SEM is used to identify the presence of IA on vascular casts of kidneys and to search for a possible link between the topography of IA and the Tie expression.

Materials and methods

Samples

Porcine gravid uteri were obtained on different occasions in a local slaughterhouse from sows that were slaughtered for human consumption. Fetuses of different fetal stages were collected, i.e. fetuses with a crown-rump length (CRL) of 5.2 cm (E41), 7.5 cm (E48), 9.5 cm (E55), 14 cm (E64), 22 cm (E100) and 29 cm (E112). Their approximate age in embryonic days post conception (E) was deduced from their CRLs (Evans and Sack, 1973). The fetuses were separated into two groups to be further used for either vascular corrosion casting in order to visualize the glomerular capillaries (group 1, 2-5 fetuses/age), or for immunohistochemistry in order to investigate the distribution of the Tie receptors (group 2, 2 fetuses/age).

Vascular corrosion casting

The fetuses from group 1 were removed from their fetal membranes and an umbilical artery in the umbilical cord still attached to the fetus was catheterized with a 26G flexible catheter. After the artery was flushed with isotonic fluid (0.9% NaCl), the second umbilical artery and the umbilical vein were clamped. Mercor II resin and catalyst (Ladd Research, Wommel, Belgium) were mixed (0.5 g of catalyst for 20 ml of resin) and colored with 0.05% (w/v) of a red dye. The resin mixture was then injected through the catheter with gentle pressure using 5 ml plastic syringes until filling of subcutaneous veins was observed or intraperitoneal leakage was discovered. When the polymerization was completed, the fetal tissues were macerated in 25% potassium hydroxide for approximately 2 days followed by rinsing of the resulting cast in running tap water. The obtained casts of the kidney vasculature were washed in multiple changes of distilled water for 2-3 days and left to dry in a fume hood. After the casts were dissected in small pieces, their surface was coated with platinum using the JEOL JFC-1300 (Jeol, Zaventem, Belgium) auto fine coater for further analysis with the JEOL JSM-5600LV (Jeol, Zaventem, Belgium) scanning electron microscope.

SDS PAGE and Western blotting

Kidney tissue from two extra fetuses of E55 and E64 was isolated and homogenized in TNE lysis buffer (50 mM Tris, pH 7.5, 140 mM NaCl, 5 mM EDTA) and a protease inhibitor cocktail (PI, Sigma, Diegem, Belgium) at 4°C. Lysates were clarified by centrifugation for 5 min at 13,000 rpm at 4°C and the supernatant was stored at -20°C until further use. Protein concentration was determined using bovine serum albumin as a standard in a Bradford reagent assay (Bio-Rad, Nazareth, Belgium). Total lysates were separated by SDS-PAGE electrophoresis in non-reducing and non-denaturing conditions, and then blotted to PVDF membranes (Thermo Scientific, Leusden, Netherlands). The membranes were blocked in 5% skimmed milk powder in PBS and then incubated with PBS (negative control) or with 1:1000 anti-Tie1 and anti-Tie2 custom-made antibodies. These affinity-purified polyclonal rabbit antibodies were raised against an antigenic determinant in the tyrosine kinase domain of porcine Tie1 and the fibronectin type III domain of porcine Tie2, respectively (GenScript, USA). Any homology regions between the two receptors were excluded from the antigenic regions chosen for the development of the two primary antibodies. After washing three times with 0.3% Tween 20 in PBS, they were incubated with EnVision labeled polymer-HRP anti-rabbit for 1 h at RT (K4010, Dako, Heverlee, Belgium). Detection was performed by Fast Western Blot Kit, ECL Substrate (Thermo Scientific, Leusden, Netherlands). Western blot signals were acquired and analyzed by ChemiDoc MP Imaging system and the Image Lab software 4.0.1 (Bio-Rad, Nazareth, Belgium).

Immunohistochemistry

The second group of fetuses was removed from the uteri and the kidneys were excised and directly fixed in 4% formalin for 24 h. The specimens were further processed using a STP 420D Tissue Processor (Microm, Prosan, Merelbeke, Belgium) and paraffin embedded with the embedding center EC 350-1 and EC 350-2 (Microm, Prosan, Merelbeke, Belgium). Sections were cut at 5 µm thickness using a HM 360 rotary microtome (Microm, Prosan, Merelbeke, Belgium), adhered to APES-coated slides, dried for 1 h at 56°C and incubated overnight at 37°C. The next day, they were dewaxed in xylene and rehydrated in decreasing alcohol series. Endogenous peroxidase activity was quenched by immersing the slides in a solution of 3% H₂O₂ in methanol for 5 min. The sections were then blocked with 30% bovine serum in PBS. The afore-mentioned primary antibodies, diluted 1:100 in PBS supplemented with 2% bovine serum albumin, were added to the sections and incubated for 1 h. After washing, EnVision labeled polymer-HRP anti-rabbit was added on the slides for 30 min. Following washing, the liquid DAB+ Substrate Chromogen system was added for 5 min. After a nuclear

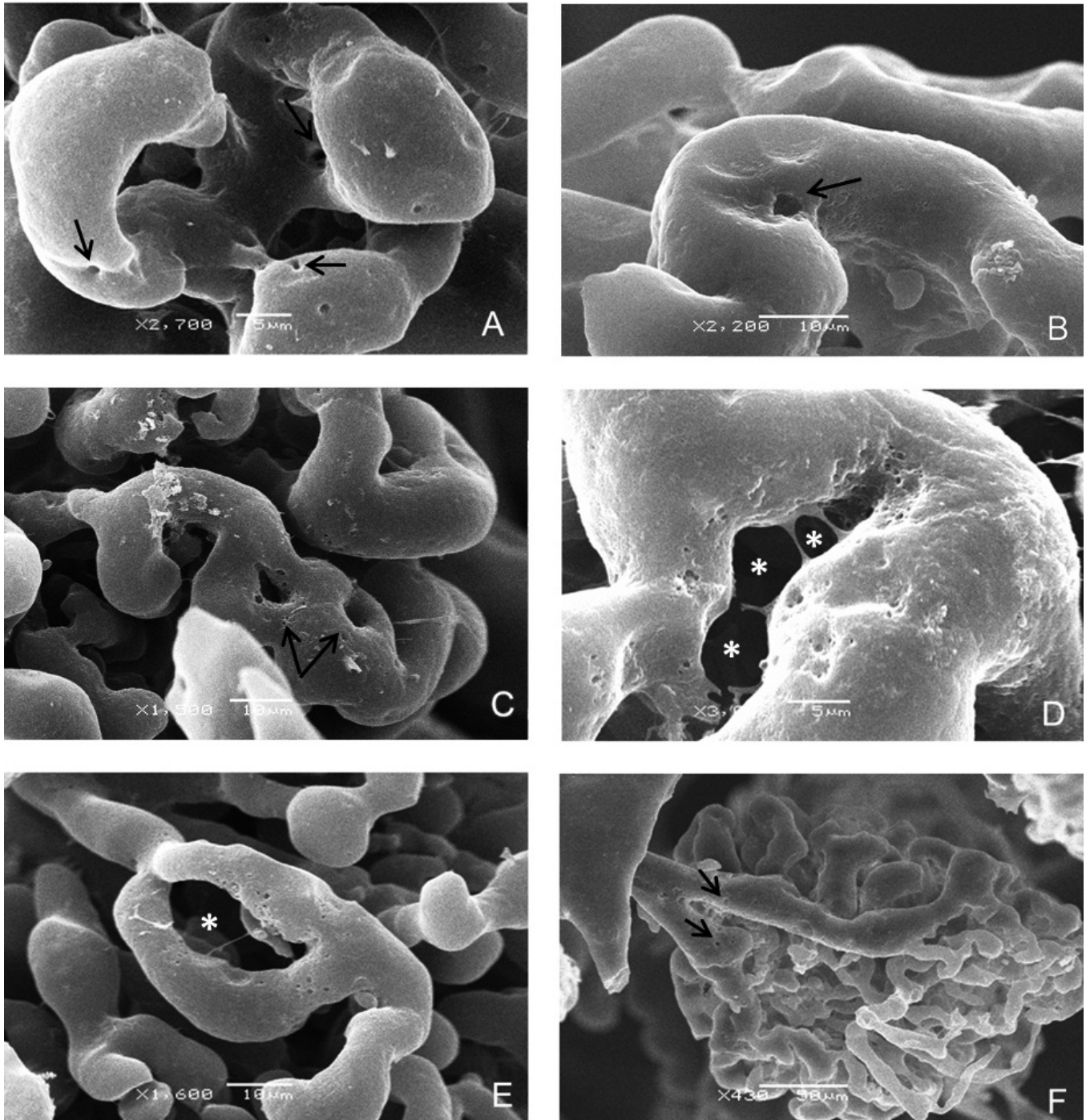
Intussusceptive angiogenesis in the kidney

Fig. 1. Vascular corrosion casts illustrating the process of intussusceptive angiogenesis in the metanephric porcine kidney. Using 2-5 corrosion casts per age, 10 fields per visible glomerulus were examined. **A.** Initially, small depressions appear on the surface of the Mercor casted glomerular capillaries (arrows). These depressions indicate early stages of pillar formation (III glomerulus, E41). **B.** Signs of IA are frequently found next to bifurcations of capillaries (arrow) (IV glomerulus, E64). **C.** As the two opposite components of the pillar approximate and subsequently fuse, the pillar is now represented by a hole that pierces through the vessel cast (arrows) (IV glomerulus, E41). Signs of pillars are often found in a linear arrangement on the casts (V glomerulus, E55). **D, E.** The newly formed vessels are separated longitudinally by pillars that have increased in girth and have fused (asterisks) (V glomerulus, E48). **F.** Pillars are depicted in the two branches of the efferent arteriole (arrows) (V glomerulus, E55).

counterstaining using Mayer's hematoxylin, the slides were mounted with DPX mounting medium (Sigma-Aldrich, Bornem, Belgium) and examined with an Olympus BX61 light microscope (Olympus Belgium NV, Aartselaar, Belgium). As negative controls, the primary antibodies were replaced with PBS or pre-incubated with the blocking peptide. As positive control, sections of human placenta were used.

Results

Evaluation of the corrosion-casted glomeruli

III, IV and V stage glomeruli were identified in the capillary casts according to their shape and diameter. The maturing metanephric V glomeruli had a larger number of capillary loops and were found deeper in the cortex as compared to III and IV glomeruli. The first developmental stage in which small casting holes with a diameter of 1-10 μm were recorded was III glomeruli (Fig. 1A). These holes were also present in IV and V glomeruli. The holes were frequently found next to bifurcations of the capillaries (Fig. 1B) and in linear arrangements (Fig. 1C). Signs of pillar formation appeared first as round holes in the casts. In further stages, the holes were slit-like and appeared to merge with each other along their longitudinal axes (Fig. 1D,E). Interestingly, holes in the cast were not only observed in the capillary bed, but also in the branches of the efferent arteriole (Fig. 1F).

Western blotting

Western Blotting results showed high specificity of the antibodies for the Tie receptors. Anti-Tie1 antibody detected a prominent ~ 120 kDa band whereas anti-Tie2 detected a ~ 250 kDa band. Control blots, in which the primary antibody was omitted, showed no immunoreaction (data not shown).

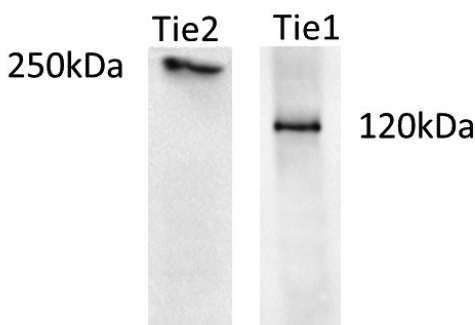


Fig. 2. Western blotting analysis of Tie1 and Tie2 in kidney tissue lysates of a fetus of E64. Images were derived from identical gels loaded with the same amount (12 μg) of the same protein extract samples. A band ~ 120 kDa is recognized by the anti-Tie1 antibody and a band ~ 250 kDa is recognized by the anti-Tie2 antibody.

Immunohistochemistry

Tie1 receptors were present in S-shaped bodies and III, IV, V stage glomeruli, as shown by the strong Tie1 staining (Fig. 3A). More specifically, Tie1 was found in endothelial cells lining the capillary loops of all glomeruli. Podocytes of IV and V glomeruli as well as the parietal epithelium of Bowman's capsule also expressed Tie1 (Fig. 3B). Tie2 receptors were also found in all developmental stages of glomeruli (Fig. 3C), specifically in endothelial cells and podocytes as well as on the epithelium of Bowman's capsule (Fig. 3D). A monolayer of cuboidal epithelial cells which is positioned apically on the immature glomeruli also showed a weak staining for Tie1 and Tie2 (Fig. 3E). In human placenta, which was used as a positive control tissue, Tie staining was present in the endothelium, the chorionic villi, the syncytiotrophoblast and the cytotrophoblast (Fig. 3F). Control sections, in which the primary antibody was either pre-incubated with the blocking peptide (Fig. 3G) or was omitted (Fig. 3H), showed no immunostaining.

Discussion

The analysis of corrosion casts with SEM provides a good approach for screening various tissues for the presence of IA. Holes of diameter ~ 2.5 μm in the vascular casts are representative landmarks for the process of intussusception. Many examples of these findings in different organs and species have been published by other authors (Caduff, 1986; Makanya et al., 2009).

Typical holes in the casts, representing transcappillary pillar formation, were observed in capillary segments of immature and maturing glomeruli. The first signs of IA appeared in III glomeruli and that is possibly when the switch from sprouting to intussusceptive angiogenesis occurs. IA was also present in larger vessels in the kidney such as the efferent arteriole. The presence of pillars in small arteries and veins is supported by other publications (Patan et al., 1993; Djonov et al., 2000). Numerous small depressions were found on the capillary casts, frequently close to bifurcations, which most likely indicate the initial stages of pillar formation. More mature pillars were represented by larger holes piercing through the cast. Further expansion of these holes delineated new vascular entities. Signs of pillar formation were found on corrosion casts of all samples, but the larger fetuses had a higher number of mature glomeruli and therefore more vessels where IA marks could be seen.

Western Blotting results showed that the custom-made antibodies display a high specificity. The anti-Tie1 recognized one ~ 120 kDa protein which has the same molecular weight as the Tie1 receptor. Anti-Tie2 recognized a protein ~ 250 kDa, although the molecular weight of the Tie2 receptor is also around 120 kDa. Similar results have been found previously by

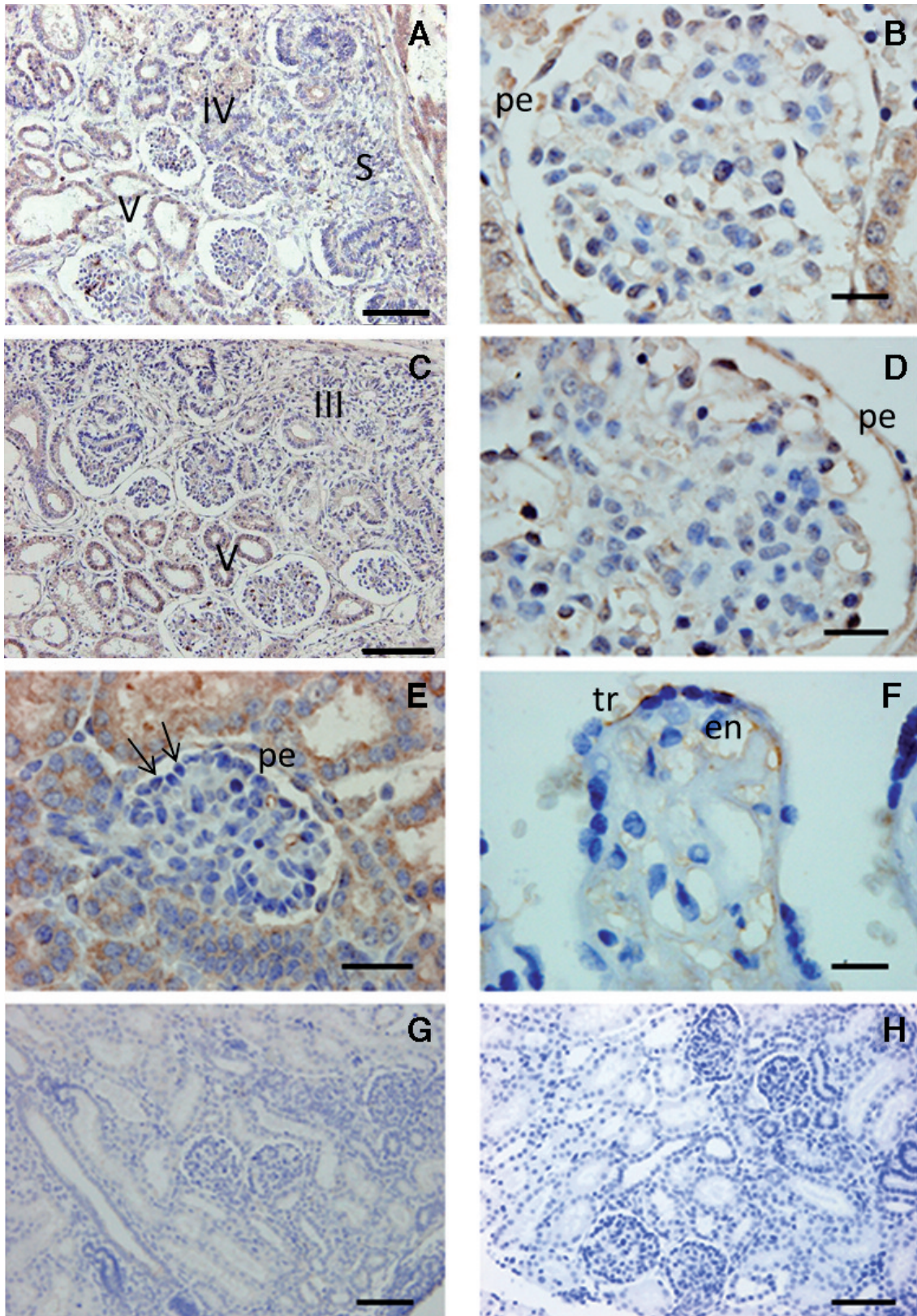


Fig. 3. Tie1 and Tie2 immunostaining of the porcine metanephros. Using 2 kidney samples per age, 10 glomeruli were examined per kidney. **A.** Staining of Tie1 is observed in the S-shaped bodies (S) and all maturation stages of glomeruli (III, IV, V) (E48). **B.** Strong staining of Tie1 is observed in the endothelial cells and the podocytes of the V glomeruli, as well as the parietal epithelial cell layer of Bowman's capsule (pe) (E64). **C.** Tie2 immunostaining is observed in all maturation stages of glomeruli (E100). **D.** Tie2 is strongly expressed in endothelial cells and podocytes of the V glomeruli and pe (E55). **E.** In a IV glomerulus Tie2 is expressed in the endothelial cells and the pe. The cuboidal epithelial cells (arrows) show light staining (E55). **F.** Human term placenta expresses Tie2 in the endothelium (en) and the trophoblast (tr). **G.** Pre-incubation of Tie2 with the blocking peptide results to no staining (E112). **H.** Negative control, in which the primary antibody Tie2 was omitted, shows no staining. Scale bars: A, C, G, H, 100 μm ; B, D, F, 20 μm ; E, 50 μm .

Bogdanovic et al. (2009) who suggested that the minimal oligomeric state of Tie2 on the cell membrane is a dimer.

Immunohistochemical results showed that Tie receptors were present on the cell membrane of endothelial cells in the more mature glomeruli where more capillaries are present. Podocytes, found in V glomeruli, and their precursor cuboidal epithelial cells found in III and IV glomeruli as well as the parietal epithelium of Bowman's capsule also express Tie. Although Tie2 as well as Tie1 were considered endothelial markers, Tie2 staining was also reported in podocytes in human (Satchell et al., 2002) and mice (Dessapt-Baradez et al., 2014) as well as the parietal epithelium of Bowman's capsule of adult human glomeruli (Satchell et al., 2002). The detection of Tie2 on podocytes suggests that there may also be an autocrine loop in the regulation of angiopoietin expression. Additionally, Tie2 was found in several cell types including smooth muscle cells, fibroblasts, epithelial cells, monocytes, neutrophils, eosinophils and glial cells (Makinde and Agrawal, 2008).

Tie1 and Tie2 showed a similar and constant expression pattern in glomeruli, regardless of the fetal age. Likewise, in mice the Tie receptor genes are also expressed from the onset of glomerulogenesis (Loughna et al., 1997; Kolatsi-Joannou et al., 2001). However, mice Tie2 levels increase during kidney development (Yuan et al., 1999), whereas our results show no difference in the expression level of the receptors. The expression of the receptors throughout the metanephric development should probably be explained by focusing on the interplay between the two receptors and the angiopoietins, rather than focusing on each receptor separately. Angiopoietin levels also increase during metanephrogenesis in mice and both show similar levels of expression (Yuan et al., 1999). However, in the study of De Spiegelaere et al. (2011) which focuses on the angiopoietin expression in the different developmental stages of the porcine glomeruli, Angpt1 staining is almost exclusively expressed in mature glomeruli of fetuses >2 cm CRL, whereas Angpt2 expression is strong in maturing III and IV glomeruli and weaker in mature V glomeruli of the developing porcine kidney. Since the receptors are constantly expressed, Angpt1 could act as a vessel maturing factor in the more developed V glomeruli, whereas Angpt2 could lead to either angiogenesis or vascular regression. On the other hand, as presented by Saharinen et al. (2008), the cellular localization of Tie2 after Angpt1 stimulation is crucial: Tie2 in the abluminal side of the endothelial cell leads to growth and proliferation of endothelial cells, whereas Tie2 close to the interendothelial junctions leads to vessel wall stabilization. Therefore it is very important to further investigate the cellular localization of the Tie receptors in the endothelial cells by means of electron microscopy, in order to understand the molecular mechanism of IA in the developing metanephros.

Acknowledgements. The authors wish to thank Patrick Vervaet, Jurgen De Craene and Lobke De Bels for their excellent technical assistance. This work was financially supported by the BOF project (01N01013) of Ghent University.

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