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Intussusceptive angiogenesis in the developing porcine kidney: morphologic characterization of the initial phases and the involvement of angiopoietin receptors

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Intussusceptive angiogenesis in the developing porcine kidney:
morphologic characterization of the initial phases and the involvement of angiopoietin receptors

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Dissertation submitted in fulfillment of the requirements for the degree of Doctor in Veterinary Sciences (PhD)

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List of abbreviations

2D:	Two-dimensional
3D:	Three-dimensional
ANGPT:	Angiopoietins
ANGPT1:	Angiopoietin 1
ANGPT2:	Angiopoietin 2
BSA:	Bovine Serum Albumin
CAM:	Chorioallantoic Membrane
Co:	Collagen
CRL:	Crown Rump Length
CT:	Computed Tomography
DAB:	Diaminobenzidine
Dll4:	Delta-like 4 protein
DOK-R:	Docking protein-R
E:	Embryonic age in days post conception
EC:	Endothelial Cell
ECM:	Extracellular Matrix
EGF:	Epidermal Growth Factor
eNOS:	Endothelial Nitric Oxide Synthase
ERK:	Extracellular Regulated Kinase
FAK:	Focal Adhesion Kinase
Fb:	Fibroblast
FE-SEM:	Field Emission-Scanning Electron Microscopy
FGF:	Fibroblast Growth Factor
FOXO1:	Forkhead box protein O1
GBM:	Glomerular Basement Membrane
GCX:	Glycocalyx
GEC:	Glomerular Endothelial Cells
HRP:	Horse Radish Peroxidase
IA:	Intussusceptive Angiogenesis

IAR: Intussusceptive Arborization Remodeling

IBR: Intussusceptive Branching Remodeling

IMG: Intussusceptive Microvascular Growth

mRNA: Messenger Ribonucleic Acid

P: Podocyte

PAX-2 Paired box gene 2

PBS: Phosphate Buffered Saline

PDGF-β: Platelet Derived Growth Factor β

PI3K-Akt: Phosphatidylinositol 3-Kinase-Protein Kinase B

PlGF: Placental Growth Factor

PP: Podocyte Process

Pr: Pericyte

RLI: Relative Labeling Index

RT: Room Temperature

SA: Sprouting Angiogenesis

SEM: Scanning Electron Microscopy

TEM: Transmission Electron Microscopy

TIE1: TIE1 Tyrosine Kinase Receptor

TIE2: TIE2 Tyrosine Kinase Receptor

TNF-α: Tumor Necrosis Factor-alpha

TGF-β: Transforming Growth Factor-beta

VE-PTP: Vascular Endothelial-Protein Tyrosine Phosphatase

VEGF: Vascular Endothelial Growth Factor

VEGF-A: Vascular Endothelial Growth Factor A

VEGFR: Vascular Endothelial Growth Factor Receptor

VEGFR-1: Vascular Endothelial Growth Factor Receptor 1

VEGFR-2: Vascular Endothelial Growth Factor Receptor 2

VEGFR-3: Vascular Endothelial Growth Factor Receptor 3

WT1: Wilms Tumor Protein 1

Chapter I: General introduction

1 Angiogenesis

The establishment of a well-organized circulatory system is considered a pivotal step in the development of large multicellular organisms. Blood vessels provide the highways for blood trafficking including the delivery of oxygen, inflammatory and progenitor cells, as well as the removal of waste products (Logsdon et al., 2014). The emergence of blood vessels occurs via vasculogenesis and angiogenesis. Vasculogenesis is the de novo formation of a primitive vascular plexus from endothelial precursor cells, whereas angiogenesis is defined as the process of further expansion and remodeling of a pre-existing vascular plexus (Carmeliet, 2000). During early embryonic development, the initial vascular plexuses are formed by vasculogenesis, but embryos require angiogenesis during organ growth and further development (Carmeliet, 2005).

Angiogenesis is an important mechanism for vascular network remodeling not only in the developing, but also in the adult animal (Riseau, 1997). In adults, angiogenesis occurs in conditions requiring an increase in blood and oxygen supply, including reproduction (e.g. cyclic organ growth), physiological repair (e.g. wound healing) and exercise (Egginton, 2009; Sung et al., 2010). Angiogenesis is also associated with several diseases, notably various cancers and ocular disorders, but also cardiovascular diseases, chronic inflammation, psoriasis and vascular malformations (Griffioen and Molema, 2000; Carmeliet, 2003). On one hand, angiogenesis can be advantageous in many diseases which are characteristized by lack or regression of blood vessels, such as pre-eclampsia, ischemia, and osteoporosis (Ferrara and Alitalo, 1999; Carmeliet, 2005; Logsdon et al., 2014). On the other hand, uncontrolled angiogenesis can also aggravate the pathology e.g. in tumors, atherosclerosis, inflammatory bowel disease, arthritis and diabetic retinopathy (Carmeliet, 2005). Taking all the above into account, the process of angiogenesis is a very important therapeutic target and a better understanding of its mechanism will facilitate the development of pro- and antiangiogenic therapies (Potente et al., 2011; De Spiegelaere et al., 2012).

1.1 Angiogenic mechanisms

To date, three different angiogenic mechanisms have been identified. Sprouting angiogenesis (SA) is the oldest reported and most investigated mechanism of angiogenesis (Clark and Clarck, 1939; Mato and Ookawara, 1982). In addition to SA, intussusceptive angiogenesis (IA) (Djonov et al., 2002) and looping angiogenesis, have been described (Benest and Augustin, 2009; Kilarski and Gerwins, 2009). Although SA (chapter 1.1.1) has been the main focus of angiogenic research, the importance of the other two mechanisms has been proved beyond doubt. Intussusceptive angiogenesis (chapter 1.1.3) has gained a lot of attention because of its role not only in embryonic development, but also because of its implication in cancer vasculature (Hlushchuk et al., 2008). Finally, looping angiogenesis (chapter 1.1.2), although only recently discovered, plays an important role in wound healing (Kilarski et al., 2009).

1.1.1 Sprouting angiogenesis

During sprouting angiogenesis a vascular sprout arises on a pre-existing vessel and forms a new vascular branch (Fig. 1). The process of capillary sprouting is initiated with the conversion of a previously quiescent endothelial cell into a tip cell via Vascular Endothelial Growth Factor (VEGF), Notch, and Delta-like 4 protein (Dll4) signalling (Metzger and Krasnow, 1999). The tip cell forms cytoplasmic projections called filopodia which probe the surrounding environment for angiogenic stimuli. These filopodia secrete large amounts of proteolytic enzymes, which digest a pathway through the extracellular matrix (ECM) for the developing sprout (Carmeliet, 2000). Meanwhile, the capillary sprout elongates because endothelial stalk cells proliferate as they follow behind the tip cell. Blood flow drives lumen expansion during sprouting angiogenesis in vivo by inducing spherical deformations of the apical membrane of endothelial cells, in a process termed inverse blebbing (Gebala et al., 2016). When the tip cells of two or more capillary sprouts converge, the tip cells fuse together creating a continuous lumen through which blood can

flow. Maturation and stabilization of the capillary require recruitment of pericytes and deposition of ECM (Schmidt and Carmeliet, 2010).

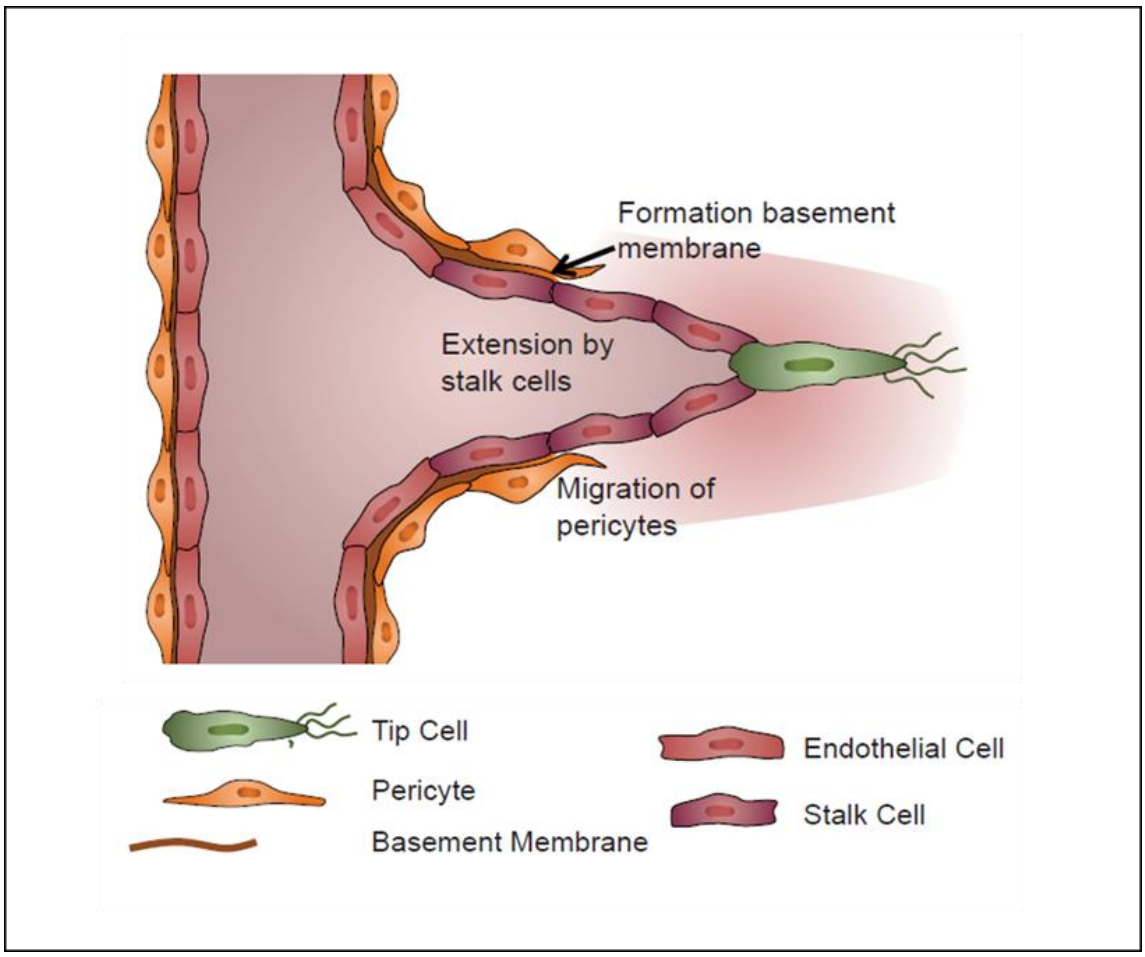

Fig. 1. Graphic representation of an angiogenic sprout. Adapted from Rice et al. (2012).

1.1.2 Looping angiogenesis

Looping angiogenesis is a mechanism of neovascularization recently identified in the chick chorioallantoic membrane (CAM) and the healing mouse cornea models (Kilarski et al. 2009). Looping angiogenesis is not regulated by vessel-specific cells, such as pericytes and endothelial cells, but is a mechanically driven mode of vessel translocation. During this mechanism, fibroblasts and proto-myofibroblasts initially migrate and populate the provisional matrix, which is surrounded by a vascularized region. Subsequently, these cells differentiate into myofibroblasts, which then remodel and contract the matrix causing translocation of the surrounding vasculature into the provisional matrix. These mechanical forces pull vessels from the preexisting vascular bed as vascular loops with functional circulation (Kilarski et al. 2009; Rice et al., 2012) (Fig. 2).

A mechanism similar to looping angiogenesis, named vascular co-option, is present in tumor vasculature. The tumor coopts the blood vessels of the surrounding tissue leading to their subsequent integration in the tumor (Holash et al., 1999; Qian et al., 2016). This way, tumors grow to a certain extent without eliciting a specific angiogenic response.

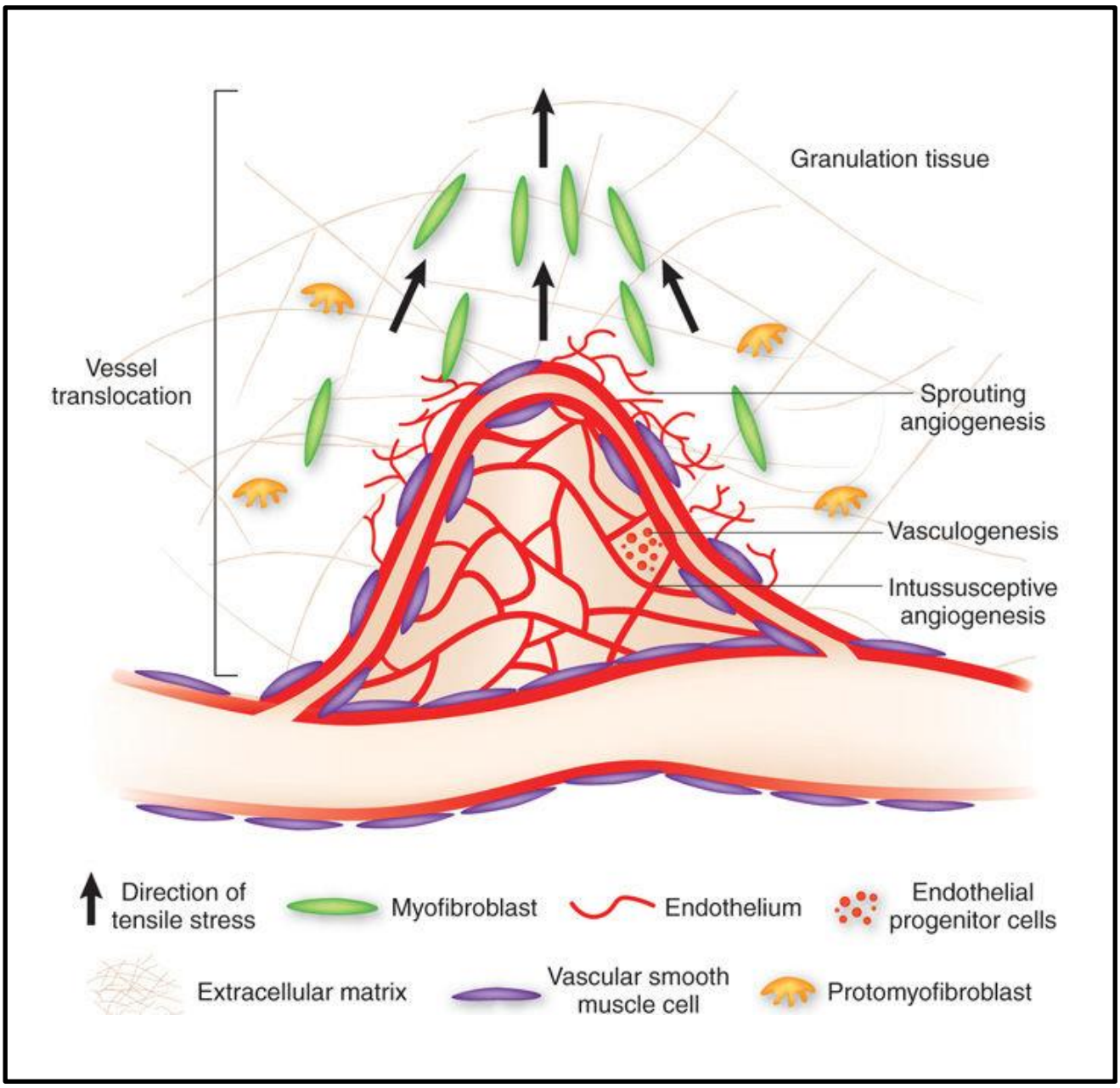

Fig. 2. Graphic representation of the mechanisms of tissue vascularization: vasculogenesis and angiogenesis (sprouting and intussusceptive). Looping angiogenesis proposes the translocation of intact vessels by biomechanical forces as a third mechanism of angiogenesis. (From Benest and Augustin, 2009).

1.1.3 Intussusceptive angiogenesis

The mechanism of IA was first discribed in the rat pulmonary microcirculation by Caduff et al. (1986) although earlier reports discovered a similar process in skeletal muscles (Ogawa Y, 1977; Appell, 1980). Using vascular corrosion casts and scanning electron microscopy (SEM), Caduff et al. (1986) observed small holes in the sheet-like alveolar microvasculature. These regular and non-random holes were temporally and spatially associated with rapid expansion of the microcirculation. Importantly, the diameter of the new alveolar capillaries was smaller suggesting that the holes were involved not only in capillary replication, but also in capillary remodeling. The authors concluded that the small holes reflected a mechanism of "in-itself" or "intussusceptional" growth. These holes corresponded to thin transcapillary (intraluminal) tissue pillars, spanning the lumen of the blood vessels. The intraluminal pillars are considered the characteristic features of the morphogenetic process of intussusception (Caduff et al. 1986; Burri and Tarek, 1990; Patan et al., 1996).

Ever since, intravascular pillars have been identified in small vessels in a variety of experimental models. A few examples of IA are the developing avian kidney (Makanya et al., 2005), the porcine mesonephros (De Spiegelaere et al., 2010), the developing chick chorioallantoic membrane (Makanya et al., 2009) and the physiologic angiogenesis associated with skeletal muscle training (Egginton et al., 2001). Similar intraluminal pillars have also been described in a variety of tumors (Ribatti and Djonov, 2012) and in the angiogenic response following chemically-induced murine colitis (Konerding et al., 2010).

1.1.3.1 Mechanism of intussusceptive angiogenesis

The process of IA can be divided in four phases. Initially, the endothelium is quiescent (Fig. 3a). Endothelial cells directly opposite from one another within the capillary wall are drawn towards each other and form a small "interendothelial transluminal bridge" (Fig. 3b). After this contact, the endothelial bilayer forms a perforation at the center of the capillary, creating a cylindrical tissue bridge that extends across the lumen and is lined by extensions of endothelial cells. An interstitial pillar core is formed and successively invaded by cytoplasmatic extensions of migrating myofibroblasts and pericytes and subsequently by interstitial fibers (Fig. 3c). In the last phase (Fig. 3d), the slender pillar grows and fuses with adjacent pillars (pillar diameter > 2.5 µm) (Burri and Tarek 1990; Burri et al., 2004). This process eventually leads to remodeling and separation of the initial capillary into two capillaries.

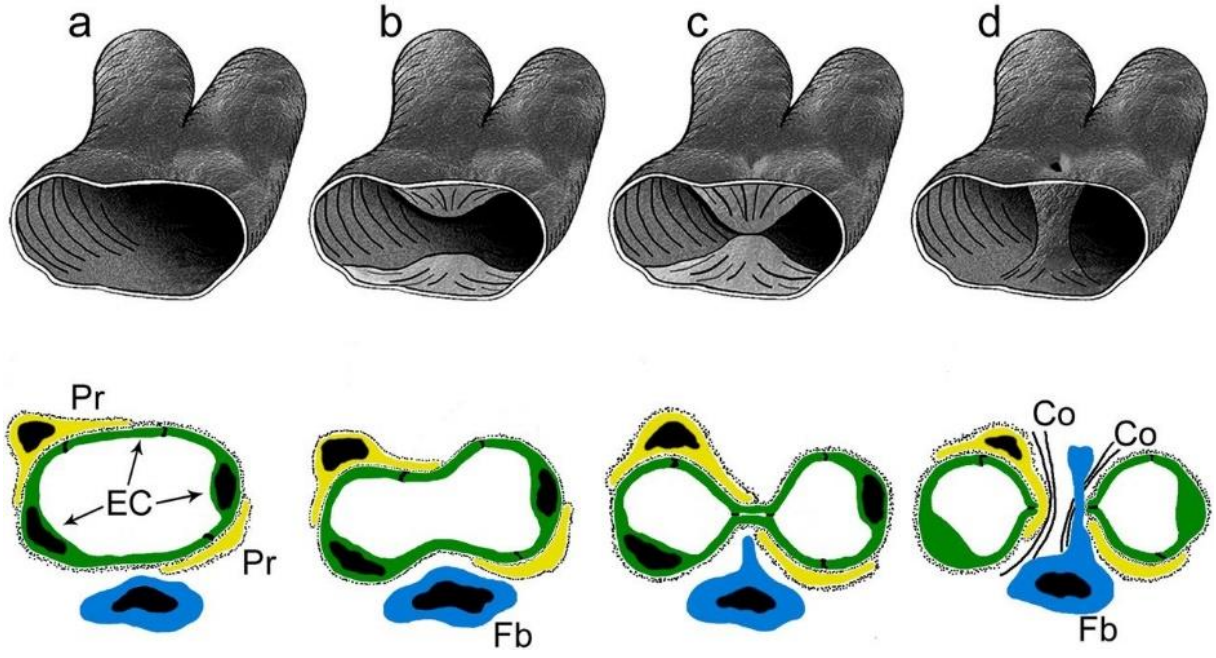

Fig. 3. 3D (top half) and 2D (bottom half) representation of the 4 phases of intussusceptive angiogenesis. (a) quiescence, (b) interendothelial bridge formation, (c) pillar core invasion by pericytes and (d) growth and fusion of pillars leading to vessel splitting. Pr: pericyte, EC: endothelial cell, Fb: fibroblast, Co: collagen. Adapted from Burri et al. (2004).

1.1.3.2 Pillar ultrastructure

Due to their intraluminal location, intussusceptive pillars have been largely defined by vascular corrosion casting and SEM (Patan et al., 1996; Makanya et al., 2009). Nevertheless, corrosion casts provide very little information regarding the cellular or extracellular composition of the pillar, since the surrounding tissue is digested away. To visualize the ultrastructural detail of the endothelial cells and the tissue comprising the pillar, the most commonly used approach is transmission electron microscopy (TEM) (Nico et al., 2007). Without a sufficiently high prevalence of intussusceptive pillars with a predictable orientation, it is difficult to reliably identify pillars in 2D (Mentzer et al., 2014). Because of the potential that other structures are misconceived as pillars, serial sections are recommended. Even though TEM is time-consuming and has a high cost, it has identified the composition of the pillars in several tissues including skeletal muscle (Williams et al., 2006), murine colitis (Konerding et al., 2010) and experimental subcutaneous tumors (Paku et al., 2011).

The mechanism of pillar formation is still not entirely understood. It was initially believed that perivascular cells or pericytes may play a role in the initial steps by exerting a pushing force on the vessel wall (Burri et al., 2004). This concept was questioned by Paku et al. (2011), who presented a detailed model of pillar formation, named inverse sprouting, in tumor-induced IA. During this process, endothelial bridges are formed and subsequently, the bridge-forming endothelium attaches to a type I collagen bundle in the underlying connective tissue. A pulling force is then exerted by the actin cytoskeleton of the endothelial cell to the collagen bundle, resulting in the transport of the latter through the vessel lumen. This model was the first to identify the force behind pillar formation, but it still remains to be proven whether the same mechanism occurs in healthy conditions too.

1.1.3.3 Types of intussusceptive angiogenesis

Pillar formation and growth can duplicate an existing vessel (Djonov et al., 2000a; Egginton et al., 2001), modify the branching angle of a bifurcating vessel (Djonov et al., 2002; Ackermann et al., 2013) and prune a redundant or energetically inefficient vessel (Lee et al., 2011). The selective growth or extension of intravascular pillars can efficiently modify vessel structure resulting in different phenotypes of the vasculature.

Intussusceptive microvascular growth (IMG) permits rapid expansion of the capillary plexus and increases the complexity of capillary beds. Intussusceptive arborization remodeling (IAR) can be recognized by the occurrence of a series of pillars and is involved in the formation of immediate pre- and postcapillary vessels. Intussusceptive branching remodeling (IBR) leads to optimization of the branching geometry and the hemodynamic conditions of the vascular tree. IBR can also lead to the removal of branches by pruning in response to changes in metabolic needs (Djonov et al., 2003) (Fig. 4).

All types of intussusceptive angiogenesis are fast and have a low metabolic cost for the organism, in contrast to sprouting angiogenesis. Blood vessels are generated more rapidly since it does not directly require cell proliferation, only migration and rearrangement of the existing vessel architecture (Djonov et al., 2003; Ribatti and Djonov, 2012).

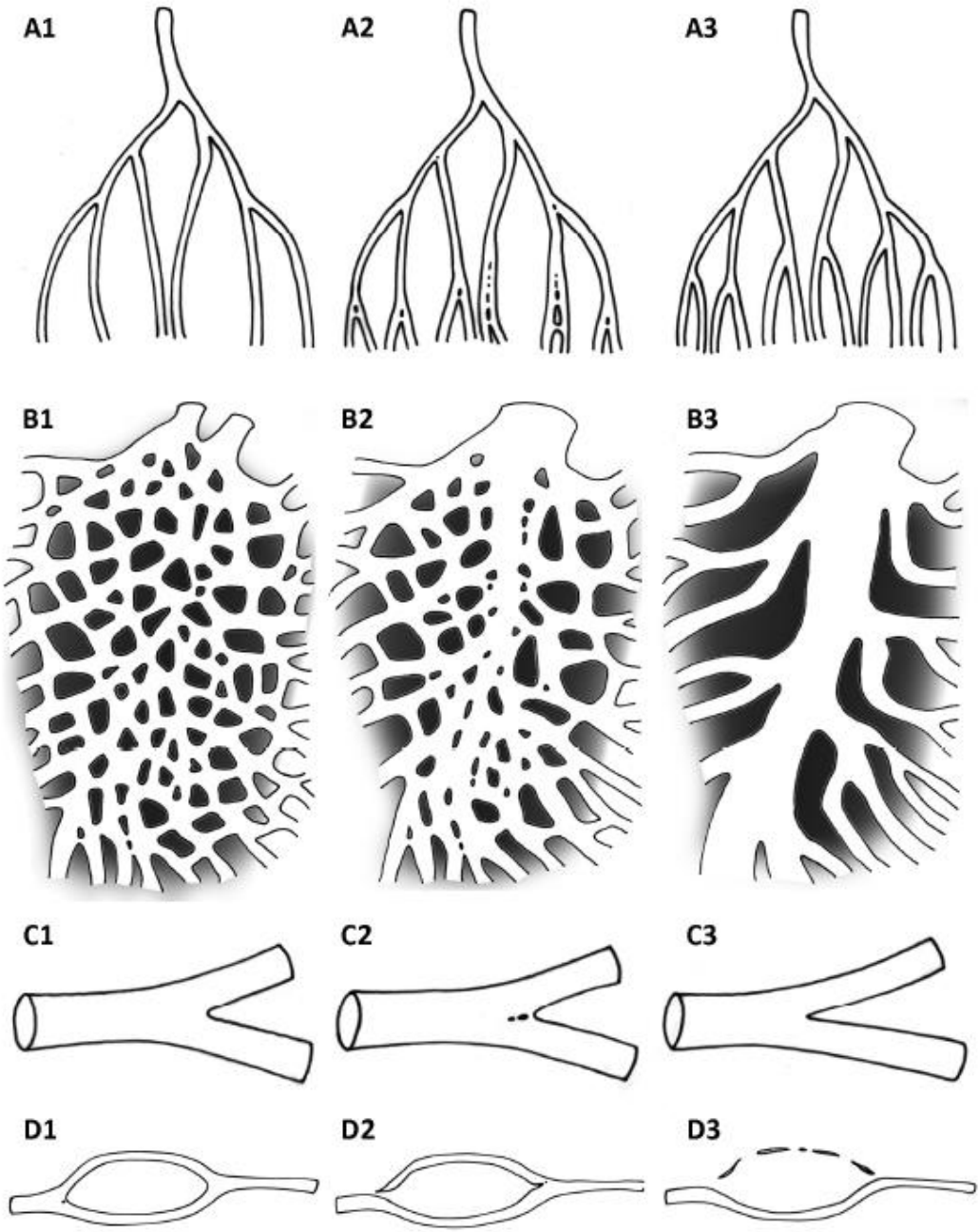

Fig. 4. Schematic illustration presenting the different types of intussusceptive angiogenesis. (A) intussusceptive microvascular growth, (B) intussusceptive arborization remodeling, (C) intussusceptive branching remodeling and (D) intussusceptive pruning. (De Spiegelaere, 2011).

1.2 Molecular mediators in angiogenesis

The process of angiogenesis is regulated by a wide spectrum of angiogenic and angiostatic factors. The most commonly described angiogenic growth factors and cytokines include vascular endothelial growth factors (VEGF), the angiopoietins (ANGPT), fibroblast growth factor (FGF), tumor necrosis factor-alpha (TNF-α), transforming growth factor-beta (TGF-β) and platelet derived growth factor (PDGF). Sources of these growth factors include endothelial cells, fibroblasts, smooth muscle cells, platelets, inflammatory cells and cancer cells (Ferrara N and Alitalo K, 1999; Kubis and Levy, 2003; Bouis et al., 2006; Ucuzian et al., 2010).

VEGF and ANGPT (Fig. 5) are key players governing the process of angiogenesis (Carmeliet, 2000; Kässmeyer et al., 2009; Jeltsch et al., 2013). VEGF receptors (VEGFR 1-4) and angiopoietin receptors (TIE1 and TIE2) are receptor tyrosine kinases largely restricted to endothelial cells, but they are also expressed in a few other cell types, such as hematopoietic progenitor cells, a subset of megakaryocytic cells and TEMs (TIE2 expressing monocytes) (Sato et al., 1998; Olsson et al., 2006; De Palma et al., 2013; Ito et al., 2016). While the current research focuses on the ANGPT-TIE system, a small introduction will be given about the VEGF family, too, due to its crucial role in angiogenesis.

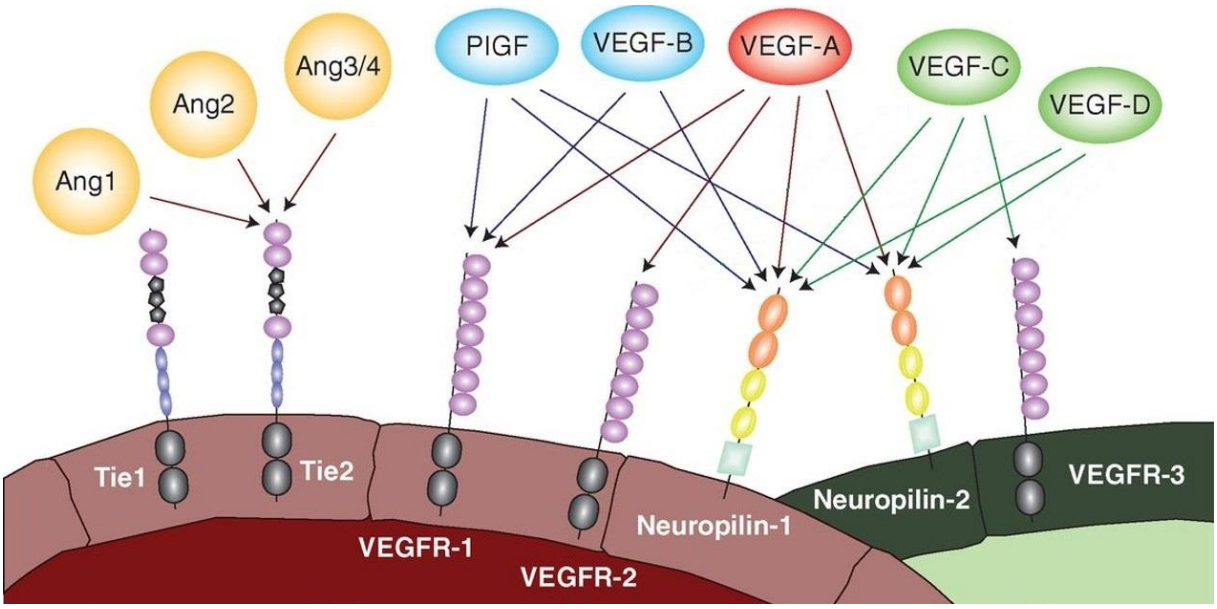

Fig. 5. Schematic presentation of TIE and VEGF receptors and their ligands in mammalian endothelial cells of blood vessels (red) and lymph vessels (green). Adapted from Jeltsch et al. (2013).

1.2.1 VEGF-VEGFR system

The mammalian vascular endothelial growth factors, VEGF-A, VEGF-B, VEGF-C, VEGF-D and placenta growth factor (PlGF) show preferential binding to the VEGF receptors (Ferrara, 2000; Jeltsch et al. 2013). There are three VEGF receptors, of which VEGFR-1 and VEGFR-2 are expressed in blood vascular endothelial cells whereas VEGFR-3 is expressed in lymphatic endothelial cells. Neuropilin-1 acts as a co-receptor for VEGFR-2 and neuropilin-2 acts as a co-receptor for both VEGFR-2 and VEGFR-3 (Favier et al., 2006; Karpanen et al., 2006).

From the three VEGFR, only VEGFR-2 and VEGFR-3 drive angiogenesis whereas VEGFR-1 mostly acts to restrict angiogenic responses (Ho et al., 2012) and to recruit macrophages for tissue remodeling (Pipp et al., 2003). Ligand binding to VEGFR-2 induces a robust tyrosine phosphorylation and results in a strong angiogenic response (Waltenberger et al., 1994). Stimulation of VEGFR-3 elicits a similar response in lymphatic endothelial cells (Tammela et al., 2008).

VEGF-A is the most potent and best described angiogenic growth factor in the VEGF family. It induces endothelial migration, functions as a survival factor for endothelial cells and enhances vascular permeability (Otrock et al., 2007). During SA, VEGF-A acts as chemoattractant to the migrating tip cells and induces proliferation of stalk cells, resulting in the guidance and growth of the newly formed sprouts (Gerhardt et al., 2003). Specific isoforms of VEGF-A play an important role in IA too, since inhibition of VEGF-signalling decelerates intussusceptive-dependent capillary maturation in the CAM vasculature (Baum et al., 2010).

1.2.2 ANGPT-TIE system

Angiopoietins and their receptors (TIE) form the second endothelial growth factor receptor signalling pathway, which regulates blood and lymphatic vessel remodeling after the VEGF-driven phase of active angiogenesis. The ANGPT-TIE system contributes to vascular homeostasis by regulating endothelial barrier function, inflammation and vessel remodeling. It also plays an important role in angiogenesis and lymphangiogenesis in mature and pathological tissues (Augustin et al., 2009; Eklund and Saharinen, 2013).

The ANGPT–TIE system consists of endothelial TIE1 and TIE2 receptor tyrosine kinases and the ligands of TIE2, ANGPT1, ANGPT2 and ANGPT4 (the latter representing a human orthologue for mouse ANGPT3) (Partanen et al., 1992; Dumont et al., 1993; Sato et al., 1993; Kim et al., 1999; Valenzuela et al., 1999). ANGPT3/4 are the least studied angiopoietins, mostly investigated in experimental models of tumor angiogenesis and metastasis. ANGPT3 inhibits pulmonary metastasis in mice (Xu et al., 2004) whereas ANGPT4 can both, promote glioblastoma progression by enhancing tumor angiogenesis (Brunckhorst et al., 2010) and inhibit angiogenesis induced by GLC19 tumor cells (Olsen et al., 2006). The opposite results of ANGPT4 in humans have derived from different studies and might be due to different tumor microenvironments.

ANGPT1-2 are the best studied angiopoietins and the moste important ligands of TIE2. ANGPT1 is an obligatory TIE2 agonist expressed by mesenchymal cells surrounding the blood vessels (Davis et al., 1996). In contrast with paracrine ANGPT1, ANGPT2 is expressed by endothelial cells and acts as an autocrine context-dependent agonist/antagonist of TIE2 (Maisonpierre et al., 1997). ANGPT2 is stored in endothelial cells in intracellular secretory granules named Weibel–Palade bodies (Fiedler et al., 2004; Scharpfenecker et al., 2005). Inflammatory and hypoxic stimuli increase ANGPT2 expression, decreasing vascular stability and promoting endothelial activation, neoangiogenesis and remodeling (Oliner et al., 2004; Fiedler et al., 2006; Benest et al., 2013; Le et al., 2015).

ANGPT2 agonist/antagonist function has not been fully elucidated, but it may depend on ANGPT2 multimerization or structural differences in the receptor-binding interface (Yu et al., 2013). Conversely, ANGPT1 promotes vessel stability in adults (Thurston et al., 2000), inhibits tissue fibrosis (Jeansson et al., 2011) and mediates vessel normalization during anti-angiogenic therapy (Koh, 2013).

TIE receptors are almost exclusively expressed in endothelial cells although TIE2 is also expressed in certain human haematopoietic cell lineages (Armstrong et al., 1993; Batard et al., 1996; Sato et al., 1998). TIE2 is also found in human and murine TIE2-expressing monocytes, macrophages and muscle satellite cells located among skeletal muscle myofibres in association with the microvasculature (Abou-Khalil et al., 2009; De Palma et al., 2013; Doan et al., 2013; Ito et al., 2016).

TIE1 remains an orphan receptor with no identified ligand, despite overall homology with TIE2, especially in the intracellular tyrosine kinase domain (Partanen et al., 1992). TIE1-TIE2 interactions have been already implicated in the regulation of ANGPT1-induced TIE2 signal transduction, indicating ligand-independent functions of TIE1 (Saharinen et al., 2005; Seegar et al., 2010). Recently, it was determined that both ANGPT1 and ANGPT2 binding to TIE2 increases TIE1-TIE2 interactions in a β1

integrin–dependent manner (Korhonen et al., 2016). TIE1 directly interacts with TIE2 to promote ANGPT-induced vascular responses under noninflammatory conditions, whereas in inflammation, TIE1 cleavage contributes to loss of ANGPT2 agonist activity and vascular stability (Korhonen et al., 2016).

1.2.2.1 ANGPT-TIE signalling

In vitro experiments on human endothelial cell lines showed that ANGPT induce translocation and activation of the TIE receptors in certain subcellular compartments, dependent on the cell microenvironment, and may partly explain versatile functions of angiopoietins during vessel quiescence and remodeling (Fukuhara et al., 2008; Saharinen et al., 2008; Pietila et al., 2012). In contacting endothelial cells, such as those in the quiescent vasculature, ANGPT1 induces the formation of trans TIE receptor signalling complexes across the endothelial junction. These junctional TIE complexes mediate cell survival signals via the phosphatidylinositol 3-kinase (PI3K-Akt) pathway, which results in activation of the endothelial nitric oxide synthase (eNOS) (Kim et al., 2000). Akt also phosphorylates the transcription factor forkhead box O1 (FOXO1), inducing its nuclear exclusion and the reduced expression of FOXO1 target genes involved in metabolic and cell growth regulation (Wilhelm et al., 2016). On the contrary, in mobile ECs, matrix-bound ANGPT1 activates TIE2 in endothelial-extracellular matrix (EC–ECM) adhesions, promoting extracellular-regulated kinases (ERK) and docking protein-R (DOK-R) activation, matrix adhesion and cell migration (Fukuhara et al., 2008; Saharinen et al., 2008) (Fig. 6).

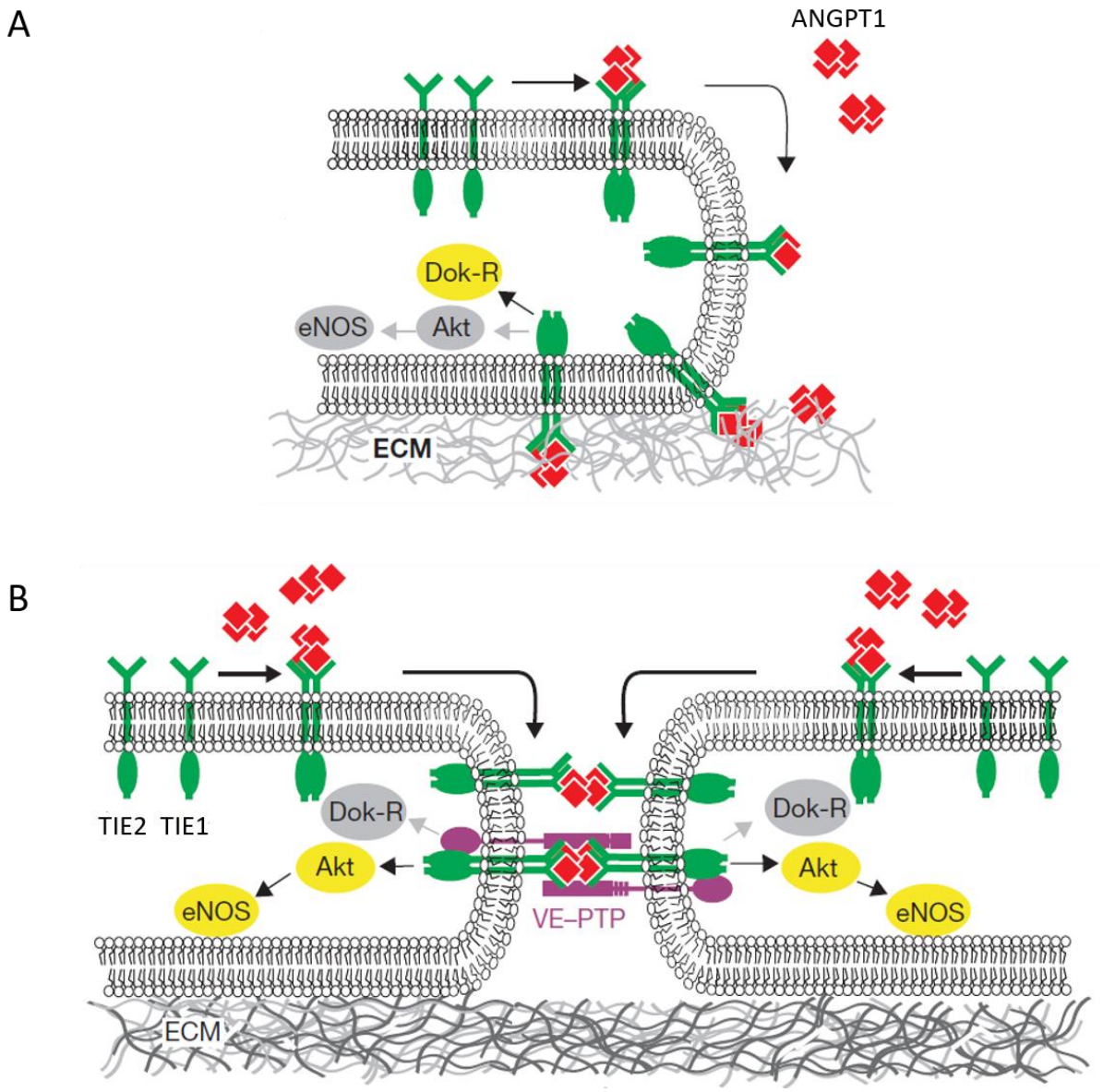

Fig. 6. Schematic representation of a model of ANGPT1–TIE2 interactions at (A) cell–matrix and at (B) cell–cell contacts in cultures of human lung microvascular endothelial cells. Akt: protein kinase B, eNOS: endothelial nitric oxide synthase, Dok-R: docking protein-R, VE–PTP: vascular endothelial protein tyrosine phosphatase. Adapted from Saharinen et al. (2008).

ANGPT2 also induces TIE2 translocation to cell–cell junctions, but activates TIE2 only weakly (Maisonpierre, 1997; Saharinen et al., 2008). Since ANGPT1 and ANGPT2 bind in a similar fashion to TIE2 (Barton et al., 2006; Yu et al., 2013), ANGPT2 binding may lead to inhibition of ANGPT1-induced TIE2 signalling, especially when the ANGPT2/ANGPT1 ratio is elevated, such as in the tumor vasculature. Attenuation of the

ANGPT1–TIE2 pathway can then lead to an increase in nuclear FOXO1, which stimulates ANGPT2 gene transcription (Kim et al., 2016; Korhonen et al., 2016). Furthermore, ANGPT2, but not ANGPT1, induces TIE2 translocation into specific extracellular matrix contact sites that may weaken EC–ECM adhesion (Pietila et al., 2012).

1.2.2.2 ANGPT-TIE cooperation with integrins

In addition to TIE receptors, angiopoietins have been reported to interact with multiple integrin cell adhesion receptors both in endothelial and non-endothelial cells. ANGPT2 can stimulate focal adhesion kinase (FAK) phosphorylation via integrins in murine TIE2-low endothelial cells and in human tumor cells, thereby promoting cell migration (Felcht et al., 2012; Lee et al., 2014). In transgenic mice, ANGPT2-induced translocation of the α5β1-integrin into the ends of actin fibers stimulates the formation of actin stress fibers, leaky endothelial junctions and destabilization of the endothelium (Hakanpaa et al., 2015). Furthermore, in bovine and human endothelial cell lines, α5β1-integrin is required for ANGPT1 induced formation of a TIE1-TIE2 receptor complex in endothelial junctions, TIE2 phosphorylation and downstream FOXO1 phosphorylation (Daly et al., 2004; Korhonen et al., 2016). In summary, these results indicate that ANGPT1, ANGPT2 and TIE2 control the EC-ECM interplay together with the integrins, which may also serve as co-receptors for angiopoietins (Eklund et al., 2017).

1.2.2.3 ANGPT-TIE during intussusceptive angiogenesis

The ANGPT-TIE system appears to act at a later stage of neovessel formation compared to the VEGF-VEGFR system (Augustin et al., 2001). Angiopoetins are likely candidates for mediating cell-cell interactions during intussusceptive angiogenesis (Augustin, 2001; Kurz et al., 2003). Indeed, there are indications that the ANGPT-TIE system is involved in controlling IMG (Sato et al., 1995; Suri et al., 1996). The vasculature

of mice lacking ANGPT1 and TIE2 remains at a primitive stage of development and fails to undergo further remodeling. Targeted deletion of TIE2 expression in mice leads to deficient pillar formation (Patan, 1998). Additionally, ANGPT1 overexpression in combination with VEGF is characterized by the presence of abundant small holes at vessel bifurcations, a finding that is symptomatic of intussusception (Burri and Tarek, 1990; Thurston et al., 1999; Thurston et al., 2005). Depending on the presence or absence of VEGF-A, ANGPT2 induces angiogenesis or vascular degeneration, respectively (Lobov et al., 2002; Scharpfenecker et al., 2005). In addition, overexpression of ANGPT2 affects ongoing intussusceptive angiogenesis in the CAM as it leads to the remodeling of a previously uniform capillary mesh into an arborized vascular tree (Winnik et al., 2009).

1.2.2.4 ANGPT-TIE signalling in inflammation

As agonist and antagonist of TIE2, ANGPT1 and ANGPT2 respectively, represent the balance between resting and activated vascular endothelium. ANGPT1 exerts potent anti-inflammatory effects (Thurston et al., 1999; Thurston et al., 2000; Baffert et al., 2006) and therefore higher expression of ANGPT1 can be considered as a switch that controls the transition from inflammatory or pro-angiogenic to resting endothelium (Imhof and Aurrand-Lions, 2006). On the contrary, ANGPT2 acts in synergy with inflammatory cytokines (Benest et al., 2013). As a result, high levels of ANGPT function as a built-in switch controlling the transition of the resting quiescent endothelium towards the activated endothelium (Fiedler and Augustin, 2006).

1.2.2.5 ANGPT-TIE signalling in disease

The ANGPT–TIE system is involved in tumor angiogenesis as well. In a wide range of tumors, although absolute levels of either angiopoietin may increase or decrease, the ratio of ANGPT1:ANGPT2 shifts in favour of ANGPT2 (Tait and Jones, 2004). Given the fact that ANGPT2 is a destabilization factor, it is suggested that tumors shift the

angiogenic balance towards a pro-angiogenic state through altering the balance between the angiopoietins. This has implicated ANGPT2 as a candidate for the angiogenic switch during tumorigenesis and extensive research has been dedicated to its role as a therapeutic target. More specifically, ANGPT2 blocking biologicals and genetic deletion of TIE1 decreased tumor angiogenesis in mice by reducing cell proliferation and endothelial sprouting and decelerated tumor growth by inducing vessel regression and endothelial apoptosis (Hashizume et al., 2010; D'Amico et al., 2014). Furthermore, anti-ANGPT2 monoclonal antibodies and peptide-Fc fusion proteins which selectively neutralize the interaction of ANGPT2 or both ANGPT1 and ANGPT2 with TIE2 have demonstrated inhibition of tumor growth and angiogenesis in human tumor xenografts and orthotopic mouse tumors (Oliner et al., 2004; Saharinen et al., 2011; Holopainen et al., 2012). Conversely, ANGPT1 blocking failed to provide tumor growth inhibition, but it prevented tumor vessel normalization, supporting the hypothesis that ANGPT1 contributes to vessel stabilization during anti-angiogenic therapy (Falcón et al., 2009; Coxon et al., 2010).

Additionally, increased ANGPT2 levels are associated with numerous human diseases, including sepsis, infectious diseases, diabetes, atherosclerosis and tissue injury. Therefore, the ANGPT–TIE system is an attractive target for the development of future vascular therapies (Parikh et al., 2006; Milam and Parikh, 2015).

1.3 Experimental models of angiogenesis

A variety of in vivo and in vitro models of SA have been developed and have contributed greatly to the understanding of this mechanism. In vitro models with isolated endothelial cell lines have allowed to study selected aspects of the angiogenic process, including endothelial migration, proliferation, proteolytic digestion of the extracellular matrix and capillary tube formation (Cimpean et al., 2010). Among the in vivo models, the most commonly used are the rabbit corneal assay (Muthukkaruppan and Auerbach, 1979), the developing mouse retina (Fruttiger, 2007) and the intersegmental vessel growth in

zebrafish (Lawson and Weinstein, 2002). Additionally, many murine models have been developed to study tumor angiogenesis (Staton et al., 2004; Eklund et al., 2013).

Conversely, there is limited availability of experimental models for IA due to the difficulty to induce and visualize it in vitro and in vivo (Augustin, 2001). To date, the chick CAM assay is the only in vivo (ex ovo) model developed to study IA in physiologic tissue (Baum et al., 2010; Belle et al., 2014). With this model, both pillar formation and the influence of angiogenic factors can be studied. Furthermore, IA has been observed in different murine disease models, including models of liver cirrhosis (Van Steenkiste et al., 2010) and inflammation (Konerding et al., 2010; Rossi-Schneider et al., 2010). Human and mouse tumor growth models have also been used to study IA (Patan et al., 1996; Paku et al., 2011). In addition, IA was observed to start glomerular repair in a model of induced Thy-1.1 nephritis in rats (Notoya et al., 2003). IA has already been visualized through descriptive methods in chick glomeruli where a switch of the angiogenic phenotype from SA to IA has been described (Makanya et al., 2005). Moreover, the numerous glomeruli in the kidney are easily identifiable and delineable regions in which IA takes place, allowing specific sampling. Since mammals are phylogenetically closer to humans and have potentially more clinical relevance compared to birds, mammalian metanephric glomeruli could be a very interesting model to study IA.

2 The mammalian kidney

Renal organogenesis of vertebrates proceeds in pairs through a series of successive phases, each marked by the development of a more advanced kidney, namely the pronephros, mesonephros and metanephros. In mammals, the pronephros is a vestigial structure which is soon replaced by the mesonephros. The mesonephros is a functional excretory organ (Ludwig and Landmann, 2005), but it is eventually replaced by the metanephros which persists as the definitive adult kidney (Carlson, 2004). The primary filtration unit in both mesonephric and metanephric kidneys is the glomerulus. The current research, though, focuses on the metanephros where glomerular development follows a predictable and topographically traceable pattern, regardless of the fetal developmental stage.

2.1 Structure of metanephric glomeruli

The glomerulus is a highly developed vascular bed that acts as a filter, allowing a filtrate of small molecules, such as water, sugars, electrolytes and small proteins, to pass through a barrier that retains high molecular weight proteins and cells in the circulation. It is a dynamic structure whose integrity depends on signalling between the three major cell lineages: podocytes, endothelial and mesangial cells (Quaggin and Kreidberg, 2008) (Fig. 7).

The glomerular endothelial cells (GEC) are highly fenestrated and form the glomerular capillaries (Haraldsson et al., 2008). GEC are covered by a glycocalyx comprising mainly proteoglycans, which appear to be important in regulating the permeability of the glomerulus (Salmon et al., 2012). The intraglomerular mesangial cells, which lay in between the capillaries, provide structural support and regulate blood flow of the glomerular capillaries due to their contractile activity (Schlöndorff, 1996).

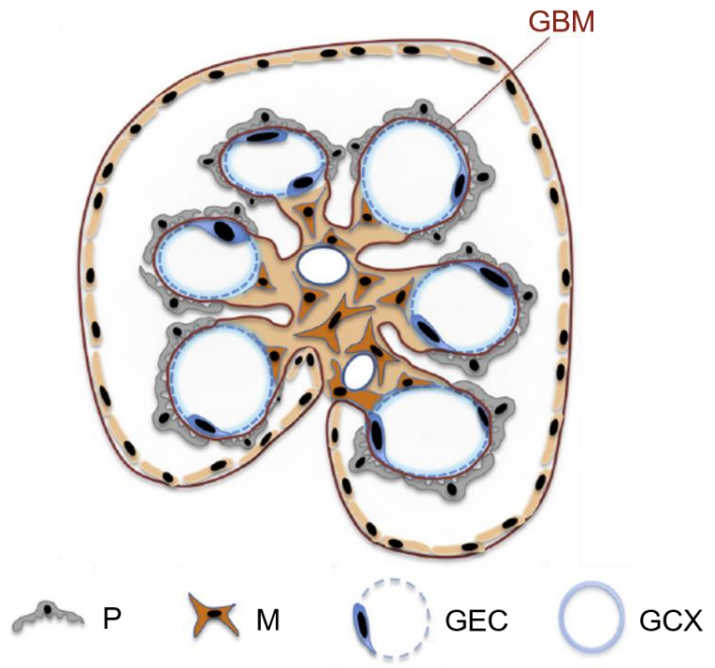

Fig. 7. Schematic representation of a metanephric glomerulus. GBM: glomerular basement membrane, P: podocyte, M: intraglomerular mesangial cell, GEC: glomerular endothelial cell, GCX: glycocalyx. Adapted from Gnudi et al. (2016).

These two cell types are enclosed within the glomerular basement membrane (GBM) which is formed by the fusion of the basement membranes of endothelial cells and podocytes. The main components of the GBM, collagen type IV, laminins, nidogen and proteoglycans, contribute to its selective permeability based on size and charge (Levidiotis and Power, 2005). Podocytes, which are specialized epithelial cells, reside on the other side of the GBM. Foot processes (pedicles), which extend from the podocytes, wrap themselves around the capillaries of the glomerulus to form the filtration slits. The pedicles increase the surface area of the cells enabling efficient ultrafiltration (Pavenstädt et al., 2003) (Fig. 8).

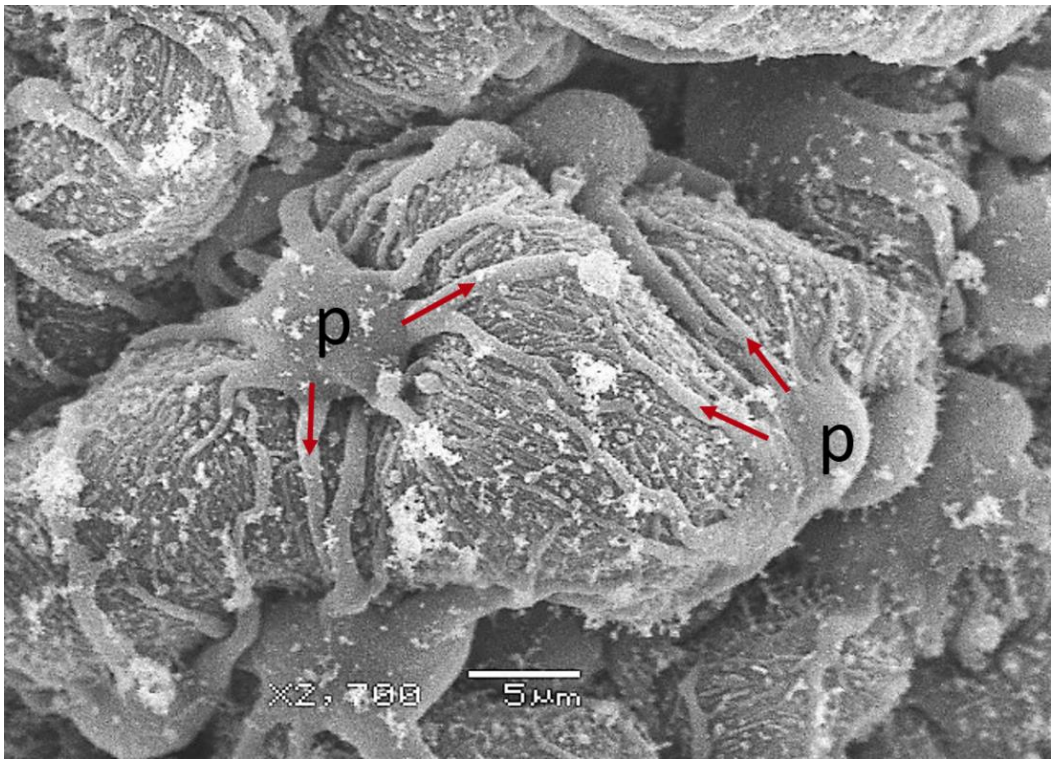

Fig. 8. Scanning electron micrograph depicting podocyte processes enveloping a mesonephric glomerulus of a porcine embryo (CRL: 10 cm). P: podocyte cell body, arrows point to the podocyte foot processes. (De Spiegelaere, 2011).

2.2 Metanephric development

The metanephric kidney develops from the union of the ureteric bud with the metanephric blastema (Potter, 1972). In porcine embryos, the metanephros appears at embryonic day (E) 20 and becomes active at E30 (Egerer et al., 1984). The formation of nephrons proceeds in a centrifugal pattern so that new nephrons are formed in the superficial cortex of the kidney and the oldest and most mature nephrons are located in the juxtamedullary area (Kazimierszak, 1971; Nash and Edelmann, 1973) (Fig. 9).

Five stages of nephron development can be discerned (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). The stage III glomerulus is spherical or oval, it has a distinct Bowman's space and a few capillary loops are present. The stage IV glomerulus has a round shape and contains more capillary loops. In both stages a monolayer of cuboidal epithelial cells (presumptive podocytes) is arranged at the outer side of the glomerulus. The stage V glomerulus contains multiple capillaries and the podocytes and endothelial cells are flattened.

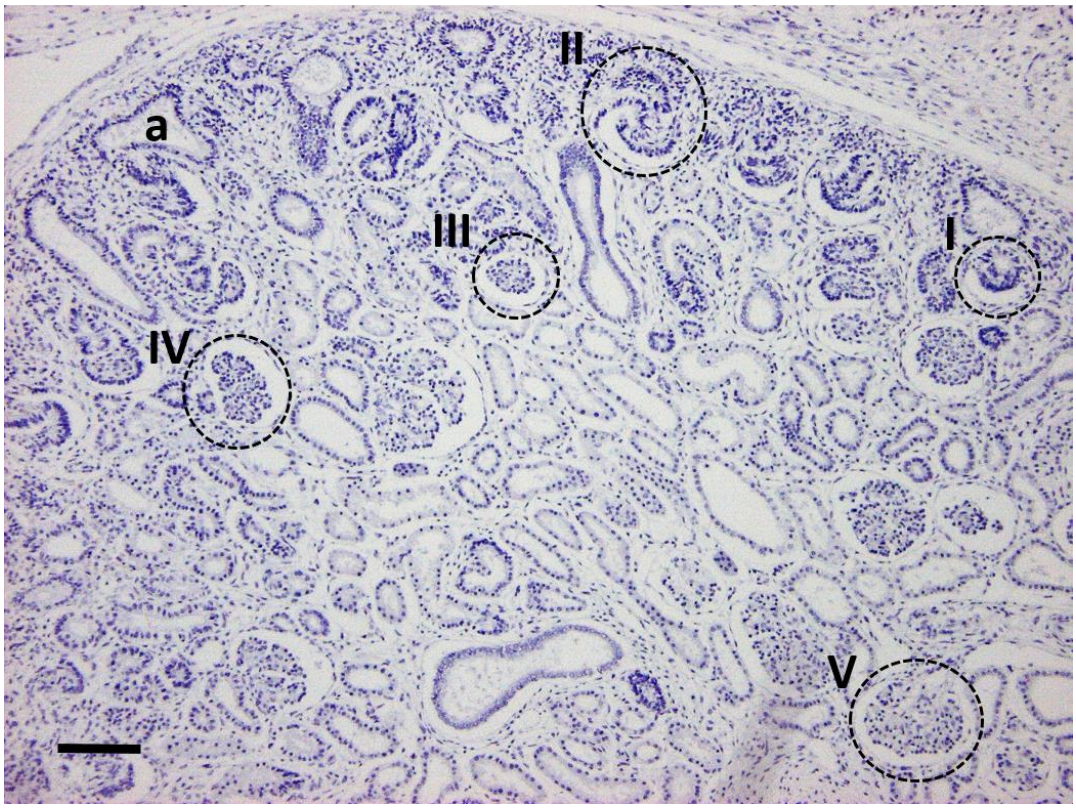

Fig. 9. Micrograph of metanephric kidney (E54) showing the centrifugal pattern of nephron formation. Stage I (renal vesicles), II (S-shaped bodies) and III nephrons are visible in the outer part of the cortex (nephrogenic zone) along with the ampullae (a) of the growing collecting duct. Stage IV and V nephrons are situated in the inner cortex, at the juxtamedullary area. Scale bar is 100 μm.

2.3 Angiogenesis during metanephric development

Subsequent to the formation of the embryonic vascular network by vasculogenesis, most of the new vessels in the developing organism arise through angiogenesis (Risau, 1997). In the avian developing kidney, the initial vascular plexuses of the renal lobules are formed via angiogenic sprouting. Later on, the vascular growth model switches to intussusceptive angiogenesis which contributes to vascular expansion and remodeling. Ultimately, the maturation of the vasculature is achieved by intussusceptive pruning and branching remodeling (Makanya et al., 2005). Similar findings are presented for the developing chick eye and CAM (Djonov et al., 2000b). The researchers proposed, that due to the increasing metabolic demands of those organs, growth of the capillary networks switches from sprouting to intussusceptive angiogenesis. Additionally, in the porcine kidney, intussusceptive angiogenesis is present in the mesonephros (De Spiegelaere et al., 2010) but no data are available for the metanephros.

2.4 ANGPT-TIE system during metanephric develoment

Information about the involvement of the ANGPT-TIE system in metanephrogenesis is insufficient. Moreover, it is mostly limited to angiopoietins, whereas little is known about their receptors. Angiopoietin and TIE genes are expressed in the normal developing kidney and the TIE genes are required for the survival of metanephric capillaries (Kim et al., 2005). TIE expressing endothelial precursors exist in the renal mesenchyme, and probably the same cells contribute to the formation of glomerular capillaries (Woolf et al., 2009). Low levels of angiopoietins and TIE2 transcripts are present from the inception of the metanephros, but they are upregulated when the first layers of vascularized glomeruli are forming (Loughna et al., 1997; Yuan et al., 1999; Kolatsi-Joannou et al., 2001). In the porcine developing metanephros, ANGPT1 is predominantly present in mature glomeruli, whilst ANGPT2 is observed in all stages of glomerular maturation (De Spiegelaere et al.,

2011). Further research regarding the TIE expression in porcine metanephric kidneys is necessary for gaining further insights into their involvement in glomerulogenesis.

2.5 ANGPT-TIE system in glomerular disease

In view of the fact that angiopoietins affect podocyte as well as glomerular endothelial biology, imbalanced angiopoietin signalling contributes to glomerular pathobiology (Woolf et al., 2009). ANGPT2 overexpression is linked to microalbuminuria due to increased protein losses in the glomerular filtrate caused by defects in the filtration barrier (Davis et al., 2007). Upregulated ANGPT2 has also been reported in a model of streptozotocin-induced diabetic nephropathy in mice (Yamamoto et al., 2004). Glomerular downregulation of ANGPT1 and upregulation of ANGPT2 correlating with glomerular endothelial apoptosis was also described in in a mouse model of anti–glomerular basement membrane glomerulonephritis (Yuan et al., 2002). ANGPT1 expression levels are important in the pathophysiology of diabetic glomerular disease and could confer protection against high-glucose-mediated glomerular capillary injury (Jeansson et al., 2011). The combination of high ANGPT1 levels and low VEGF-A signalling in diabetic nephropathy is likely to represent an important mechanism that favours a more stable capillary wall paralleled with a reduction in glomerular endothelial cell proliferation (Gnudi et al., 2016).

To summarize, angiopoietins could represent pharmacologic targets for the treatment of glomerular diseases and modulation of their signalling system could be very beneficial for treatments. However, such therapies may need to be tailored to specific primary kidney diseases, otherwise they could also cause side effects such as enhanced fibrosis and inflammation (Long et al., 2008). Therefore, it is of high importance to further explore the role of the ANGPT-TIE system in glomerular diseases.

Chapter II: Aims of the study

Intussusceptive angiogenesis (IA) is not easily approachable experimentally and major questions about its mechanism remain unanswered. Especially the mechanism leading to the initiation of IA and the morphological evolution of intraluminal pillars still need to be elucidated. Furthermore, angiopoietins are important mediators of IA, since these factors are directly linked to vascular homeostasis and activation. However, the role of the angiopoietin receptors (TIE) in relation to IA needs to be further investigated.

In the present thesis, we used the developing porcine metanephric glomerulus as a model to investigate the involvement of the ANGPT-TIE system in IA. This model was used to gain insight into the expression pattern of the TIE receptors glomeruli, and to study the morphology, formation and ultrastructure of the intussusceptive pillar in the specific setting of the glomerulus.

The specific aims addressed in this thesis were:

- to identify a possible correlation between IA and fetal development by studying the temporal and spatial presence of IA in the porcine metanephric kidney and to investigate how the expression of TIE1 and TIE2 progresses during development (Chapter III).
- to pinpoint the exact subcellular localization of TIE1 and TIE2 in the endothelial cells of the glomerular capillaries and test whether the differential localization of TIE receptors presented in in vitro conditions can be also seen in in situ metanephric kidney samples during development. (Chapter IV).
- to determine the ultrastructure of intussusceptive pillars and the cells which participate in their formation and to test the hypothesis if pillar formation in the specific vascular setting of the glomerulus occurs according to the original described process or if another mechanism is involved. (Chapter V).

Chapter III

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

Adapted from: Logothetidou A, Vandecasteele T, Van Mulken E, Vandevelde K, Cornillie P (2017) Intussusceptive angiogenesis and expression of TIE receptors during porcine metanephric kidney development. Histol Histopathol. 32:817-824

3.1 Abstract

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 the TIE receptors and IA during porcine metanephric development and during glomerulogenesis in particular.

3.2 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 TIE1/LacZ transgenic mice to follow kidney endothelial cell development. TIE1 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 involves angiogenic sprouting and then switches to intussusception which contributes to vascular amplification and 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 (Caduff et al., 1986). 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 μm in diameter being considered to represent tissue pillars (Makanya et al., 2009).

Vascular endothelial growth factors (VEGF) and angiopoietins (ANGPT) form two families of growth factors which are crucial for the development of renal vasculature (Wakelin et al., 2004; Woolf et al., 2009). 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 rectae. 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). In order to gain more insight into the molecular mechanism of IA, the present study focuses on the localization of the angiopoietin 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.

3.3 Materials and methods

3.3.1 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).

3.3.2 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. Mercox II resin and catalyst (Ladd Research, Wemmel, 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.

3.3.3 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, 5mM 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 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 1h 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).

3.3.4 Immunohistochemistry

The second group of fetuses was removed from the uteri and the kidneys were excised and directly fixated in 4% formalin for 24h. 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 (Mircom, 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% H_2O_2 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 1h. 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 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.

3.4 Results

3.4.1 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 (Figure 1A). These holes were also present in IV and V glomeruli. The holes were frequently found next to bifurcations of the capillaries (Figure 1B) and in linear arrangements (Figure 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 (Figures 1D, 1E). Interestingly, holes in the cast were not only observed in the capillary bed, but also in the branches of the efferent arteriole (Figure 1F).

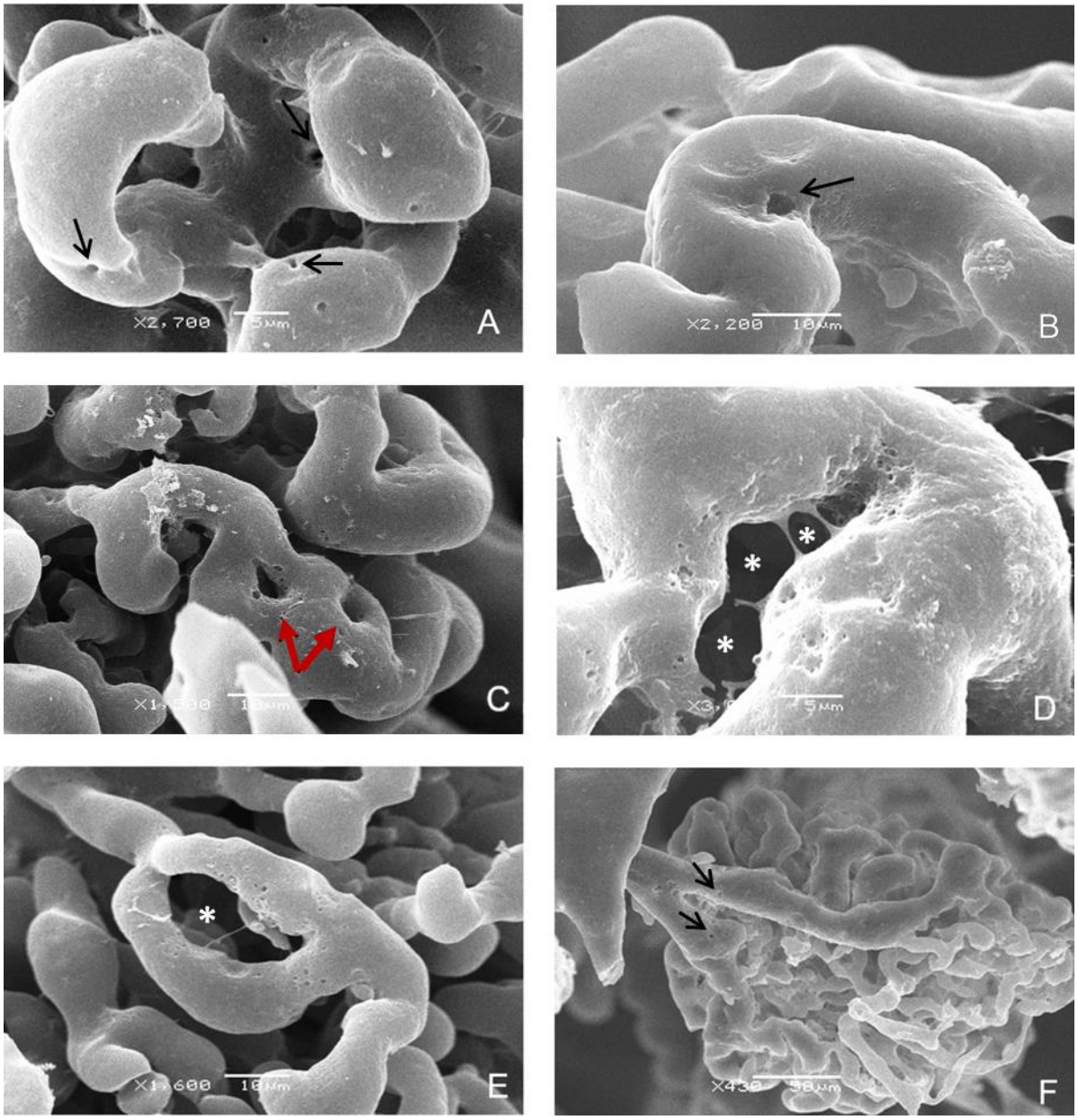

Figure 1. Vascular corrosion casts illustrating the process of intussusceptive angiogenesis in the metanephric porcine kidney. Using 2-5 corrosion casts per age, 10 fields per visible glomerulus were examined. A) Initially, small depressions appear on the surface of the Mercox casted glomerular capillaries (arrows). These depressions indicate the 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).

3.4.2 Western Blotting

Western Blotting results showed the 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).

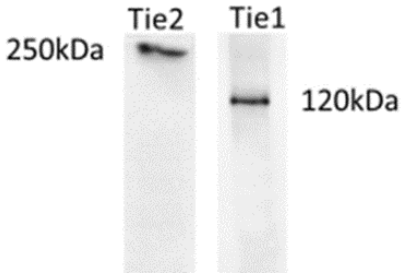

Figure 2. Western blotting analysis of TIE1 and TIE2 in kidney tissue lysates of a fetus of E64. Images were derived from identical gels loaded with the same amount (12 μg) of the same protein extract samples. A band ~120 kDa is recognized by the anti-TIE1 antibody and a band ~250 kDa is recognized by the anti-TIE2 antibody.

3.4.3 Immunohistochemistry

TIE1 receptors were present in S-shaped bodies and III, IV, V stage glomeruli, as shown by the strong TIE1 staining (Figure 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 (Figure 3B). TIE2 receptors were also found in all developmental stages of glomeruli (Figure 3C), specifically in endothelial cells and podocytes as well as on the epithelium of Bowman's capsule (Figure 3D). A monolayer of cuboidal epithelial cells which is positioned apically on the immature glomeruli also showed a weak staining for TIE1 and TIE2 (Figure 3E). In the 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 (Figure 3F). Control sections, in which the primary antibody was either pre-incubated with the blocking peptide (Figure 3G) or was omitted (Figure 3H), showed no immunostaining.

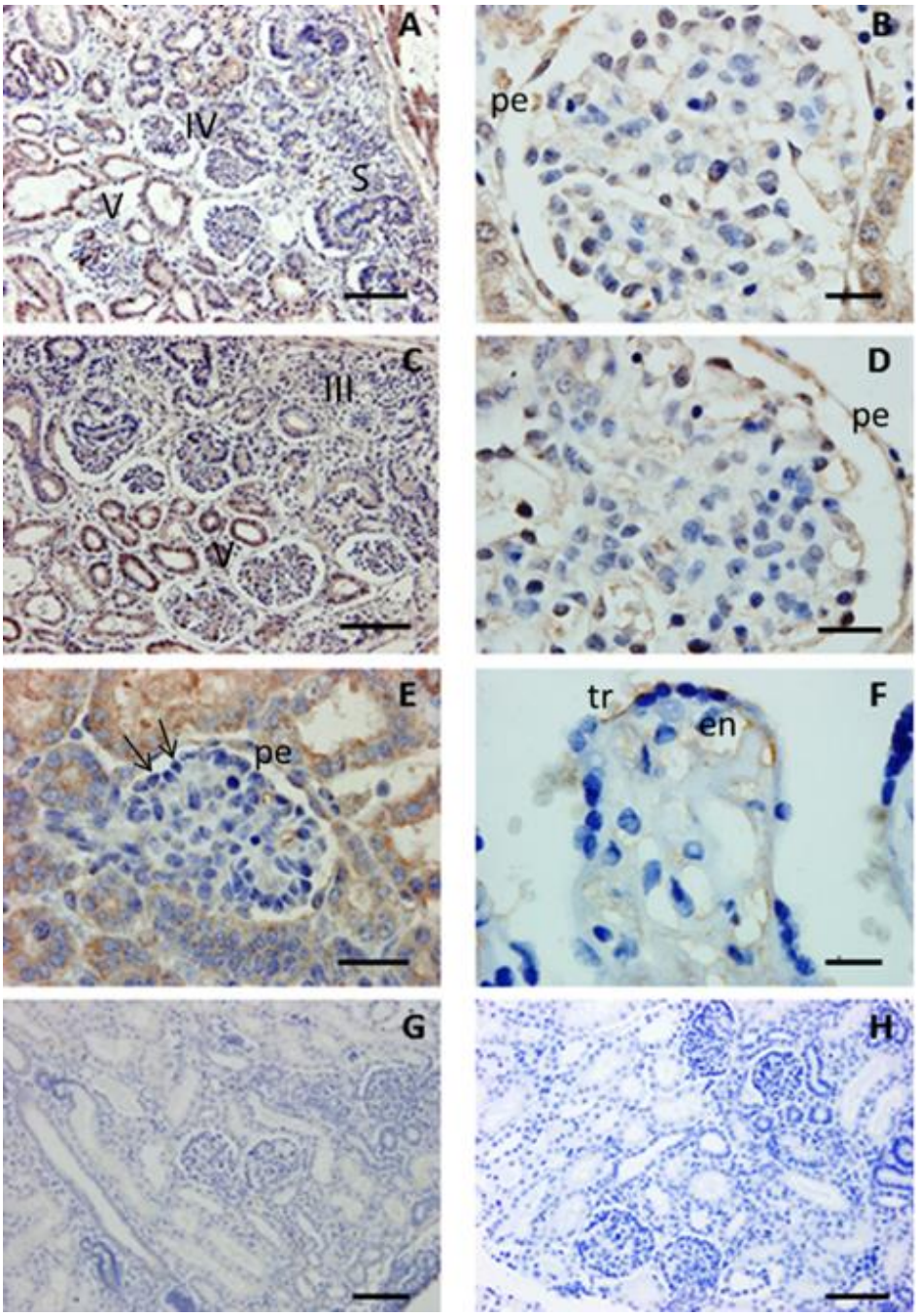

Figure 3. TIE1 and TIE2 immunostaining of the porcine metanephros. Using 2 kidney samples per age, 10 glomeruli were examined per kidney. A) Staining of TIE1 is observed in the S-shaped bodies (S) and all maturation stages of glomeruli (III, IV, V) (E48). B) Strong staining of TIE1 is observed in the 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. In panels A, C, G and H the scale bar is 100 μm. In panels B, D and F the scale bar is 20 μm. In panel E the scale bar is 50 μm.

3.5 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 transcapillary pillar formation, were observed in capillary segments of immature and maturing glomeruli. The first signs of IA appeared in G1 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., 2000b). 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 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 receptors. 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.

3.6 Acknowledgments

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.

Chapter IV

Stereological and immunogold studies on TIE1 and TIE2 localization in porcine metanephric glomeruli indicate angiopoietin signalling in podocytes

Adapted from: Logothetidou A, De Spiegelaere W, Van den Broeck W, Vandecasteele T, Couck L, Simoens P, Cornillie P (2017) Stereological and immunogold studies on TIE1 and TIE2 localization in porcine metanephric glomeruli indicate angiopoietin signaling in podocytes. Micron. 97:6-10

4.1 Abstract

Angiopoietins (ANGPT) and their TIE receptors are important regulators of vascular stability and remodeling. These molecules are involved not only in the normal development of kidney glomeruli, but also in disease, thus making them promising targets for therapies. Although TIE receptors are mainly found in endothelial cells, some reports observed TIE2 expression in glomerular podocytes as well. This suggests a role of angiopoietins in the regulation of podocytes. In the present study, we aimed to map the subcellular localization of TIE receptors in metanephric glomeruli of fetal pigs using high-resolution immunogold electron microscopy and the relative labeling index stereological approach. TIE1 and TIE2 antibody labeling was detected on the abluminal side of endothelial cell membranes. In endothelial cells, 4.5% of TIE2 was observed close to cell-cell contacts and 11.9% of TIE2 was found in closely associated pairs, which suggests the presence of homodimers. Interestingly, TIE1 and TIE2 were also expressed in podocyte foot processes indicating that they may play a similar role in podocytes as in endothelial cells.

4.2 Introduction

Angiogenesis, the process of vascular network formation and remodeling, is regulated by a combination of hemodynamics and growth factors (Burri and Djonov, 2002; De Spiegelaere et al. 2012). The ANGPT–TIE ligand–receptor system is a key regulator for the developing renal vasculature (Wakkelin et al., 2004; Woolf et al., 2009) and glomerulogenesis in particular (De Spiegelaere et al., 2010; Kolatsi-Joannou et al., 2001, Yuan et al., 2000, 1999). Furthermore, angiopoietins play an important role in glomerular diseases, but they have also been proposed as therapeutic targets for the treatment of these diseases (Davis et al., 2007; Dessapt-Baradez et al., 2014).

In developing glomeruli, ANGPT expression has been found mainly in podocytes and mesangial cells. This led to the hypothesis that mesangial cells and podocytes regulate the glomerular endothelium through a paracrine signalling of ANGPT1 and ANGPT2 (Woolf et al., 2009). However, Satchell et al. (2002) have shown expression of TIE2 on glomerular podocytes, which suggests that the ANGPT-TIE signalling may also be important for podocytes. In the present study, our objective was to investigate the exact location of both receptors in glomeruli of porcine fetal kidneys in order to give insight into their function in each cell type.

4.3 Materials and methods

4.3.1. Sample collection, fixation and embedding

Porcine gravid uteri were obtained on different occasions in a local slaughterhouse from sows that were culled for human consumption. Fetuses of different fetal stages were collected, i.e. fetuses with a crown-rump length (CRL) of 7 cm (E47), 12.5 cm (E62) and 22 cm (E98). Their approximate age in embryonic days post conception (E) was deduced from their CRLs (Evans and Sack, 1973). Two fetuses/age were removed from their fetal membranes and their kidneys were dissected. Small pieces of kidney cortex ~ 1 mm^3 were

excised and placed in a mild fixative (2.5% paraformaldehyde and 0.3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2). After overnight fixation at 4 °C, the samples were washed with 0.1 M cacodylate buffer and were then dehydrated in a series of alcohol dilutions. Subsequently, the samples were impregnated in graded mixtures of LR white embedding medium (Aurion Immuno Gold Reagents & Accessoires, Wageningen, Netherlands) and alcohol and finally in pure LR white for 24 h. Afterwards, they were placed in gel capsules and were embedded in LR white, in which they remained for at least 14 days at 37 °C to allow resin polymerization.

4.3.2. Immunogold labeling of TIE1 and TIE2 receptors

Ultrathin sections were cut with a Leica EM UC6 ultramicrotome (Leica Microsystems, Diegem, Belgium) using a DiATOME ultra 45° diamond knife (Diatome AG, Nidau, Switzerland) and collected on single slot formvar pre-coated copper grids. All steps of the immunolabeling were performed at room temperature and during each step the grids were floated upside-down on 25 µl of aliquots. Initially, the sections were incubated with 50 mM glycine/PBS in order to inactivate free aldehyde groups of the fixative. Following a step with a blocking solution containing bovine serum, the sections were then incubated for 1 h with 1:200 anti-TIE1 or 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, New Jersey, USA). Any homology regions between the two receptors was excluded from the antigenic regions chosen for the development of the two primary antibodies. Afterwards, the sections were incubated for 30 min with protein A conjugated to colloidal gold particles of 10 nm diameter. After every step, the sections were washed with BSA-c, which is prepared by acetylation of bovine serum albumin and prevents a charge based background by interacting with polycationic areas in

the tissue samples. Finally, the sections were contrasted with 2% uranyl acetate/ethanol for 15 min and then rinsed. Examination of the sections was performed at 80 kV with a JEM-1400 Plus transmission electron microscope (Jeol, Zaventem, Belgium). Sections where the primary antibodies were omitted, served as negative controls. All materials and reagents were purchased from Aurion (Aurion Immuno Gold Reagents & Accessoires, Wageningen, Netherlands).

4.3.3. Quantitative and statistical analysis

A simple and efficient method by Mayhew et al. (2002) was implemented in order to quantify immunogold labeling of antigens localized in different cellular compartments and to statistically evaluate the resulting labeling distributions. Briefly, the labeling of each TIE1 and TIE2 was analyzed in 18 capillaries which were randomly selected. Since TIE2 was previously found on the membranes of endothelial cells and podocyte processes, the region of interest consisted of the following compartments: podocyte foot processes, the glomerular basement membrane, the fenestrated endothelium and the extracellular space (Fig. 1). Test-point lattices (grids) were superimposed on capillaries and design-based stereology was used to count the corresponding test points (p) for the different compartments of the region of interest. The observed gold particles (n_0) were quantified in these compartments. Using the total number of observed gold particles and the relative surfaces of each compartment, an expected gold particle count distribution (n_e) assuming a random staining could be obtained. The ratio of observed gold particles to randomly expected gold particles (n_0/n_e) provided a relative labeling index (RLI) for each compartment. For random labeling, the predicted RLI = 1 and for preferential labeled, the RLI > 1. Chi-squared test (χ^2) was used to test if the observed gold distributions differed significantly from a random staining. The null hypothesis of random gold particle distribution must be rejected at a probability level of $P < 0.001$.

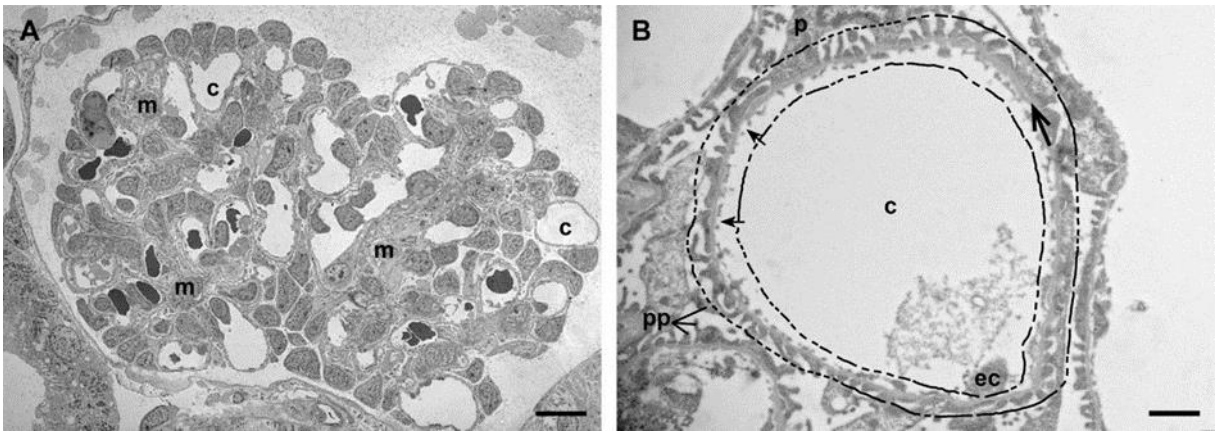

Fig. 1. Transmission electron micrographs showing the ultrastructure of a mature glomerulus (porcine fetus E62). A) The glomerulus is composed of a capillary network. Between the capillaries (c) few intraglomerular mesangial cells (m) are present. Scale bar: 10 µm. B) Endothelial cells (ec), lining the capillaries (c), are fenestrated (small arrows). The associated glomerular basement membrane (gbm) (thick arrow) surrounds the capillary and is enveloped by the interdigitating foot processes (pp) of podocytes (p). The region of interest lies between the two demarcation lines and consists of endothelium, podocyte processes, glomerular basement membrane and extracellular space. Scale bar: 1 µm.

4.4 Results

Anti-TIE1 (Table 1) revealed preferential labeling in podocyte processes and endothelium, with the podocyte processes being the most important contributor to total χ^2. The RLI of the glomerular basement membrane and the extracellular space are < 1 meaning that TIE1 labeling is not preferential in these compartments (non-specific labeling). Anti-TIE2 (Table 2) demonstrated also preferential labeling in podocyte processes and endothelium, but this time endothelium was the most important contributor to total χ^2. Similarly to TIE1, TIE2 labeling in the glomerular basement membrane and the extracellular space was non-specific. For 3 degrees of freedom (2-1 columns by 4-1 rows) the null hypothesis of random gold distribution must be rejected at a probability level of $P < 0.001$.

Table 1. Quantification of TIE1 antibody labeling. Observed and expected distributions of gold particles in the compartments of the region of interest and calculation of relative labeling index (RLI) and χ^2 values. Asterisks (*) identify compartments that are preferentially labeled.

Compartment	Observed points, p	Observed particles, n_0	Expected particles, n_e	RLI, n_0/n_e	χ^2 values
Podocyte processes	380	100	61	1.64*	24.9*
Glomerular basement membrane	308	6	49	0.12	37.7
Endothelium	237	62	38	1.63*	15.2*
Extracellular space	146	3	23	0.13	17.4
Totals	1071	171	171	1	95.2

Table 2. Quantification of TIE2 antibody labeling. Observed and expected distributions of gold particles in the compartments of the region of interest and calculation of relative labeling index (RLI) and χ^2 values. Asterisks (*) identify compartments that are preferentially labeled.

Compartment	Observed points, p	Observed particles, n_0	Expected particles, n_e	RLI, n_0/n_e	χ^2 values
Podocyte processes	402	107	69	1.55*	20.9*
Glomerular basement membrane	301	4	51	0.08	43.3
Endothelium	218	67	37	1.81*	24.3*
Extracellular space	138	3	24	0.13	18.4
Totals	1059	181	181	1	106.9

Regarding the localization of the receptors in endothelial cells, TIE1 was always found at the cell membrane facing the glomerular basement membrane, i.e. the abluminal side of the cell. (Fig. 2). TIE2 was also expressed predominantly at this side of the endothelial membrane, although 4.5% or 3/67 gold particles were localized close to endothelial cell-cell contacts (Fig. 3). Furthermore, 11.9% or 8/67 gold particles were in very close proximity to each other (~ 10 μm) (Fig. 4). Overall, the expression of TIE receptors showed similar levels in both endothelial cells and podocytes. Finally, for both TIE1 and TIE2, staining occurred scarcely in the nuclei and cytoplasm of glomerular cells

(Fig. 5) and on mesangial cell membranes (Fig. 6). In control experiments where the primary antibodies were omitted, no labeling was observed in any cell compartments (data not shown).

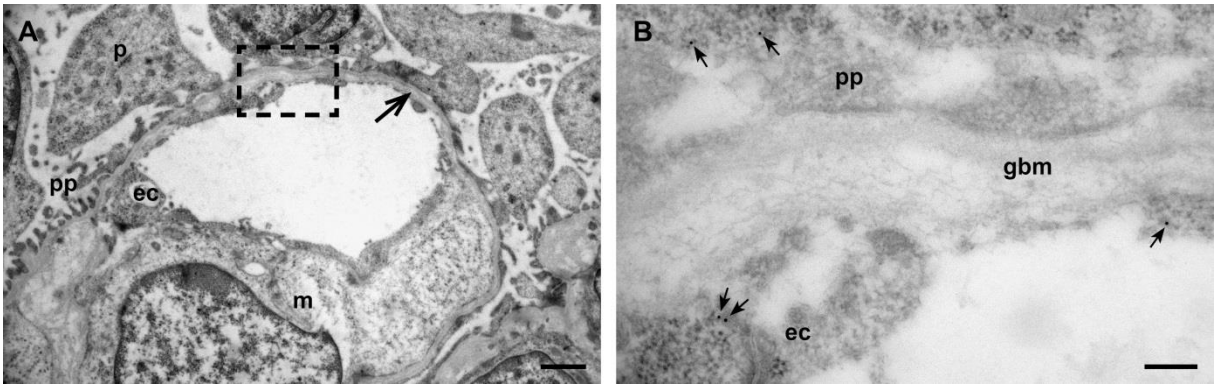

Fig. 2. TIE1 subcellular localization in porcine fetal glomeruli (E47). A) Endothelial cells (ec) of a glomerular capillary are surrounded by the glomerular basement membrane (arrow) and foot processes (pp) of podocytes (p). A mesangial cell (m) and its nucleus are also visible. Scale bar: 1 μm. B) Inset of A: Immunogold labeling (arrows) in endothelial cells is localized on the cell membrane that faces the glomerular basement membrane (gbm) and it is also observed in podocyte processes. Scale bar: 200 nm.

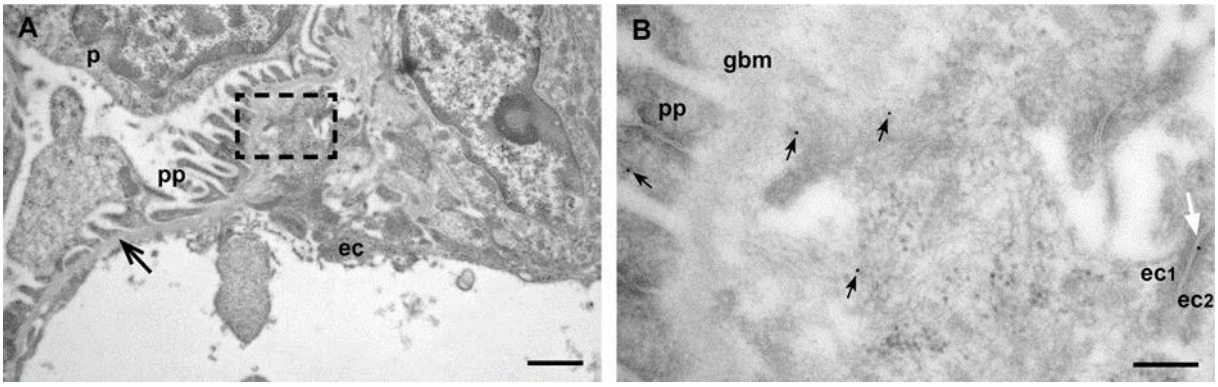

Fig. 3. TIE2 subcellular localization in porcine fetal glomeruli (E62). A) Region of interest showing the lumen of a capillary, its endothelial cell (ec), the glomerular basement membrane (arrow) and the foot processes (pp) of podocytes (p). Scale bar: 1 μm. B) Inset of A: TIE2 (arrows) is primarily expressed in the abluminal side of the endothelial cell facing the glomerular basement membrane (gbm) but also close to cell-cell contacts (white arrow) between two endothelial cells (ec1, ec2). Additionally, TIE2 is expressed in podocyte processes. Scale bar: 200 nm.

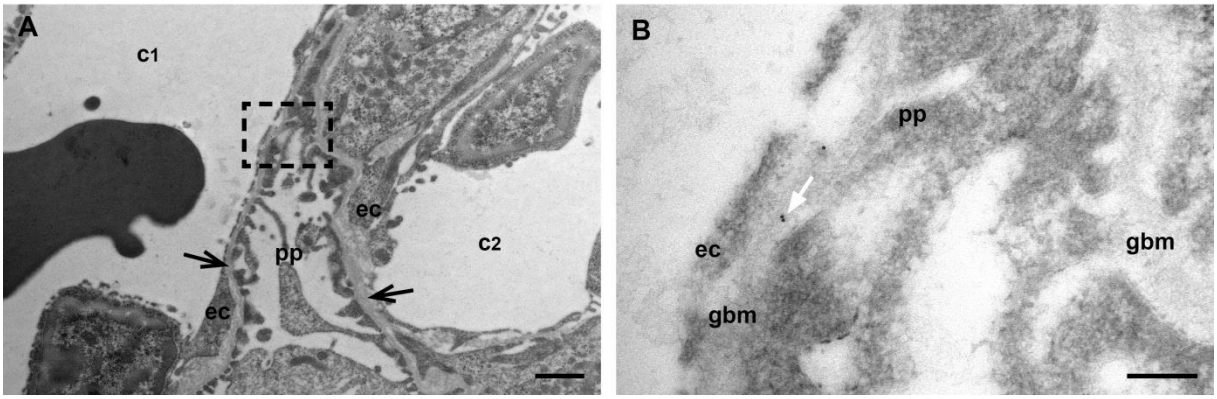

Fig. 4. Immunoreactivity of TIE2 in endothelial cells of porcine fetal glomeruli (E98). A) Region of interest with two capillaries (c1, c2), their endothelial cells (ec), the glomerular basement membrane (arrows) and the interdigitating podocyte processes (pp). Scale bar: 1 µm. B) Inset of A. Gold particles can be found in pairs (white arrow) and in close proximity of ~ 10 nm. Scale bar: 200 nm.

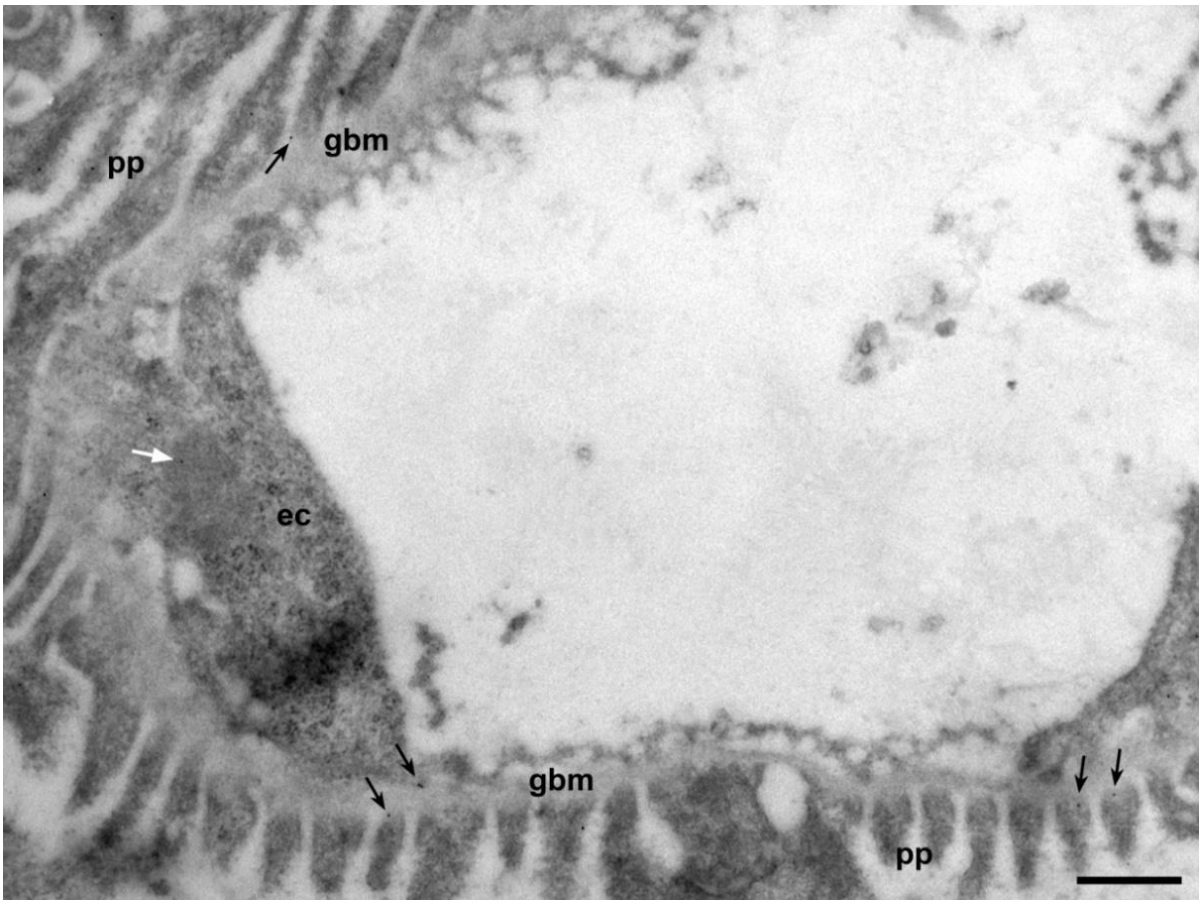

Fig. 5. Immunoreactivity of TIE2 in porcine fetal glomeruli (E47) shows staining (arrows) on the membranes of endothelial cells (ec) and podocyte processes (pp) and scarse staining (white arrow) in the cytoplasm of an endothelial cell. The associated glomerular basement membrane (gbm) is also visible. Scale bar: 500 nm.

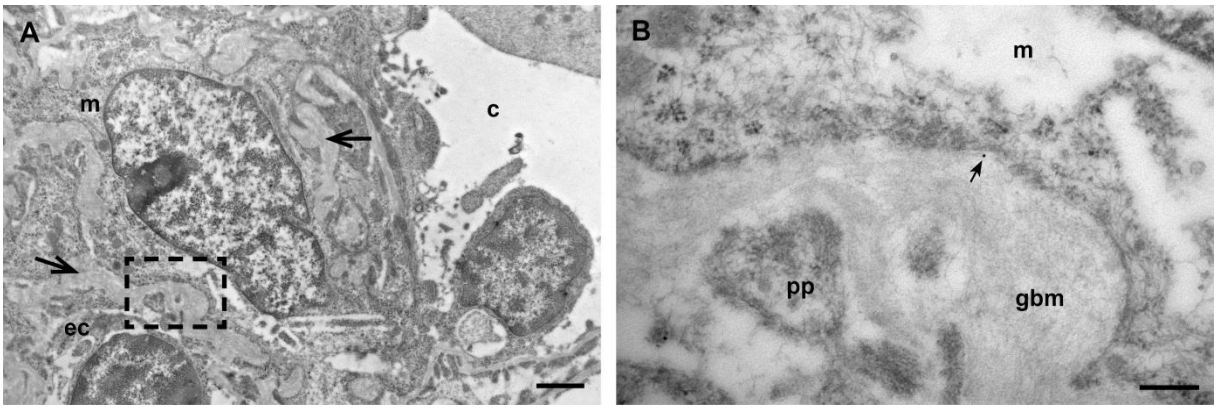

Fig. 6 Subcellular localization of TIE2 in mesangial cells in porcine fetal glomeruli (E62). A) A mesangial cell (m) adjacent to a capillary (c) is surrounded by glomerular basement membrane (thick arrows). Endothelial cells (ec) are also visible. Scale bar: 1 μm. B) Inset of A. TIE2 labeling (arrow) is observed on the mesangial cell membrane. Podocyte processes (pp) are also present. Scale bar: 200 nm.

4.5 Discussion

Electron microscopy, when combined with molecular detection methods, is the only technique with sufficient resolution to localize proteins in intracellular compartments and small membrane domains (Koster et al., 2003). Immunogold labeling offers great sensitivity and the relative amounts of labeling can be quantified. Nevertheless, this technique has also some limitations such as background staining and/or less penetration of the immunoreagents through the resin. In the present study, visualization of TIE1 and TIE2 receptors in the developing fetal glomeruli was achieved with high resolution immunogold labeling. Using the stereological approach of Mayhew et al. (2002), we quantified the immunogold labeling distributions of the receptors, expressing them as indices of relative labeling and testing whether or not their distributions indicate random or differential labeling. Since there was already available information of TIE2 possible antigen sites in endothelial cells and podocytes close to the glomerular filtration barrier (Satchell et al., 2002), we restricted the number of compartments to these two cell types. In order to make the statistical testing effective, we sampled additional compartments unlikely to be specifically labeled, such as the extracellular space.

Our results provide strong evidence that both TIE1 and TIE2 are expressed in endothelial cells and podocyte foot processes, which is in accordance with previous results of immunohistochemistry, stating that these receptors were constantly expressed in the metanephric glomeruli (Logothetidou et al., 2016). Although TIE receptors were considered to be mainly restricted to endothelial cells, TIE2 has been previously immunolocalized in rat podocytes in vivo (Satchell et al., 2002) and murine podocytes in vitro (Davis et al., 2007). The present study is the first report for TIE1 expression in podocytes. The expression of both receptors in these cells further promotes the importance of their interactions during angiopoietin signalling. Previously, the study of Davis et al. (2007) indicated a direct effect of ANGPT2 on podocytes. However, it was not clear whether this effect was mediated through TIE receptor signalling on the podocytes or through integrin interactions with angiopoietins. The hypothesis behind the presence of TIE receptors in podocytes is that the effect of angiopoietins on podocytes and endothelial cells might be similar (Woolf et al., 2009). Our findings of both receptors in podocytes further support this hypothesis. The expression of TIE receptors in podocytes could represent a direct autocrine feedback loop or a paracrine pathway through ANGPT expression from mesangial or endothelial cells. It remains to be determined how podocytes react to angiopoietin stimulation. Scarce staining was also found in mesangial cells. However, we cannot be sure whether this is a genuine labeling of TIE receptors since non preferential staining was also observed in the nuclei of glomerular cells.

Numerous studies demonstrated that TIE receptors and angiopoietins play an important role in metanephric development and glomerulogenesis (De Spiegelaere et al., 2011, Kolatsi-Joannou et al., 2001, Loughna et al., 1997, Yuan et al., 2000, 1999). The present study reveals that TIE1 and TIE2 expression in endothelial cells is predominantly observed at the abluminal side of the cell, namely the side that faces the glomerular basement membrane. TIE receptors found on this site is in agreement with the hypothesis that ANGPT1 can bind to the extracellular matrix and that ANGPT-TIE signalling interacts with integrin cell adhesion receptors (Eklund et al., 2017). Although TIE2 is

mainly found at the abluminal side, TIE2 expression was also found close to endothelial cell-cell contacts. This localization indicates the in vivo existence of receptor signalling complexes at cell junctions between neighboring endothelial cells. Previous work by Fukuhara et al. (2008) and Saharinen et al. (2008) showed that ANGPT1-activated TIE2 signalling complexes at endothelial cell junctions lead to PI3K-Akt mediated cell survival and increased vessel stabilization (Eklund et al., 2017; Saharinen et al., 2008).

In conclusion, the present study shows clear evidence of TIE1 and TIE2 expression both in endothelial cells and podocytes in the developing porcine glomerulus. Their presence indicates that angiopoietins might have an effect on podocytes too.

4.6 Acknowledgments

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Chapter V

Intussusceptive pillar formation in the porcine metanephric glomeruli

Adapted from: Logothetidou A, De Spiegelaere W, Vandecastele T, Tschulenk W, Walter I, Van den Broeck W, Cornillie P (2017) Intussusceptive pillar formation in the porcine metanephric glomeruli. Manuscript in preparation

5.1 Abstract

Intussusceptive angiogenesis (IA) is a dynamic process which contributes to vascular expansion and remodeling. Intraluminal pillars have long been the distinctive structural indicator of intussusceptive angiogenesis. However, the mechanism of their formation has not been fully elucidated yet. Using light and electron microscopy, we studied intussusceptive vascular growth in the developing porcine metanephric kidney. Utilizing serial semithin sectioning, we observed intraluminal pillars formed by endothelial cells in the vasculature of developing glomeruli. Their diameter was < 2.5 μm, consistent with the diameter of nascent pillars. Transmission electron microscopy revealed that the majority of these pillars consisted only of endothelium. However, a central core of extracellular matrix (ECM) covered by endothelium, reminiscent of a mature intussusceptive pillar, was also found in the lumen of glomerular capillaries. Interestingly, although collagen fibers were not detected in this matrix, pillar formation proceeded in the absence of fibrillar collagen indicating that the mechanism of pillar formation might be tissue-specific. In addition, perivascular cells or pericytes were not involved in the pillar structure during these stages of formation. This study suggests that ECM deposition preceeds pericyte/perivascular cell involvement in the intraluminal pillars during intussusceptive angiogenesis in porcine metanephric glomerular capillaries.

5.2 Introduction

Angiogenesis, the process through which the vascular system expands, occurs mostly during development and cyclic organ growth but also in pathological conditions involving tissue repair, organ regeneration, chronic inflammation and tumorigenesis (Paku and Paweletz, 1991; Risau, 1997; Augustin, 1998, 2001; Conway et al., 2001; Bergers and Benjamin, 2003; Carmeliet, 2003; Rajashekhar et al., 2006; Eming et al., 2007; Konerding et al., 2010; Song et al., 2011). The two best known angiogenic mechanisms, i.e. sprouting and intussusceptive angiogenesis (IA), lead to the expansion of the capillary network. However, they involve different cell types and are regulated by different molecules (Augustin, 2001). Sprouting angiogenesis is responsible purely for vascular growth; yet, IA can also involve vascular remodeling through pruning of excessive blood vessels (De Spiegelaere et al., 2012). Intraluminal pillars are considered the characteristic features of the morphogenetic process of IA (Patan et al., 1996, 2001; Djonov et al., 2001, Makanya et al., 2005).

In pathological tissues such as tumors, various mechanisms of angiogenesis have been identified (Döme et al., 2007). In murine ascites tumor vessels, intraluminal bridging has been described during which endothelial cytoplasmic processes extend into and across the vessel lumen, forming transluminal bridges that divide blood flow into multiple smaller-sized channels (Nagy et al., 1995). More specifically, in the vasculature of experimental subcutaneous tumors, a detailed model of vascular division due to endothelial bridging was proposed by Paku et al. (2011). During this mechanism, endothelial bridges are formed and subsequently the bridge-forming endothelium attaches to a type I collagen bundle in the underlying connective tissue. The actin cytoskeleton of the endothelial cell then exerts a pulling force to the collagen bundle, resulting in the transport of the latter through the vessel lumen. This process, named inverse sprouting, generates a connection between the processes of endothelial bridging and intussusceptive angiogenesis and identifies the collagen-pulling force behind pillar formation.

In the present study, the presence of intussusceptive angiogenesis and the pillar ultrastructure were investigated in the glomerular capillaries of the porcine metanephric kidney at different developmental stages using serial sectioning combined with light and electron microscopy. Previous studies have shown that IA is active during renal and glomerular development in vertebrates (Makanya et al., 2005; Logothetidou et al., 2016). Moreover, the numerous glomeruli in the kidney are easily identifiable and delineable regions in which IA takes place, allowing specific sampling. The developing kidney enables the investigations of different stages of vascular development, as the formation of nephrons proceeds in a centrifugal pattern so that the the newly formed nephrons are found in the superficial cortex whereas the oldest and most mature nephrons are located in the juxtamedullary area regardless of the fetal age (Kazimierszak, 1971; Nash and Edelmann, 1973).

5.3 Materials and methods

5.3.1 Sample collection and processing

Porcine fetuses of different developmental stages were obtained from gravid uteri collected in a local slaughterhouse and their approximate age in embryonic days post conception (E) was calculated from their crown-rump length (CRL) (Evans and Sack, 1973). The fetuses used in this research had a CRL of 6.5 cm (E46), 9.5 cm (E55), 14 cm (E64) and 22 cm (E98). For both light and electron microscopic studies, small pieces ± 1 mm^3 of fetal kidney cortex were fixated overnight in Karnovsky fixative (2% paraformaldehyde and 2.5% glutaraldehyde in 0,1 M sodium cacodylate buffer, pH 7.2). The tissue blocks were then postfixated in 1% reduced osmium tetroxide for 1.5 h, dehydrated through ascending concentrations of ethanol and embedded in EPON 812 resin.

5.3.2 Light microscopy and 3D reconstruction of semithin sections

Four kidney samples of different embryonic ages were used for serial semithin sectioning which was performed as described by Ruthensteiner (2008). Briefly, after the application of glue on one side of the EPON block, a diamond knife with a big boat (DiATOME, histo jumbo, ultra 45° 8 mm) was used to cut serial semithin sections of 0.5 µm. As the sectioning progressed, the sections formed a ribbon due to the glue in between and were later detached from the knife and transferred onto pretreated glass slides. After stretching and drying, the ribbons were stained with toluidine blue, mounted with DPX and observed with an Olympus BX61 (Olympus, Belgium) microscope. All the glomeruli present in each serial section were captured by an Olympus BX-UCB camera, followed throughout > 200 semithin sections and analyzed for the presence of pillars. Endothelial protrusions in the capillary lumen were considered as intraluminal pillars only when they appeared and then disappeared in consecutive sections, spanning from one endothelial wall towards the opposite one. In that way, other structures such as vessel bifurcations, endothelial folds or artifacts were not accidentally attributed as pillars. Digitized images of areas of interest were transferred to the Amira 6.1.1 software program (FEI, France) using the protocol described by Cornillie et al. (2008) in order to perform three-dimensional (3D) reconstruction of the pillar formation.

5.3.3 Transmission electron microscopy (TEM)

Two additional fetal kidney samples were used for ultrathin sectioning starting at a random point in the tissue block. An ultra 45° 2.5 mm diamond knife (DiATOME, Switzerland) was utilized to cut 150 consecutive ultrathin sections of ± 80 nm thickness which were then collected on precoated formvar copper grids. Sections were contrasted with 1% uranyl acetate in 10% ethanol and afterwards with a lead citrate buffer (133 mg lead nitrate and 175 mg sodium citrate in 10 ml double distilled water). The sections were viewed at 80 kV with a JEM-1400 Plus transmission electron microscope (Jeol, Belgium)

equipped with a Quemesa TEM CCD camera (Olympus, Belgium). Image analysis was performed with the Radius software (EMSIS, Belgium). All reagents and materials were purchased from Aurion (Aurion ImmunoGold Reagents & Accessoires, the Netherlands).

5.4 Results

5.4.1 Intussusceptive pillars in metanephric glomerular capillaries

The semithin sections of the different fetal kidney samples showed that the metanephros consisted of several tubules in the medullar area and S-shaped bodies and glomeruli in the cortical area. Glomeruli of different developmental stages, i.e. stages III, IV and V as described by Friis (1980) were easily discerned in the cortex (Fig. 1A). The glomerular tuft, the Bowman's capsule and the vascular and urinary poles of the renal corpuscle were clearly identifiable (Fig. 1B). From the numerous endothelial protrusions found in the glomerular capillaries, we were able to prove that several of them were indeed pillars. Analysis of the serial sections demonstrated endothelial cytoplasmic processes which elongated in the lumen towards the opposite side of the vessel wall and finally disappeared. Their diameter was always < 2.5 µm which is consistent with the diameter of a nascent pillar. By following serial sections, the point at which a pillar started and its intraluminal extent until the end point could be clearly identified (1C-F). Three-dimensional reconstruction was performed using these serial sections in order to highlight the recreated structures (Fig. 1G).

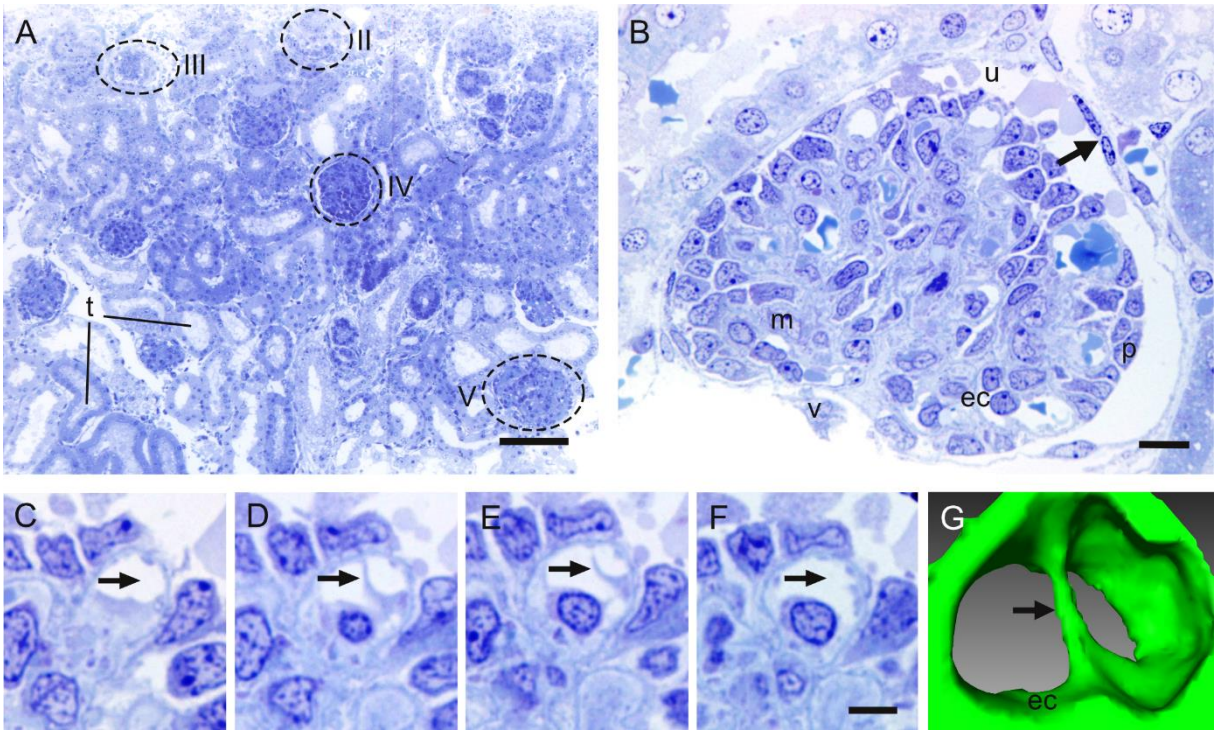

Fig. 1. Semithin sections (0.5 µm) showing the kidney morphology of a porcine fetus of E55. **A)** S-shaped bodies (II) and metanephric glomeruli of different maturation stages (III, IV and V) are located in the cortex of the metanephros. Tubuli (t) are located in the kidney medulla. Scale bar: 100 µm. **B)** Higher magnification of a stage IV metanephric glomerulus in which the vascular (v) and urinary poles (u) are depicted. The glomerular tuft is enclosed in Bowman's capsule (arrow). Endothelial cells (ec), intraglomerular mesangial cells (m) and podocytes (p) are indicated. Scale bar: 10 µm. **C-F)** Consecutive semithin sections demonstrating an intraluminal tissue pillar within a glomerular capillary. The open lumen of the vessel in C and F indicates the emergence and end of the pillar, respectively. The width of the pillar is 1.2 µm close to the endothelium and 0.59 µm in the middle of the lumen. Scale bar: 5 µm. **G)** 3D reconstruction of the transcapillary pillar represented in C-F. The endothelial cell wall (ec) and the surrounding tissue are indicated in green. Arrow points to the intraluminal pillar.

5.4.2 Ultrastructural changes in glomerular capillaries during development

The TEM analysis revealed ultrastructural changes in the capillaries during glomerular development. The immature capillaries of the metanephros showed endothelia with a large amount of cytoplasm and abundant euchromatin in their nuclei. The endothelium lacked fenestrations and the podocyte processes were not formed yet (Fig. 2A). As the capillaries mature, a more prominent glomerular basement membrane was formed and interdigitations of podocyte pedicles were developed showing primary and

more developed foot processes (Fig. 2B). Finally, the mature capillaries exhibited fenestrated thin endothelium with relatively scanty cytoplasmic organelles and well-developed glomerular basement membrane and podocyte processes (Fig. 2C).

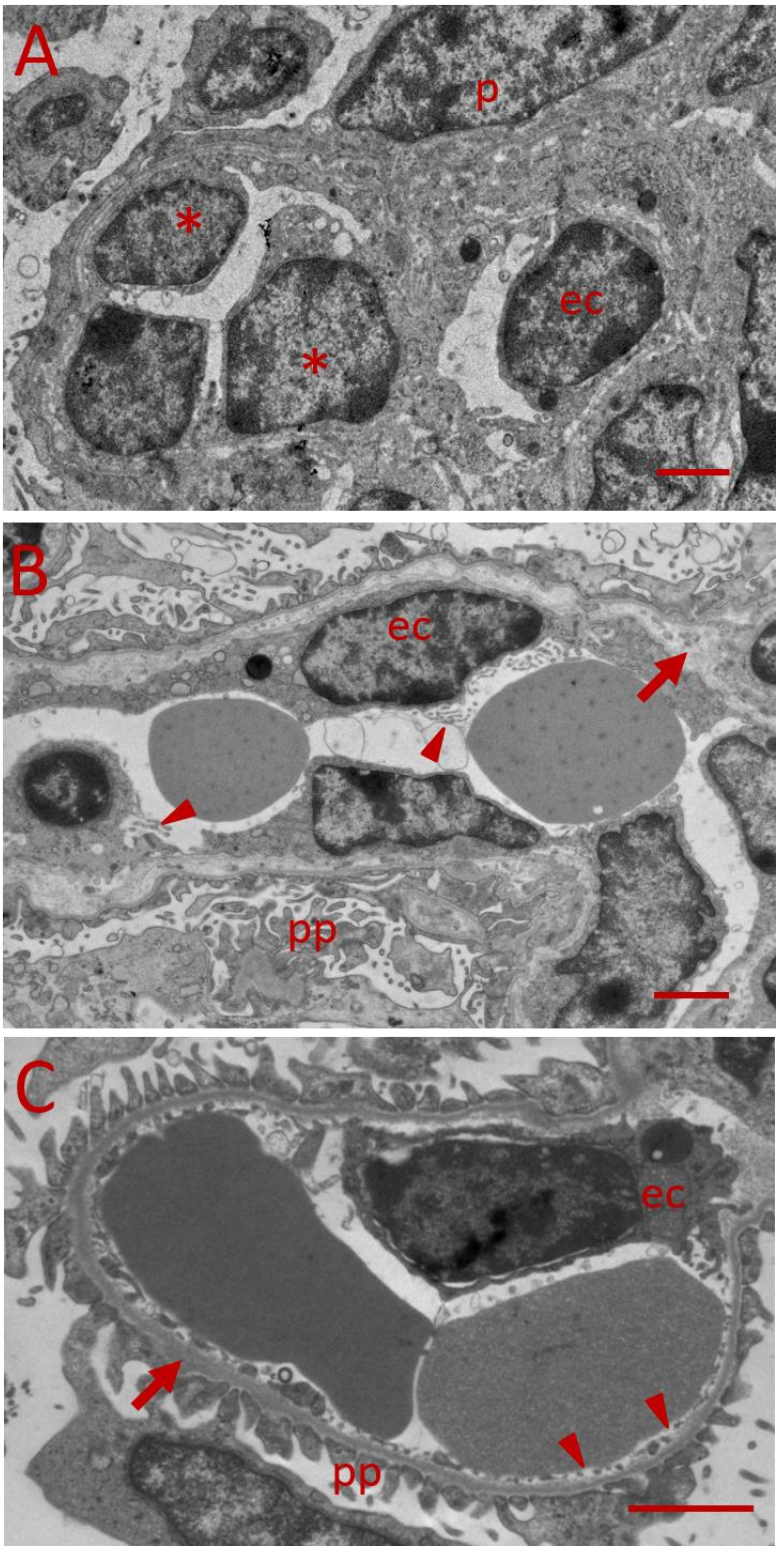

Fig. 2. Transmission electron micrographs illustrating various capillary phenotypes in the developing porcine metanephric glomeruli of a fetus of E64. **A)** Immature capillary showing different signs of activation: thickened endothelial cells (ec), numerous organelles in the cytoplasm, nuclei with enlarged amount of euchromatin (*). The endothelium is continuous and the podocytes (p) have not developed foot processes yet. **B)** Maturing capillary with a narrow lumen exhibiting a few intraluminal protrusions (arrowheads). The endothelium is not fenestrated. The podocyte processes (pp) show different morphology (primary and more developed foot processes). The glomerular basement membrane (thick arrow) is prominent and its three layers are visible (from podocyte towards endothelium: lamina rara externa, lamina densa, lamina rara interna). **C)** Mature glomerular capillary displaying thin endothelial cells (ec) with sparse organelles. Endothelial fenestrations (arrowheads) are present. The glomerular basement membrane (arrow) is prominent and the podocyte processes (pp) are fully developed. p: podocyte. Scale bars: 2 µm.

5.4.3 Ultrastructure of intussusceptive pillars

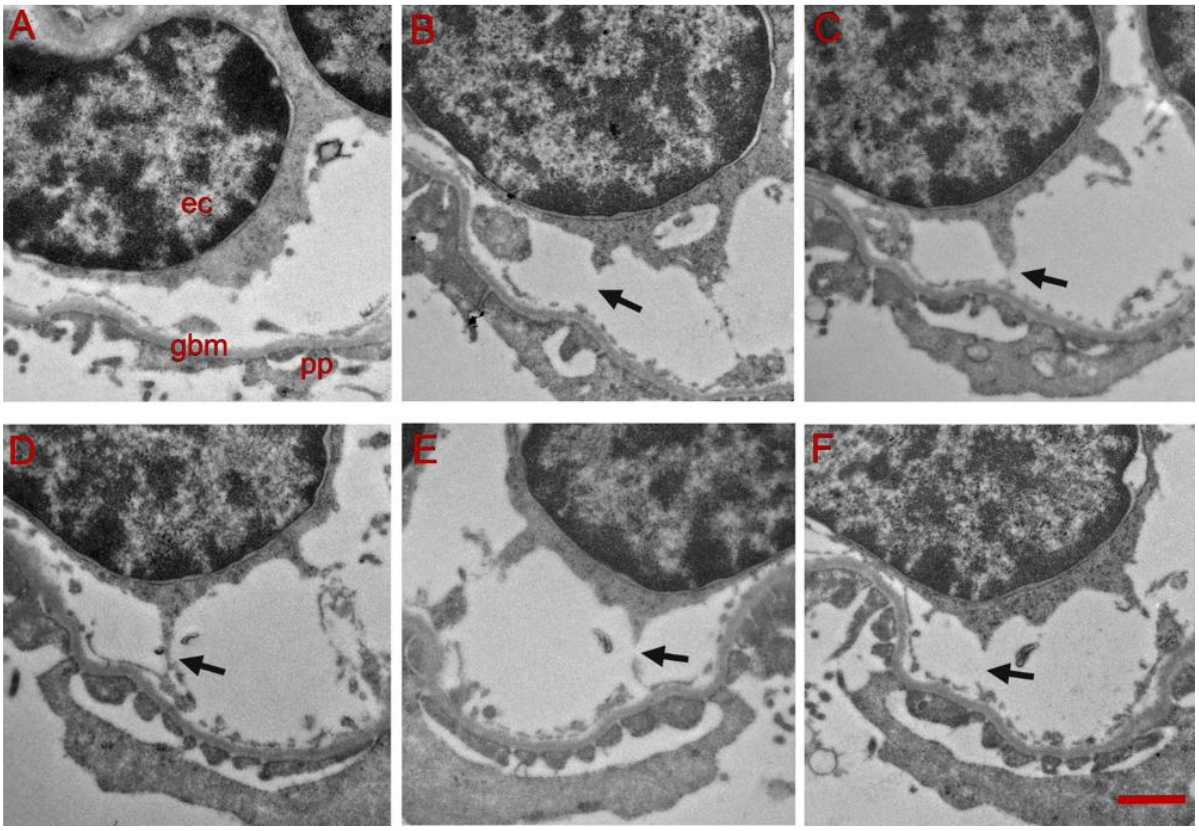

Fig. 3. Serial ultrathin sections (approximately 80 nm thick) demonstrating a glomerular capillary of a fetus of E64. The capillary consists of multiple endothelial cells (ec) with a fenestrated endothelium. Podocyte foot processes (pp) envelop the capillary. The nascent pillar consists of endothelial cytoplasm and is present in 16 sections. **A)** section 24, **B)** section 29, **C)** section 33, **D)** section 35, **E)** section 37, **F)** section 39. Scale bar: 1 µm.

Most of the intraluminal pillars were formed by single endothelial cell processes. These processes connected to a different part of the vessel wall on the opposite side of the lumen. Interstitial tissue was not present within these nascent pillars (Fig. 3).

A structure resembling a more mature intraluminal pillar was also identified in the capillary lumen. This structure consisted of extracellular matrix covered by endothelium and its diameter was ± 1.2 μm (Fig. 4). Moreover, no other types of cells except endothelial cells participated in the formation of both aforementioned pillar structures.

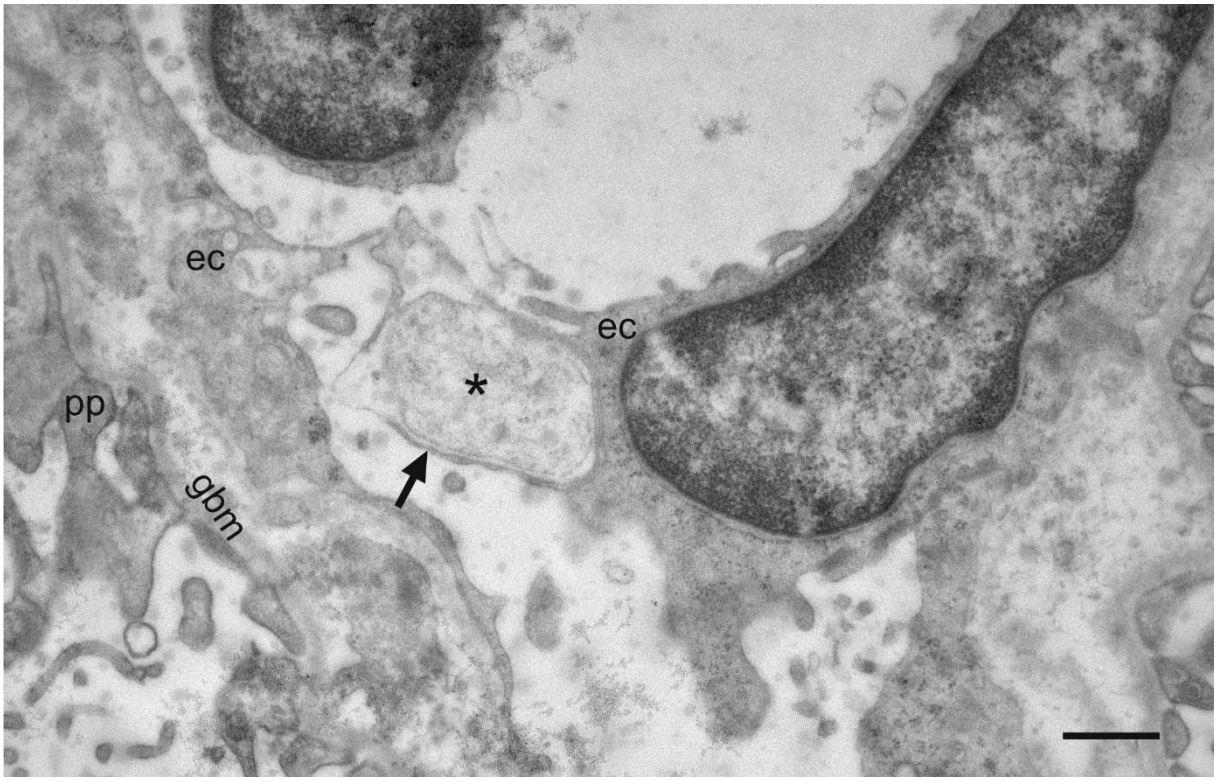

Figure 4. Transmission electron micrograph depicting a pillar-like structure in a glomerular capillary of a porcine fetus of E98. A) Capillary consisting of multiple endothelial cells (ec) with a continuous endothelium. The podocytes enveloping the capillary have interdigitated foot processes (pp). The pillar-like structure (*) consists of extracellular matrix and is covered by endothelium. Arrow points to the endothelial cell membrane covering the pillar structure. Scale bar: 500 nm.

5.5 Discussion

It is established that many changes occur within the glomerular cells during glomerulogenesis. More specifically in developing glomeruli, capillary loops increase in number and endothelial and podocyte cell layers begin to resemble their fully mature counterparts (Abrahamson, 2009). In the current study, we observed similar structural changes in the capillary phenotype, and there seems to be a correlation between the observed pillars and the maturation of the capillaries, since pillars were found in more mature capillaries. On the other hand, the identified pillars were rather scarce due to the strict criteria of our methodology. Consequently, we do not have enough data to undeniably establish a correlation between pillar formation and glomerular developmental stage.

The vasculature in the glomerular tuft showed characteristics of intussusceptive angiogenesis, i.e. intraluminal pillar formation, at various stages of development. The regions of interest were followed throughout serial sections to exclude vessel bifurcations or other structures, which may look like intraluminal pillars in single sections. Thus, it was ensured that they represented endothelial pillars with a determined starting and ending point, connecting opposite sides of the endothelial wall. These pillars were the two-dimensional depiction of the holes seen in the Mercox casts in our previous research (Logothetidou et al., 2016).

Although the formation of transluminal pillars is considered the most characteristic feature of intussusceptive angiogenesis (Djonov et al., 2003; Burri et al., 2004; Makanya et al., 2009), the exact mechanism of this process has yet to be completely clarified. In the glomerular capillaries, the pillars originated from an endothelial cell and extended in the lumen towards the opposite side of the vessel wall. This is in contrast with the original hypothesis of intussusceptive angiogenesis, during which opposite endothelial walls form a bridging contact (Burri and Tarek, 1990). Furthermore, both maturation stages of pillars demonstrated in the current research showed no involvement of perivascular cells.

Therefore, we can hypothesize that, in the very specific vascular setting of the glomerulus, perivascular cells are not a driving force in the initiation of pillar formation mechanism. This is in agreement with the original hypothesis of intussusceptive angiogenesis (Burri and Tarek 1990) and the mechanism presented in tumor-induced intussusceptive angiogenesis (Paku et al., 2011).

Most pillars identified in this research were nascent pillars, consisting only of endothelium. During pillar maturation, interstitial tissue is involved in the pillar formation. In both physiological and pathological situations, the core of a mature pillar reveals a bundle of collagen fibers (Burri and Tarek, 1990; Burri and Djonov, 2002; Paku et al., 2011). The current study demonstrated a structure resembling a mature pillar and its cross section showed extracellular matrix covered by endothelium. However, the core of this structure did not contain collagen fibers. Since the only type of collagen found in glomeruli is type IV (Ishimura et al., 1989; Miner, 1999), it is most likely that this type is present in the ECM of the pillar. Collagen IV forms meshworks, not fibers, and it is found in the glomerular basement membrane and the mesangial extracellular matrix (Shoulders and Raines, 2009). Furthermore, endothelial cells, mesangial cells and podocytes of developing glomeruli are known to synthesize different alpha chains of collagen IV (Abrahamson et al., 2009).

In conclusion, this study demonstrates the presence of intussusceptive pillars in glomerular capillaries of the porcine developing kidney. Perivascular cells or pericytes do not participate in the initial stages of pillar formation and ECM invasion seems to precede their involvement in the mature pillar. The absence of collagen fibers in the core of the pillar proves that collagen bundles are not necessary for intussusceptive angiogenesis in the metanephric glomerulus and indicates that the mechanism of pillar formation might be tissue-specific.

5.6 Acknowledgements

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Chapter VI: General discussion

Research in angiogenesis is very important, not only because of the role of angiogenesis in physiologic conditions e.g. during embryonic development and wound healing, but also because of its implication in numerous diseases such as cardiovascular diseases, ocular disorders, chronic inflammation and cancer (Griffioen and Molema, 2000; Carmeliet, 2005; Andres and Djonov, 2010; Rajappa et al., 2010; Eklund et al., 2017). Of the main angiogenic mechanisms, sprouting angiogenesis, has been intensely studied with a plethora of models (Fantin et al., 2010; Unterleuthner et al., 2017), whereas only a small number of research groups has focused on intussusceptive angiogenesis (Djonov et al., 2003; Konerding et al., 2010). Previous research on the role of angiopoietins during porcine mesonephric development, conducted in our department, indicated that angiopoietins are involved in intussusceptive angiogenesis (IA). With the intent to contribute to the current knowledge on intussusceptive angiogenesis, this research setup aimed to address the following topics (1) pinpoint the occurrence of IA in the porcine metanephros, (2) identify the mechanism of pillar formation in the glomeruli and (3) investigate the expression pattern of TIE receptors during glomerular development and find a possible link between the receptors and IA.

The first priority was to identify the spatial and temporal characteristics of IA in order to gather topographic data for the following experiments (section 3.4.1). Vascular corrosion casts of fetal kidneys provided evidence of IA, in the form of capillary holes when viewed with SEM. Holes corresponding to intussusceptive pillars were identified in capillaries of stages III, IV and V in all tested developmental stages, namely E41 until E112. This proves that intussusceptive angiogenesis is active throughout the porcine fetal development and ensures the continuous growth of functional metanephric glomeruli and is in line with previous research in other species. During the initial steps of its organogenesis, the avian metanephros is characterized by intensive sprouting angiogenesis but early on the vascular growth mode switches to IA (Makanya et al., 2005) which is energetically and metabolically more economical than sprouting (Djonov et al., 2003).

Although SA was not investigated in the current study, a similar switch could also happen in the porcine metanephric glomeruli since developing avian and mammalian kidneys show great similarities in the process of maturation in analogous nephron types between these two classes of vertebrates (Gambaryan, 1992). Additionally, glomerular filtration is not jeopardized, since no basement membrane degradation occurs during IA, as seen in sprouting angiogenesis (Ribatti and Djonov, 2012). Although no quantification of IA occurrence was performed, the holes were more frequently observed in mature stage V glomeruli, possibly because they consist of more capillaries than the developing III and IV stages. Moreover, some of these holes were located close to vessel bifurcations, which might indicate their involvement in remodeling and pruning of these microvessels (Djonov et al., 2002). Holes reminiscent of IA have been previously observed in the porcine mesonephros (De Spiegelaere et al., 2010) and in a range of tissues and species, such as the rat lung (Burri and Tarek, 1990), the CAM (Patan et al., 1993) and the chick kidney (Makanya et al., 2005).

Since the mechanism of pillar formation is not entirely understood, we focused on the morphology and the mechanism behind pillar formation during metanephric glomerulogenesis (sections 5.4.1 and 5.4.3). Taking into account our previous results, we chose to investigate III, IV and V glomeruli of different fetal stages. Our experimental setup combined serial sectioning and TEM which allowed us to identify in great detail structures which were certainly pillars, since they were followed throughout several sections ensuring their starting and ending points. The majority of the intraluminal pillars consisted of endothelial processes and had a diameter < 2.5 μm, corresponding to nascent pillars. Along the immature pillars, a more mature pillar was also identified. This structure contained extracellular matrix and was covered by endothelium. According to the original hypothesis of IA, mature pillars are invaded by collagen fibers (Burri and Tarek, 1990). In a model of tumor-induced intussusceptive angiogenesis, after subcutaneous transplantation of tumors in mice, it was proved that the collagen in the pillar core is type I (Paku et al., 2011). This could be explained by the fact that type I collagen is abundant in all dermal layers (Meigel

et al., 1977). Notably, the pillar found in our research did not contain collagen fibers. This finding indicates that collagen bundles are not necessary for pillar formation and the mechanism of intussusceptive angiogenesis might be tissue-specific, depending on the characteristics of the microenvironment. Moreover, it can be hypothesized that the ECM in the pillar found in the current research could contain collagen type IV since it is the only type found in the glomerulus (Ishimura et al., 1989; Miner, 1999). Immunstaining against collagen type IV would help validate this hypothesis.

Except for the involvement of endothelial cells and extracellular matrix, our TEM study did not reveal any contribution of glomerular pericytes, namely intraglomerular mesangial cells (Smith et al., 2012) or perivascular cells (podocytes) to the pillar formation during these stages of pillar maturation. This finding indicates that these cells are not the driving force behind the initial phases of pillar development in the metanephric glomeruli. This is in agreement with the original hypothesis of IA and the previously mentioned model of tumor induced IA, where it is stated that pericytes invade the pillar at later stages of its maturation and therefore are not driving its initial formation (Burri and Tarek, 1990; Paku et al., 2011). Although a fully mature pillar was not identified in our samples, we can hypothesize that podocytes would be involved later in the pillar formation and subsequent vessel splitting. Based on our observations of vascular bifurcations in the glomerular capillaries, the splitting is initiated at the outer side of the capillary, opposite of the side where the mesangial cell resides. Glomerular basement membrane and podocytes follow the invagination of the endothelium, leading to the formation of two capillaries from a pre-existing one (Fig. 10).

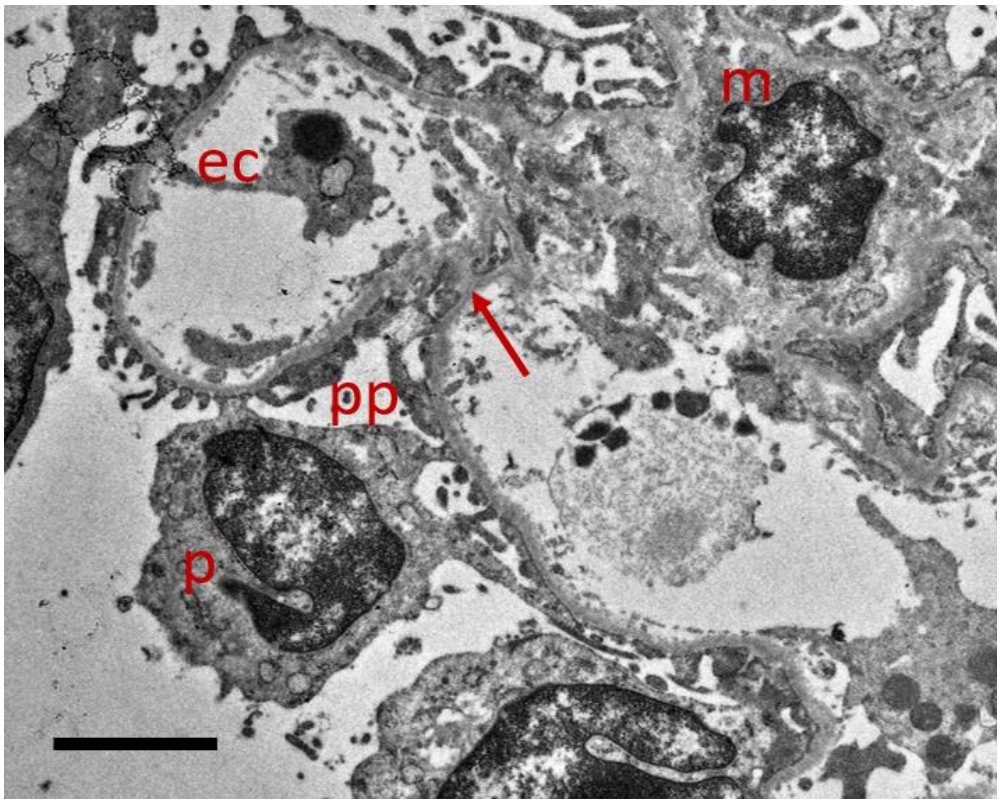

Fig. 10. TEM micrograph depicting a capillary bifurcation in a porcine metanephric glomerulus (E64). Vessel splitting begins from the endothelial wall opposite from the mesangial cell (m). Glomerular basement membrane (arrow) and podocyte processes (pp) follow the endothelial invagination. Ec: endothelial cell, p: podocyte. Scale bar is 2 μm.

A great number of consecutive sections and different samples were used in this TEM study but our criteria were very strict in order to avoid misattribution of other structures to pillars. This could explain why we couldn't identify many examples of different maturation stages of pillars. While TEM analysis is very reliable in tissues with a high density of intraluminal pillars, such as tumors (Paku et al., 2011), using it in a normally developing tissue, such as our model can be very time-consuming and laborious. Alternative techniques, which could somewhat minimize the effort and time needed, are the recently invented automatic tape-collecting ultramicrotome-SEM (ATUM-SEM) process (Schalek et al., 2011) and the serial sectioning combined with field emission SEM (FE-SEM) allowing 3D reconstruction (Kremer et al., 2015). With the first technique, the ultrathin sections are immediately and automatically collected from the knife's water boat onto the

surface of a partially submerged conveyor belt. Low resolution SEM imaging of the series of sections is then used to map the dataset so that the time consuming process of high resolution imaging can be intelligently targeted and automatically executed. A semi-automatic microscope control software package can orchestrate all of these steps to produce volume EM image sets from an ATUM tape (Hayworth et al., 2014). The second technique combines automate sectioning (Gatan 3View2 ultramicrotome) with image capture of 3D ultrastructure using (FE-SEM). This system that enables the analysis of specimens up to 400 μm^3. Although both of these techniques are promising and facilitate a more effortless way of sample analysis, they require special equipment and are expensive.

TIE1 and TIE2 receptor tyrosine kinases are expressed during embryonic development (Sato et al., 1995) and regulate embryonic angiogenesis by the mechanism of intussusceptive microvascular growth (Patan, 1998). Therefore, the molecular part of this study was initiated by immunohistochemically mapping the expression pattern of the TIE receptors in the developing metanephros (section 3.4.3). In our research, we found that both receptors were expressed not only in endothelial cells in the glomerular capillaries, but also in podocytes, namely specialized epithelial cells. TIE1 and TIE2 showed a similar and constant expression pattern in glomeruli and S-shaped bodies, regardless of the fetal age. Therefore, a clear link between TIE expression and intussusceptive angiogenesis could not be identified. It is possible that, whereas TIE receptors are expressed throughout fetal development, the differential expression of angiopoietins controls the balance between quiescence and angiogenesis. ANGPT2, which promotes angiogenesis, is strongly expressed early during glomerular development and its expression is attenuated in mature glomeruli (De Spiegelaere et al., 2011). ANGPT1 is expressed in later stages of glomerular development (De Spiegelaere et al., 2011) and in mature glomeruli (Satchell et al., 2002), where it is thought to promote vascular maturation and maintenance

Although TIE expression is considered largely restricted to endothelial cells, there are a few publications demonstrating that some epithelial cells express TIE genes. Both

receptors have been previously found in the the primary cilia in the female reproductive organs (Teilmann and Christensen, 2005) and TIE2 has been found in rodent cholangiocytes in polycystic liver diseases (Fabris et al., 2006). Interestingly, TIE2, but not TIE1, expression was shown in human (Satchell et al., 2002) and murine (Dessapt-Baradez et al., 2014) podocytes. The role of TIE1 and TIE2 in endothelial cells has been investigated to a great extent (Dumont et al., 1994; Puri et al., 1995). TIE receptors mediate interactions between endothelial cells with their extracellular matrix and with surrounding mesenchymal cells. These interactions are crucial for normal endothelial cell motility and/or attachment and also for recruitment of periendothelial cells (Patan et al., 1998). Conversely, the role of TIE receptors in podocytes remains a mystery. Since ANGPT-TIE2 signalling was also described in nonendothelial cells, such as monocytes (De Palma et al., 2013) and muscle satellite cells (Abou-Khalil et al., 2009), angiopoietin signalling could be an important regulator in podocytes, too. A first hypothesis might be that TIE2 receptors in podocytes act as decoy receptors, controlling the overexpression of ANGPT. Another possibility is that TIE2 in podocytes could play a special role in patterning and/or maintaining the glomerular vessels through angiopoietin signalling. This offers a very promising perspective, because ANGPT-TIE signalling in podocytes could help explain some nonendothelial effects observed when angiopoietins are overexpressed (Davis et al., 2007). The function of TIE1 remains somewhat obscure in the current literature and our research is the first report of TIE1 in podocytes. Our finding further supports the hypothesis that the effect of angiopoietins on podocytes and endothelial cells might be similar (Woolf et al., 2009). Therefore, the role of TIE1 could be the same in podocytes as in endothelial cells, namely acting as an inhibitory co-receptor of TIE2. Within sites of active angiogenesis, endothelial TIE1 and TIE2 associate to form heterodimers which inhibit TIE2 activation and clustering (Kim et al., 2006; Seegar et al., 2010). ANGPT2 is unable to dissociate the inhibitory TIE2-TIE1 complexes upon binding TIE2 and, therefore, does not induce TIE2 clustering and signalling, yet behaves as a competitive antagonist by blocking further binding of ANGPT1. This antagonistic effect of ANGPT2 opposes the

activation of downstream signalling generated by ANGPT1, promoting either vascular degeneration or angiogenesis (Seegar et al., 2010). This hypothesis is in accordance with the observations in the present study, in which TIE1 was continuously expressed in developing metanephric glomeruli, which kept on growing throughout development.

Previous in vitro studies have linked translocation of TIE receptors in different areas of the endothelial cell membrane with the activation of different molecular pathways. More specifically, TIE2 receptor signalling complexes across the endothelial junction mediate cell survival signals, whereas TIE2 at cell–substratum contacts elicits a potent angiogenic response (Fukuhara et al., 2008; Saharinen et al., 2008). Therefore, it was interesting to investigate the distribution of TIE receptors on the endothelial cell membrane of metanephric glomerular capillaries (section 4.4). Using high-resolution immunogold labeling and the relative labeling index stereological approach, this study demonstrated that both receptors were predominantly expressed on the abluminal side of endothelial cell membranes, namely the side that faces the glomerular basement membrane. Our finding that the vast majority of TIE2 is close to the production site of ANGPT1, namely the podocytes, could indicate that these receptors are able to interact with ANGPT1 to further promote angiogenesis. According to the same hypothesis, the small number of TIE2 receptors detected close to interendothelial junctions, could be ANGPT-activated translocated TIE2 receptors which would promote vascular quiescence. Although such an explanation makes sense in our model, where angiogenesis is indeed active, it has to be further investigated since the original studies showed that the activation of different molecular pathways depends on the presence or absence of neighboring endothelial cells. However, this was not the case in our experiments, since our study was an in situ investigation and neighboring cells were always present.

We recognize that immunogold has some drawbacks such as limited penetration of the immunoreagents through the resin which can influence the labeling intensity (Mayhew,

2011). Nevertheless, the expression of TIE receptors in podocytes, at similar levels to endothelial expression, is very intriguing and deserves further research.

The kidney is a good model to study cellular and molecular mechanisms of organogenesis for most known developmental processes are involved in its morphogenesis (Kuure et al., 2000). Kidney development requires reciprocal inductive signalling between mesenchyme and epithelium (Little and McMahon, 2012). It is noteworthy that similar cellular interactions (mesenchyme-to-epithelial transition) and signalling molecules e.g. paired box gene 2 (PAX-2), Wilms tumor protein (WT-1), VEGF and platelet derived growth factor β (PDGF-β) seem to be involved in the development of all the kidney types (Carroll and Vize, 1996; Heller and Brandli, 1997; Little and cMahon, 2012). This may suggest some degree of conservation in the genetic program of kidney organogenesis. More specifically, the ultrastructural components of mesonephros and metanephros show a high degree of similarity, although the vascularization pattern of the organs differs markedly (Tiedemann and Egerer, 1984). This makes the mesonephros, along with the metanephros, interesting models in organogenesis, especially in pigs where active mesonephroi are present in a relatively large embryos (Gersh, 1937). Additonally, the numerous and easily identifiable and delineable glomeruli in these kidneys offer interesting models not only for nephrogenesis but also angiogenesis.

Conclusions and future perspectives

The present PhD thesis provided strong evidence that intussusceptive angiogenesis occurs throughout the fetal development of the porcine metanephros. Initially, the intussusceptive pillars consist only of endothelial processes whereas extracellular matrix with no collagen fibers invades the pillar at a later stage. Because podocytes were not associated with these pillars, they are not considered the driving force behind pillar formation in the glomerulus. Nevertheless, their role and the timeframe of their involvement in the intraluminal pillar formation has to be further clarified, since the

glomerular capillaries are a very specialized setting of blood vessels. Additionally, this study proved that TIE receptors, which are crucial for the growth and morphogenesis of the glomerular vasculature, are present in the developing porcine metanephros in both endothelial cells and podocytes. However, their functions in podocytes remain hypothetical and should still be further investigated in future studies.

The complexity of angiogenesis, a morphogenetic process with numerous stimuli, multiple steps and several cell types involved, makes its study difficult using reductionist techniques alone. For this reason, it is very important to develop a method to investigate intussusceptive angiogenesis in its full complexity. Hlushchuk et al. (2016) have developed a model of in vivo-imaging down to single cells within the living organism, followed with the light and electron microscopy of the site of interest harvested at the chosen time-point. However, this model was developed in the zebrafish caudal fin, which offers the possibility for repetitive in vivo observation at high resolution because the distal tip of the fin is less than 150 μm thick and is organized in an almost single plane (Huang et al., 2003). Such technique cannot be utilized in a mammalian kidney, e.g. the porcine metanephros used in the current research. An example of a model with a sheet-like organization which would allow such investigations is the CAM vasculature. Intussusceptive angiogenesis was previously reported in the CAM (Patan et al., 1993) and this fact makes it a very good candidate for this correlative microscopy method or the 3D FE-SEM system, previously mentioned as an alternative method (Kremer et al., 2015).

The zebrafish and the CAM models could be useful tools to further investigate ANGPT-TIE involvement in IA, although there are some major drawbacks which should be mentioned. Both organisms, zebrafish and chicken, are phylogenetically more distant from human compared to mammals. Moreover, the CAM model is restricted only to avian species and the CAM vasculature is highly angiogenic and this fact might hamper the extrapolation of results to other species (De Spiegelaere et al., 2012). Another point that should be raised is that the adult zebrafish have mesonephric kidneys whereas mammals

and birds have metanephric (Swanhart et al., 2011), a difference which should be taken into consideration when extrapolating date to mammalian species. Nevertheless, there is a striking similarity in the molecular and segmental organization of the mammalian and zebrafish nephrons (Wingert et al. 2007; Wingert and Davidson, 2008) and the zebrafish has become an important model system for studying renal disease thanks to the anatomical simplicity of the embryonic kidney (Drummond, 2005).

The current research indicated a possible link between TIE receptors and intussusceptive angiogenesis, but additional research is needed to prove their role in the mechanism of IA. A good experimental approach would be to generate conditional knockout models targeting TIE1, TIE2, and/or angiopoietins. Specifically in zebrafish, a strategy allowing tissue- or temporal-specific disruption of genes has been developed (Maddison et al., 2012). After identifying embryos with the insertional mutants, their vasculature can be studied for growth restriction, vascular defects, absence of patterning in the targeted tissue, but in the whole embryo, too. Moreover, knockouts generated with site-specific recombinase technology can be induced in the pregnant dam in order to knock out the targeted allele at each embryonic day, as presented in mice (Jeansson et al., 2011).

Furthermore, the fact that the ANGPT-TIE system might not be exclusive in endothelial cells of the blood and lymphatic vasculature could provide new insights into the development and function of renal glomeruli. The discovery that both TIE1 and TIE2 are found in podocytes, indicates a mechanism of podocyte regulation via angiopoietin signalling. Preliminary Western Blotting results from our research group already showed TIE1 expression in an immortalized mouse podocyte cell line (Shankland et al., 2007). Additionally, a human podocyte cell line will also be used to validate that the obtained results are not specific to the cell-line or parent species. Future investigations of human and murine pococyte cell lines should focus on the effect of ANGPT-TIE signalling on podocyte survival, proliferation, migration and permeability after treatment with recombinant ANGPT1 and ANGPT2. Moreover, the molecular pathways that are

involved in angiopoietin signalling should be also explored. Since ANGPT1 mediates endothelial survival through the PI3K/AKT pathway (Kim et al., 2000), its possible activation in podocytes upon angiopoietin stimulation should be tested. Finally, in order to clarify whether TIE1 acts as an inhibitory TIE2 co-receptor in podocytes, as in endothelial cells, TIE1 knockdown can be used to investigate changes in TIE2 phosphorylation levels.

Summary

Summary

Glomeruli are a part of the primary filtration units of the vertebrate kidney. Although the molecular pathways behind glomerular development have not been fully elucidated, intussusceptive angiogenesis (IA) has a major contribution to vascular expansion and remodeling of the glomerular vasculature. The ANGPT-TIE system signals between mesangial cells, endothelial cells and podocytes to maintain the structure and integrity of the glomeruli. These molecules are involved not only in the normal development of kidney glomeruli, but also in disease, making them promising targets for angiogenic or anti-angiogenic therapies.

The general introduction (**Chapter I**) of this doctoral thesis provides a comprehensive overview of the current literature on angiogenesis and its significance during development and disease. This research focuses on the mechanism of IA, which is responsible for the expansion and remodeling of most vascular beds but is not fully elucidated yet. One of the most important angiogenic growth factor systems linked with IA are the ANGPT and their receptors TIE. Numerous studies have focused on ANGPT-TIE involvement in angiogenesis, but the conflicting functions of ANGPT and the unclear role of TIE1 still represent big obstacles. Intussusceptive angiogenesis and the ANGPT-TIE pathway are important during metanephric development and specifically during glomerulogenesis. Metanephric glomerular development follows a predictable and topographically traceable pattern in all fetal stages, making the metanephros a good model for a study on IA.

Chapter II describes the objectives of the experimental work which was performed during this PhD thesis. The focus of this thesis was to gain insight into the expression pattern of the TIE receptors in developing porcine glomeruli, and to study the morphology, formation and ultrastructure of the intussusceptive pillar in the metanephric kidney.

In **Chapter III**, the presence of IA and the TIE expression in metanephric glomeruli are investigated, in order to find a link between the receptors and IA. The temporal and

spatial occurence of IA was identified in capillaries of stage III, IV and V glomeruli throughout fetal porcine development, using vascular corrosion casting and SEM. Additionally, TIE1 and TIE2 were immunohistochemically detected in endothelial cells and podocytes in all developmental stages of glomeruli. To our knowledge, this is the first report of TIE1 in glomerular podocytes. The presence of these receptors during active IA indicates an association between the TIE receptors and IA during glomerulogenesis in the porcine metanephros.

Since the localization of TIE2 receptors plays an important role in eliciting differential responses towards quiescence or angiogenesis, we aimed to map the subcellular localization of TIE receptors using high-resolution immunogold electron microscopy (**Chapter IV**). Quantitative analysis showed that TIE1 and TIE2 antibody labeling was predominantly detected on the abluminal side of endothelial cell membranes. This could indicate that these receptors are able to interact with ANGPT1 from the adjacent podocytes, further promoting angiogenesis. Similar expression levels of the receptors in both endothelial cells and podocytes promotes the hypothesis that TIE1 and TIE2 interplay, as described in endothelial cells, might also be present in podocytes.

In **Chapter V**, the mechanism of intraluminal pillar formation, the structural indicator of intussusceptive angiogenesis, was studied. Using serial semithin sectioning and TEM, intraluminal pillars were observed in the vasculature of developing glomeruli. The majority of these pillars were nascent, consisting of endothelial processes, although a more mature pillar was also identified. The mature pillar had a central core of extracellular matrix covered by endothelium. Collagen fibers were not visible in the pillar core indicating that they are not necessary for pillar formation or maturation. Such finding might mean that the mechanism of IA is tissue-specific. Additionally, perivascular cells and pericytes were not involved in the pillar structure during these stages of its formation, suggesting that they are not the driving force behind pillar initiation. This is in agreement with the

currently described mechanisms of IA, stating that the pillar is only invaded by pericytes at the last stage of its maturation.

The general discussion (**Chapter VI**) tackles the most important points which raised questions. Additionally, it highlights the strengths and weaknesses of the techniques and the experimental model used in this research and eventually, suggests future perspectives that could further aid angiogenic research. Overall, the results of this study indicated a link between IA and TIE receptors, but further investigations are needed to prove the role of these receptors in the mechanism of IA. In addition, the presence of TIE receptors in podocytes, led us to the hypothesis of ANGPT-TIE signalling in podocytes, a possibility that should be explored in the future. Furthermore, the study of pillar formation in the metanephric glomeruli showed similarities, but also differences with the current described mechanisms in other tissues, such as the absence of collagen fibers in the mature pillar. This led us to postulate that IA might be a tissue-specific mechanism.

Samenvatting

Samenvatting

Glomeruli vormen bij de gewervelde dieren een onderdeel van het primaire filtratieapparaat in de nieren. Ondanks het feit dat het moleculaire mechanisme dat de glomerulogenese aanstuurt nog onvoldoende opgehelderd is, is het wel zeer duidelijk dat intussusceptieve angiogenese (IA) een belangrijke rol speelt in de ontwikkeling, uitbreiding en remodellering van het bloedvatenkluwen waaruit de glomerulus is opgebouwd. Hierbinnen is signaaltransductie binnen het ANGPT-TIE-systeem tussen mesangiale cellen, endotheelcellen en podocyten van belang voor de instandhouding van de structuur en integriteit van de glomeruli. De ANGPT en hun receptoren zijn evenwel niet enkel van belang tijdens de normale ontwikkeling van de nieren, maar hebben ook een duidelijke rol in een aantal pathologische processen, waardoor ze een interessant doelwit vormen voor angiogene therapieën.

In de algemene inleiding van deze doctoraatsthesis (**Hoofdstuk I**) wordt een bondig overzicht gegeven van de huidige wetenschappelijke literatuur rond angiogenese, met bijzondere aandacht voor de rol ervan in fysiologische en pathologische processen. Hierbij gaat de aandacht voornamelijk naar de focus van het onderzoek die ligt op het mechanisme van IA. IA is verantwoordelijk voor de uitbreiding en de remodellering van de meeste vasculaire netwerken, maar ondanks zijn belang en algemeen voorkomen is het proces tot op heden nog onvoldoende uitgeklaard. Een van de belangrijkste angiogene groeifactoren die betrokken zijn in IA zijn de angiopoietines en hun TIE-receptoren. Hun rol hierin wordt bevestigd door talrijke studies, al leverden deze contradictorische gegevens op voor wat betreft de functies van de ANGPT en blijft er ondanks alles redelijk wat onduidelijkheid over de exacte rol van de TIE1-receptor, wat het verwerven van diepere inzichten in hun actiemechanisme bemoeilijkt. Intussusceptieve angiogenese en de ANGPT-TIE reactieweg zijn belangrijk tijdens de ontwikkeling van de metanefros of definitieve nier, en in het bijzonder tijds de glomerulogenese. De ontwikkeling van glomeruli in de metanefros vindt plaats volgens een voorspelbaar en topografisch

lokaliseerbaar patroon, ongeacht de foetale leeftijd, waardoor de metanefros een geschikt model vormt voor het diepgaand bestuderen van IA.

De specifieke doelstellingen van de experimentele studies binnen dit doctoraat werden opgelijst in **Hoofdstuk II**. Het algemeen doel was verdere inzichten te verwerven in het expressiepatroon van de beide TIE-receptoren in ontwikkelende glomeruli in de metanefros bij het varken, en de morfologie, ontstaanswijze en ultrastructuur van de intussusceptieve intraluminale zuil te beschrijven.

Hoofdstuk III omvat de studie waarin de aanwezigheid van IA en de expressie van TIE-receptoren en de correlatie tussen beiden in de metanefrotische glomeruli onderzocht werd. Zowel de plaats als tijdstip van voorkomen van IA in de capillairen van glomeruli van stadium III, IV & V van de ontwikkeling werd in kaart gebracht aan de hand van vaatgietsels die via SEM onderzocht werden. Daarenboven werden in alle glomerulaire ontwikkelingsstadia TIE1 en TIE2 immunohistochemisch gedetecteerd ter hoogte van de endotheliale cellen en de podocyten. De aanwezigheid van TIE1 in podocyten is hierbij opmerkelijk, want voor zover we konden nagaan werd dit nog nooit eerder in de literatuur beschreven. De aanwezigheid van deze receptoren gedurende actieve IA toont alvast een verband aan tussen de TIE-receptoren en IA gedurende de glomerulogenese in de metanefros van het varken.

Aangezien de specifieke lokalisatie van de TIE2-receptoren op de endotheliale celmembraan bepalend is of uitgelokte respons na activatie leidt tot angiogenese dan wel tot vasculaire stabiliteit, hadden we in **Hoofdstuk IV** tot doel de subcellulaire lokalisatie van de TIE-receptoren via immunogoud-transmissie-elektronenmicroscopie in hoge resolutie in kaart te brengen. Via kwantitatieve analyse werd aangetoond dat anti-TIE1 en anti-TIE2 kleuring ter hoogte van de celmembraan voornamelijk voorkwam aan de abluminale zijde van de endotheelcellen. Dit is een aanwijzing dat deze receptoren mogelijk interageren met ANGPT1 afkomstig van de podocyten, die hiermee de angiogenese aansturen. Gelijkaardige expressiepatronen van TIE1 en TIE2 zowel in de

endotheelcellen als de podocyten kan er op wijzen dat het samenspel van beide receptoren, zoals al eerder beschreven ter hoogte van de endotheelcellen, zich ook voordoet ter hoogte van de podocyten.

In **Hoofdstuk V** werd het mechanisme van de vorming van de intraluminale zuil, een morfologisch kenmerk tekenend voor intussusceptieve angiogenese, nagegaan. Via lichtmicroscopische observatie van semidunne seriecoupes alsook van ultradunne seriecoupes via TEM werd de ruimtelijke configuratie van de intraluminale zuilen in de ontwikkelende glomeruli in beeld gebracht. Het merendeel van de waargenomen zuilen waren pas ontstane, primitieve structuren, die uitsluitend uit endotheliale uitlopers waren opgebouwd. Een meer mature zuil werd evenwel ook waargenomen. Deze bestond uit een centrale as van extracellulaire matrix die omgeven was door endotheel. In deze centrale as werden geen collageenvezels waargenomen, wat er op kan wijzen dat dezeniet noodzakelijk zijn voor de vorming en verdere maturatie van de intraluminale zuil. Voorts kan dit er op wijzen dat het mechanisme van intussusceptieve angiogenese op een weefselspecifieke manier verloopt. Daarenboven waren perivasculaire cellen en pericyten niet betrokken in de zuilvorming, althans niet voor de geobserveerde stadia, wat kan betekenen dat zij niet de stuwende kracht zijn in de initiële aanleg van de intraluminale zuil. Deze bevinding komt overeen met de huidige inzichten in de mechanismen van IA, waarbij aangegeven wordt dat de invasie van de zuil door pericyten slechts optreedt in de laatste stadia van de ontwikkeling van de zuil.

In de algemene discussie (**Hoofdstuk VI**) worden de belangrijkste twistpunten en de nieuwe vragen die voortvloeien uit het gevoerde onderzoek gepareerd. Ook de sterktes en tekortkomingen van de gehanteerde technieken en het gebruikte experimentele model worden belicht en er wordt vooruit gekeken naar mogelijke vervolgonderzoeken. Algemeen gesproken kon aan de hand van huidig onderzoek de betrokkenheid van de TIE-receptoren in intussusceptieve angiogenese aangetoond worden, maar de exacte rol van hun interacties met elkaar en hun liganden dient nog verder uitgeklaard te worden. De

complexe interactie van de ANGPT en de TIE-receptoren zoals beschreven op het niveau van het endotheel, kan, dankzij onze waarneming van de TIE-receptoren ter hoogte van de podocyten, ook op dit niveau een belangrijke rol spelen in de angiogenese. Ook deze hypothese verdient verder onderzoek. Ten slotte vertoonde het proces van intraluminale zuilvorming niet enkel gelijkenissen maar ook enkele markante verschillen met de vormen van IA die momenteel in de literatuur beschreven zijn, zoals de afwezigheid van collageenvezels in de groeiende zuil. Hieruit leidden we af dat het mechanisme van IA mogelijk weefselspecifiek is.

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Curriculum Vitae & Bibliography

Curriculum Vitae

1.1 Personal Information

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1.2 Education

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Doctoral school of life sciences & medicine (2011-2017)

- Laboratory animal science I and II
- TEM sectioning of serial semithin and ultrathin sections
- Advanced academic English: Conference skills
- Fostering responsible conduct of research
- Personal effectiveness

Aristotle University of Thessaloniki

Master of Science – Biology (2008-2010)

"Germination of clinical strains of Dermatophytes and its inhibition"

Laboratory of Microbiology, Department Genetics, Development and Molecular Biology

1.3 Experience

Research in bovine neonatal pancytopenia (2011-2012)

Laboratory of Immunology, Faculty of Veterinary Medicine, Ghent University

Practical training in "Clotting tests - Control of thrombophilia"

Ippokrateio Hospital of Thessaloniki, Greece

Bibliography

Publications in peer-reviewed international journals

Logothetidou A, Vandecasteele T, Van Mulken E, Vandevelde K, Cornillie P (2017) Intussusceptive angiogenesis and expression of Tie receptors during porcine metanephric kidney development. Histol Histopathol. 32:817-824

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Participation in international conferences

ECMIS Symposium, E. coli and the Mucosal Immune System. 2- 5 July, 2011. Ghent, Belgium. (participation)

Logothetidou A, Van den Broeck W, Cornillie P (2016) Ultra-microscopic and molecular characterization of intussusceptive angiogenesis. Joint Conference EAVA & WAHVM. 27-30 July 2016. Vienna, Austria. (oral presentation)

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Now is probably the part where I am expected to quote some famous Greek philosopher stating something profound about education or life. But, I will just leave here the following quote from H.P. Lovecraft which is dedicated to my **NixieWixie**, probably my strongest anchor to sanity: "In its flawless grace and superior self-sufficiency I have seen a symbol of the perfect beauty and bland impersonality of the universe itself, objectively considered; and in its air of silent mystery there resides for me all the wonder and fascination of the unknown". Because cats!

Natassa,

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