The *FoxF2* Gene in Development and Disease

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To my family

The *FoxF2* gene in development and disease

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Abstract

In this thesis I present our recent data on the involvement and the mechanism of action of the forkhead transcription factor Foxf2 in development of the brain microvasculature, formation of the blood-brain barrier, control of the intestinal stem cell niche, and fusion of the secondary palate. The potential clinical significance of these findings is strengthened by a correlation between *Foxf2* expression and intestinal adenoma formation, and by association between genetic variants in human *FOXF2* and incident stroke.

We showed that *Foxf2* is expressed in brain pericytes, but not in mural cells of other organs. *Foxf2* null mutants have a defective brain vasculature and suffer from intracranial hemorrhage and a leaky blood-brain barrier with increased endothelial vesicular trans-cytosis. *Foxf2*$^{-/-}$ brain pericytes have diminished Pdgfrβ expression, and the cerebral vasculature a reduced activity of the Tgfβ – Alk5–Smad2/3 signaling pathway, associated with decreased expression of integrins, *Tgfb2*, *Tgfbr2*, *Alk5* and other pathway components.

In a large GWAS performed by an international consortium, we identified a genome-wide significant association of common variants near *FOXF2* with risk of stroke. Conditional knockout mice, in which *Foxf2* was deleted in healthy adults, developed clinical signs of stroke and exhibited cerebral ischemia, reactive gliosis and microhemorrhage. The animal model results thus corroborate the human genetic association and identifies *FOXF2* as a novel risk locus for stroke.

In the intestine we showed that *Foxf2* is expressed by subepithelial fibroblasts and restricts the size of the stem cell niche, and thereby the number and proliferation of Lgr5^{+} stem cells. *Foxf2* is a target of epithelial hedegehog signaling and inhibits the Wnt pathway by increasing the expression of the extracellular Wnt inhibitor Sfrp1. As a consequence, reduced *Foxf2* expression significantly increases both initiation and growth of intestinal tumors.

Reduced proliferation and decreased extracellular matrix production in the neural crest-derived mesenchyme of the palatal shelves was found to be responsible for the cleft palate phenotype in *Foxf2* null mutants. Mechanistically, the defect is associated with reduced canonical Tgfβ signaling and integrin expression. The *Tgfb2* mRNA level was not affected, but the amount of Tgfβ2 protein was significantly decreased in mutant palatal shelf mesenchyme.

Keywords: *Foxf2*, Pericyte, Pdgfrβ, Blood-brain barrier, Stroke, Wnt signaling, *sFRP-1*, Intestinal stem cell niche, Lgr5, Palatogenesis, Cleft palate, Tgfβ signaling

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Papers discussed

This thesis is based on the following publications, referred to by roman numerals in the text:

I.

Foxf2 is required for brain pericyte differentiation and development and maintenance of the blood-brain barrier

Reyahi A, Nik AM, Ghimai M, Gritli-Linde A, Pontén F, Johansson BR, Carlsson P.

Developmental Cell (2015) 34, 19-23.

II.

FOXF2, a novel risk locus for stroke and small artery disease

Ganesh Chauhan, Corey R Arnold, Audrey Y Chu, Myriam Fornage, Azadeh Reyahi, Joshua C Bis, Aki S Havulinna (equal contribution first authors) … *additional co-authors excluded for brevity*… (joint senior authors:) Lenore J Launer, M Arfan Ikram, Peter Carlsson, Daniel I Chasman, Sarah J Childs, William T Longstreth, Jr, Sudha Seshadri, Stéphanie Debette.

Submitted

III.

Foxf2 in intestinal fibroblasts reduces numbers of Lgr5(+) stem cells and adenoma formation by inhibiting Wnt signaling

Nik AM, Reyahi A, Pontén F, Carlsson P.

Gastroenterology (2013) 144(5), 1001-11.

IV.

Foxf2 enhances Tgfβ signaling in secondary palate development

Ali M.Nik, Jeanette Astroga-Johansson, Azadeh Reyahi, Mozhgan Ghiami, Fredrik Pontén and Peter Carlsson.

Manuscript

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The *FoxF2* gene

FoxF2 encodes a transcription factor

Forkhead genes encode transcription factors, which contain "winged helix" DNA-binding domains consisting of 110 amino acids. They are highly conserved and present in mammals and other metazoans, as well as in some lower organisms like fungi, but absent in plants, protists, archaea, and bacteria (Carlsson and Mahlapuu 2002). The mammalian *FoxF* group consists of two genes, *FoxF1* and *FoxF2*. Sequence similarities and genomic locations suggest that a duplication of an ancestral metazoan gene created the *FoxC* and *FoxF* homologues found for example in *Drosophila*. A second duplication of the entire locus then gave rise to the two gene pairs seen in vertebrates: *FoxF1*/*FoxC2* – in human located at chr 16q24 – and *FoxF2*/*FoxC1*. *FOXF2* and *FOXC1* are located approximately 200 kb apart, in human on chromosome 6 (6p25) (Larsson, Hellqvist et al. 1995, Kaestner, Bleckmann et al. 1996, Blixt, Mahlapuu et al. 1998).

Murine *Foxf1* and *Foxf2* are located on chromosome 8 and 13 respectively. Both consist of two exons with the DNA-binding domain encoded by exon 1 (Miura, Kakinuma et al. 1998, Chang and Ho 2001). The proteins encoded by the two *FoxF* paralogues have identical DNA binding domains, but differ in the localization and properties of transcriptional activation domains (Hellqvist, Mahlapuu et al. 1996, Blixt, Mahlapuu et al. 1998, Hellqvist, Mahlapuu et al. 1998).

Foxf2 expression

During murine embryonic development, the expression patterns of the two *Foxf* genes overlap, but also exhibit important differences, which are reflected in distinct mutant phenotypes. Both genes are expressed in the splanchnic mesoderm and its derivatives, such as the mesenchyme, muscle and connective tissue of the gastrointestinal tract, and organs derived from the primitive gut (Mahlapuu, Pelto-Huikko et al. 1998, Aitola, Carlsson et al. 2000, Mahlapuu, Ormestad et al. 2001, Ormestad, Astorga et al. 2004, Ormestad, Astorga et al. 2006). *Foxf1* expression during mouse embryonic development has been described in detail elsewhere (Peterson, Lim et al. 1997, Blixt, Mahlapuu et al. 1998, Mahlapuu, Ormestad et al. 2001, Kalinichenko, Gusarova et al. 2003) and will not be discussed further here.

The expression pattern of *Foxf2* during mouse embryonic and postnatal development has been studied by *in situ* hybridization (Aitola, Carlsson et al. 2000, Ormestad, Astorga et al. 2004). In the prenatal stages *Foxf2* is detected in the mesenchymes lining the endodermal epithelium of the gastrointestinal tract, surrounding the tooth germ and beneath the epithelia of the respiratory system and genitourinary tract. In the embryonic gastrointestinal tube, the expression follows an anteroposterior gradient – lowest in foregut to highest in hindgut – together with a radial gradient, with the highest mRNA concentration close to the epithelium. *Foxf2* is also expressed in the developing central nervous system (CNS), eye, ear, limb buds and intervertebral discs. In postnatal development, *Foxf2* mRNA was mainly detected in eye, intestine, stomach and lung (Aitola, Carlsson et al. 2000, Ormestad, Astorga et al. 2004).

At embryonic day 9.5 (E9.5), *Foxf2* is expressed in the head mesenchyme surrounding Rathke's pouch, next to the oropharynx and stomodeum, and also in the mandibular component of the first branchial arch. At E10.5, sclerotomes and neural crest-derived cells start to express *Foxf2*. Expression is also seen in the mesenchyme posterior of the optic stalks and in cells associated with blood vessels invading the neuroectoderm of the brain (Ormestad, Astorga et al. 2004).

In Paper I, we investigated the identity of *Foxf2* expressing cells in the developing brain by Foxf2 immunostaining and identified them as pericytes and precursors of vascular smooth muscle cells. In contrast, no Foxf2 was detected in cells associated with blood vessels outside the brain.

Embryonic phenotype of Foxf2 null mutants

Foxf2 null mice are born with several severe developmental defects and die shortly after birth. Most *Foxf2* mutant pups suffer from cleft palate, which appears to be the immediate cause of death. A cleft in the secondary palate interferes with the ability of the newborn to breath and suckle and causes air filling of the gastrointestinal tract (Wang, Tamakoshi et al. 2003 and Paper IV). Gut malformations such as anal atresia, muscular hypoplasia, disintegration of the intestinal epithelium, and Hirschsprung's disease with megacolon compromise feeding and digestion, which contribute to the mortality of pups born with a normal, fused palate (Ormestad, Astorga et al. 2006). In Paper I, we showed that *Foxf2* mutants also have a defective brain vasculature and suffer from intracranial hemorrhage and a leaky blood-brain barrier (BBB), malformations that are likely to contribute to their lack of viability.

Foxf2 conditional knockout mice

Targeted disruption of forkhead genes has provided information about the function of Fox proteins in metabolic regulation and physiology. However, a number of forkhead transcription factors are important regulators of embryonic development, and loss-of-function mutations often lead to early embryonic lethality. Therefore, the conventional knockout approach is often not useful to study the gene function in postnatal stages.

Foxf2 mutants survive birth, but die immediately after. We generated a conditional knockout allele of *Foxf2* to address the role of this gene in different organs during pre- and postnatal development. Our strategy to design a conditional knockout was based on the *Cre-loxP* system, which allows us to control *Foxf2* gene inactivation in a desired spatial and temporal pattern by choice of different promoters to drive the *Cre* gene.

Foxf2 consists of two exons. Exon 1 encodes the DNA-binding domain of the Foxf2 protein. To generate a *Foxf2* allele flanked by *loxP* sites (a "floxed" allele), genetic modifications were performed on a BAC clone containing the *Foxf2* gene using RedET recombination (Muyrers, Zhang et al. 2004). A *loxP* sequence was inserted upstream (5') of exon 1, and an *Frt*-flanked selection cassette (*Pgk-Neo^R*) together with a *loxP* site in the intron (Fig 1). The targeting construct was integrated in the genome of R1/E murine (129) embryonic stem cells by homologous recombination. The resulting *Foxf2* $^{fl\text{-}neo/+}$ mice were crossed with an *FLPe* transgenic germ-line deleter strain (Rodriguez, Buchholz et al. 2000) to remove the selection cassette (*Pgk-Neo^R*). *Foxf2* $^{fl/+}$ offspring were bred for several gen-

erations on C57Bl/6, and then interbred to generate a homozygous floxed strain. *Foxf2* $^{fl/fl}$ mice were fertile and indistinguishable from wild type in terms of phenotype, fertility and life span. *Wnt1-Cre; Foxf2* $^{fl/fl}$ E18.5 embryos developed a cleft palate indistinguishable from that of *Foxf2*$^{-/-}$ embryos (Paper IV).

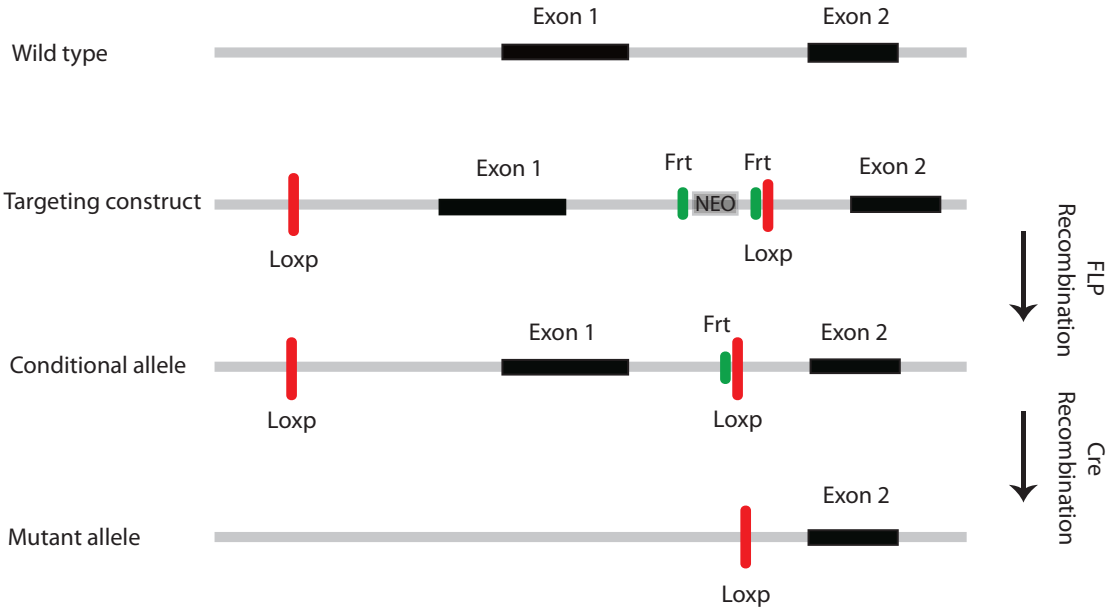

Fig 1. Generation of the conditional *Foxf2* ^{fl} allele in mice

The conditional knockout strain was used to analyze the role of *Foxf2* in various postnatal processes by crossing these mice with tissue-specific *Cre* lines, or the tamoxifen-inducible *Cre* strain *CAGG-Cre* ^{ERT2} (Hayashi and McMahon 2002). As judged by PCR (Fig 2), the recombination efficiency of the floxed *Foxf2* locus in adult animals after tamoxifen induction was close to 100%.

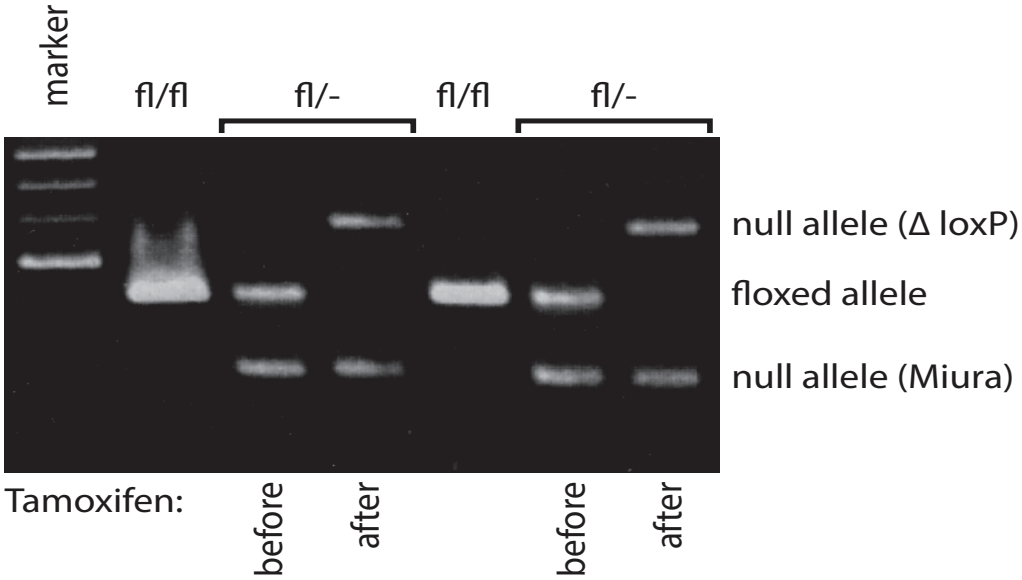

Fig 2. PCR genotyping of the conditional *Foxf2* ^{fl} allele in mice, before and after tamoxifen induction of CreERT2

Cerebrovascular development and disease

The neurovascular unit

Brain, the most complex organ of the mammalian body, has a high metabolic demand and is unique with respect to nutritional and physiological requirements. Neurovascular coupling ensures adequate blood supply to the neurons, while at the same time providing restricted permeability and highly selective transport of metabolites. The structural basis for this blood-neuron interface is an intimate contact and communication between cells associated with brain capillaries, in what is known as the neurovascular unit.

The neurovascular unit consists of neurons, glia cells (microglia and astrocytes), endothelial cells, pericytes and the vascular basement membrane (Muoio, Persson et al. 2014) (Fig 3).

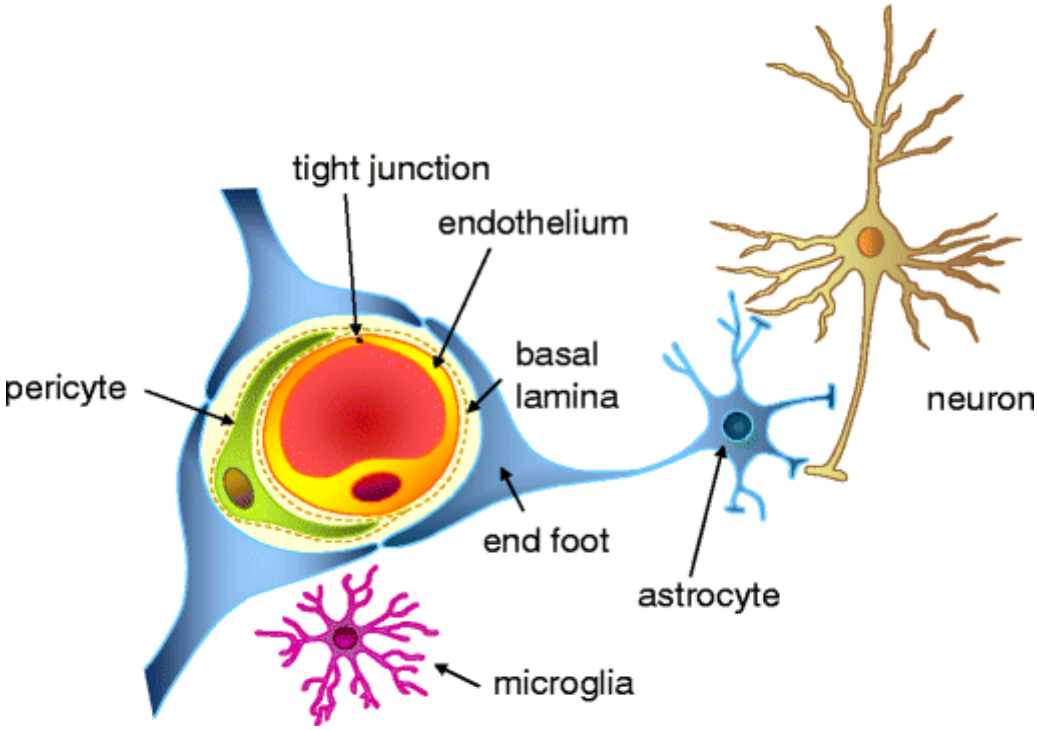

Fig 3. A schematic view of the neurovascular unit, which consists of vascular endothelial cells, pericytes, astrocytes, microglia and neurons. Brain endothelial cells constitute the BBB and are partially covered by pericytes, with which they share the basal lamina. The astrocytic end-feet engulf the capillaries and astrocytes also provide a link between the neurons and the microvasculature. Microglia are CNS-resident immune cells. Image adopted from (Abbott 2013).

Endothelium

Endothelial cells are squamous cells that form the thin, interior layer of all blood vessels. During embryonic development, the vasculature of the CNS forms from endothelial sprouts that invade the neuroectoderm from the perineural vascular plexus in response to a gradient of neural-derived vascular endothelial growth factor (VEGF) (McCarty 2009).

Brain microvascular endothelial cells have many features similar to peripheral endothelium, including the expression of glycoproteins, adhesion molecules and integrins, but also unique properties such as reduced density of caveolae,

presence of circumferential tight junctions between endothelial cells, and increased density of mitochondria (Nag 2011).

Pericytes

Pericytes, or vascular myofibroblasts, are the mural cells of capillaries. They are in close contact with the endothelium, embedded within the basement membrane, located abluminal to the endothelial cell layer, and – in brain – luminal to astrocyte end-feet along the capillary wall (Bagley, Weber et al. 2005). Pericytes form cell-to-cell contacts with endothelial cells through gap junctions (Armulik, Genove et al. 2011).

Capillaries are partially coated by pericytes and the degree of coverage varies between tissue types. Of all organs, retina and brain have the highest pericyte density, and the pericyte coverage on capillaries correlates with the tightness of the endothelium (Dalkara, Gursoy-Ozdemir et al. 2011).

The ontogeny of cerebral mural cells has been most rigorously traced in avian embryos, where those of the forebrain are exclusively of neural crest origin, whereas mural cells of the posterior CNS are mostly mesodermal (Etchevers, Vincent et al. 2001, Korn, Christ et al. 2002, Kurz 2009). However, available data suggest that in mammals most, and perhaps all, mural cells are neural crest derived (Heglind, Cederberg et al. 2005, Armulik, Genove et al. 2011). Apart from in the CNS, neural crest derived pericytes are also found in the thymus (Foster, Sheridan et al. 2008) (Fig 4).

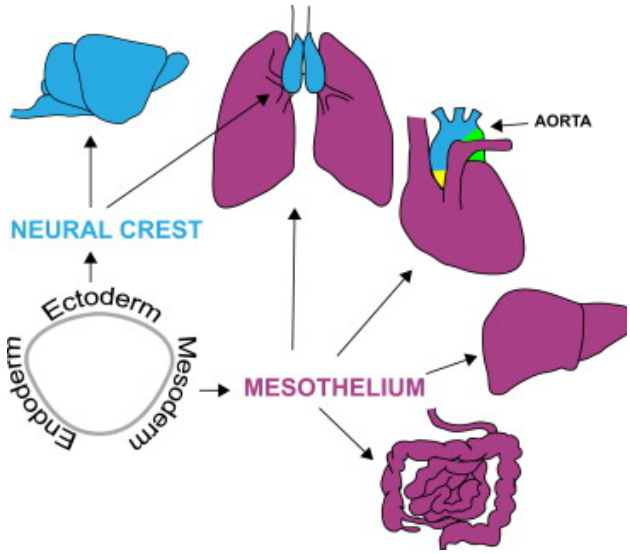

Fig 4. Developmental origin of mural cells. Mural cells of the CNS and thymus are derived from embryonic neural crest, whereas those of the coelomic organs are of mesodermal origin. Mural cells of the aorta are of mixed origin. Image adopted from (Armulik, Genove et al. 2011).

Pericytes are defined by location, rather than by molecular characteristics, and it has been difficult to define a single, entirely pericyte specific marker (Dore-Duffy, Katychev et al. 2006). Several molecular markers are used to identify pericytes, but not all are useful in the CNS. Validated and often-used brain pericyte markers include platelet-derived growth factor receptor β (Pdgfrβ), chondroitin sulfate proteoglycan 4 (Ng2), alanyl aminopeptidase (Cd13) and desmin (Armulik, Genove et al. 2011 and Paper I)

Pericytes are multi-functional cells, which contribute to several neurovascular unit key functions, including formation of BBB, vascular stability, and control of blood flow through regulation of capillary diameter. They decrease the permea-

bility of brain capillaries by limiting the rate of the endothelial transcytosis (Armulik, Genove et al. 2010, Daneman, Zhou et al. 2010).

Basement membrane

The basement membrane, or the vascular basal lamina, covers the endothelial cells, encloses the pericytes, and provides an attachment interface between the vasculature and the surrounding brain resident cells (Carvey, Hendey et al. 2009). It is built from different types of extracellular matrix (ECM) molecules such as structural proteins (collagens and elastin), integrin ligands (fibronectin and laminin), and proteoglycans. Apart from its structural role, it is also important for sequestering growth factors, such as PdgfB and Tgfβ (Cardoso, Brites et al. 2010).

Astrocytes

Astrocytes are glia cells, recognized by their numerous foot processes containing glial fibrillary acidic protein (GFAP). Astrocytic end-feet form a lacework of fine lamellae that covers the basement membrane of the outer surface of the microvessel wall.

Astrocytes cover more than 99% of the brain endothelial cells. Interaction between astrocytes and endothelial cells enhances endothelial cell tight junctions and reduces the gap junctional area, thus supporting the integrity of the blood-brain barrier (Abbott, Ronnback et al. 2006). Until recently, it was thought that astrocytes were responsible for induction of BBB properties in endothelial cells, but Daneman et al (2010) showed that formation of the BBB occurs already at the embryonic stage, before differentiation of astrocytes. They concluded that BBB formation is dependent on pericytes, but proposed that once integration of differentiated astrocytic end-feet into the neurovascular unit is complete, BBB maintenance is taken over by astrocytes. In Paper I we show that Foxf2 is required for upholding the barrier function also in the mature CNS vasculature, which implies properly differentiated pericytes in the process.

Microglia

Microglia are resident immunocompetent and phagocytic cells inside the brain that play important roles in the response to brain injury, trauma and neurological disorders like stroke and Alzheimer's disease (Kim and de Vellis 2005). They originate from circulating monocytes, which enter the brain during embryogenesis and differentiate into resident microglia. The brain microglia are present in two forms: a resting form with small cell bodies and long, thin processes, and an activated form with an amoeboid, phagocytic morphology and short processes, typically associated with pathologic conditions (Kim and de Vellis 2005).

Neurons

Neurons are extremely vulnerable cells, characterized by a central cell body with long axon and dendrites. The fact that in the human brain, nearly every neuron has its own capillary illustrates the importance of the close neuronal-vascular relationship for normal function of the brain. Communication between neurons, astrocytes and endothelial cells within the neurovascular unit adjusts the blood supply to ensure optimal neuronal function (Sa-Pereira, Brites et al. 2012). Brain microvascular endothelial cells and astrocytic processes are inner-

vated by noradrenergic, serotonergic, cholinergic and GABA-ergic neurons (Cardoso, Brites et al. 2010).

Blood brain barrier

In the late 19th century, Paul Ehrlich, German bacteriologist, observed that vital dye administered intravenously stained all organs of the animal except the brain. Thirty years later, his student, Edwin Goldmann, injected trypan blue into the cerebrospinal fluid and stained the entire brain, but the dye did not enter the bloodstream. These observations indicated the presence of a barrier between the blood and CNS, which was called the blood-brain barrier (BBB).

For optimal function of the neurons in the CNS, their environment needs to be protected against toxic compounds and pathogens. The BBB is a dynamic and physical interface between blood and CNS required to preserve brain homeostasis, protect it from hazardous substances in the environment, but still allow transfer of nutrients and inflammatory cells through specific transport systems (Zlokovic 2008) . The BBB functional components include the brain microvascular endothelial cells with their tight junctions and diminished transcytosis, the basement membrane, and other cells of neurovascular unit such as pericytes, neurons and astrocytes (Persidsky, Ramirez et al. 2006).

The elaborate intercellular junction complexes between endothelial cells include tight junctions and adherence junctions. Tight junctions are composed of occludin, claudins and junctional adhesion molecules, which are transmembrane proteins that connect to the cytoskeleton by ZO-1, ZO-2 and ZO-3 anchoring proteins and restrict the paracellular flux of hydrophilic molecules (Doolittle, Abrey et al. 2005). Endothelial cells bind to the surrounding basement membrane through integrins, which contribute to BBB properties (Wolburg, Noell et al. 2009).

There are two independent molecular trafficking pathways across the BBB: the paracellular pathway (passage between two adjacent endothelial cells) and the transcellular pathway (passage through the endothelial cells). The opening and closing of the paracellular cleft is a dynamic interaction between the tight and adherence junctional elements and is controlled by signaling pathways involved in regulation of intercellular junctions, such as protein kinases A, B, C and G and also Ca^{2+}-mediated signal transduction (Kumar, Shen et al. 2009).

Different means of transcellular transportation are used, based of the nature of the molecules to be transported. Passive diffusion, receptor-mediated shuttling and also transcytosis through caveolae are some examples (Abbott, Ronnback et al. 2006). The endothelial cell membrane provides a diffusive route for lipid-soluble agents. The endothelial membrane contains transporter proteins for glucose, amino acids and nucleosides. Certain proteins, such as insulin and transferrin are bound by specific receptors, internalized through caveolae, and transferred by transcytosis (Wolburg, Noell et al. 2009) (Fig 5). Caveolae are microinvaginations in the endothelial cell membrane, covered by the coat protein caveolin, and sites of endocytosis for transport through the intracellular space (Wolburg, Noell et al. 2009) .

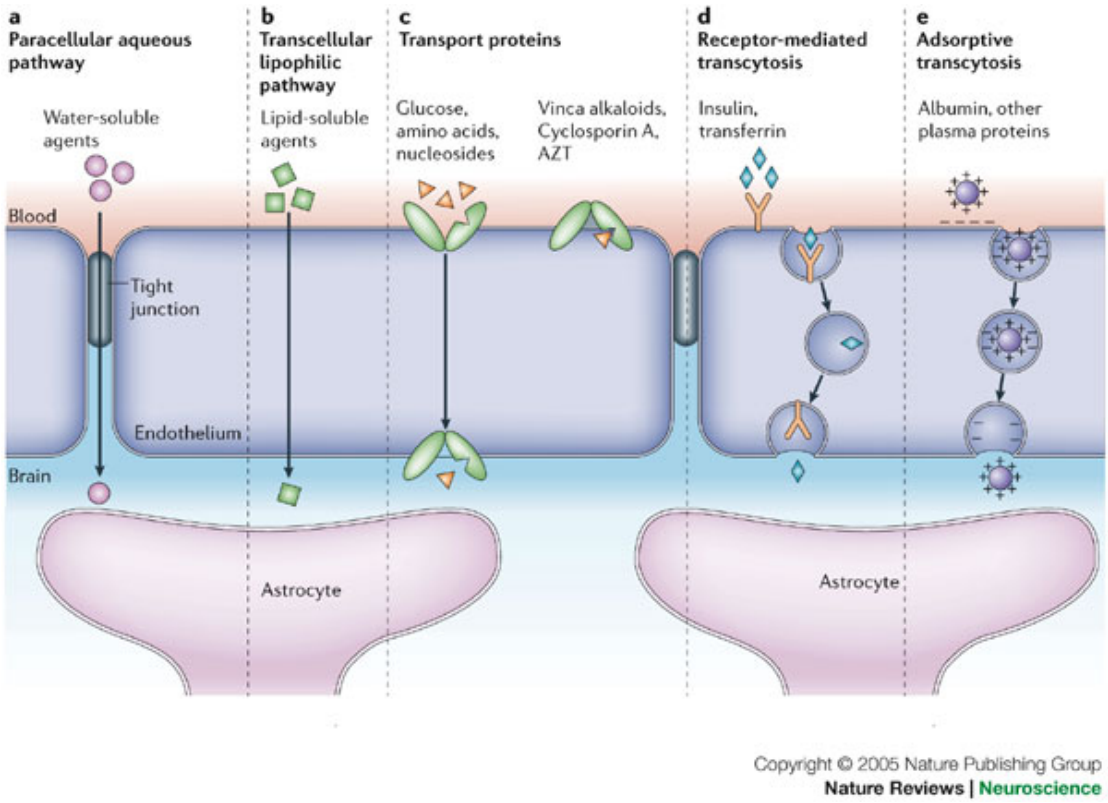

Fig 5. A schematic diagram showing the brain endothelial cells, which constitute the BBB, and various means of molecular trafficking across the endothelial cells. Image adopted from (Abbott, Ronnback et al. 2006).

Pericyte interactions at the neurovascular unit

As a result of their physical and biochemical interactions in the neurovascular unit, pericytes play essential roles in neurovascular unit development and maturation, and also in brain homeostasis.

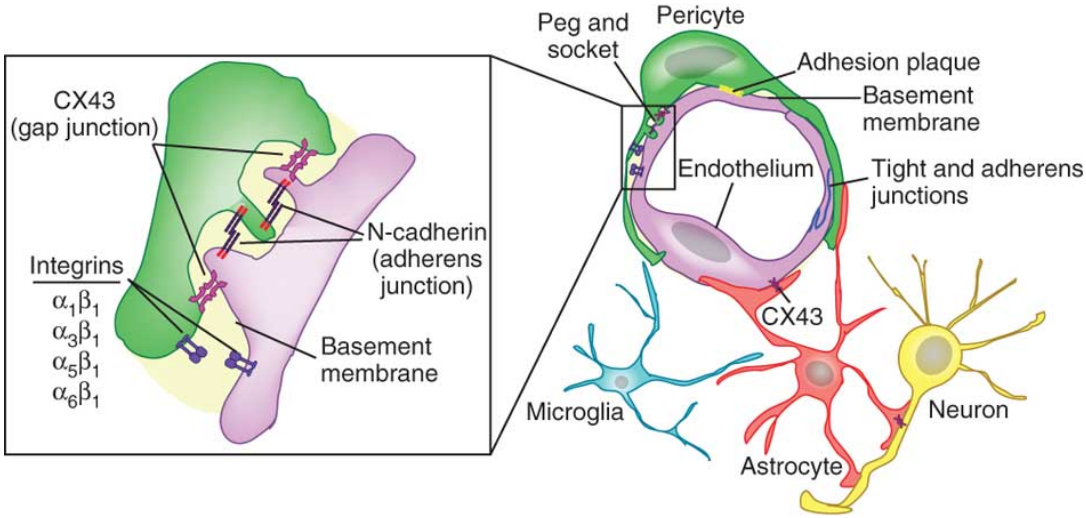

Fig 6. Schematic view of the intimate contact between the brain endothelial cells and pericytes. Pericytes and endothelial cells share the same basement membrane, and gaps in this membrane allow direct cell to cell connection in the form of peg-socket contacts. Here, cells connect through trans-membrane junction molecules such as Cx43 and N-cadherin. The space between endothelial cells and pericytes is filled with adhesion plaques containing fibronectin. Image adopted from (Winkler, Bell et al. 2011).

Cell-to-cell interactions

Pericytes are embedded within the basement membrane and their elongated processes cover the endothelial cells, establishing specialized cell-cell contacts called peg-socket contacts (Armulik, Genove et al. 2011), which contain junction proteins such as N-cadherin and the gap junction protein connexin 43 (Cx43). Chemicals pass between pericytes and endothelial cells through the gap junctions formed by Cx43 hemichannels (Gerhardt, Wolburg et al. 2000, Bobbie, Roy et al. 2010) (Fig 6).

Signaling pathways

Several signal transduction pathways are important for communication between endothelial cells and pericytes. Examples, which are essential for mural cell development and function, and BBB formation, are platelet derived growth factor B (PdgfB), transforming growth factor β (Tgfβ), sphingosine-1-phosphate, Notch and angiopoietin-1/Tie2 signaling (Gaengel, Genove et al. 2009) (Fig 7).

PdgfB

Endothelial cells secret PdgfB in its active homodimer form PdgfBB, which binds to the cell surface or ECM heparan sulfate proteoglycans through its retention domain. This retains the freely diffusible PdgfBB and creates a high local concentration that can bind to Pdgfrβ receptor on pericytes. Ligand binding induces receptor dimerization, autophosphorylation and activation of signal transduction cascades resulting in pericyte proliferation, as well as cytoskeletal rearrangement and pericyte migration (Abramsson, Lindblom et al. 2003, Lindblom, Gerhardt et al. 2003, Tallquist, French et al. 2003, Andrae, Gallini et al. 2008).

Deletion of *PdgfB* or *Pdgfrβ* in knockout mice leads to mural cell deficiency, vascular leakage and perinatal lethality (Hellstrom, Kalen et al. 1999, Hellstrom, Gerhardt et al. 2001).

Tgfβ

Tgfβ signaling has critical roles in many aspects of brain vascular development, such as pericyte differentiation, proliferation and adhesion, as well as endothelial cell proliferation and differentiation.

Both endothelial cells and pericytes express a latent form of Tgfβ, which binds to ECM. Latent Tgfβ can be activated by integrins, thrombospondin and proteases. Two different models have been suggested regarding the contribution of integrins to activation of latent Tgfβ. The first model suggests that binding of integrins to matrix metalloproteinases and latent Tgfβ1 facilitates enzymatic cleavage of the latter, and release of active Tgfβ1. The second model is based on transmitting cell traction forces that change the conformation of the latent Tgfβ1 complex to liberate the active form in a non-proteolytic way (Wipff and Hinz 2008). The importance of integrins for Tgfβ signaling is illustrated by the effects of genetic ablations of integrin α5 and/or β8, which cause defective angiogenesis associated with attenuated Tgfβ signaling (Cambier, Gline et al. 2005, Arnold, Niaudet et al. 2014).

Activated Tgfβ binds Tgfβ receptor II (TgfβrII), leading to activation and phosphorylation of two distinct type I Tgfβ receptors called activin receptor like kinase 1 and 5 (Alk1 and Alk5). These two receptors are expressed in both endothelial cells and pericytes and trigger two different pathways with opposing ef-

fects on proliferation, migration and differentiation. In endothelial cells, Alk1 inhibits Alk5, whereas Alk5 is required for Alk1 signaling (Goumans, Valdimarsdottir et al. 2002).

Activated Alk5 phosphorylates Smad2/3, which then binds Smad 4. The Smad 2/3-Smad4 complex translocate to the nucleus to regulate the expression of Tgfβ target genes. Activation of Tgfβ signaling through Alk5 in the brain microvasculature inhibits pericyte proliferation and migration, and instead promotes pericyte differentiation and vessel maturation. On the other hand, activation of Alk1 leads to phosphorylation of Smad 1/5, which in turn triggers cell migration and proliferation but inhibits vessel maturation and pericyte differentiation (Goumans, Valdimarsdottir et al. 2002, Ota, Fujii et al. 2002, Chen, Kulik et al. 2003).

Null mutants in most of the genes involved in the Tgfβ pathway in mice leads to embryonic lethality with severe vascular abnormalities (Armulik, Genove et al. 2011).

Notch

In embryonic vascular development, Notch signaling is important for arterial cell fate determination and angiogenesis. Mammals have five Notch ligands (Jagged 1 and 2, Delta-like 1, 3 and 4) and four receptors (Notch 1-4), all of which are transmembrane proteins. Signaling therefore requires direct cell to cell contacts. Ligand-receptor binding triggers a series of proteolytic cleavages to release the Notch intracellular domain (NICD) in the cytoplasm, which translocates to the nucleus and binds to RBP-Jκ and changes the transcription of Notch-dependent genes (Kume 2012) Several studies show the importance of Notch signaling in pericyte recruitment and survival (Liu, Kennard et al. 2009, Liu, Zhang et al. 2010). Notch signaling regulates the expression of Pdgfrβ in mural cells and Notch3-driven proliferation of pericytes requires Pdgfrβ function (Jin, Hansson et al. 2008, Wang, Pan et al. 2014). Cooperation of Tgfβ and Notch signaling in endothelium facilitates proper pericyte-endothelial attachment and prevents perinatal hemorrhage by up-regulation of the adhesion molecule N-cadherin (Li, Lan et al. 2011).

Sphingosin-1-phosphate

Sphingosine-1-phosphate (S1P) is a secreted sphingolipid or blood-borne lipid mediator which signals through a specific cell surface G-protein-coupled receptors to mediate cytoskeletal and junctional alternations, leading to activation of endothelial N-cadherin, a critical cell adhesion molecules for connecting endothelial cells to pericytes (Paik, Skoura et al. 2004). Furthermore, S1P/$S1P_1$/Gi/Rac signaling cascades modulate vascular barrier integrity and permeability by affecting the junctional stabilization (McVerry and Garcia 2005).

Angiopoietin1/Tie2

Ang1 is expressed by perivascular mesenchymal cells including pericytes. It binds to the Tie2 receptor on endothelial cells to create a paracrine loop of signaling with inverted orientation compared to PdgfB/Pdgfrβ signaling (Gaengel, Genove et al. 2009). The effect of Ang1/Tie2 signaling on vessel maturation is mediated through up-regulation of endothelial cytokines, PdgfB and Tgfβ, which in turn lead to endothelial stabilization, differentiation, inhibition of vascular leakage and barrier formation (von Tell, Armulik et al. 2006).

Studies on *Tie2* knockout mice, revealed cardiac defects, edema and hemorrhage because of impaired angiogenic sprouting, which resulted in embryonic lethality between E9.5 to E10.5 (Sato, Tozawa et al. 1995). *Ang1* null mice have a similar, but less severe, phenotype (Suri, Jones et al. 1996).

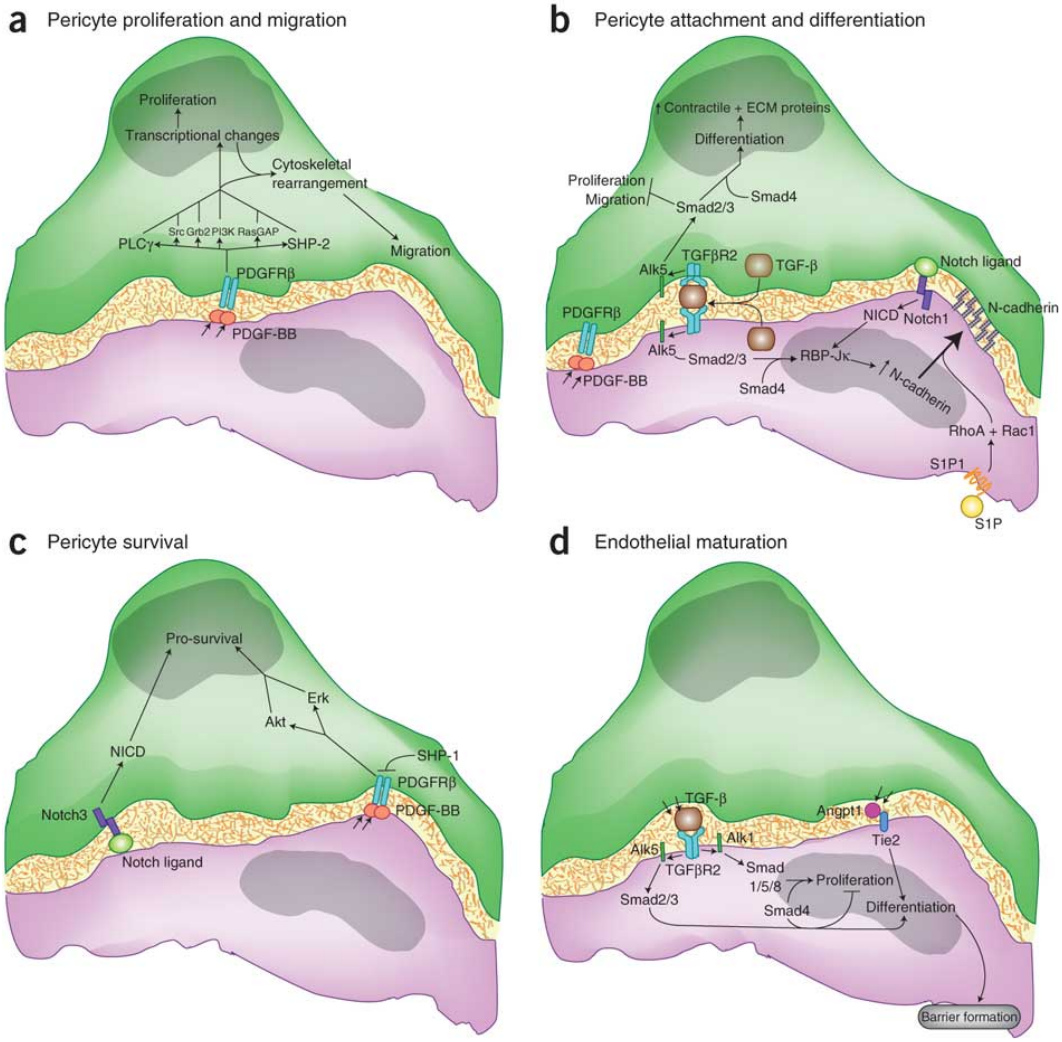

Fig 7. Different effects of PdgfB and Tgfβ signaling on the brain pericytes and endothelial cells. a. Pericyte proliferation and migration. Activation of the PdgfB signaling in the pericyte leads to activation of several downstream signaling cascades (such as Src, the Grb2 adaptor protein, phosphatidylinositol-3-OH kinase (PI3K), Ras GTPase activating protein (RasGAP), phospholipase C (PLC)-γ and SHP-2 tyrosine phosphatase), which in turn lead to increased proliferation of pericytes. The other consequence is rearrangement of the pericyte's cytoskeleton, which facilitates the cell motility and enhances the cell migration. b. Pericyte attachment and differentiation. Activation of the canonical Tgfβ signaling through Alk5 has distinct effects on pericytes and endothelial cells. In the pericytes, active canonical Tgfβ signaling triggers the production of contractile and ECM proteins and enhances the attachment of pericytes to the ECM and consequently leads to pericyte differentiation. In the endothelial cells, activated canonical Tgfβ together with Notch signaling leads to production and deposition of N-cadherin in the intercellular space between pericytes and endothelial cells and in turn enhances the attachment between these two cell types. c. Pericyte survival. Activation of Akt and Erk serine/threonine kinases and downstream survival pathways is a result of activated PdgfB signaling in the pericyte. Notch3 signaling is also implicated to have a role in survival of the pericytes. d. Endothelium maturation. Activation of TgfrβII in the endothelial cells can result in both activation of Alk5- Smad2/3/4 and Alk1-Smad1/5/8, which can have opposing effects on the maturation of the endothelial cells. Image adopted from (Winkler, Bell et al. 2011).

Stroke

Stoke or brain attack is a clinical neurological deficit of vascular origin. Stroke is the second most common cause of death and it is the major cause of disability in adults worldwide (Johnston, Mendis et al. 2009).

Stroke can be categorized into two types: ischemic and hemorrhagic. Ischemic strokes are caused by arterial occlusion and accounts for over 80% of strokes. The remaining almost 20% are hemorrhagic and result from bleeding within the cranial vault. Since both interacerebral hemorrhage and ischemic stroke have similar clinical features, they cannot be distinguished without brain imaging. Ischemic stroke is further subdivided into large-vessel disease stroke, small-vessel disease stroke, and cardioembolic stroke based on the underlying pathophysiological mechanisms (Jerrard-Dunne, Cloud et al. 2003).

Common known risk factors for stroke include hypertension, diabetes, ischemic heart disease, atrial fibrillation, alcohol intake and cigarette smoking, together with age and genetic factors (Donnan, Fisher et al. 2008).

Stroke genetics

Stroke is a clinical end-point, and from an etiological point of view it is a heterogeneous group of diseases. To better understand the biological mechanisms behind cerebrovascular diseases, one approach is to compare the genetic constitution of individuals who experienced a stroke with healthy controls (Markus 2011, Falcone, Malik et al. 2014).

Studies of twins, family history, and epidemiological data provide strong evidence for a genetic component of stroke susceptibility (Falcone, Malik et al. 2014), but, with a few exceptions, the genes responsible remain poorly understood. Genome wide association studies (GWAS) have transformed the field of complex genetic conditions and have begun to be applied to stroke (Ikram, Seshadri et al. 2009).

Foxf2 *in differentiation of brain pericytes and development of BBB (Paper I)*

In Paper I, we investigated the role of Foxf2 in differentiation of brain pericytes and also on development and maintenance of the BBB using *Foxf2*$^{-/-}$ mouse embryos, adult *Foxf2* conditional knockouts, heterozyogtes and a transgene with an extra copy of *FOXF2*.

As detailed above, pericytes are brain mural cells essential for formation of the BBB. In this article, we show that *Foxf2* is expressed in neural crest-derived cerebrovascular pericytes and negatively regulates their proliferation. Reduction in *Foxf2* gene dosage results in an increased number of pericytes associated with brain capillaries. We also studied the status of CNS pericytes in the *Tg(FOXF2)* transgenic strain, which harbors an extra copy of human *FOXF2* (described in Paper III), and observed a reduction in pericyte number compared to wild type.

Foxf2 $^{-/-}$ embryos have abnormal cerebral capillaries with narrow lumen, a thickened and irregular endothelium and weaker basal lamina. In contrast, capillary density and branching frequency were normal. Intracranial hemorrhage occurred both in the form of large macroscopically visible bleeds, and in the form of scattered extravascular erythrocytes in the brain parenchyma. Importantly,

the BBB did not develop and the cerebral vasculature remained leaky in the *Foxf2* null embryos, in spite of the high pericyte coverage.

Adult inactivation of *Foxf2* did not lead to the severe structural defects observed in *Foxf2* $^{-/-}$ embryos, but increased vascular permeability significantly. These results demonstrate that persistent Foxf2 expression is required to maintain the barrier function in the mature cerebral vasculature.

Inactivation of *Foxf2* leads to attenuation of Pdgfrβ and Tgfβ-Smad2/3 signaling. These are two of the major paracrine signaling pathways involved in pericyte-endothelial communication. Based on mutant mouse phenotypes PdgfB/Pdgfrβ signaling has been reported to play a crucial role in CNS pericyte recruitment (Andrae, Gallini et al. 2008). We detected a dramatic reduction of Pdgfrβ at both protein and mRNA levels in the absence of Foxf2, but associated with increased, rather than decreased, pericyte density. This observation contradicts the firmly established requirement of Pdgfrβ signaling for pericyte proliferation and migration, which is apparently lost in Foxf2 mutants.

Tgfβ signaling, which is an essential pathway in vascular development, endothelial and pericyte differentiation and ECM production, is attenuated in the absence of Foxf2. Tgfβ signaling through Alk5 and Smad2/3 has an antagonistic effect on the Alk1 – Smad1/5 pathway (Goumans, Valdimarsdottir et al. 2002), as well as on the non-canonical pathway mediated by p38 (Iwata, Hacia et al. 2012). In both embryonic and adult conditional *Foxf2* knockout brain, a reduction in Smad 2/3 phosphorylation and a corresponding increase in phosphorylation of Smad 1/5 and p38 were detected. Furthermore, integrin αv and β8, which are important activators of latent extracellular Tgfβ complexes also showed reduced expression. We concluded that in *Foxf2* mutants the diminished Pdgfrβ is responsible for BBB breakdown, whereas reductions in Tgfβ signaling and integrin expression lead to vascular instability and hemorrhage.

Regulation of the intestinal stem cell niche

Histology of the intestine

The small intestine is the largest part of the alimentary canal, located between the stomach and cecum and divided into three sequential segments called duodenum, jejunum and ileum. The wall of the small intestine, like the rest of the alimentary tube, consists of four layers. From innermost to outer named mucosa, submucosa, muscularis and serosa. The mucosa consists of a single layer of columnar epithelial cells and lamina propria, which is a loose layer of connective tissue. The submucosa is a dense layer of connective tissue, surrounded by muscularis and mesothelial layer of serosa, or peritoneum (Fig 8).

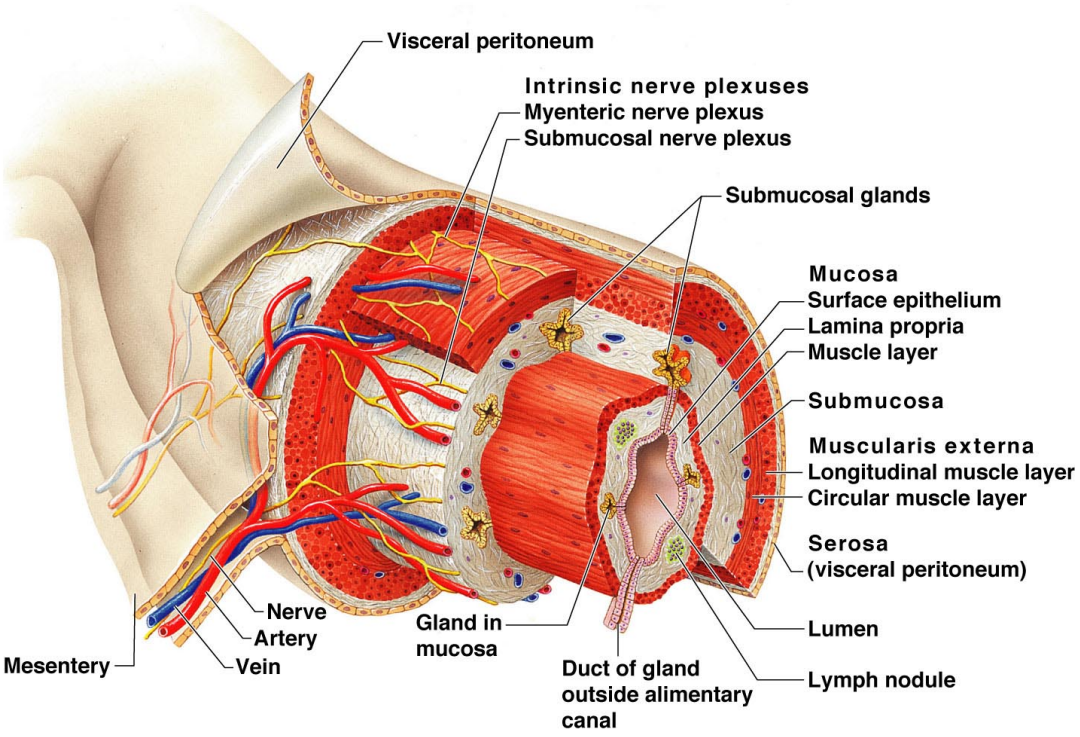

Fig 8. Schematic view of the small intestine. The small intestine consists of four distinct layers: mucosa (epithelium + lamina properia), submucosa, muscle layers (inner circular and outer longitudinal layer), and serosa.

The mucosa undergoes different degrees of folding to amplify its absorptive surface area, including the plicae circulares, intestinal villi, intestinal glands and microvilli. Villi are finger-like projections of the mucosa into the lumen, covered by epithelium. The core consists of loose connective tissue, as an extension of the lamina propria, which contains fibroblasts, smooth muscle cells, plasma cells, immune cells and a network of blood and lymph capillaries to mediate transport of absorbed nutrients into the body (van der Flier and Clevers 2009).

The crypts of Lieberkühn, or intestinal glands, are simple tubular glands, formed by invaginations of the mucosa between adjacent intestinal villi ending at the muscularis mucosa. Intestinal mucosal epithelium, which covers the villi and crypts, contains the following types of cells: enterocytes, goblet cells, paneth cells, enteroendocrine cells, microfold – or M – cells, and intestinal stem cells.

Intestinal stem cells and paneth cells reside at the base of the crypts. The differentiated epithelial cell types of the villi have distinct functions: enterocytes absorb nutrients from the chyme, goblet cells secret mucus, and enteroendocrine cells release hormones. Paneth cells at the base of the crypts secret anti-bacterial peptides (Clevers 2013). The villi and associated crypts constitute the functional units of the small intestine.

The proliferative epithelial compartment consists of a population of undifferentiated, rapidly cycling cells located in the crypts of Lieberkuhn. At the bottom of the crypts two types of stem cells are located: $Bmi1^+$ and $Lgr5^+$ cells. $Bmi1^+$ stem cells are normally quiescent, but activated in response to tissue damage. The $Lgr5^+$ stem cells on the other hand are rapidly dividing and responsible for everyday epithelial renewal (Barker, van Es et al. 2007, van der Flier, van Gijn et al. 2009) (Fig 9).

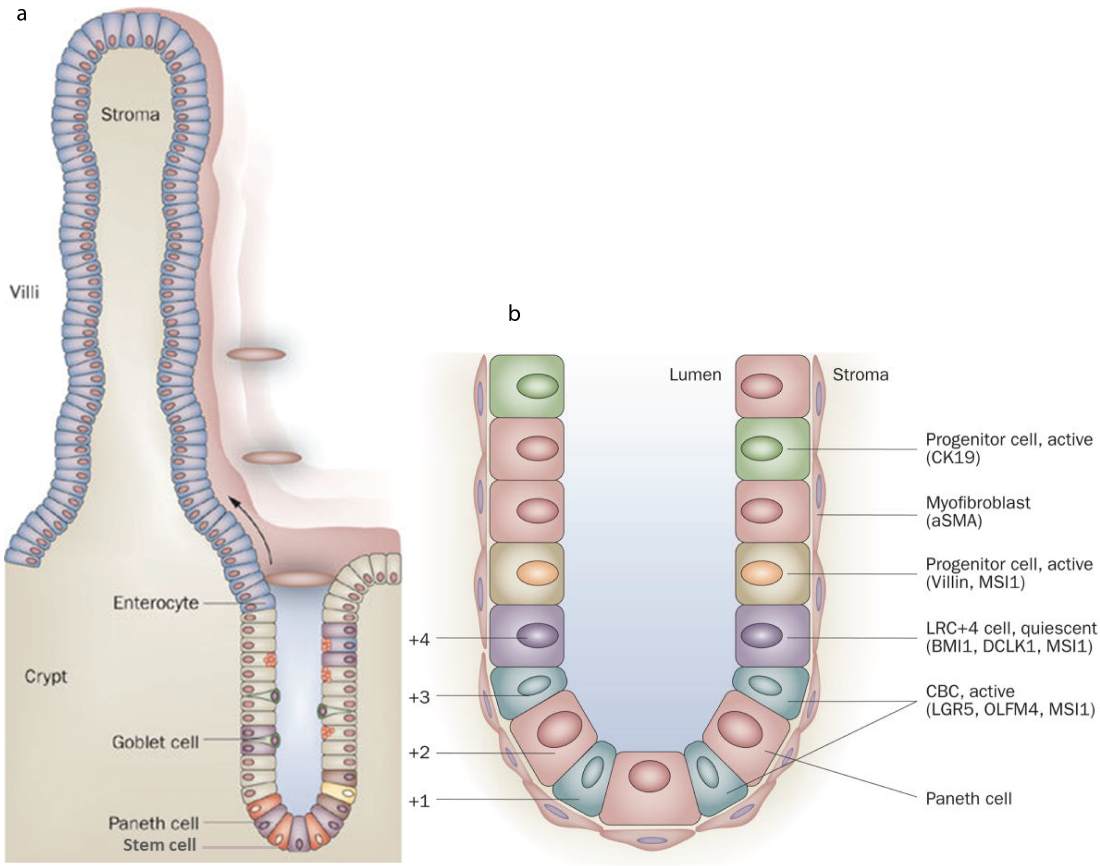

Fig 9. Schematic diagram of the intestinal crypt and villus. a. Cell arrangement along the crypt villus axes. Epithelial cell proliferation in the intestine is restricted to the crypt compartment. Cells that leave the crypt undergo differentiation. b. Cell arrangement in the intestinal crypt. Actively proliferating LGR^+ stem cells reside at the bottom of the crypts and surrounded by the paneth cells. Another population of quiescent stem cell resides close to the bottom of the crypts. These cells are referred to +4 or $Bmi1^+$ stem cells. Image adopted from (Quante and Wang 2009).

The intestinal stem cell niche

Proper function of the intestine depends on its epithelial homeostasis, which is maintained through self-renewal, obtained by proliferation of undifferentiated intestinal stem cells in the crypts, and subsequent migration along the crypt-villus axis to generate all differentiated cell types.

The stem cell niche is a special tissue microenvironment that maintains the stem cells for a non-limited period of time. A feed-back interaction between the villus mesenchyme and crypt epithelial cells is required for regulation of the stem cell niche, leading to a balance between differentiation, quiescence and proliferation (Moore and Lemischka 2006). A large number of signaling pathways, including but not limited to: Wnt, Bmp, Hedgehog, Egf and Notch play roles in the epithelial-mesenchymal cross talk. Two of these will be discussed below:

Wnt

Wnt signaling is an essential activator of stem cell renewal and proliferation. It is a short range paracrine signal, mostly between touching cells, due to the biochemical structure of Wnt proteins: all Wnt proteins harbor a covalent lipid modification, palmitate, which renders the protein hydrophobic, leading to limited distribution and range of biological action after secretion (Pinto and Clevers 2005).

Upon interaction with target cells, Wnt proteins bind Frizzled receptors and Lrp5/6 transmembrane co-receptors, forming a complex with conformational changes that leads to phosphorylation of receptors by associated protein kinases. This results in inhibition of glycogen synthase kinase 3 (Gsk3) and binding of axin to the cytoplasmic tail of Lrp6, all resulting in inhibition of the β-catenin destruction complex that includes axin, adenomatous polyposis coli (APC) and Gsk3. In the absence of Wnt signaling, the destruction complex phosphorylates β-catenin and targets it to be degraded by the proteasome. Wnt-Frizzled-Lrp interactions and inhibition of the destruction complex lead to accumulation of β-catenin in the cytoplasm and its translocation to the nucleus. Association of β-catenin to Tcf/Lef transcription factors results in transcriptional upregulation of Wnt target genes (Li, Ng et al. 2012, Clevers, Loh et al. 2014) (Fig 10).

Intestinal stem cell markers like Lgr5, Cd44 and Msi1 are direct Wnt targets, which confirms the importance of this pathway in regulation of stem cell self-renewal (Barker, van Es et al. 2007, Rezza, Skah et al. 2010, Hou, Yang et al. 2011). Abrogation of crypt Wnt signaling *in vivo*, either by transgenic expression of Dickkopf 1, an inhibitor of Wnt, or by deletion of Tcf4 or β-catenin, results in reduced proliferation of small intestine epithelial cells, together with loss of crypts (Pinto, Gregorieff et al. 2003, Fevr, Robine et al. 2007).

As a consequence of the importance of Wnt signaling for stem cell maintenance, gain-of-function mutations in the Wnt pathway are frequently observed in cancers (Clevers and Nusse 2012). The importance of limiting Wnt signaling is reflected by the large number of Wnt inhibitors. Small, secreted inhibitory proteins such as Dickkopf (Dkk1), Wise/Sost, Frizzled related proteins (Sfrps), Kremen, and Wnt inhibitory protein (Wif) and also membrane bound glycoprotein Apcdd1 prevent activation of Frizzled receptors by Wnt ligands through a plethora of mechanisms (Shimomura, Agalliu et al. 2010).

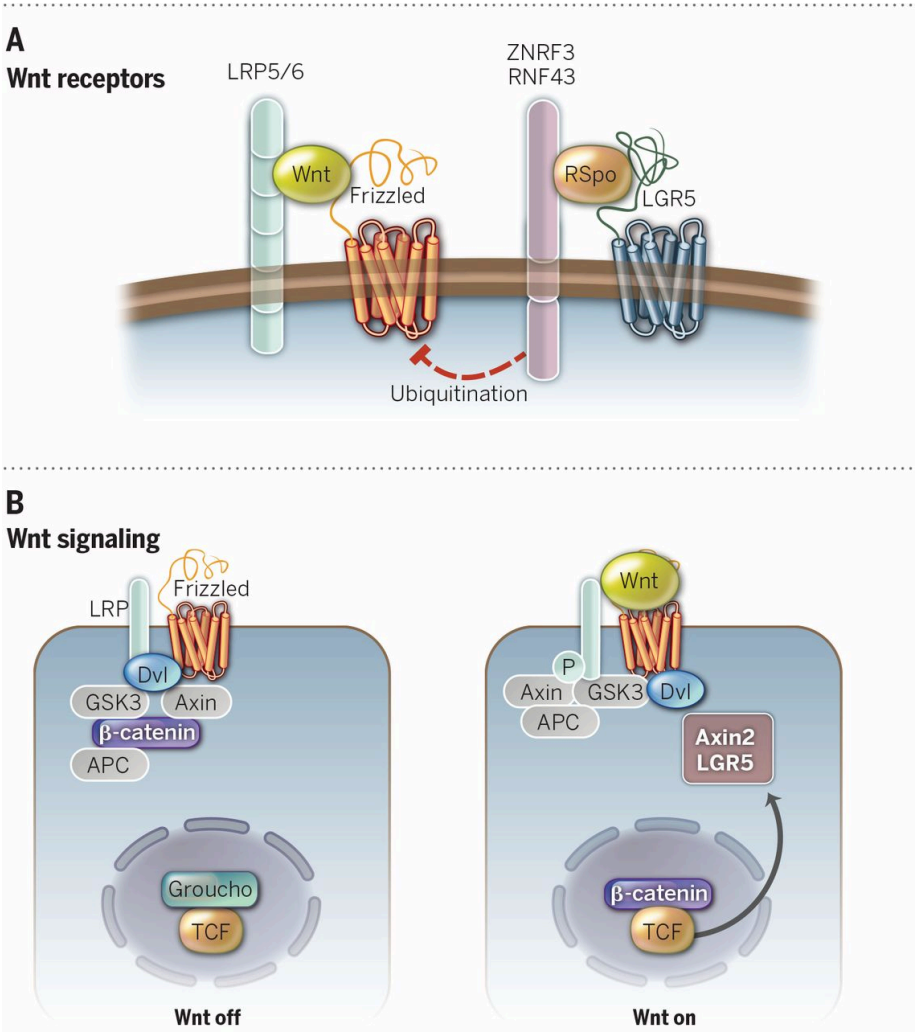

Fig 10. Wnt signaling regulation. A. Wnt reception on the cell surface is compromised through constant down regulation of the Wnt receptor Frizzled. Two surface proteins Znrf3 and Rnf43 constantly ubiquitinate the frizzled. Binding of R-spondins to LGR4/5/6 and to Znrf3 and Rnf43 relieves Znrf3 and Rnf43 and stabilizes the Frizzled. B. Wnt signaling in the target cell. In the absence of Wnt ligand, β-catenin is phosphorylated and degraded by the destruction complex (left). Up on the binding of Wnt ligand to Frizzled, destruction complex falls apart and β-catenin stabilizes. Binding the β-catenin to the TCF in the nucleus activates the transcription of Wnt targets. Image adopted from (Clevers, Loh et al. 2014)

Hedgehog

Hedgehog (Hh) is another class of paracrine signaling proteins of importance for the intestinal crypt-villus axis formation and stem cell homeostasis (Madison, Braunstein et al. 2005). In the absence of Hh ligand (Sonic (Shh), Indian (Ihh), or Desert (Dhh) hedgehog), the Smoothened signal transducer (Smo) is inhibited by Patched receptors leading to formation of the Gli degradation complex and phosphorylation of Gli proteins. Phosphorylation of Gli is followed by ubiquitination that release the intact N-terminal half of the Gli, which functions as a transcriptional repressor. Hh-binding to Patched receptors releases the Smo signal transducer, which inhibits the assembly of the Gli degradation complex and leads to nuclear accumulation of the activating version of Gli and transcription of Hh target genes (Varjosalo and Taipale 2008).

Studies on Ihh and Shh mutant mice revealed complex intestinal phenotypes, which implicate both proteins in small intestine morphogenesis. $Ihh^{-/-}$ shows

reduction in proliferation in the intervillus region and depletion of the progenitor cell compartment, whereas $Shh^{-/-}$ mutants have overgrowth of the duodenal villi (Ramalho-Santos, Melton et al. 2000). Hh signaling is paracrine from the epithelium (secreting Hh ligand) to the mesenchyme (expressing Ptch and Smo). Hh signaling controls the size of the crypt compartment, indirectly via mesenchymal signals that inhibit epithelial proliferation (Buller, Rosekrans et al. 2012).

Wnt signaling is active in the progenitor region around the bottom of intestinal crypt, whereas Hh signaling is highest in the villi. Several studies have found an antagonistic relationship between Hh and Wnt signaling, mediated by Gli1 transcription factor (van den Brink, Bleuming et al. 2004, Akiyoshi, Nakamura et al. 2006) (Fig 11), but the mechanistic link between these two pathways has not been understood. In Paper III, we identify a pathway by which *Foxf2* in villus fibroblasts in response to Hh from the epithelium inhibit canonical Wnt signaling in the epithelium through the extracellular Wnt inhibitor Sfrp1 (Fig 11). This will be discussed further below.

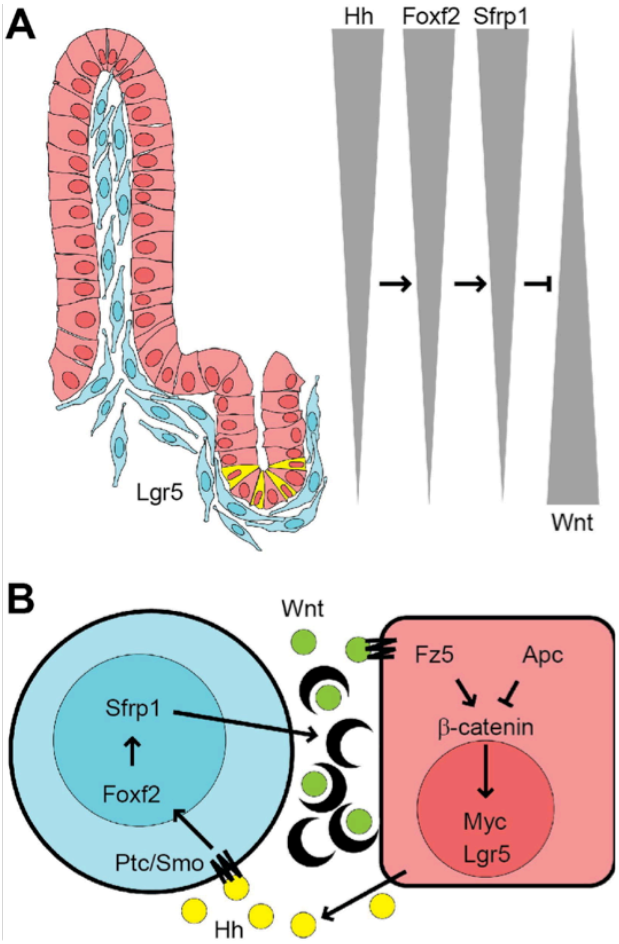

Fig 11. A. Schematic illustration of gradients formed by signaling molecules of the crypt-villus axis, and the proposed mechanism for how Foxf2 in fibroblasts limits the stem cell niche for Lgr5+ cells by inhibition of Wnt signaling. *B*. Simplified summary of paracrine signaling between epithelium and fibroblasts, and a proposed mechanism through which Foxf2 in fibroblasts inhibits Wnt signaling in adjacent epithelial cells. "Wnt" is used as a generic term for different Wnt ligands produced by epithelium and fibroblasts and "Hh" for Shh and Ihh. Image adopted from Paper III.

Intestinal adenocarcinoma

Under normal physiological conditions, the epithelial layer of the small intestine is renewed every 3 to 4 days. Cells generated by the intestinal stem cells at the bottom of the crypts and undergo rapid proliferation in the upper part of the crypt, as transit amplifying (TA) cells. The newly produced cells migrate upwards, become post-mitotic at the crypt-villus boundary, and continue into the villus where they differentiate into the distinct intestinal cell types. After reaching the villus tip epithelial cells undergo apoptosis and are shed to the intestinal

lumen. This balance between cell death and epithelial renewal is maintained by strict control of the proliferation of intestinal stem cells. If daughter cells of the stem cells retain a high level of Wnt pathway activity, the undifferentiated cells continue to divide and form an adenoma, which occurs when the Wnt pathway becomes constitutively active as a result of mutations in one of its components. An adenoma consists of multiple stem cell-like cells that maintain their ability to proliferate and grow uncontrollably (Zeilstra, Joosten et al. 2008).

Adenomas are benign neoplasms, but will accumulate additional mutations in genes such as *p53*, and components of the Tgfb and Bmp pathways, which will eventually transform the adenoma into a carcinoma, *i e* an invasive cancer.

Foxf2 in regulation of the intestinal stem cell niche and adenoma formation (Paper III)

In Paper III, we describe a molecular mechanism by which Foxf2 regulate the number of intestinal stem cells through control of the size of the stem cell niche. The experimental system consisted of an allelic series that varied the *Foxf2* gene dosage: *Foxf2* heterozygote, wild type, and the *Tg(FOXF2)* transgene which carries an extra copy of human *FOXF2*.

Foxf2 is expressed in subepithelial fibroblasts of the small intestine. There is a gradient of expression, highest in the villus mesenchyme, lower around the base of the villus and around the crypt, and disappearing at the crypt base. This gradient corresponds to the level of Hh signaling from the epithelium, consistent with *Foxf2* being a Hh target (Ormestad et al, 2006). Expression of the extracellular Wnt inhibitor Sfrp1 by villus fibroblasts correlates linearly with *Foxf2* expression, in a cell autonomous manner, which suggests that *Sfrp1* is a direct Foxf2 target. Sfrp1 has been shown by others to inhibit the Wnt pathway, which provides a plausible explanation for the observed negative correlation between, on one hand, *Foxf2* expression and, on the other, epithelial proliferation and expression of the Wnt target *Myc*.

Alteration in *Foxf2* gene dosage also lead to changes in the number of Lgr5+ stem cells in the crypts, again with a negative correlation. Foxf2 thus controls the production of epithelial cells by limiting the extent of the stem cell niche for Lgr5+ cells, which is determined by a threshold level of Wnt signaling. The alteration in stem cell number and proliferation that resulted from differences in *Foxf2* gene dosage also translated into significant differences in both growth and initiation rate of intestinal adenomas . On the mouse equivalent of Familial Adenomatous Polyposis Coli, Apc^{Min}, animals with one and three *Foxf2* alleles differed 24-fold in tumor burden, which illustrates the importance of stroma for tumor growth, and of Foxf2 as a tumor suppressor.

Development of the secondary palate

Palatogenesis

Palatogenesis is a highly regulated morphogenetic process in higher vertebrate development, which results in separation of the oral from the nasal cavity by formation of the secondary palate. This "roof of the mouth" consists of a bony hard palate in the anterior part, and a muscular soft palate in the posterior (Bush and Jiang 2012).

Palatogenesis in mice begins at E11.5 by symmetrical outgrowths from the maxillary prominences of the first pharyngeal arch. They consist of a mesenchymal core, which is mainly derived from neural crest, covered by a thin layer of ectodermal epithelium. The palatal shelves continue to grow vertically down the sides of the tongue. Accumulation of hygroscopic ECM (mainly glucoseaminoglycans and collagens) produced by the mesenchyme leads to a dramatic expansion and elevation of the shelves, which by E12.5 have reached a horizontal position above the tongue. By E13.5 their edges meet at the midline, and by E14.5 they start to merge (Murray and Schutte 2004). Palatal fusion is complete when the midline epithelial seam (MES) disappears around E16. There are three different hypotheses about the fate of the epithelia of MES, including apoptosis (Cuervo and Covarrubias 2004), migration (Jin and Ding 2006), and epithelial-mesenchymal transformation (Vaziri Sani, Hallberg et al. 2005). After fusion, ossification starts to form the hard palate in the anterior two-thirds of the secondary palate.

Proper palatal development depends on many factors, such as growth factors, efficient production of ECM proteins, and cell adhesion molecules. Disturbance in the production of any of these proteins may cause failure in palatal fusion and lead to cleft palate.

Cleft palate

The hard palate is crucial for feeding and normal speech in humans, while the soft palate closes the nasal airway during swallowing. Cleft palate, which is the most common congenital craniofacial malformation in humans, results from a failure of palatal shelves to grow and fuse during the first trimester of embryonic development. Fusion of the palatal shelves is a sensitive developmental procedure and can be disturbed and affected by many genetic and environmental factors. Correction of cleft palate requires surgical intervention shortly after birth.

In rodents, which are obligatory nose breathers, cleft palate is fatal because of the interference with breathing and suckling. Genetic manipulations in mice, followed by detailed morphological and molecular analysis of mutant embryos, have been applied to investigate the molecular mechanisms of palatogenesis and to identify genetic mutations that lead to cleft palates. The molecular basis of cleft palates in mice have been reviewed recently (Funato, Nakamura et al. 2015).

Tgfβ signaling in palatogenesis

As described above, a normal palatogenesis involves cell proliferation, differentiation, and adequate ECM production, all of which are coordinated by secreted growth factors and their signaling pathways. Of these, the most extensively

studied in palatal development is the Tgfβ pathway. Tgfβ signals through several distinct and often antagonistic pathways, such as the Smad2/3-dependent, or canonical, pathway and Smad-independent, or non-canonical pathways. Non-canonical Tgfβ signaling acts trough transducers such as MAPK, JUN, PI3K, PP2A, Rho, and PAR6 (Iwata, Parada et al. 2011).

The Tgfβ superfamily includes Tgfβ cytokines, activins and bone morphogenic proteins (Bmps). Tgfβ exist in three isoforms: Tgfβ1, Tgfβ2 and Tgfβ3, all of which are secreted as inactive (latent) forms that cannot bind to their receptors without an activation process to release the active 25 kD Tgfβ ligand. The Tgfβ precursor forms a homodimer covalently bound to latency associated peptide (LAP) and a Tgfβ binding protein (Ltbp), all together forming the large latency complex (LLC). Binding of Ltbp incorporates the LLC into the ECM. Tgfβ activation involves the release of the LLC from the ECM, followed by proteolytic cleavage of LAP. Matrix metalloproteinase 2 and -9 (Mmp2 and Mmp9), and thrombospondin 1 (Thbs1) are responsible for cleavage of latent Tgfβ. Integrin α5β6 and α5β8 can bind the RGD motif of LAP and release the mature Tgfβ from its latency complex (Munger, Huang et al. 1999, Shi, Zhu et al. 2011).

The active form of Tgfβ binds to the trans-membrane threonine/serine kinase receptor heteromeric complexes of Tgfβ receptors I and II. Binding of the ligand induces conformational changes in the receptors and their auto-phosphorylation. Activation of the type I receptor, triggers signaling in two ways: in the canonical pathway the type I receptor phosphorylates the Smad2/3 signal transducer, which after association with Smad4 translocates to the nucleus and activates the transcription of target genes. The best characterized non-canonical pathway acts through phosphorylation of p38 mitogen-activated protein kinase (p38 MAPK) (Iwata, Parada et al. 2011).

In palatogenesis, the canonical and non-canonical Tgfβ pathways have antagonistic effects, and the cleft palate that results from diminished Smad2/3 pathway activity can be rescued by abrogation of p38 signaling (Iwata, Hacia et al. 2012).

The importance of Tgfβ signaling in palatogenesis is confirmed by mouse craniofacial phenotypes after deletion of genes related to Tgfβ signaling. Tgfβ1 and Tgfβ3 ligands are expressed only in the epithelium of palatal shelves, whereas Tgfβ2 is expressed in the mesenchyme. Mutant phenotypes of *TgfβrII* reveal the distinct roles of Tgfβ signaling in epithelium and mesenchyme. Targeting *TgfβrII* in the palatal shelves mesenchyme leads to cleft palate due to reduction in cell proliferation (Ito, Yeo et al. 2003), whereas *TgfβrII* removal in the epithelium also leads to cleft palate, but due to failure of the palatal shelves to fuse at the MES (Xu, Han et al. 2006).

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