Building The Blood-Brain Barrier in a Dish: Design of Two Innovative In Vitro Models

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Building The Blood-Brain Barrier in a Dish: Design of Two Innovative *In Vitro* Models

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To Rim and my children,

To my family

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1. ABBREVIATIONS

aa	Amino acid
ABC	ATP-binding cassette
AJ	Adherens junctions
α-SMA	α-smooth muscle actin
Ang-1/-2	Angiopoietin-1/-2
ARNT	Aryl-hydrocarbon receptor nuclear translocase
AQP4	Aquaporin-4
BBB	Blood-brain barrier
BED	Biotin ethylendiamin
bFGF	Basic fibroblast growth factor
bHLH	Basic helix-loop-helix
BM	Basement membrane
BMP	Bone morphogenic protein
CAM	Cell adhesion molecule
CM	Conditioned medium
CNS	Central Nervous System
COX	Cyclooxygenase
CVO	Circumventricular organs
DDC	Dystroglycan dystrophin complex
ECs	Endothelial cells
ECF	Extracellular fluid
ECM	Extracellular matrix
CLUT-1	Glucose transporter-1
GDNF	Glia derived neurotrophic factor
HA	Hyaluronic acid
HACE	High altitude cerebral edema
HIF-1	Hypoxia inducible factor-1
HRP	Horseradish peroxidase
HRE	Hypoxia responsive element
iBRB	Internal blood-retinal barrier
ICAM-1	Intercellular adhesion molecule-1
IgSF-CAM	Immunoglobulin super family cellular adhesion molecule
JAM	Junctional adhesion molecule
LIF	Leukemia Inhibitory Factor
MCAO	Middle cerebral artery occlusion
MMP	Matrix metalloproteinase
MRP	Multidrug resistant protein
NPC	Neuronal precursor cell
NO	Nitric oxide
NT	Neural tube
ODD	Oxygen dependant degradation domain
PDGF	Platelet-derived growth factor
PECAM-1	Platelet endothelial cell adhesion molecule-1 (CD31)
P-gp	P-glycoprotein
PHD	Prolyl-4-hydroxylase domain
PNVP	Perineural vascular plexus
pVHL	Van-Hippel Lindau factor
SPARC	Secreted protein acidic rich in cysteine
SLC	Solute carrier
Shh	Sonic hedgehog
SSeCKS	Src suppressed C-kinase substrate
SVZ	Subventricular zone
TAD	Trasnactivation domain
TEER	Transendothelial electrical resistance
TGF-β	Transforming growth factor β
Tie2	Angiopoietin receptor
TJ	Tight junction
VEGF	Vascular endothelial growth factor
VEGFR-1/2	Vascular endothelial growth factor recptor-1/-2
VSMC	Vascular smooth muscle cell
Wnt	Wingless/Int
ZO-1	Zonula occludens-1

2. SUMMARY

The blood-brain barrier (BBB) is a neurovascular unit formed by specialized brain endothelial cells (ECs) surrounded by pericytes and astrocytes end-feet processes. The blood-brain barrier plays a crucial role in the maintenance of brain homeostasis by avoiding fluctuation of neuron extracellular fluid composition but also penetration of any harmful substances that might compromise neuron integrity. Despites breakdown of the blood-brain barrier is associated with many neurological diseases, efficient therapies to fight such barrier disruption are still missing. In addition, current *in vitro* BBB models still suffer from comparison with *in vivo* situation and thus allow only a poor translation of *in vitro* results to *in vivo* situation. Two major issues limit current in vitro BBB models: absence of astrocytes and/or pericytes in brain EC cultures and limitations in intercellular interactions due to an absence of a 3-dimensional structure.

The aims of this thesis were to elaborate two innovative *in vitro* BBB models (a 2-and 3-dimensional models respectively) that consider the presence of both astrocytes and pericytes but also that allows adequate cellular interactions. Using these models we addressed the respective roles of astrocytes and pericytes on endothelial barrier function during physiological conditions and during hypoxia, an important stress factor involved in barrier breakdown.

Our study performed in the 2-dimensional model showed that brain EC barrier function was compromised only after prolonged exposure or during severe O_2 deprivation (near-anoxia) insult. Loss of barrier function was associated with increased permeability to water-soluble compounds, cellular junctions disruption and increased apoptosis by activation of caspase-dependant pathway. In addition we observed that both astrocytes and pericytes induced barrier function during normal

conditions and play important roles as modulators of the barrier function during O_2 deprivation stress, promoting both EC barrier function and cell survival.

In addition we observed that pericytes, but not astrocytes, were able to provide decent EC protection during severe insult suggesting that astrocytes and pericytes may have distinct response to O_2 deprivation stress. We showed that VEGF signaling plays an important role in induction of barrier breakdown during hypoxic stress, as subsequent inhibition of such signaling resulted in enhancement of the barrier function during hypoxic stress.

The second study focused on the characterization of a 3-dimensional BBB model based on collagen matrix to study the influence of a 3-dimensional structure on EC morphology and its interactions with astrocytes and/or pericytes. We observed that EC were able to form tube-like structures. These tubes showed many similarities with *in vivo* capillaries, especially by presence of a lumen within these structures. In addition, we observed that presence of astrocytes and pericytes were able to interact directly with these tubes by formation of cellular processes similar to *in vivo* observations. Such interactions resulted in modulation of tube morphology but also in induction of a BBB phenotype as observed by localization of tight junction complexes and localization of ABC transporters in the lumen of these structures.

Taken together this work provided design of enhanced *in vitro* BBB models and shows the importance to consider the BBB as a neurovascular unit and the importance of cellular interactions as astrocytes and pericytes induce BBB phenotype and partially maintain the barrier function during hypoxic stress.

3. ZUSAMMENFASSUNG

Die Blut-Hirn-Schranke ist eine neurovaskuläre Einheit, bestehend aus spezialisierten cerebralen Endothelzellen, die von Perizyten und den Endfortsätzen der Astrozyten umgeben sind. Die Blut-Hirn-Schranke spielt eine wichtige Rolle bei der Aufrechterhaltung der Homeostase des Gehirns und verhindert Fluktuationen in der Zusammensetzung der extrazellulären Flüssigkeit, welche die Neuronen umgibt. Im Weitern verhindert sie, dass für die Neuronen schädliche Substanzen ins Hirn gelangen. Obwohl verschiedenste neurologische Krankheiten mit der Beeinträchtigung der Blut-Hirn-Schranke verbunden sind, fehlen bisher effiziente Therapien, welche die Schranke schützen oder wiederherstellen könnten.

Die vorhandenen *in vitro* Modelle entsprechen nur ansatzweise der *in vivo* Situation, was die Interpretation von *in vitro* Forschungsergebnisse erschwert. Zwei Hauptaspekte schränken die aktuellen Blut-Hirn-Schranken-Modelle ein: fehlende Astrozyten und/oder Perizyten in cerebralen Endothelzellkulturen und limitierte interzelluläre Interaktionen durch die 2-dimensionale Kultur.

Das Ziel dieser Arbeit war es, zwei innovative *in vitro* Blut-Hirn-Schranken-Modelle zu schaffen, (ein 2-dimensionales und ein 3-dimensionales), die sowohl Astrozyten und Perizyten beinhalten als auch adäquate interzelluläre Interaktionen erlauben. Mit Hilfe dieser Modelle erforschten wir den Einfluss von Astrozyten bzw. Perizyten auf die endotheliale Schrankenfunktion unter physiologischen Bedingungen und während Hypoxie, einem bedeutenden Stressfaktor für die Schrankenfunktion.

Unsere Resultate aus dem 2-dimensionalen Modell zeigten, dass nur langanhaltender oder (nahezu) kompletter Sauerstoffmangel die Schrankenfunktion der Endothelzellen beeinträchtigt. Der Verlust der Schranke führte zu einer erhöhten Durchlässigkeit für wasserlösliche Stoffe, Öffnung der Zell-Zell-Verbindungen und vermehrte Apoptose

durch die Aktivierung von caspase-abhängigen Signalwegen. Im Weiteren beobachteten wir, dass sowohl Astrozyten als auch Perizyten die Schrankenfunktion unter normalen Bedingungen induzierten. Unter Sauerstoffmangel spielten sie eine wichtige Rolle als Modulatoren der Schranke indem sie die Funktion und das Überleben der Endothelzellen förderten. Zusätzlich hatten Perizyten eine schützende Rolle unter erhöhtem Sauerstoffmangel, was darauf hindeutet, dass Astrozyten und Perizyten auf unterschiedliche Sauerstoffkonzentrationene verschieden reagieren.

Wir zeigten, dass der VEGF Signalweg (mit-)verantwortlich ist für die Beeinträchtigung der Schrankenfunktion unter Hypoxie, da die pharmakologische Hemmung dieses Signalwegs zur Verbesserung der Schranke führte.

Die zweite Studie fokussierte auf der Charakterisierung des 3-dimensionalen Blut-Hirn-Schranken-Modells. Dieses Modell basiert auf einer Kollagenmatrix und erlaubt die Erforschung der 3-dimensionalen Interaktionen zwischen Endothelzellen, Astrozyten und Perizyten. Die 3-dimensionale Struktur erlaubte es den Endothelzellen röhrchen-ähnliche Strukturen zu bilden. Diese Röhrchen sind vergleichbar mit *in vivo* Kapillaren und enthalten ein Lumen. Im Weiteren stellten wir fest, dass Astrozyten und Perizyten einen direkten Einfluss auf die Bildung dieser Röhrchen hatte, so wie es auch *in vivo* bei der Bildung von Kapillaren beobachtet wurde. Die Interaktionen zwischen den verschiedenen Zelltypen beeinflussten die Morphologie der Röhrchen, aber auch die Induktion des Blut-Hirn-Schranken-Phenotyps, das heisst, der Lokalisierung von Zell-Zell-Verbindungen (tight junction complexes) und ABC-Transportern im Lumen dieser Strukturen.

Zusammenfassend beschreibt diese Arbeit die Erschaffung von innovativen, erweiterten *in vitro* Blut-Hirn-Schranken-Modellen und betont die Wichtigkeit, die Blut-Hirn-Schranke als neurovaskuläre Einheit zu betrachten. Die interzellulären

Interaktionen zwischen Endothelzellen, Astrozyten und Perizyten sind essenziell um die Schrankenfunktion zu errichten und unter hypoxischem Stress aufrechtzuerhalten.

4. INTRODUCTION

4.1. The blood-brain barrier

4.1.1. Introduction

The central nervous system (CNS) is a highly specialized multicellular structure responsible of the coordination of both cognitive and non-cognitive processes in vertebrates. The brain represents the central organ of the CNS. It represents one of the densest organs in vertebrates, both by the number of cells (~100 billions neurons in a average human brain) and by the number of cellular interactions (one neuron establish up to 10^4 contacts with other neurons by synaptic connections) and thus we may consider it as one of the most important organ in vertebrates.

It is clearly understandable that such organ has an important dependency towards O_2 and nutrients to ensure its proper function, therefore the presence of an efficient O_2 and nutrient supply is definitively required. Such nutrients need is complied by the presence of a rich cerebral vascular density, providing an optimal brain tissue perfusion. In addition to their important dependency to O_2 and nutrients, neuron activity is susceptible to variation in the extracellular fluid (ECF) compartment but also to presence of any harmful and lethal agents. Thus, in order to protect neurons from such insults, vertebrate cerebral vasculature has developed a barrier phenotype that impeach any fluctuation in ECF composition and penetration of any agents that might compromise neuron integrity. This physical and chemical barrier, formed by a neurovascular unit in which cerebral endothelial cells (ECs) plays a pivotal role is classically referred as the “blood-brain barrier” (BBB).

4.1.2. Historical aspects of the BBB

The first description of the BBB dates from the end of the 19th century by Paul Ehrlich (1854-1915). Ehrlich, a famous bacteriologist and physiologist, had a certain attraction concerning the use of chemical stains for observation of histological and cellular preparation. His first observation of the barrier was first described in 1885. Intravenous injection of Trypan blue, a water-soluble dye, resulted in the absence of staining inside the CNS whereas all other organs were stained. However, Ehrlich attributed such remarkable feature to low affinity of the CNS tissue towards the dye rather than to presence of a physical barrier. In 1900, Lewandowsky investigated potassium ferrocyanide penetration into the brain tissue by intravenous injection. Like Ehrlich, Lewandowsky observed absence of staining within the CNS. Unexpectedly, Lewandowsky attributed his observations to the presence of a barrier between the blood and CNS tissue, denominating such barrier "*bluthirnschranke*". Edwin Goldmann confirmed the concept in 1909 and 1913, by performing two experiments. In the first experiment Goldmann, one of Ehrlich's students, repeated Ehrlichs experiment and also noted the absence of staining in the CNS. In the second experiment, Goldmann injected directly the dye into the CSF and observed staining of the CNS but an absence of dye in other organs. These experiments showed the presence of a barrier between the blood and the CNS compartment, but also an absence of barrier between the CNS and the CSF. As no other studies showed any membranes that might explain such observations, Goldmann attributed the barrier role to the cerebral blood vessels. However, the presence of a BBB was still rejected by a certain part of the scientific community until the end of the 1960s. In fact, Goldmann and colleagues experiments raised controversies. The main controversy was that CSF and plasma compositions differ and thus may induce diffusion artifacts. Therefore

injection of dyes into the CSF cannot be compared with dyes injected into the bloodstream. Another point of controversy concerned the aniline based dyes. Aniline has two electrochemical forms: one neutral basic form and one positively charged acidic form. The basic form is able to cross the apparent BBB whereas the acidic form cannot. Friedemann reviewed such observations in 1942 (Friedemann, 1942). In this review, Friedemann reviewed known permeability of various substances and concluded "*the ability of substances to pass the capillaries of the C-N-S is determined by their electrochemical properties. The cerebral capillaries are permeable to substances carrying a positive or no charge at the pH of blood while they are impermeable to those carrying a negative charge…the problem is only part of the more general problem of capillary permeability…*"

Although this review pinpointed the problem of electrochemical status of certain molecules such hypothesis has been since shown to be not strictly true. Further hypothesis described the physicochemical nature of the molecules that can cross the BBB include molecular weight, size, affinity and dissociation coefficients, lipophilicity and electric charges (Tschirgi, 1962). In addition to the apparent chemical selectivity of the BBB, anatomical studies that were performed to explain the determination of solute diffusion constituted a supplementary step in the controversy. Despites the emergence of electron microscope in 1931 by Ernst Ruska and Max Knoll would bring crucial informations about cerebral microvessels ultrastructure and thus clarify the controversy, the absence of adequate staining techniques did not provide experimental arguments advocating the presence of a BBB.

In 1957, Maynard and colleagues (Maynard et al., 1957) suggested that the BBB was only a experimental artifact, justifying their argument by the following citation: "*The*

present authors believe that the 'blood-brain barrier' may be mainly an illusion. Physiologists have been led to postulate its existence on the assumption that there are considerable extracellular tissue spaces. Which would be in equilibrium with blood plasma if a barrier did not exist. We see, however, that the basic assumption is incorrect and these tissue spaces simply do not exist." Later on, the authors wrote, "*When the physiologist quantitatively studies the penetration of ions, organic crystalloids, proteins, vital dyes, etc. into nervous tissue, he must actually be studying the penetration (or lack of it) into the glial nervous cells… Thus, we believe that the physiological data available should be re-interpreted on the assumption that nearly all substances are within cytoplasmic compartments. Under these circumstances there would be no expectation of a simple correlation with their distribution into the blood plasma. The commonly accepted laws of permeability, which apply in the brain without important exception and without invoking a specialized barrier*."

However in 1961 Dobbing counteracted such statement. Relying on the previous findings of Hodgkin and others, Dobbing argued that the argumentation provided by Maynard were in contradiction with the gradient need of electrolytes to support neural function. At the same time, Davson and colleagues (Davson and Spaziani, 1959) argumented that the hypothesis evocated by Maynard would apply only if diffusion of molecules within the cerebral compartment would remain constantly low, even after removal of the brain from the cranium.

Using different water-soluble compounds such as iodide, *p*-aminohippurate and sucrose, Davson showed that it was not the case. Peripheral injection of these compounds showed that their penetration occurred at "*barely measurable rates by contrast with their penetration into skeletal muscle*" as expected. However diffusion of these molecules into excised brain tissue was comparable to excised skeletal

muscle tissue, indicating that the presence of an extracellular space inside the brain tissue, diffusion of water-soluble compounds from the blood to the brain were therefore limited by the presence of a barrier between the blood vessel and the brain tissue. Hoffman and Olszewski (Hoffman and Olszewski, 1961) supported such argumentation by injecting fluorescein inside the vascular bed from the area postrema. They showed diffusion of fluorescein into the area, but also in the regions surrounding the area postrema, demonstrating the presence of an extracellular space and concluded that the barrier function is carried by the brain vasculature rather than an absence of extracellular space.

Another point of discord concerned the anatomical location of the BBB. Scientific community was split between scientists believing that the barrier function was carried by the capillary endothelium itself, whereas other scientist attributed the barrier feature to the astrocytic end-feet processes or to the basal lamina.

Astrocytic end-feet processes have been described as a continuum with the basal lamina and thus overlap most of the capillary surface, several observations agreed with the hypothesis that astrocytes *per se* were responsible of the barrier function (Gershenfeld et al., 1961). However, such hypothesis was eliminated by the study of Reese and Karnovsky (Reese and Karnovsky, 1967). In this study, using high-resolution electron microscopy and HRP staining, they showed that intravenously injected HRP activity was restricted to the lumen, whereas no trace of HRP activity was detectable in the perivascular space. Furthermore, these authors described presence of tight contacts at the interendothelial clefts, showing similarities with epithelial “tight” junctions. Thus the authors proposed that the capillary lumen presents tight junctions that form a continuous, impermeable membrane and therefore constitute the corner stone of the BBB. This statement was later confirmed by

injection of HRP into the brain tissue that brought the final proof that endothelial cells but not astrocytes end-feet processes, mediate the anatomical barrier function (Brightman and Reese, 1969).

4.1.3. Development of the BBB

Formation of the BBB occurs at late embryonic and early post-natal stages. Thus formation of the cerebral vasculature fundamentally follows identical developmental pattern as the vasculature of other organs. Chick and mouse embryos provided us with crucial information on the different cellular stages occurring during vascular development in vertebrates. However, further investigations to understand the nature of the different genes involved in vertebrate vascular development are limited in such models as they represent an important technical challenge. More recently, the scientific community raised its interest on zebrafish model, as it constitute a model of choice for genetic studies and therefore provided an important quantum leap in our understanding of the nature of the genes involved during development of the vasculature. This chapter summarizes the formation of the vasculature in vertebrates based on different studies performed in zebrafish (Baldessari and Mione, 2008), chick and mouse embryos (Flamme et al., 1997; Kurz, 2000; Kurz et al., 1996; Risau, 1997; Risau and Flamme, 1995; Risau and Wolburg, 1990).

Development of vasculature occurs by two major physiological processes:

- **Vasculogenesis:** that consists of the formation of a primitive vascular network from non-existing vasculature.

- Angiogenesis: that extends and develops the vascular tree by relying on the pre-existing vascular network.

Vasculogenesis occurs during gastrulation phase (see Fig.1), when the three germ layers are formed. Haemangioblasts constitute the first cells of the endothelium differentiation lineage. Haemangioblasts are directly derived from migrating cells of ventral mesoderm.

Differentiation of mesodermic cells into haemangioblasts occurs under influence of the notochord and endoderm. The notochord secretes Sonic hedgehog (Shh) (Lawson et al., 2002) whereas the endoderm secretes vascular endothelial derived growth factor (VEGF) (Gale and Yancopoulos, 1999).

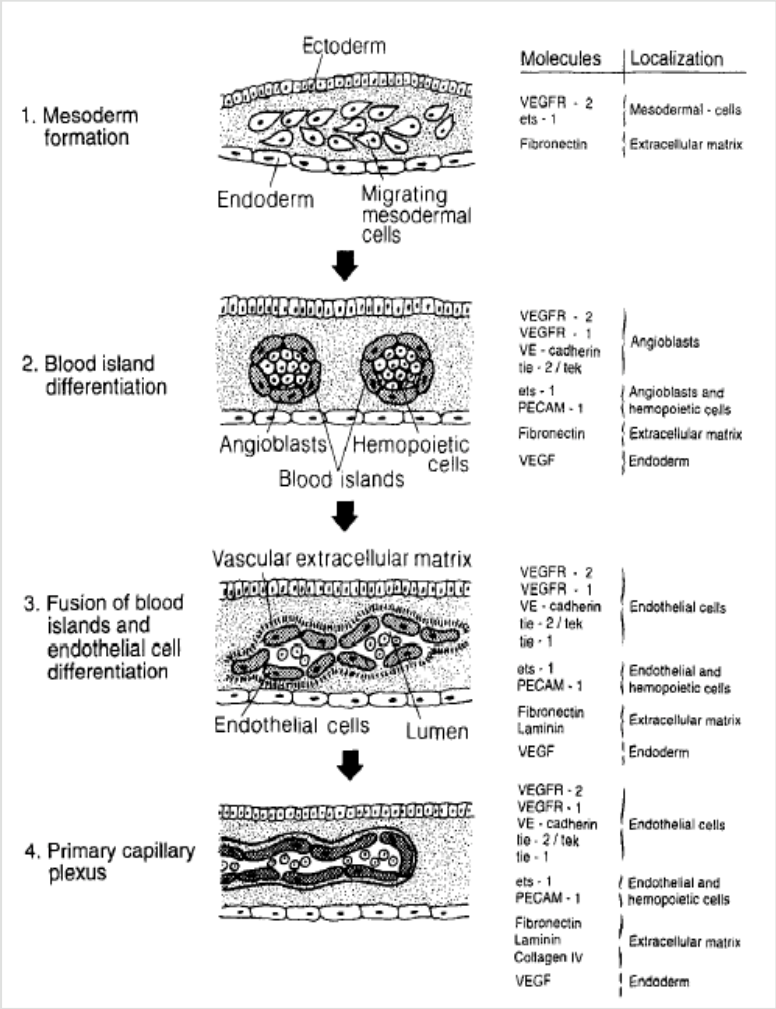

Figure 1: Schematic view of vasculogenesis in vertebrates
(From (Risau and Flamme, 1995)

A certain population of mesodermic cells, mostly located in the ventral mesoderm, express VEGF receptor-2 (VEGFR-2, also known as Flk-1/KDR) (Liao et al., 1997) and under the influence of VEGF, these cells initiate a migration from the lateral plate mesoderm to the notochord, by sliding over the endoderm (Jin et al., 2005). In parallel, these VEGFR-2 positive mesodermic cells intitate their differentiation into haemangioblasts, such differentiation already occurs in the lateral plate mesoderm. Haemangioblasts constitute the common progenitor of both blood cells (e.g. red blood cells, leukocytes, platelets…) and endothelial cells (ECs).

Haemangioblasts that reach the notochord initiate the formation of spherical structures comparable to cysts: the blood islands. Haemangioblasts contained within the blood islands initiate their differentiation between endothelial cell lineage (angioblasts) and hematopoietic cell lineage. Under the influence of bone-morphogenic protein family (BMP) (Gupta et al., 2006) and by Wingless/Int (Wnt) (Lindsley et al., 2006) gradients, haemangioblasts initiate their differentiation into angioblasts (Feinberg and Noden, 1991; Noden, 1989; Noden, 1990) and hematopoietic cells. The haemangioblasts that initiated differentiation into angioblasts express different sets of EC-specific genes such as friend leukemia integration (fli1), growth differentiation factor 5 (gdf5) and endothelium-specific receptor tyrosine kinase (tie1, tie2). In addition, angioblasts initiate the secretion of a fibronectin-rich primitive extracellular matrix (ECM) essential for further development of the vasculature (Jin et al., 2005). In parallel, formation of interconnections between blood islands allows formation of a primitive network, classically referred in the literature as vascular cord or primitive plexus. The primitive plexus formed by differentiating angioblasts consists of a primitive capillary network formed by filled tubular structures. ECs and pericytes originate from the mesoderm EC lumen formation is initiated by the formation and

the fusion of pinocytic intracellular vacuoles (Bayless et al., 2000; Davis and Camarillo, 1996). Recent studies performed by Koh and colleagues (Koh et al., 2008a; Koh et al., 2008b) demonstrated *in vitro* the importance of Cdc42 and Rac1 (two small GTPases involved in cytoskeleton regulation) during lumen formation in EC vessel-like structures.

Besides lumen formation, the primitive plexus extends the vascular network density by angiogenesis. Angiogenesis differs from the vasculogenesis by different aspects: it requires the presence of a pre-existing vascular network and involves formation of hollow tubular structures. Angiogenesis occurs by two different mechanisms: sprouting angiogenesis and non-sprouting angiogenesis (Risau, 1997). Non-sprouting angiogenesis is defined by formation of new vessels by a split of pre-existing vessels (Patan et al., 1996), whereas sprouting angiogenesis forms new vessels by branching processes based on pre-existing vessels. Interestingly, sprouting angiogenesis constitutes the main mechanisms by which brain angiogenesis occurs. Brain angiogenesis is initiated after embryonic day 9.5 (E9.5) in mice. At this embryonic stage, the perineural vascular plexus (PNVP) that surrounds the neural tube (NT, Fig.2) constitutes the basement for the formation of cerebral vasculature.

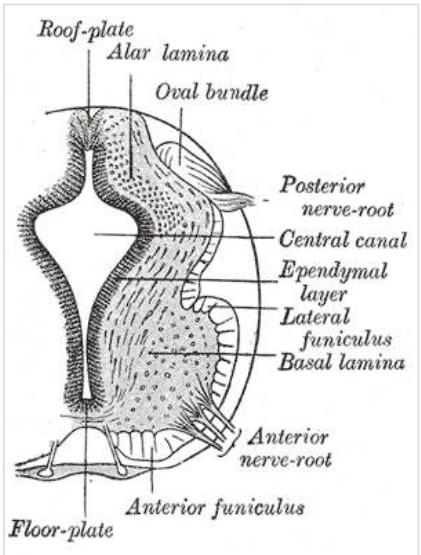

Figure 2: Structure of the neural tube (Source: http://upload.wikimedia.org/wikipedia/commons/8/89/Gray642.png)

Formation of cerebral arteries occurs from the medial portion of the PNVP, whereas formation of cerebral veins occurs from the lateral portion of the PNVP (Kurz et al., 1996). The floor-plate and the ependymal layer mediate primitive cerebral artery invasion by secretion of two important angiogenic factors: VEGF (Aitkenhead et al., 1998; Breier et al., 1992; Plate, 1999; Strong, 1961) and angiopoietin-1 (Ang-1) (Koblizek et al., 1998; Suri et al., 1998). Interestingly, the guidance of the newly formed vascular structures inside the NT relies on similar cues used by the nervous system for axonal guidance such as ephrins/EphB (Kuijper et al., 2007) or semaphorin/plexin (Bussolino et al., 2006) signaling pathways. In addition to cue signaling molecules, the ECM plays also an important role in the blood vessel guidance. The loose mesenchyme and mitogenic fibronectin ECM found in the PNVP (Poelmann et al., 1990) greatly contrast with the densely populated NT, in which hyaluronic acid constitutes the main ECM component (Turley et al., 1994). Such dramatic changes in the ECM composition results in a decreased EC proliferation rate compared to other developing vasculature, despite the persistence of VEGFR-2 expression in EC and the intense VEGF secretion by the neural tube (Plate, 1999). In addition brain EC must face another important challenge, the attachment to the ependymal layer.

Interestingly, the ependymocyte and cerebral EC do not show any common adhesion molecules and therefore need to find a method to form cell-cell contacts. A mechanism hypothesized by Kurz (Kurz, 2000) may explain how ECs and ependymocytes are able to interact without using any adhesion molecules. Kurz suggests that ECs migrate between the neuroblasts and the radial glia by formation of filipodia (Fig.3), reaching the ependymal neuroblasts and anchors to them by formation of TJ strands. Tip cells that successfully anchored the ependymal layer

serve as a “pillar” for surrounding EC filipodia and lead to the formation of a vascular cord and lumen formation. Once the cerebral vascular tree is formed and stabilized, formation of the BBB can occur.

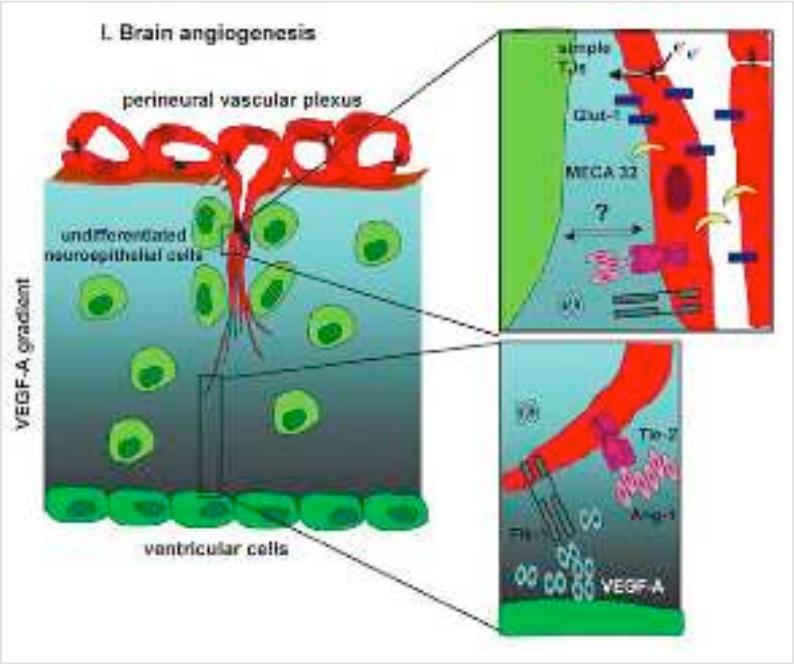

Figure 3: Brain EC migration during cerebral angiogenesis (From Engelhardt, 2003)

However, the question addressing the presence of a BBB or not during embryonic stages is still debated. Since Wislocki’s experiments (Wislocki, 1920) performed on immature animals, investigations of the presence of a BBB during embryonic development continue to rely on extravasation of century-old markers such as Trypan Blue, Evans Blue or HRP. In fact, use of such techniques might constitute a major stress for fragile immature brain vasculature and therefore might result in the apparent leakiness of the vasculature (Saunders et al., 2000).

More recently, Ek and colleagues (Ek et al., 2006) proposed an alternative technique to assess vascular permeability in immature mammals that induce less damage to the vascular structures by using biotin ethylendiamin (BED), a small water-soluble organic molecule (molecular weight: 286 Da). Ek and colleagues demonstrated that

Opossum (*Monodelphis domestica)* brain vasculature was impermeable to BED, suggesting that brain ECs in the forming vasculature already display a vascular phenotype. However, such study needs to be carefully extrapolated. In this study, Ek and colleagues used Opossum, a marsupial mammal at post-natal day 5 (P5). It is well known that rodents show already a mature BBB at P5 (Butt et al., 1990; Senjo et al., 1986). The question that can be therefore addressed in such a study is how comparable is a P5 opossum compared to a P5 rodent? Therefore additional studies using the same technique should be conducted on rodent embryos in order to provide a clear statement on the presence or not of a BBB in mammals during development. In parallel to such studies that are mainly focused on the assessment of barrier function during development, other investigators rather focused on expression of tight junctions protein, another marker of the BBB phenotype in adults. Virgintino and colleagues (Virgintino et al., 2004) investigated occludin and claudin-5 expression and localization during human gestation. They described positive staining against both occluding and claudin-5 in 12 weeks-old fetal brain microvessels. However, the staining for both proteins appeared diffuse within the cytoplasm, suggesting that at this gestational time period, brain EC were not able to form a cohesive barrier. However, in 14-weeks-old fetuses, occludin and claudin-5 staining shifted from the cytoplasm to the cell borders, suggesting that brain ECs started formation of a barrier phenotype.

The debate generated by the question on the presence or not of a BBB during development reflects our limitation on the techniques available so far to address such questions. In one hand we have *in vivo* models that might give us useful information but limited by usage of century-old permeability assays. In the other hand, we do not have yet any suitable *in vitro* model that reproduce simultaneously brain angiogenesis

and cellular interactions as they occurs during development. Therefore usage of other animal models or innovative cell culture techniques is definitively required to provide a definite answer to such questions.

4.1.4.The blood-brain barrier: a neurovascular unit

The BBB (Fig. 4) is a neurovascular unit formed by specialized EC delimitating the blood compartment from the cerebral tissue. The basement membrane (BM) that lines the EC allows induction of cell polarity by formation of luminal and abluminal sides. The luminal side (apical side) of the endothelium is directly in contact with the circulating blood flow, whereas the abluminal side (basolateral side) directly faces the BM. Pericytes constitutes the unique cell type that makes direct contact with EC, facing directly the EC and sharing the inner side of the BM. In addition to EC and pericytes, the perivascular cells constitute the third cell type located in the inner side of the BM. It is located between the pericytes and the inner face of the BM.

On the outer side of the BM, astrocytes, a cell type that belongs to the glia, indirectly interact with EC through formation of astrocytic end-feet processes over the vasculature. Neurons constitute the last member of the neurovascular unit. Despites the presence of axon terminal branches close to the EC, the possible involvement of neurons in the BBB remains debateful. The role of different cell types within the neurovascular results in the presence of complex cellular interactions, the feature of each cell type and its respective interaction with the EC will be subject to more detailled discussion in the following chapters.

Interestingly, the presence of a BBB does not prevail in all cerebral blood vessels. Song and Patscher (Song and Pachter, 2003) observed that the highest expression of various BBB markers (i.e. transferrin receptor (TfR), P-glycoprotein (P-gp), glucose

transporter-1 (GLUT-1)) occurs in small caliber cerebral blood vessels (<40µm) including microvessels, arterioles and venules, suggesting that the BBB phenotype may be carried on by brain EC located in microvessels.

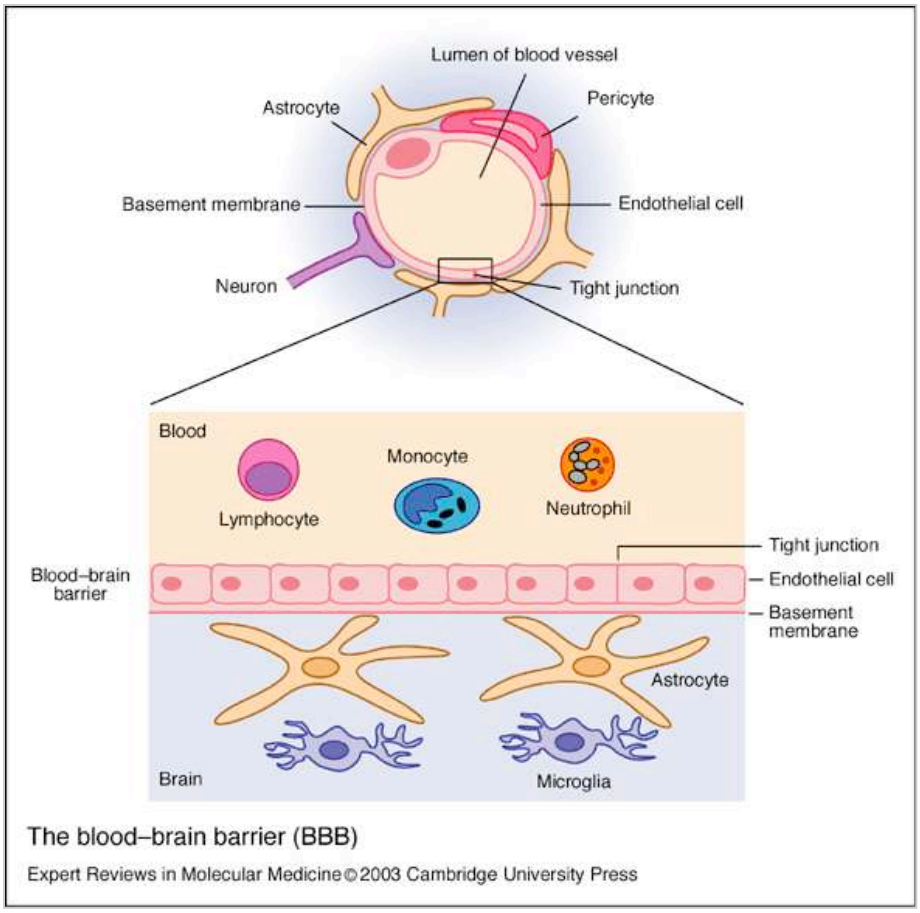

Figure 4: Schematic representation of the BBB (From (Francis et al., 2003)

4.1.5. The brain EC

Brain ECs constitute the corner stone of the neurovascular unit, as it is responsible for the barrier function. Brain ECs differ from other ECs by the presence of the BBB phenotype. The BBB phenotype is based on the presence of different features:

- Tight junction complexes that allow brain ECs to act as a selective filter against diffusion of small water-soluble compounds.

- Low vesicle-associated transcytosis that considerably limits the exchange between the compartment either by pinocytosis or by receptor-mediated transcytosis.

- Solute carriers and ABC transporters that provide an important restriction in amino acids and glucose diffusion from the blood to the brain tissue, but also limit the penetration of lipophilic drugs.

a) EC cell-cell contacts:

EC cell-cell contacts play an important role for the cohesion of the vascular tissue, but also to ensure the barrier function. The contacts are formed by interactions of transmembrane proteins through their extracellular domains. These transmembrane proteins are anchored to the actin cytoskeleton by presence of cytosolic proteins that play the role of coupling between the transmembrane protein and the actin cytoskeleton respectively.

Cell-cell contacts are divided into two major types distinguished by the nature of proteins involved, the adherens junction (AJ) and tight junction (TJ) proteins.

AJ are present in all EC. The primary role of AJ is to maintain cohesion of the vasculature during physical stress (i.e. shear stress), but also be physical barrier against circulating blood cells and proteins (Brown and Davis, 2002; Corada et al., 2001; Corada et al., 1999). The second role of the AJ is to regulate EC proliferation during vascular growth by inducing contact inhibition. The third role of AJ in EC is to initiate cell polarity by the delimitation on a luminal and abluminal domains in the plasma membrane. AJ are divided two superfamilies: The **cadherin superfamily** and the **immunoglobulin superfamily cellular adhesion molecules (IgSF-CAM)**.

The cadherin superfamily consists of a variety of transmembrane proteins that requires presence of Ca^{2+} ion to ensure their function. Members of the cadherin superfamily include cadherins, protocadherins, desmogleins and desmocollins.

Cadherins (Fig.5) contain an extracellular domain containing an extracellular Ca^{2+} binding domain and interact with other cadherins through homophilic interactions. Until now, 18 different family members have been described in humans (Hill et al., 2001). ECs possess a specific cadherin, the vascular endothelial (VE)-cadherin (Breier et al., 1996), but express also isoforms found in other tissues, such as epithelial (E-cadherin) and neuronal (N-cadherin) cadherin isoforms (Abbruscato and Davis, 1999; Gerhardt et al., 1999).

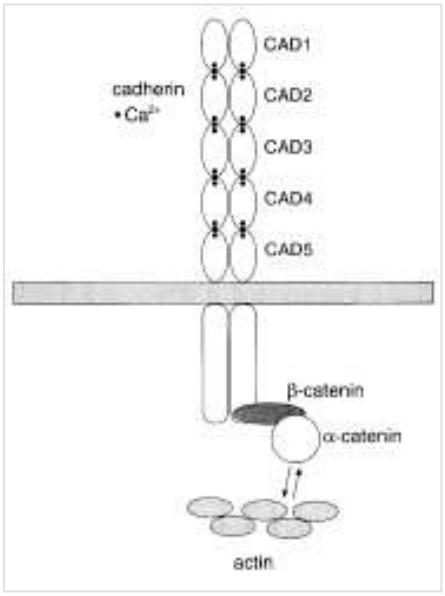

Figure 5: Schematic representation of cadherin structure
(Source: http://calcium.uhnres.utoronto.ca/cadherin/images/pub_pages/general/regulation.jpg)

In the BBB, VE-cadherin mRNA and protein levels are remarkably low compared to other vasculature during embryonic development and it is even further reduced during the adulthood (Breier et al., 1996), suggesting that VE-cadherin role in the BBB phenotype is minimal. However, a possible compensatory effect between the different cadherin isoforms has not been demonstrated yet and still needs to be addressed. Recently, Williams and colleagues (Williams et al., 2005) described the expression of a novel kind of cadherin in brain microvessels, the cadherin-10. Interestingly, cadherin-10 expression was found in "BBB" microvessels, but was absent in the

vasculature found around the circum ventricular organs (CVO), a vascular territory marked by the absence of barrier phenotype in EC. In this region, ependymal cells, the epithelium that delimits the choroid plexus, plays the role of barrier. Interestingly, these ependymal cells also expressed cadherin-10 at the cell borders. This study raises therefore the possible direct or indirect roles of cadherins in the BBB phenotype.

The IgSF CAMs constitute the second member of the CAM superfamily. EC express three different members of the IgSF CAMs: intercellular CAM-1 (ICAM-1), vascular CAM-1 (VCAM-1) and platelet-endothelial CAM-1 (PECAM-1). ICAM-1 and VCAM-1 functions are classically associated with EC-leukocyte interactions and therefore with neuroinflammation (Greenwood et al., 2002).

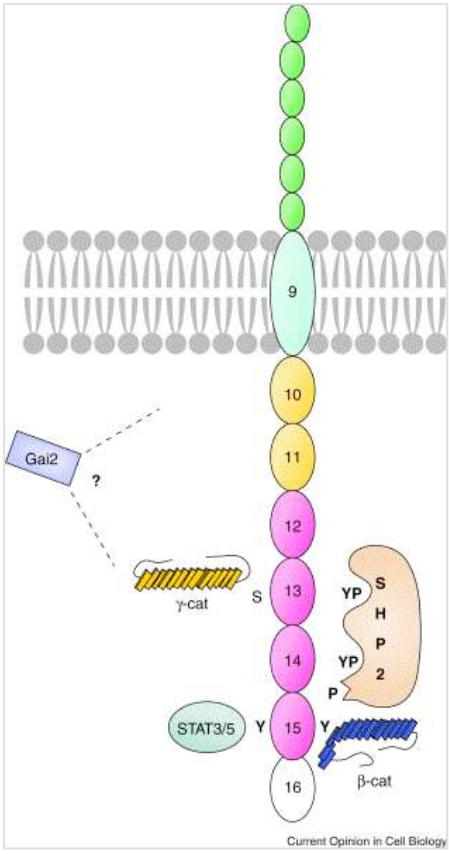

Figure 6: Schematic representation of PECAM-1
(From (Ilan and Madri, 2003))

PECAM-1 (Fig.6) expression is widely distributed on ECs but also found in leukocytes, including monocytes, granulocytes and lymphocytes population (Albelda

et al., 1990; Muller et al., 1989; Ohto et al., 1985; Stockinger et al., 1990). PECAM-1 may have two major functions: maintenance of EC cell-cell contacts, but also ensures EC-leukocyte interactions. Interestingly, PECAM-1 and ICAM-1 levels in brain EC are rapidly down-regulated to minimal levels during post-natal stages in developing mice (Lossinsky and Wisniewski, 1998; Lossinsky et al., 1997) and therefore suggest that these proteins might not play a role in the barrier function at the BBB. However, since these molecules are involved in EC-leukocyte interactions, we can speculate that down-regulation of these proteins might be tightly involved in the CNS immune privilege phenomenon (for review see (Carson et al., 2006)). Subsequent alteration in either protein expression levels or activation might suggest their possible implication in neuroinflammatory diseases.

The role of PECAM-1 in the BBB came out from a study performed on PECAM-1 knock-out (KO) mice subjected to experimental autoimmune encephalomyelitis (EAE) (Graesser et al., 2002), a mouse model of multiple sclerosis, a neuroinflammatory disease. In this study, Graesser and colleagues observed that PECAM-1 KO animals presented an early onset of the clinical symptoms EAE compared to wild-type animals, but also presented increased leukocytes infiltration and increased paracellular permeability to Evans Blue dye. These results suggest that PECAM-1 may acts as an inhibitor of leukocyte infiltration, such inhibition may occurs by modulation of ICAM-1 signaling by PECAM-1 (Couty et al., 2007). We can therefore imagine that PECAM-1 may have a positive role during neuroinflammation. However other investigations rather suggest that PECAM-1 up-regulation may occurs in neuroinflammatory disease and other neurological diseases (Kalinowska and Losy, 2006; Schnell et al., 1999). Apart from the contradictory results obtained with PECAM-1 studies, we can speculate that inhibition of IgSF-

CAMs activity or their signaling at the BBB might constitute a source of investigation to counteract neuroinflammatory disorders.

Cadherins and IgSF-CAMs superfamilies interact with the cytoskeleton by a variety of proteins that allows coupling between the CAMs and the actin cytoskeleton. β-catenin constitutes a common coupling protein for both cadherins and for PECAM-1. In addition to its role in the cytoskeleton, β-catenin acts as a signal transducer in the Wnt signaling pathway (Kuhl and Wedlich, 1997). Wnt/β-catenin signaling plays an important role in vascular development by promoting angiogenesis (Goodwin and D'Amore, 2002) and more recently in a study performed by Liebner and colleagues (Liebner et al., 2008), in the maturation of the BBB during development by inducing expression of claudin-3, a protein involved in TJ complex. This study raises the hypothesis of a possible link between and AJ and TJ complexes, and thus an indirect contribution of AJ to the BBB phenotype.

On the other side of cell-cell contacts, TJ constitute the most important and thus the most studied cell junctions of the BBB as they represent the molecular structure responsible for the barrier phenotype in brain EC (Schneeberger and Lynch, 1992; Schneeberger and Lynch, 2004). In contrast to AJ, TJ proteins are divided into three major families: **occludin, claudins and JAMs.** TJ proteins show the presence of several transmembrane domains (with the exception of junctional adhesion molecules (JAMs) that share similarities in their structures with the CAM superfamilies).

Occludin (Fig.7) constitutes the unique member of the occludin family and the first TJ protein discovered in epithelial and endothelial TJ complexes (Furuse et al., 1993). It has a molecular weight (MW) of 65 kDa (504 amino acids (aa)) and possesses four transmembrane domains with two extracellular loops and two intracellular domains.

The N-terminal intracellular domain (149 aa) is associated with the barrier function as the truncated form results in loss of TJ complexes (Bamforth et al., 1999). Such deleterious effect suggests that N-terminal domain may be involved in the clustering of occludin with other TJ proteins to form the TJ complex.

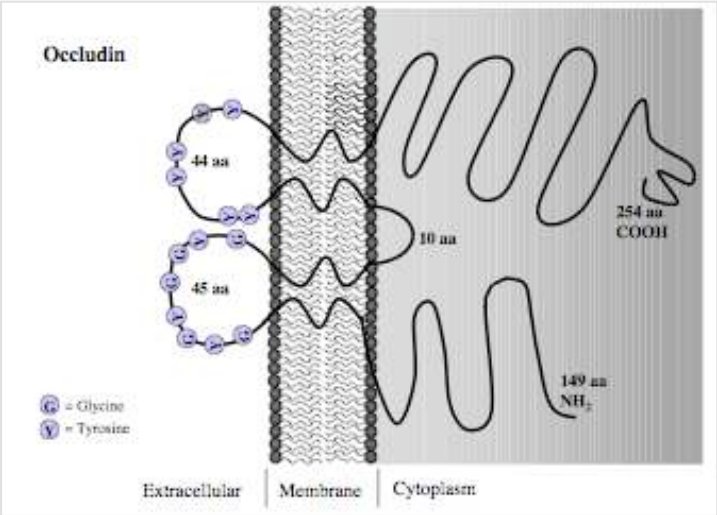

Figure 7: Schematic representation of occludin
(From Feldman et al., 2005)

The C-terminal intracellular domain (254 aa) is involved in the interaction with the actin cytoskeleton, but also in the TJ complex assembly (Chen et al., 1997). Interactions between occludin are achieved by homophilic interactions via their extracellular domains. Interestingly, Bazzoni and colleagues (Bazzoni et al., 2000) demonstrated that occludin and JAMs form clusters in the TJ complex, suggesting presence of occludin-JAM oligomeric complex in the TJ strands. Notably occludin expression is high in the BBB and very low in non-BBB vasculature (Hirase et al., 1997). In addition, occludin expression increase during rat brain development. Occludin appears to be weakly expressed in P8 rats and constantly increase its expression levels during development (Hirase et al., 1997). These studies reflect that occludin constitutes an important TJ protein involved in the BBB. Surprisingly, generation of occludin KO animals by Saitou and colleagues (Saitou et al., 2000)

resulted in the absence of TJ-associated epithelial and endothelial barrier leakage, these animals were viable and fertile. It is important to notice that although this study did not investigate the barrier function in the BBB, the authors have observed a calcification of the brain, with important calcium mineral deposits around the cerebral vasculature, suggesting that occludin barrier function may be associated with selective ion permeability. This study also shows that occludin is not the sole TJ protein to mediate the barrier function and likely other proteins may be involved in the formation of the BBB.

Claudins (Fig.8) constitute the second protein family involved in TJ (Furuse et al., 1998; Krause et al., 2008). Claudin family comprises 24 different members divided into classic and non-classic claudins according to their degree of similarity.

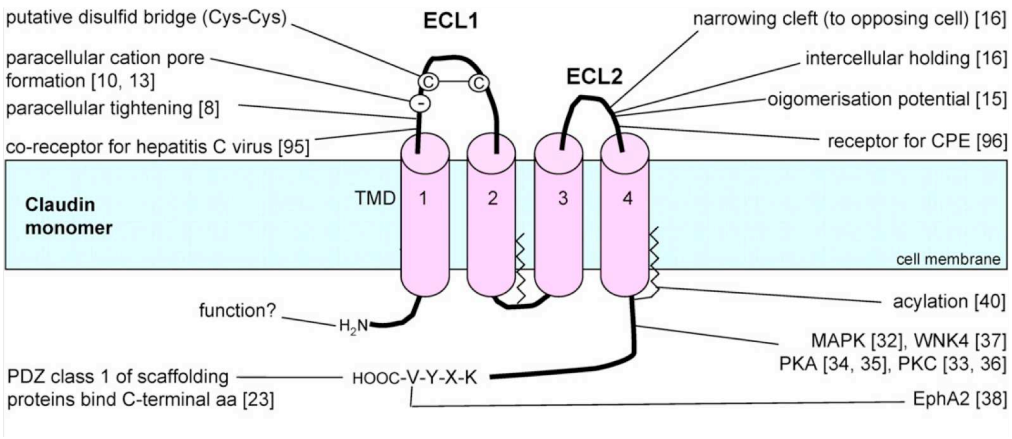

Figure 8: Schematic representation of claudin
(From Krause et al., 2008)

Brain EC expresses four different claudins: claudin-1 (Liebner et al., 2000), claudin-3 (Wolburg et al., 2003), claudin-5 (Morita et al., 1999; Nitta et al., 2003) and claudin-12 (Nitta et al., 2003). Remarkably, claudin-5 expression is restricted to brain EC and thus constitutes a classical marker of BBB phenotype. Claudins constitute smaller molecules than occludin, with an average molecular weight between 20-27 kDa (180-245 aa), but share the same structures. Claudins form a four-transmembrane protein

with 2 extracellular loops and 2 intracellular domains. N-terminal intracellular domain constitutes the shortest domain present in mammalian claudins (~7 aa) and no function has been associated yet. The C-terminal intracellular domain (25-55 aa) constitutes the interaction domain with the actin cytoskeleton through coupling with ZO-1. As occludin, claudins interact with the same claudin members by homophilic interactions through their extracellular loops (Furuse et al., 1999). The function of claudins in the barrier function has been obtained by generating KO animals; claudin-5 deletion study by Nitta and colleagues (Nitta et al., 2003) raised most curiosity on the roles of claudins in the BBB phenotype. Interestingly, KO animals were not embryonic lethal and showed normal morphology after birth. However, these animals rapidly died few hours after birth. Surprisingly, microscopic analyses of claudin-5 deficient brain vessels did not show any differences in TJ complexes, but also no changes in other claudins such as claudin-12, suggesting that TJ complexes were still present. However, permeability assays provided more information and showed clear differences between wildtype (wt) and KO mice. Using a perfusion mixture of rhodamine-dextran (10 kDa), microperoxidase (1.9 kDa) and Hoechst 33258 (562 Da), Nitta and colleagues showed that brain frozen-sections obtained in wt animals were not stained with any of the markers used. However, claudin-5 KO animals were positively stained against the Hoechst staining (562 Da), suggesting that the BBB in these animal was compromised compared to the wt, but also that BBB leakiness occurs for size-specific molecules. These results suggest that claudin-5 and probably other claudins define a size-selectivity of the TJ complexes that form the BBB, but also may occur in other barriers. Furthermore it is not a single TJ molecule that induces the barrier phenotype, but rather the accumulation of different TJ proteins.

JAMs (Fig.9) constitute the third TJ protein involved in the BBB (Orlova and Chavakis, 2007; Weber et al., 2007). Interestingly, JAMs do not share the occludin and claudin structures, but present a structure comparable to the proteins of the immunoglobulin-like superfamily. The JAM family present three different members: JAM-A, JAM-B and JAM–C. JAM extracellular portion consists of two Ig-like domains, with one transmembrane domain and a small cytoplasmic C-terminus tail that allow interactions with the actin cytoskeleton through ZO-1.

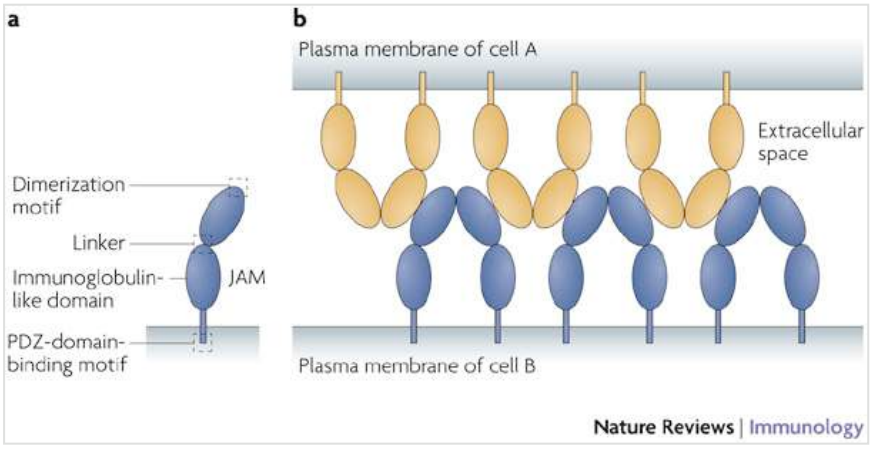

Figure 9: Schematic representation of JAM (From (Weber et al., 2007)

Expression of these three different isoforms has been described in EC. Notably JAM-B was highly expressed in venules. As described for occludin and claudins, JAMs interactions occur by homophilic interactions. Interestingly, down-regulation of JAM-A activity by soluble JAM-A (Ostermann et al., 2005) resulted in increased permeability. Conversingly, JAM-C inhibition by soluble JAM-C (Orlova et al., 2006) resulted in decreased permeability, suggesting that JAM-A and JAM-C play antagonistic roles in EC regulation of paracellular permeability. Unlike occludin and claudin-5, no exhaustive studies performed on JAM KO animals have been performed yet, thus the roles of JAMs in the BBB remain unclear. However, some studies have shown that JAM-A down-regulation is associated with BBB breakdown (Yeung et al., 2008), whereas abnormal increased JAM-A expression has been reported in the

cerebral vessels of multiple sclerosis patients (Padden et al., 2007). These studies suggest that JAMs may contribute in the BBB phenotype, but also may play important roles in neuroinflammatory diseases.

As observed with β-catenin and the AJ proteins, TJ proteins share the same adapter proteins in order to interact with the actin cytoskeleton. ZO-1, an important member of the ZO family, seems to allow interactions between occludin, claudins and JAMs and actin cytoskeleton. Thus, the strategic position occupied by ZO-1 suggests an important role for this protein in terms of regulation of the barrier function.

For example, it has been shown that ZO-1 phosphorylation impairs barrier function and results in increased permeability in various barriers (Howarth et al., 1994; Singer et al., 1994; Stevenson et al., 1989) including the BBB (Antonetti et al., 1999; Fischer et al., 2002; Wong, 1997). Therefore, ZO-1 plays not only important roles in the function of the BBB but also is involved in BBB breakdown. Another remarkable feature that ZO-1 shares with β-catenin is nuclear translocation and it may act as a transcription factor (Gottardi et al., 1996). Unlike β-catenin in which the signaling pathway and target genes have been clearly identified, the possible mechanism and genes regulated by ZO-1 remains completely unknown. Therefore the role of ZO-1 nuclear translocation in the BBB remains largely hypothetical and speculative.

b) Solute carriers and transporters

As described before, the BBB acts as a formidable barrier against diffusion of water-soluble compounds from the blood compartment to the cerebral tissue. In addition to the physical barrier formed by the TJ complexes, the BBB acts as a metabolic barrier towards water- and lipid-soluble molecules. In order to play such a role the BBB has developed an array of different features. Firstly, brain ECs display a very low

pinocytic activity, resulting in a very low exchange between the plasmatic compartment and the ECF, contribute to the maintenance of a constant ECF composition. The second feature performed by the BBB is the presence of different solute carriers (SLC) that allow the transport of different water-soluble compounds necessary for proper neuron activity such as amino acids, glucose or lactate, but also the recycling and the purge of the neurotransmitter metabolites, contributes therefore to the CNS homeostasis. The third major feature allows the BBB to act as a barrier to lipophilic molecules, by expression of an array of ATP-binding cassettes (ABC) efflux transporters both at the luminal and abluminal sides (Soontornmalai et al., 2006). These transporters ensure an important limitation of lipid-soluble toxins diffusion from the blood to the brain compartment, thus protecting the neurons from any harmful substances. However, an important number of drugs including neuroprotective drugs are substrates for different ABC transporters. Therefore their penetration inside the CNS is largely compromised, resulting in a penetration of only a fraction of the plasmatic dose. Thus drug delivery to the CNS tissue constitutes an important challenge in elaboration of novel therapies targeting the CNS (Neuwelt et al., 2008) and requires us a better understanding of the BBB transporters physiology in order to optimize their delivery in the brain.

4.1.6. Astrocytes and the BBB: roles of the glio-vascular interactions

Glial cells constitute the second important cell type present in animal nervous system after neurons. Interestingly, glial cells and neurons share the same common neuroepithelial progenitor cells, although respective morphology and function of these two cell types differ during development. Glial cells are classically divided into two major families: macroglia and microglia. Astrocytes constitute a major member of the

macroglia family. For decades astrocytes were simply considered as “brain glue” cells, their roles in the CNS were thought to be strictly limited to the maintenance of the cerebral cytoarchitecture and neuron microenvironment. In addition, as astrocytes are unable to produce action potential and thus are not considered as excitable cells, they were thought not to be able to convey any signals inside the brain. It is only recently that our vision of astrocytes changed. Recent studies mostly performed by Volterra and colleagues (Bezzi et al., 2001; Bezzi and Volterra, 2001; Volterra and Meldolesi, 2005) revealed presence of glutamate receptors but also presence of secretion machinery in the astrocytes, showing presence of vesicles comparable to the ones found in neurons. These studies suggest that astrocytes may respond to synaptic activity, act as signal transducer and release glial transmitters or “gliotransmitters” towards neurons and other glial cells and ultimately regulate activity of both. However, such hypothesis remains mostly speculation and therefore more investigation need to be performed to understand the relevance of gliotransmitters in astrocyte-neuron interactions.

Interactions between astrocytes and ECs constitute the most studied cellular interactions of the neurovascular unit. The different microscopical studies performed by Brightman and Reese (Brightman and Reese, 1969) and Kacem and colleagues (Kacem et al., 1998) provided us important information concerning these important interactions. Certain astrocyte populations, classically referred in the literature as the perivascular astrocytes, invest the cerebral microvasculature by establishment of astrocytic “end feet processes” (Fig.10). The onset of astrocyte-endothelial interactions during development differs between human and rodents. Whereas astrocyte-endothelial interactions already occur in human brain vasculature during the 12^{th}-18^{th} gestation week (Bertossi et al., 1999; Virgintino et al., 1998), they appear in

brain rodents during post-natal stages as suggested by Zerlin and Goodmann (Zerlin and Goldman, 1997), when gliogenesis occurs.

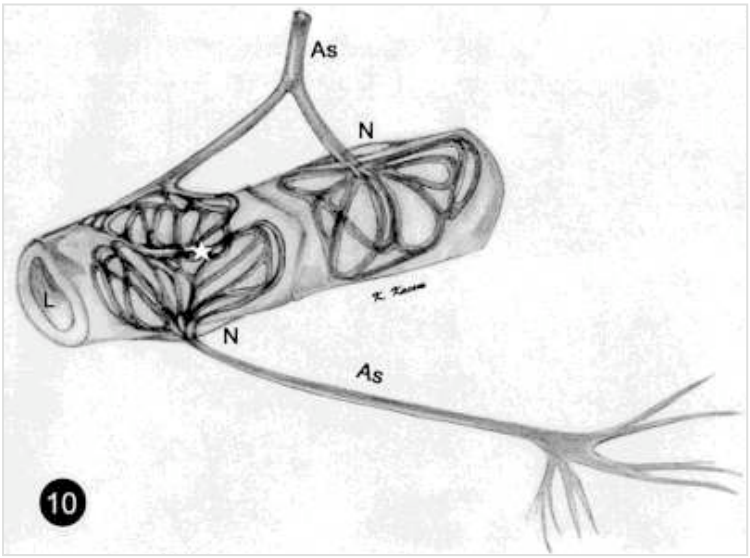

Figure 10: Schematic representation of astrocytic end-feet process
(From (Kacem et al., 1998))

The authors described that astrocyte-endothelial interactions were initiated between blood vessels and immature progenitor cells originated from the sub-ventricular zone (SVZ) during the first post-natal day. Under the influence of the microenvironment nearby the blood vessels, but also by the influence of paracrine factors secreted by ECs, the migrating SVZ progenitors cells that contacted the blood vessels begin their differentiation into perivascular astrocytes. In parallel, these differentiating astrocytes increase their interactions with the blood vessels, by increasing the number of end-feet processes formed but also by increasing the average end-feet surface overlap over the vasculature.

Astrocytic end-feet processes invest the vascular structure by direct apposition on the BM, forming a dense and complex rosette-like structure (Fig.10) according to the description performed by Kacem and colleagues (Kacem et al., 1998). One end-feet process may contact different EC locally by the rosette-like structure but also contacts

distal ECs by formation of different end-feet processes. On the other hand, different end-feet processes can contact a single EC. Notably, it is important to remember that astrocytes end-feet do not directly anchor to ECs, the BM that delimit the vasculature impeaching direct physical interactions, suggesting that endothelial-astrocyte cross-talk occurs mostly by secretion of paracrine factors. Astrocyte end-feet interactions with the BM form a highly organized structure (Amiry-Moghaddam and Ottersen, 2003; Wolburg et al., 2008), sharing many morphological similarities with the neuromuscular junction (Fig 11).

As the neuromuscular junction, astrocyte end-feet process interacts with the basal lamina by formation of a dystrophin/dystroglycan complex (DDC) that allows direct interactions between the astrocyte end-feet process with laminin and agrin included within the BM. Formation of such astrocyte junctions with the basal membrane reflects that astrocyte end-feet processes may form stable static structures over the vasculature and the maintenance of such structures might play important roles in astrocytes differentiation but also for the maturation and the maintenance of the BBB. In addition, these end-feet processes lead to formation of specialized micro domains on the astrocytes cell surface, restricting the localization of certain proteins at the end-feet processes. Aquaporin-4 (AQP4) constitutes one of these proteins restricted to the end-feet processes (Badaut et al., 2007). AQP4 is a water channel protein formed by six transmembrane domains with N- and C-terminal intracellular domains. Interestingly, AQP4 expression in astrocytes is restricted in the astrocytic synaptic and astrocytic end-feet processes (Badaut et al., 2000a; Badaut et al., 2000b; Nielsen et al., 1997). Like other AQPs, AQP4 is involved in regulation of water movements between the intracellular and extracellular compartments and therefore plays a crucial role in the maintenance of the brain tissue osmotic pressure. In addition to regulation

of the osmotic pressure in the extracellular compartment, AQP4 plays a role in cell adhesion between astrocytes and the other cell types of the neurovascular unit (Guadagno and Moukhles, 2004; Warth et al., 2004), however the role of AQP4 as a cell adhesion molecule on the endothelial barrier function remains unclear and requires further investigations.

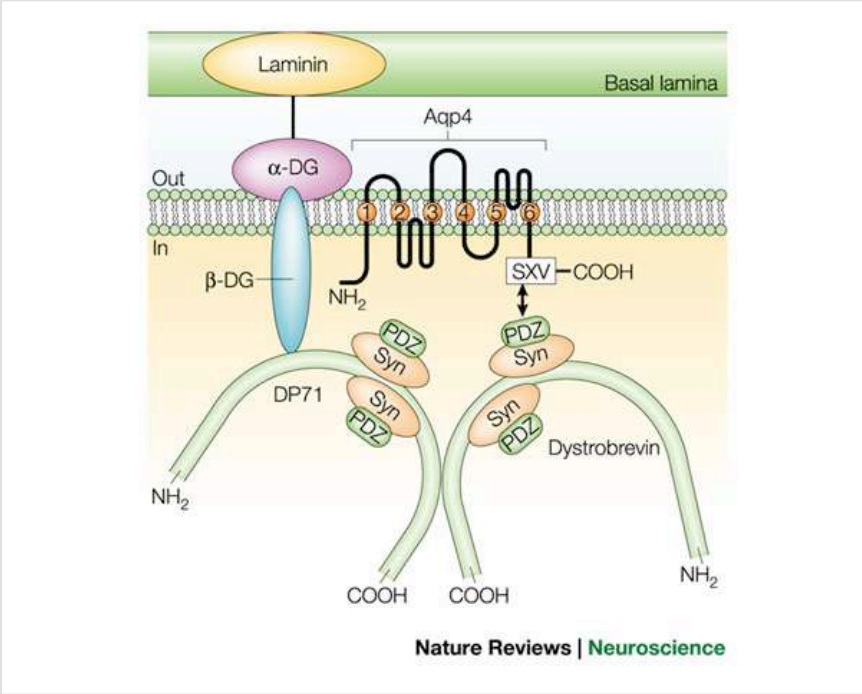

Figure 11: Schematic representation of the dystrophin/dystroglycan complex located in astrocyte end-feet process (From(Amiry-Moghaddam and Ottersen, 2003)

Interestingly, increased AQP4 expression levels occur in different neurological pathologies involving cerebral edema formation such as brain trauma (Sun et al., 2003a) or cerebral ischemia (Kaur et al., 2006; Ribeiro Mde et al., 2006). In addition, brain edema volume was lower in AQP4 KO-mice compared to wild type after an ischemic insult (Manley et al., 2000), suggesting that AQP4 induction during pathological state may overload water transport across the BBB, resulting in cerebral edema formation. However a study performed by Papadopoulos and colleagues (Papadopoulos et al., 2004) suggests that AQP4 rather has a protective role during cerebral edema by clearing water from the brain the blood compartment. Therefore

the contribution of AQP4 during edema formation remains debateful and requires further investigations.

Apart from the physical closeness between astrocyte end-feet process and the ECs, secretion and release of factors by both cells constitute the second remarkable feature of such interface. The existence of soluble factors released by astrocytes towards ECs in order to induce BBB phenotype has not been extensively studied yet. Most of the studies have been performed *in vitro* using astrocyte-EC co-cultures. However, the first study that demonstrate the induction of BBB phenotype by astrocytes was performed *in vivo* by Senjo and colleagues (Senjo et al., 1986). In this study, a correlation between the increase of glutathione-S-transferase activity, an astrocyte specific marker, and decreased permeability in rat embryos during embryonic and post-natal development was demonstrated. This study suggested that maturation of the barrier phenotype in the BBB was correlated with astrocyte differentiation. Later on, Janzer and Raff (Janzer and Raff, 1987) described a similar phenomenon using chick embryos. In this study, Janzer and Raff implanted rat primary astrocytes cultures over the allantoidal vasculature of chick embryos. They showed that presence of astrocytes significantly decreased allantoidal vascular permeability compared to the control group. Again, this study suggested that astrocytes might be necessary to induce barrier phenotype in EC. However, these *in vivo* studies were performed in immature organisms and therefore the induction of barrier function might be due to other differentiating cells. Therefore *in vivo* models based on differentiating embryos might not constitute adequate models to address the specific roles of astrocytes in barrier phenotype induction.

Generally, *in vitro* models constitute the major model of choice for deciphering the roles of astrocytes in the induction of barrier function. Arthur and colleagues

performed the first *in vitro* study (Arthur et al., 1987) based on astrocyte-endothelial co-cultures. In this study, the investigators showed that astrocyte-conditioned medium (CM) was able to restore ZO-1 localization in prolonged primary brain EC cultures that had lost adequate ZO-1 localization, suggesting that astrocytes constitutively release a soluble factor that induces barrier properties in brain EC. Further *in vitro* experiments performed by Tao-Cheng and colleagues (Tao-Cheng et al., 1987) showed that astrocytes-EC mixed co-cultures or astrocytes CM induced TJ complexes *in vitro*, showing a significant increase in the TJ length and width but also a decrease in gap junctions. Later on, Rauh and colleagues (Rauh et al., 1992) showed similar effects of direct astrocyte co-cultures on the restoration of BBB phenotype in primary brain EC. However, these studies need to be criticized as they established their statement on changes in structures but did not provide any evidence of changes in the barrier function. Therefore, the study performed by Dehouck and colleagues (Dehouck et al., 1990) constitutes the seminal work that defined actual *in vitro* BBB models. More details about such *in vitro* models will be discussed in a later section (see section 4.2.2.)

Different molecules have shown to up-regulate barrier function in brain EC. Growths factors such as basic fibroblast growth factor (bFGF/FGF-2) (el Hafny et al., 1996) or glial cell-derived neurotrophic factor (GDNF) (Igarashi et al., 1999) have been shown to induce barrier function in brain ECs. Garcia and colleagues (Garcia et al., 2004) recently showed that astrocyte contacts were necessary to significantly increase TEER in co-cultures and such contacts were required to induce transforming growth factor-β (TGF-β) production, providing direct evidence of an inducible factor released by astrocytes towards ECs. Another important factor that has been associated with increased barrier function is Ang-1. Ang-1 release in astrocytes is under control of

src-suppressed C-kinase substrate (SSeCKS), a protein kinase C substrate (Lee et al., 2003). Although the description of a signaling cascade that activates SSeCKS and ultimately results in release of Ang-1 is still missing, recent evidence of Ang-1 up-regulation at mRNA and protein levels by dexamethasone has been described in astrocytes (Kim et al., 2008), suggesting that glucocorticoids activate a signaling pathway resulting in astrocytes Ang-1 secretion.

This section provided a detailed description of very limited astrocyte-derived secreted factors and should not exclude the probability that a number of other factors involved in barrier induction may be involved. Although the identification of such factors is constantly requested by the scientific community (Haseloff et al., 2005), it appears clearly that current *in vitro* models cannot answer such request yet. Therefore the design of more sophisticated *in vitro* co-culture models is definitively required in order to achieve an identification of these unknown astrocyte-derived factors.

4.1.7. Pericyte-EC interactions in the BBB

Pericytes constitute the third type of cell involved in the BBB. They were firstly discovered by Rouget in 1873 (Rouget, 1873), followed by a detailed description by Zimmermann (Zimmermann, 1923) and attributed the name of "pericytes". From the different cell types constituting the BBB, pericytes represent the most mysterious and therefore the most fascinating one. Pericytes share many similarities with vascular smooth muscle cells (VSMC) and thus they are classically assumed to share similar embryonic cell lineage. Pericytes are present in all capillaries, including fenestrated and non-fenestrated ones. Like the other cell types that form the neurovascular unit, pericytes share the same BM than brain ECs. However the BM layer between ECs and pericytes is fenestrated, allowing ECs to invest pericytes with invagination

processes usually referred in the literature as “peg-socket interactions” (Rucker et al., 2000). These interactions are constituted by AJ, TJ and gap junction complexes (Cuevas et al., 1984; Gerhardt and Betsholtz, 2003; Gerhardt et al., 1999). Such closeness between pericytes and EC may underlie an important cross talk between the two cell types. Pericytes are found around arterioles, venules and in capillaries, with an endothelial-pericyte ratio varying considerably between vascular beds. Pericytes originate from the mesoderm (Hungerford and Little, 1999), however CNS pericytes derive from the neurectoderm (Etchevers et al., 2002) and suggests that CNS pericytes may have additional functions than other pericytes. Interestingly, CNS vasculature displayed the highest pericyte-EC ratio, showing values of 1:1-1:3 and 1:1-1:5 in the retina and the brain respectively, whereas their presence is limited to a ratio of 1:100 in skeletal muscle (Frank et al., 1987; Shepro and Morel, 1993). Such variation in pericytes numbers would suggest their presence is elevated in barrier vasculature and thus suggests that pericytes may modulate EC barrier properties. Like astrocytes, pericytes were considered for decades just as “mural cells” and thus playing a static role, but recent studies have highlighted the role of pericytes in physiological (e.g. vascular development) and pathophysiological (i.e. tumor-associated angiogenesis, diabetic retinopathy) situations (Hammes et al., 2004; Hellstrom et al., 2001; Lindahl et al., 1997; Sundberg et al., 2002). The contribution of CNS pericytes during pathological states is becoming more and more accepted, especially for pericytes of the inner blood-retinal barrier (iBRB) during diabetic retinopathy in which a massive loss of pericytes is classically described during the onset of the disease (Gerhardt and Betsholtz, 2003; Hammes et al., 2004).

This section highlights the phenotype diversity within pericytes and how such heterogeneity constitutes an important challenge for endothelial-pericyte interaction

studies. Firstly, pericytes display a heterogeneous cellular morphology according to the culturing conditions (Balabanov et al., 1996; D'Amore, 1990). For instance, on uncoated dishes, pericytes appear as large cells with a stellar shape whereas they adopt a round morphology on collagen-coated dishes. Secondly, pericytes originate from different vascular beds and display important differences in cell proliferation. Wong and colleagues (Wong et al., 1992) have observed that brain pericyte growth was significantly higher than retinal pericytes in medium with normal or high glucose levels. It is therefore important to establish standardized protocols in order to allow direct comparison between the different studies involving pericytes cultures.

Unlike the other cells of the BBB, characterization of pericytes by immunological markers constitutes an important challenge and still remains debated, as no specific marker has been described so far. Balabanov and Dore-Duffy (Balabanov and Dore-Duffy, 1998) investigated expression of different markers in brain pericytes compared to the other cell types present in the BBB (Table 1). It is interesting to note that pericytes positively stained against an array of different markers, thus overlapping with other cell types such as perivascular, microglial or smooth muscle cells (SMC).

In addition to such diverse expression of immunological markers, expression of these markers varies between pericytes of different vascular beds, but also within the same vasculature. (Armulik et al., 2005). Thus the definition of a pericyte-specific marker is still unclear and molecular markers such as α-smooth muscle actin (α-SMA) or heparan-sulfate proteoglycan (NG2) constitute until now the conventional markers for pericytes identification.

Cerebral pericyte-endothelial interactions take place already between the 17^{th} and 22^{nd} gestation week in humans (Braun et al., 2007; Virgintino et al., 2007). However such interactions may already occur earlier during development but the absence of

homogenous markers constitutes a limitation for the determination of interactions during earlier stages. It is interesting to note that pericyte coverage in the brain vasculature is unequal, according to Braun and colleagues (Braun et al., 2007).

Marker/cell	α-SMA	NG2	Desmin	Vimentin	VIII	GSA	CD11b	ED-2	CD45	MHC Class II	GFAP
PC	+	+	-	+	-	-	+	+/-	-	-	-
SMC	+	-	+	-	-	-	-	-	-	-	-
EC	-	-	-	+	+	+	-	-	-	-	-
PvM	-	-	-	+	-	+	+	+	+	+	-
AC	-	-	-	-	-	-	-	-	-	-	+

a-SMA = a-smooth muscle actin; GSA = Griffonia Simplicifolia Agglutinin; GFAP = Glial Fibrillary Acidic Protein; PC = Pericytes, SMC = Smooth Muscle Cell; EC = Endothelial Cell, PvM = Perivascular Macrophage; AC = Astrocytes.

Table 1: CNS pericytes markers (Adapted from (Balabanov and Dore-Duffy, 1998)

Pericyte coverage of the germinal matrix vasculature appeared significantly lower than the cortical and the white matter vessels, persisting even after the 25th gestation week, Presence of greater vascular density in the germinal matrix than the cortex or the white matter suggests that pericyte-endothelial interactions may be important for regulation of cerebral vascular density (Ballabh et al., 2004). In addition, pericyte-endothelial interactions are crucial for the stabilization of the cerebral vascular network (Abramsson et al., 2007; Armulik et al., 2005; Gerhardt and Betsholtz, 2003). It has been demonstrated that impairment of pericytes recruitment in the cerebral vasculature resulted in formation of rupturing microaneurysms (Lindahl et al., 1997). In addition to a role purely "vascular", different studies (Dohgu et al., 2005; Hayashi et al., 2004; Nakagawa et al., 2007) highlighted the roles of pericytes as possible inducer of barrier function in brain EC. However, as described in the astrocyte section, the nature of the different factors involved remains largely unclear.

Notably, pericyte-endothelial interactions rely on multifaceted communications, involving gap junctions, cell adhesion and secretion of soluble factors. Two secreted factors shown to induce barrier phenotype in brain EC. Interestingly these factors are

also found in astrocyte-endothelial interactions: Ang-1 and TGF-β. Ang-1 constitutes the first factor shown to induce barrier properties (Thurston et al., 1999), both in the iBRB (Park et al., 2003b) and the BBB (Hori et al., 2004). The signaling pathways that modulate Ang-1 expression in pericytes are not yet fully known. However, Kim and colleagues reported that dexamethasone up-regulated Ang-1 in dose-dependant manner in brain pericytes (Kim et al., 2008) whereas Park and colleagues described hypoxia and VEGF as inducer of Ang-1 secretion in retinal pericytes (Park et al., 2003b). Presence of angiopoietin receptor Tie2 on both EC and pericytes may suggest that Ang-1 acts both as a paracrine and autocrine factor. However, the possible regulation of Ang-1 expression by such autocrine loop remains obscure.

TGF-β constitutes a second important factor involved in barrier induction. Dohgu and colleagues (Dohgu et al., 2005) observed that close contacts between pericytes and brain ECs were necessary to provide maximal induction of the barrier function, suggesting that physical contacts are crucial for pericytes to induce their effects. Inhibition of TGF-β signaling by blockade of TGF-β receptor binding or receptor activity resulted in significantly increased permeability and decreased P-gp activity, demonstrating that TGF-β/TGF-β receptor signaling pathway is essential for pericyte-induced BBB phenotype in ECs. Presence of both Ang-1 and TGF-β signaling in astrocytes and pericytes suggests that these two cells might use similar mechanisms to induce barrier phenotype, but also raises the question of possible additional factors specific to each cell type and may participate in a possible cumulative effect when both astrocytes and pericytes are present.

4.1.8.Neurons and their possible contribution to the BBB

The BBB plays a crucial role to neurons by providing them with efficient O_2 and nutrient perfusion but also by isolating them from any fluctuation occurring outside the brain. However, we can question whether neurons themselves can modulate O_2 and nutrient supply as well as the BBB phenotype. The ability of neurons to regulate local blood flow to ensure optimal O_2 and nutrient supply is documented, either for the retinal (Pournaras et al., 2008) or the cerebral vasculature (Filosa and Blanco, 2007; Filosa et al., 2004; Filosa et al., 2006; Iadecola and Nedergaard, 2007). Regulation of cerebral and retinal local blood flow occurs through neurovascular coupling (Fig.13). Neurovascular coupling can be effected directly or indirectly. In the direct neurovascular coupling, neurons directly modulate local blood flow by modulating the vascular tone through the release of nitric oxide (NO) and prostaglandins (PGs). In the indirect neurovascular coupling, release of both NO and PGs are performed by astrocytes. The release of these vasoactive factors is directly modulated by the local neuronal activity. Glutamate, an important neurotransmitter, and other signaling molecules (i.e. ATP, PGs) released in the synaptic cleft by active neurons can initiate astrocyte phospholipase C/inositol-3-phosphate (PLC/IP3) signaling pathway. IP3 induces intracellular Ca^{2+} increase resulting in synthesis of epoxyeicosatrienoic acid, NO and PGs by cytochrome P450, NO synthase and cyclooxygenase respectively by activation of the phospholipase A2 pathway. Release of these compounds by astrocytes induces vasodilation by pericytes/VSMC relaxation (Filosa and Blanco, 2007; Filosa et al., 2004; Filosa et al., 2006). With such mechanisms, neurons can very efficiently adapt local blood flow according to their needs. The question of possible neuronal modulation of the BBB phenotype is more disputable. Stewart and Wiley (Stewart and Wiley, 1981) performed the first *in vivo*

experiment that raised the possible role of neurons in induction of BBB phenotype in EC. In this study, they used quail and chick embryos to prove the ability of neural tissue to induce a barrier phenotype. They grafted quail neural tissue into the chick coelomic cavity and allowed vascularization of the graft by the chick blood vessels to occur.

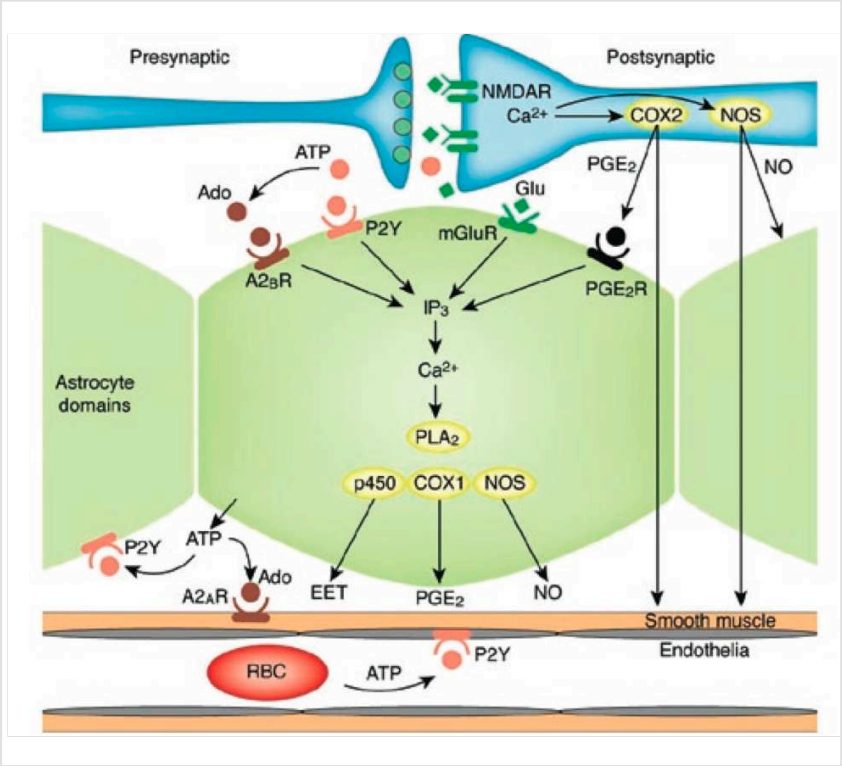

Figure 12: Cellular and molecular basis of the neurovascular coupling in the CNS vasculature (from Iadecola and Nedergaard, 2007)

They observed that blood vessels that invested the graft developed TJ complexes, suggesting that neural tissue induced BBB properties in the invading EC. However, this study was performed on a developing brain with immature neurons and glial cells and thus does not prove that neurons alone were responsible since the induction might be due to astrocyte presence.

In order to verify that neuron-derived factors induce barrier phenotype in EC, it is important to use a simplified model that allows us to test such hypothesis. Schiera and colleagues (Schiera et al., 2003; Schiera et al., 2005) have extensively addressed such

questions by using a three-cell *in vitro* BBB model, based on the *in vitro* BBB model developed by Dehouck and colleagues (Dehouck et al., 1990). It is important to note that these experiments were performed on non-contacting primary neurons cultures. However the results obtained with such models are debateful and rather suggest that neurons may only have a modest contribution to barrier induction in EC. For instance, the authors showed an increased occludin localization in EC-neuron co-cultures, whereas no change in occludin was observed at protein levels. In addition, permeability to sucrose decreased modestly compared to EC monocultures with no cumulative effects when cells were co-cultured with astrocytes. Weidenfeller and colleagues (Weidenfeller et al., 2007) took a different approach to answer this question and hypothesized that only immature neurons may induce BBB phenotype. In their study, they used an *in vitro* BBB model consisting of neural progenitor cells (NPCs) co-cultured with EC in a non-contact model. The authors showed that NPCs were able to induce BBB phenotype in EC, as shown by increased TEER, decreased permeability to water-soluble tracers and by increased formation of TJ complexes. However, a further analysis of NPC cell population obtained after 24h of co-cultures with EC revealed that NPC population initiate a mixed population of neurons and astrocytes. Again, we cannot ascribe to neurons the possible effects on the BBB phenotype. So, should we consider that neurons have only limited effect on the EC biology?

The answer is certainly negative. Neurons plays important roles in other functions associated to EC. Indeed, neurons and EC share the same signaling pathways for both vessel and axon guidance. Furthermore, although neurons and EC have completely different embryonic origins, they share much anatomical similarities. For instance, the tip cell (the EC present in front of forming vessel) acts very similar to axon growth

cones. Like axon growth cones, the tip cell filipodia probe the extracellular environment and dynamically respond to positive and negative cues resulting in proper orientation inside the tissue.

EC and neurons share four major guidance signaling pathways: Ephrins/Eph (Helbling et al., 2000), semaphorins/plexins (Torres-Vazquez et al., 2004), Netrin/Unc5b (Park et al., 2004) and Slit/Robo (Park et al., 2003a) signaling pathways. Neurons can guide tip cells and established vasculature can guide migrating axons to reach the target tissue (Autiero et al., 2005) showing that both systems are interconnected and require the help of the other to achieve guidance to the target tissue. In conclusion, neurons might not play an important role in BBB compared to astrocytes and pericytes but definitively play crucial roles in vascular patterning and vessel guidance.

4.1.9. The basement membrane: the matrix reloaded

The BM does not simply constitute a scaffold structure, but definitively represents the foundation of the whole neurovascular unit. Four different molecules (Tilling et al., 2002; Tilling et al., 1998) compose it: collagen IV, laminin, fibronectin and a secreted protein acidic rich in cystein (SPARC). Tilling and colleagues (Tilling et al., 2002; Tilling et al., 1998) performed a series of studies to determine which BM components play roles in the barrier function. In the first study they compared the possible TEER increase when EC were grown on collagen IV-, laminin-, fibronectin- or SPARC-coated inserts. The authors observed that from the four components tested, laminin showed the highest increase in TEER either alone or in combination with fibronectin, suggesting that laminin may constitute an important component of the BM for induction of barrier phenotype. As astrocytes and pericytes have direct contacts with

the BM, their capacity to induce BBB phenotype might involve ECM secretion and thus provide modification of EC BM composition. In a second study, Tilling and colleagues demonstrated that astrocytes and pericytes secrete some ECM and both secreted matrices were able to increase TEER in brain ECs. However this study did not provide any information on the composition of such matrices. Thus the nature of the ECM secreted by astrocytes and pericytes is still not defined, composition of such matrices will definitively help us to understand more the inductive effects carried on by astrocytes and pericytes.

In addition to the ability to induce barrier properties in brain EC, the BM may also act as a barrier under certain circumstances. Willis and colleagues have observed (Willis et al., 2004) that loss of astrocyte contacts induced disruption of the BBB towards small molecules (500Da), but it remained impermeable to mid-size molecules (10kDa) suggesting the presence of another barrier, that gives a higher size-selectivity than brain EC. In order to test whether the barrier was the BM, Willis and colleagues (Willis et al., 2007) investigated the barrier properties of the BM located in the area postrema, a region of the brain belonging to the circumventricular organs (CVO), a brain area that physiologically show an absence of barrier phenotype in EC. This region of the brain is unique as EC that form the vasculature show a total absence of BBB phenotype, but also an absence of astrocyte end-feet processes. Ependymal cells that line the cerebral ventricles play the role of barrier in this region. Willis and colleagues showed that the BM can acts as a size-selective filter in cerebral vascular regions in absence of the BBB and suggest that such filter may constitutes an additional line of defense in cerebral vasculature displaying a BBB phenotype.

4.1.10. The perivascular cells and the microglia: the immune cells of the BBB

Perivascular cells and microglia constitute the two last cell types present in the BBB (Guillemin and Brew, 2004). Perivascular cells are present in the perivascular space located between pericytes and the BM, whereas microglia share same location with astrocytes or neurons (see Fig.4). Perivascular cells may derive from both monocytes and pericytes (Balabanov et al., 1996; Thomas, 1999) whereas microglial cells are derived mostly from migrated monocytes. Unlike astrocytes and pericytes, perivascular and microglial cells appear not to have a role on EC barrier phenotype during physiological condition. However, these two cells may play crucial roles during pathological states. Activation of perivascular and microglial cells induces release of important pro-inflammatory cytokines, including interleukin-1β (Stoll, 2002), monocytes chemoattractant protein-1 (Guillemin et al., 2003) or tumor-necrosis factor-α (Poritz et al., 2004), responsible for the BBB breakdown during neuroinflammatory diseases as well as other neurological diseases. Both activated cells facilitate leukocyte migration through the BBB by TJ breakdown and EC activation. Therefore we need to have a better understanding of the cellular cross talk between these two cells and ECs to develop new anti-inflammatory drugs that specifically inhibit activation of these cells.

4.2. Models of the blood-brain barrier

4.2.1. *In vivo* models

In vivo models of the BBB constitute the model of choice in order to understand human BBB physiology and pathophysiology. Until now, most of the models are based on mammalian systems that allow a direct comparison with humans. Mouse, rat and in lesser extent cat and dog constitute the most common species used as *in vivo*

models to reproduce human neurological diseases involving barrier breakdown such as cerebral ischemia (mouse middle cerebral artery occlusion model) or multiple sclerosis (mouse EAE model) (Webb and Muir, 2000),. In addition to usage of these models for studying BBB alteration during adulthood, it is important to note the usage of mouse, dog, marsupial or sheep models in the literature constitute as *in vivo* the BBB study models during development (Ek et al., 2006; Ment et al., 1992; Ment et al., 1986a; Ment et al., 1986b; Saunders et al., 2000; Stonestreet et al., 1996; Stonestreet et al., 2006). However, usage of mammalian models as in vivo models of the BBB constitute an important technical approach as it allows only to observe macroscopic changes of the barrier function but also need to face ethical constrains, thus limit the usage of these models routinely. Recently, non-mammalian BBB *in vivo* models, based on zebrafish and drosophila, have been suggested as an alternative to mammalian models (Daneman and Barres, 2005; Jeong et al., 2008). Apart that such animals constitute excellent genetic models to discover the nature of the genes underlying the barrier function in brain endothelium, it is important to note that such models still lack a complete characterization of the BBB and requires further investigations to assess the relevance of such non-mammalian models.

4.2.2. *In vitro* models

In vitro models constitute, by their accessibility and reproducibility, useful alternative to *in vivo* models for the study of cellular and molecular mechanisms at the BBB. Most of *in vitro* models rely on usage of mammalian brain ECs isolated from either primary cultures or immortalized brain EC lines (Deli et al., 2005; Roux and Couraud, 2005). Primary cultures are classically isolated from bovine, porcine or murine brain microvessels. Despites brain EC isolation techniques are standardized and highly

reproducible, the yield and cell contamination (e.g. pericytes) constitute major issues for broaden usage of primary cultures routinely.

For this purpose many different groups established immortalized brain EC lines. Among the different cell lines established, it is important to mention that only a fraction of such cell lines are extensively used in the literature. The cell lines classically used in the literature are the mouse (b.End3, b.End5), rat (RBE4, GPNT) and human (ECV304, HBMEC) brain EC lines (Al Ahmad et al., 2009; Brown and Davis, 2005; Neuhaus et al., 2008; Rist et al., 1997; Roux and Couraud, 2005; Roux et al., 1994; Weksler et al., 2005).

Most *in vitro* BBB models rely on the usage of a 2-dimensional cell culture model similar to the model initially described (Dehouck et al., 1990; Rubin et al., 1991). It consists on the usage of a modified Boyden chamber (Fig.13) that separates two compartments by a presence of a semi-porous membrane. Such membrane allows solute diffusion between the two compartments. ECs are seeded on the top of the membrane, thus allows the formation of a monolayer and the induction of cell polarity.

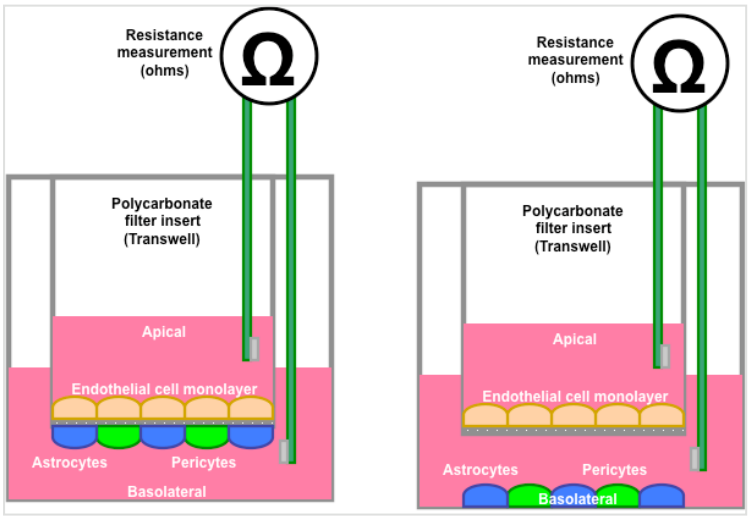

Figure 13: Schematic representation of in vitro BBB contact (left) and non-contact (right) co-culture models

Formation of a barrier phenotype can be easily quantified by determining the transendothelial electrical resistance (TEER) across the monolayer but also by measuring the diffusion of water-soluble labeled compounds (e.g. sucrose, inulin, dextran) across the monolayer. However, these models based on EC monocultures rapidly demonstrated its limitation due to an important discrepancy between TEER values obtained *in vitro* (10-100 $\Omega.cm^2$) compared to *in vivo* (1000 $\Omega.cm^2$) situation due to a rapid loss of barrier phenotype in cultured brain ECs (Butt et al., 1990; Rubin et al., 1991). Dehouck and colleagues (Dehouck et al., 1990) proposed an enhanced model by developing a co-culture model by addition of astrocytes of the opposite face of the membrane and thus recreate the astrocyte-endothelial interactions as they naturally occur *in vivo*. In most studies found in the literature, addition of astrocytes were able to significantly induce higher TEER values than EC monocultures (Deli et al., 2005), however the degree of induction remains significantly lower than *in vivo* situation. Treatment of the cultures by different drugs such as cyclic adenosine monophosphate (Rist et al., 1997), dexamethasone (Kim et al., 2008) or hydrocortisone (Hoheisel et al., 1998) have shown to artificially elevate TEER in mono- and co-cultures, however such empirical methods may alter EC response to various stimuli. More recently addition of pericytes showed the ability to increase of the barrier function (Hayashi et al., 2004; Lai and Kuo, 2005), presence of both astrocytes and pericytes appear to be necessary to obtain optimal induction of barrier phenotype in brain ECs (Al Ahmad et al., 2009; Nakagawa et al., 2007). In addition to the presence of the different cell types, some authors suggest that enhancement of current *in vitro* models require the addition of a flow-induced shear stress over the EC monolayer (Cucullo et al., 2008; Cucullo et al., 2002). In conclusion, it appears clearly that an oversimplification of the BBB *in vitro* cannot reproduce efficiently in

vivo phenomenon and demonstrates that the different components found in the neurovascular unit (e.g. astrocytes, pericytes, basement membrane, flow) are indeed necessary in order to obtain satisfactory *in vitro* BBB models.

4.2.3. Designing BBB models: an important challenge

The BBB constitutes probably one of the most difficult vascular bed to model, as it forms a cellular and molecular complex involving different cell types. In addition, the BBB distribution within the cerebral vasculature is heterogeneous but also difficult to access physically as it requires invasive techniques. On the other hand, current *in vitro* models importantly reduced the *in vivo* situation by omitting many parameters including the presence of both astrocytes and pericytes or appropriate cellular interactions between ECs and the other cells of the neurovascular unit. Such extreme simplification constitutes an important pitfall, as obtained *in vitro* results are poorly transferable to *in vivo* situation. Therefore it is important to design elaborated *in vitro* models to allow a better reproduction of *in vivo* situation.

In conclusion, design of *in vitro* models that allow both the ease of actual *in vitro* models and the complexity of *in vivo* situation are urgently required to provide a significant quantum leap within the field.

4.3. Hypoxia

4.3.1. Hypoxia inducible factor-1: an overview

Hypoxia is defined as a deficit of O_2 availability for a tissue compared to O_2 need. Hypoxia occurs in many physiological states such as embryonic development or high-altitude exposure but also during pathological conditions such as ischemia or cancer. Physiological adaptation to hypoxia involves different steps in order to increase O_2 availability towards the hypoxic tissue. At the cellular level, adaptation to hypoxia

involves hypoxia inducible factor (HIF) (Hopfl et al., 2003; Hopfl et al., 2004; Wang et al., 1995). HIF-1 transcription factor constitutes the best known transcription factor described in the mammalian response to hypoxia and therefore this chapter will limit the discussion to the HIF-1 pathway. HIF-1 is a heterodimer formed by HIF-1α/HIF-1β subunits. Whereas HIF-1α protein degradation rapidly occurs under normal O_2 concentration (normoxia), HIF-1β protein levels remain constant. HIF-1α and HIF-1β (Fig.14) N-terminal domains present a basic helix-loop-helix (bHLH) sequence involved in DNA binding activity of the heterodimer complex and a Per-Arnt-Sim (PAS) domain responsible for the interactions between the α and β subunits. On the C-terminus, HIF-1α presents additional domains absent in HIF-1β. HIF-1α presents an O_2-dependant degradation domain (ODD), responsible for its degradation under normoxic conditions. HIF-1α also has a N-terminal and C-terminal transactivation domains (N-TAD and C-TAD respectively) involved in the recruitment and interactions with co-activators necessary for the transcriptional activity of the HIF-1 complex.

HIF-1α degradation and hypoxic stabilization mechanisms may represent one of the most interesting molecular mechanisms involved in transcription factor regulation. As we mentioned previously, HIF-1α is constantly produced and degraded under normoxia. HIF-1α degradation occurs by different molecular steps (Fig.15). The first step is the prolyl-hydroxylation step (Ivan et al., 2001; Jaakkola et al., 2001). Prolyl-hydroxylation involves modification of two proline residues (Pro^{402} and Pro^{564}) present in the HIF-1α ODD domain. Such biochemical step is processed by three different prolyl-4-hydroxylase (PHD) isoforms known as PHD1, PHD2 and PHD3 respectively (Bruick and McKnight, 2001; Huang et al., 2002). Whereas PHD1 expression appears to be hypoxia independent, PHD2 and PHD3 are upregulated by

hypoxia, both at the mRNA and protein levels, suggesting that these two PHDs are involved in a negative feedback loop for regulating HIF-1α protein level even during hypoxia (Metzen et al., 2003; Stiehl et al., 2006).

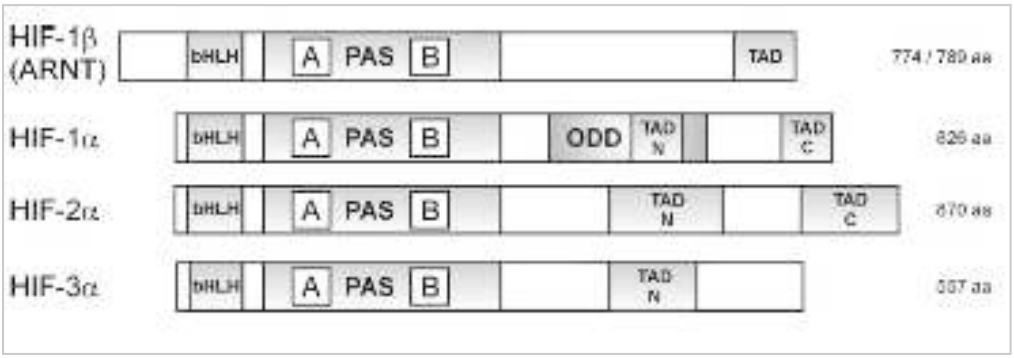

Figure 14: HIF-1α, -2α , -3α and HIF-1β structures
(From (Hopfl et al., 2004))

Proline hydroxylation by the three different PHD isoforms requires presence of O_2 but also presence of co-substrates such as 2-oxoglutarate, ascorbate and Fe^{2+}. The Von Hippel-Lindau protein (pVHL)-E3 ubiquitin ligase complex recognizes prolylhydroxylated HIF-1α and induces ubiquitinylation of HIF-1α at different sites (Ivan et al., 2001; Jaakkola et al., 2001). Finally, the proteasome degrades the polyubiquitinylated HIF-1α (Krek, 2000) resulting in its complete inactivation. Presence of O_2 in the environment is crucial for PHDs activity; therefore the absence or reduction of O_2 environment results in a substantial decrease in their enzymatic activity (Stiehl et al., 2006). When O_2 concentration decreases in the environment, PHD activity is drastically reduced, resulting in HIF-1α stabilization. Once stabilized, HIF-1α can translocate to the nucleus and associate with HIF-1β, forming the HIF-1 heterodimer complex. The HIF-1 heterodimer complex recognizes a specific consensus sequence formed by six base pairs classically referred to "hypoxia responsive elements" (HRE) located in promoter or enhancer regions of HIF target genes. Interactions between HIF-1 and HREs allow recruitment of transcription

machinery and initiation of transcription of a number of important HIF target genes. HIF-1α stabilization/degradation cycle constitutes a remarkable mechanism for O_2 sensing. It allows a very rapid response to hypoxia resulting in a very fast nuclear translocation (<2 min), whereas reoxygenation very rapidly causes HIF-1α degradation (~5min) (Jewell et al., 2001). In addition to such transient switch in feedback loops, HIF-1α levels vary exponentially according to O_2 levels. Whereas HIF-1α protein level is undetectable under normoxic condition, stabilization and therefore its detection increase exponentially as O_2 levels decrease (Jiang et al., 1996). In conclusion, HIF-1 pathway constitutes an efficient O_2 sensing system in the mammalian organisms to allow a rapid adaptation to environmental changes in O_2 concentration.

4.3.2. HIF target genes and their role in EC response to hypoxia

As described in the previous chapter, the HIF-1 heterodimer acts a transcription factor and activates transcription of many hypoxia-regulated genes. More than 100 genes have been described as hypoxic-responsive (Semenza, 2002), the number of genes described as hypoxia-inducible genes is in constant increase. The gene products play different roles in the organisms by acting on glucose metabolism, O_2 transport and delivery or cell survival/cell death. This chapter will primarily discuss on hypoxia-induced genes in EC during hypoxic conditions and the next chapter will focus on the BBB response to hypoxic stress.

A variety of different genes are up regulated in EC to allow cellular adaption to hypoxia but the most striking and visible EC response is certainly the initiation of angiogenesis in hypoxic tissue, in order to re-establish appropriate O_2 levels to the tissue. Tumor growth and its subsequent neo-vascularization by newly formed blood

vessels constitute the study model of choice to understand the hypoxia-induced angiogenesis.

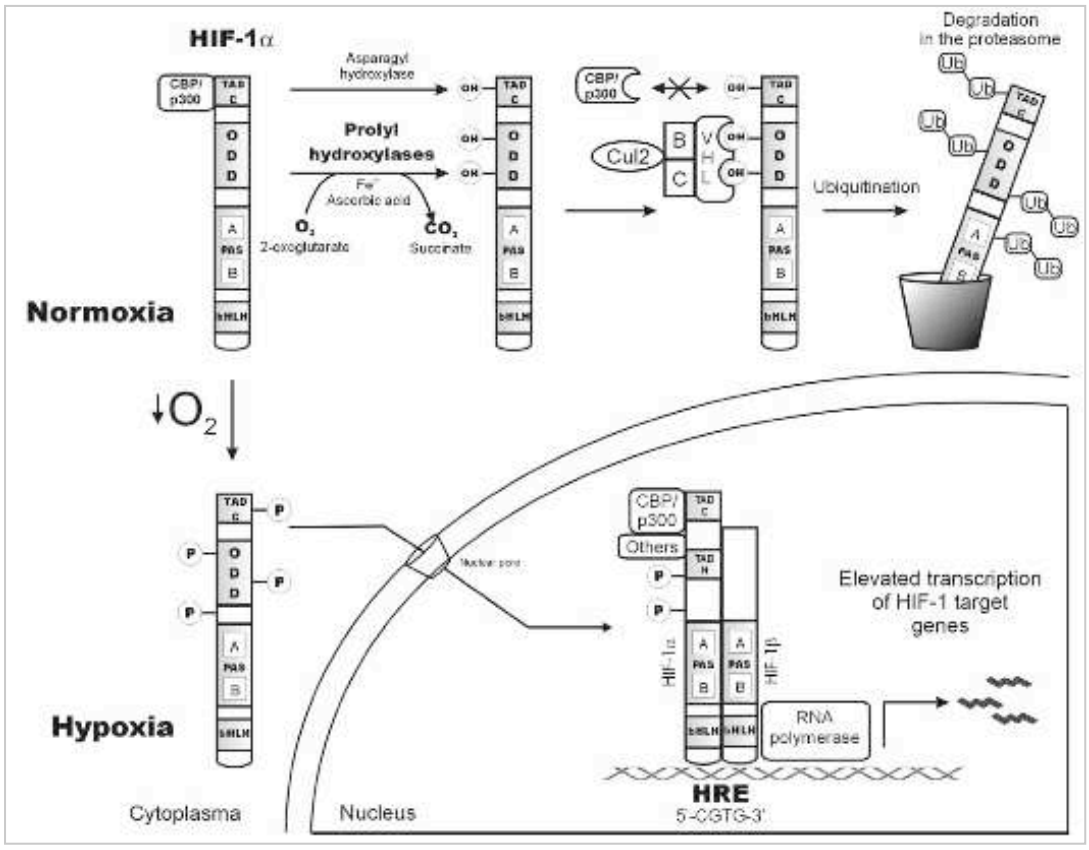

Figure 15: HIF-1α degradation and stabilization during normoxic and hypoxic conditions (From (Hopfl et al., 2004))

Tumor angiogenesis occurs by different steps (Fig.16): Hypoxic tumor cells secrete angiogenic factors reaching neighboring capillaries (i), initiating EC activation. Activated EC (aEC) induce expression of secreted and membrane-bound matrix-metalloproteinases (MMPs) that allow degradation of the basement membrane and the surrounding ECM (ii). EC present at the degradation site sprouts and migrates (iii) following the gradient formed by the angiogenic factor released by the tumor cells. Neighboring EC show an intense mitogenic activity to allow extension of the vasculature. In parallel newly formed vessels synthesize a newly formed basement

membrane (iv) that allows recruitment of mural cells and induce stabilization of the newly formed blood vessels (v).

Among the different genes induced by HIF-1α stabilization, an important amount of gene products are involved in angiogenesis including the following ones: angiopoietin-2 (Ang-2) (Pichiule et al., 2004), angiopoietin-receptor 2 (Tie2) (Willam et al., 2000), bFGF (Phillips et al., 1995), endostatin (Wu et al., 2001), platelet-derived growth factor-B (PDGF-B) (Yoshida et al., 2006), TGF-β (Sakuda et al., 1992), VEGF and VEGF receptor-1 (VEGFR-1/Flt-1) (Gerber et al., 1997; Iyer et al., 1998).

The case of Ang-2 is very interesting. Ang-2 is directly secreted by EC under hypoxia (Pichiule et al., 2004). In addition, Ang-2 signaling occurs by interactions with Tie-2 receptor and therefore shares the same receptor as Ang-1, another important factor involved in vessel stabilization. Whereas Ang-1/Tie-2 interactions result in Tie2 autophosphorylation, Ang-2 acts as a negative factor, inhibiting Tie-2 autophosphorylation and subsequent activation of the signaling cascade (Maisonpierre et al., 1997). Inhibition of Tie2 signaling cascade results in detachment of pericytes/VSMC and vasculature destabilization. Ang-2 plays an important role in tumor angiogenesis as it involved in the disruption of endothelial-matrix (Lauren et al., 1998).

As other fragments found in the BM such as endorepellin (Bix et al., 2006; Bix et al., 2004; Bix and Iozzo, 2005) or collagen IV fragments (Marneros and Olsen, 2001), endostatin plays an important role as anti-angiogenic factor and therefore its down-regulation by HIF-1 allows EC activation and angiogenesis. Endostatin constitutes one of the vasoactive molecules down-regulated by HIF-1 (Wu et al., 2001). Endostatin is a 20-kDa C-terminal proteolytic fragment obtained from the collagen

XVIII, a matrix component secreted by EC and pericytes and is present in almost all EC BM (Marneros and Olsen, 2005; Wu et al., 2001).

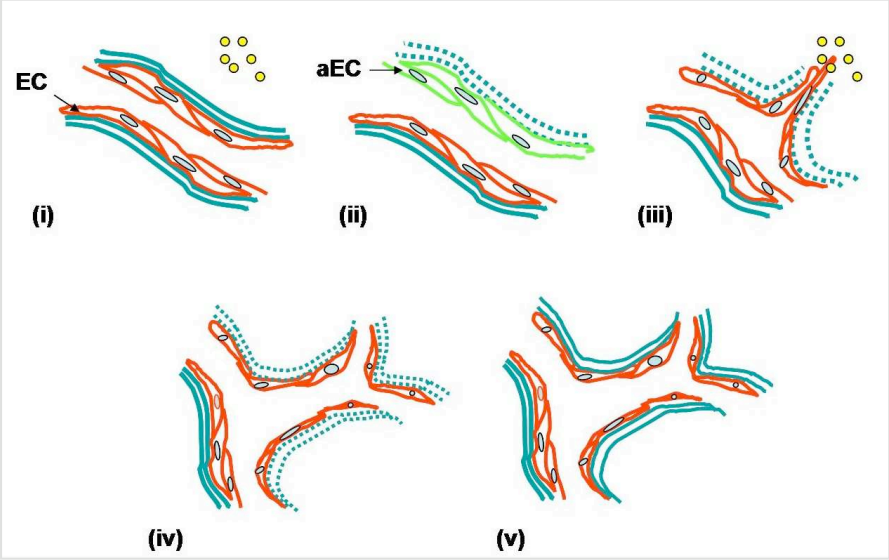

Figure 16: Hypoxia-induced angiogenesis in tumor
(From Yue et al., 2007)

PDGF-B belongs to the PDGF family (Fredriksson et al., 2004). The active form of PDGF-B consists of homodimer (PDGF-BB) or heterodimer (PDGF-AB) formation. PDGF-A and -B bind to PDGF (PDGFR)-α (PDGF-AB) or PDGF-β (PDGF-BB) dimerized receptor. Secretion of PDGF-BB by the tip cell allows pericytes recruitment to the developing vasculature. Such recruitment occurs by activation of PDGFR-β in migrating pericytes (Armulik et al., 2005). PDGF-BB/PDGFR-β is important for the stabilization of newly formed blood vessels during angiogenesis (Abramsson et al., 2007). Induction of PDGF-B expression by HIF-1 (Yoshida et al., 2006) during hypoxic exposure might therefore constitute an adaptation mechanism to induce stabilization during hypoxic-driven angiogenesis.

VEGF constitutes probably the best-described hypoxia-driven angiogenic factor. VEGF (VEGF-A) belongs to the VEGF family formed by six members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placental growth factor (PlGF) (Ferrara,

2004). Whereas VEGF-C and –D play important roles in lymphangiogenesis, VEGF-A plays crucial roles in vasculogenesis and angiogenesis. VEGF-A gene product can be spliced into four major splice variants that differs by the number of aa present in the sequence: $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$ (Rini and Small, 2005). Despite these splice variants displayed different heparin and ECM binding affinity, they showed similar biological effects (Rini and Small, 2005), Among all these splicing isoforms, $VEGF_{165}$ constitutes the most prominent splice variant by its bioavailability and potency (Rini and Small, 2005). Initially described as a tumor-secreted vascular permeable factor (Rosenthal et al., 1990; Shing et al., 1985), the demonstration of its induction by hypoxia through HIF-1 by Shima and colleagues (Shima et al., 1995) revealed that VEGF expression occurs in a numerous number of cells including astrocytes (Chow et al., 2001; Hossain et al., 2000; Salhia et al., 2000), CNS endothelium (Fischer et al., 1999; Josko et al., 2001; Nag et al., 2002; Rajah and Grammas, 2002), neurons (Famiglietti et al., 2003; Ment et al., 1997; Ogunshola et al., 2002; Ogunshola et al., 2000), retinal pigmented epithelium (Adamis et al., 1993) and CNS pericytes (Kim et al., 2008; Park et al., 2003b). It appears indeed that VEGF constitutes an important housekeeping gene, as one VEGF allele deletion is embryonic lethal showing abnormal vasculature development (Carmeliet et al., 1996; Ferrara et al., 1996). Indeed, VEGF plays a crucial role in vascular development inducing vasculogenesis and angiogenesis. In addition to its important on the blood vessels, VEGF plays important roles as a neuroprotective factor in many neurological diseases (Ogunshola et al., 2002; Plaschke et al., 2008; Sun et al., 2003b; Wang et al., 2007; Wang et al., 2005; Yasuhara et al., 2005), but also contributes to endothelial and astrocytes survival during hypoxic/ischemic stress (Chow et al., 2001; Schmid-Brunclik et al., 2008). However VEGF also acts as a hyperpermeable factor resulting

in barrier disruption in the CNS, the effect of VEGF on the BBB will be addressed in detail in the next chapter. In conclusion, HIF-1 pathway induces important number of genes responsible for cellular adaptation to hypoxic stress (metabolism, cell survival) but is also remarkably visible in the endothelium by increasing vascular density in hypoxic tissue thus restoring optimal O_2 delivery. However, this increased vascular density may not be only beneficial but may cause detrimental effects in EC functions.

4.3.3. The BBB and the hypoxic stress: the road to barrier disruption

The brain is an important blood flow and glucose consumer, with an average of 13% and 20% respectively under resting condition (Vander et al., 1995), showing its important dependency of an adequate O_2 and nutrient supply. According to Sick and colleagues (Sick et al., 1982), rat brain show a heterogeneous O_2 partial pressure ($_PO_2$) inside the brain area, showing values ranging from 1-2 mmHg to 50 mmHg and more, with an average around 10 mmHg. It shows that the brain constitutes one of the organs in mammals with the lowest physiological pO_2 levels compared to other tissues and suggests that the brain tissue is highly susceptible to any decrease in O_2 supply level, in particularly during hypoxic stress.

Adaptation of the CNS to hypoxia is therefore crucial to maintain the organism integrity. Such adaptation occurs with slight differences whether the CNS faces an acute or a chronic mild hypoxia. In the rat brain, different mechanisms provide adaptation to hypoxia (LaManna et al., 2004). During acute hypoxia, an increase in both cerebral blood volume and flow is observed, accompanied by an increase in glucose metabolic rate. Prolonged mild hypoxia involves increase in glycolysis and glucose transport by up-regulation of GLUT-1 expression at the BBB but also important structural changes in the cerebral vasculature including vascular remodeling

that allow restoration of the normal tissue oxygenation profile at long-term. Hypoxic stress occurs in the BBB during two major situations: when changes in O_2 concentration occur in the environment (Hackett and Roach, 2004) or during an ischemic situation when O_2 and glucose supply are both impaired (del Zoppo, 2006; del Zoppo and Mabuchi, 2003; Zlokovic, 2008), most of the time such stress result in BBB breakdown.

In vivo studies indicate us that hypoxia-driven BBB disruption occurs in rats exposed for 1-24 hours at 6-12% O_2 (Kaur et al., 2006; Schoch et al., 2002; Witt et al., 2003; Witt et al., 2008) or after 90-mins in the middle cerebral artery occlusion (MCAO) models (Kilic et al., 2006; Kumai et al., 2007; Yang et al., 2007b), whereas *in vitro models* showed barrier breakdown occurring between 2-48h at 0-3% O_2 levels (Abbruscato and Davis, 1999; Al Ahmad et al., 2009; Brown and Davis, 2005; Fischer et al., 2004; Fischer et al., 2000; Fischer et al., 2002; Hayashi et al., 2004; Kuhlmann et al., 2007; Schroeter et al., 1999; Yang et al., 2007a). Regarding such variations between the different experimental paradigms observed in the literature, it is obvious that direct comparison between the different studies cannot be totally performed and provide us with fragmented information on the onset of the barrier breakdown. However these studies have provided us with important information concerning different mechanisms occurring in the BBB during such insult that may lead to barrier disruption.

Barrier disruption involves dramatic changes at different levels: cytoskeleton, cell-cell and cell-matrix interactions but also cell-cell communications. At the cytoskeleton level, hypoxia induces F-actin stress fiber formation (Brown and Davis, 2005) and myosin light chain kinase activation (Kuhlmann et al., 2007), and results in formation of actin/myosin contractile machinery. EC contraction during injury may therefore

contribute to barrier breakdown. In addition to changes in cytoskeleton disruption of both AJ and TJ occur during hypoxic stress, mostly characterized by decrease in E-cadherin (Abbruscato and Davis, 1999), claudin-3 (Witt et al., 2003), occludin (Brown and Davis, 2005; Fischer et al., 2004; Witt et al., 2003), and ZO (Fischer et al., 2004; Fischer et al., 2002) protein expression levels, delocalization of proteins from the membrane to the cytoplasm and increased phosphorylated state (Fischer et al., 2002). Thus BBB disruption involves multi-modal alterations of the cell junctions that ultimately result in compromised barrier function.

Hypoxia also induces dramatic changes in the ECM by up-regulating MMP-2, MMP-3 and MMP-9 (Cunningham et al., 2005; Fukuda et al., 2004; Yang et al., 2007b) resulting in both TJ complexes and BM degradation. A fragilized and partially degraded BM will compromise BBB recovery during reoxygenation. Autocrine and paracrine release of various cytokines constitutes an important aspect of the cellular communication during hypoxic stress and different factors have been involved in BBB breakdown following hypoxic/ischemic insult. For instance, VEGF constitutes one of the most important secreted factors that induces BBB breakdown (Fischer et al., 2002; Schoch et al., 2002). Hypoxic-driven VEGF secretion is up regulated during hypoxia in ECs (Argaw et al., 2006; Fischer et al., 1999; Fischer et al., 2002; Josko et al., 2001), but also by astrocytes (Chow et al., 2001; Schmid-Brunclik et al., 2008) and pericytes (Park et al., 2003b), suggesting that such transient VEGF overexpression by all the members of the neurovascular unit might induce barrier breakdown. Inhibition of VEGF/VEGFR signaling might therefore contribute to protect the BBB opening during hypoxic insult (Vogel et al., 2007). In addition of VEGF signaling, down-regulation of Tie2 signaling by Ang-2 during ischemic insult

(Beck et al., 2000) suggest that both VEGF and Ang-2 might contribute to the barrier breakdown during hypoxia/ischemia situation.

A question that needs to be addressed concerns the role of astrocytes and pericytes during hypoxic stress. Surprisingly, although their role in induction in the barrier function during physiological conditions is relatively well described, their importance during hypoxic situation remains more obscure.

Astrocytes appear to have beneficial roles towards EC during hypoxia by partially maintaining high TEER values (Kondo et al., 1996), low paracellular permeability (Fischer et al., 2000), low oxidative stress (Schroeter et al., 1999) and maintenance of AJ expression at the protein level (Abbruscato and Davis, 1999). On the other hand, pericytes have shown similar behavior as astrocytes, by moderating decrease in TEER (Hayashi et al., 2004) and by secretion of Ang-1 and PGs (Dore-Duffy et al., 2005; Park et al., 2003b). However, these experiments were addressed at defined hypoxic timepoints and do not allow to appreciate how such protection is maintained over time and at different O_2 concentrations, or how astrocytes and pericytes co-operate to provide optimal barrier protection during hypoxic insult. Importantly this thesis will address a number of these important yet unanswered questions.

5. OBJECTIVES

Alteration of the BBB constitutes a hallmark of many neurological diseases including stroke, neurodegenerative diseases and neuroinflammation. Notably, hypoxia also constitutes an important stimulus in induction of the BBB breakdown. Therefore, it is important to understand by which mechanisms hypoxia induces BBB breakdown and how the different component of the neurovascular unit may modulate the response of the BBB during insult.

Despite usage of *in vivo* models constitutes a necessary step to understand the effect of hypoxia on the BBB, such studies remain technically difficult for the elucidation of the cellular and the molecular mechanisms underlying the hypoxic response of the neurovascular unit. However current *in vitro* models are also limited. These models, based on 2-dimensional approach, do not allow formation of 3-dimensional structures and cellular interactions as they normally occur *in vivo*. Furthermore, many researchers have neglected the presence of both astrocytes and pericytes in EC cultures. Thus these models cannot directly address the roles of astrocytes and pericytes in the modulation of the barrier function during physiological and pathological states. The aim of this thesis is to understand the roles of astrocytes and pericytes as modulators of the BBB phenotype by using two innovative *in vitro* BBB models.

In the first part of the thesis, the role of astrocytes and pericytes as modulators on the barrier function in brain EC during normal and hypoxic states was investigated. In order to perform such investigation, brain EC cultured alone or in co-cultures with astrocytes and/or pericytes using the classical *in vitro* model developed by Dehouck (Dehouck et al., 1990) and described in the previous chapters (see Chapter 4.1.6) was employed. We firstly observed EC response to hypoxia stress by investigating

alterations in barrier function by TEER measurements and barrier permeability to different water-soluble compounds. In addition we investigated alterations in EC cell integrity by investigating AJ and TJ protein localization, cell viability and caspase-dependant cell death activation during hypoxic stress. Notably we investigated the possible roles of astrocytes and pericytes as modulators of EC response to hypoxic stress. We particularly focused on their modulation of EC barrier function and cell viability during hypoxic insult.

In the second part of the thesis, we designed an innovative *in vitro* BBB model that reproduced the 3-dimensional structure of the BBB as observed *in vivo*. This model was also used to investigate the roles of astrocytes and pericytes during BBB maturation. The model is based on a 3-dimensional EC culture based on collagen matrix, a classical *in vitro* model used in the literature that allows EC to form tube-like structures comparable to *in vivo* capillaries (Bayless et al., 2000; Davis and Camarillo, 1996; Koh et al., 2008a; Koh et al., 2008b; Madri and Pratt, 1986). We firstly investigated the effect of astrocytes and pericytes on EC the tube-like structure morphogenesis. In addition, the ability of astrocytes and pericytes to induce BBB phenotype on these tubes was observed. We also investigated cell junction (AJ & TJ) formation and localization of ABC transporters in the luminal side by immunocytochemistry. This model showed that new *in vitro* systems might provide valuable information on the BBB induction and disruption.

The design of such innovative *in vitro* models will provide an enhancement of current *in vitro* models and raise the importance to recreate the BBB environment as it is described *in vivo* and boost the usage of these models to find novel therapeutic target to fight neurological diseases and ultimately help patients to fight against such diseases.

6. OWN RESEARCH

The following sections summarize the published or submitted data as shown in the manuscripts enclosed with this work.

6.1. 2-dimensional study (manuscript 1): Pericytes perform better than astrocytes in maintaining barrier function during prolonged oxygen deprivation

The aim of this study was to understand the roles played by astrocytes and pericytes on the barrier function during hypoxic insult.

6.1.1. Prolonged hypoxic or near-anoxic exposure induces barrier breakdown and cell death in RBE4 cells

Hypoxic exposure of endothelial monolayers results in alterations of cell junction proteins and loss of barrier function. Severity and duration of O_2 deprivation stress might constitute important parameters that need to be considered during BBB breakdown. In our study we demonstrate that RBE4 cells, a rat brain endothelial cell line, were able to efficiently maintain barrier function during 24h of hypoxic exposure (1% O_2), followed by dramatic opening after 48h. Notably, reoxygenation allowed barrier function recovery. However exposure of RBE4 cells to near-anoxia (0.1% O_2) dramatically accelerated barrier breakdown and reoxygenation allowed only a slight recovery of barrier function in RBE4 cells. In addition, we have demonstrated that such discrepancy in barrier function was accompanied by major disruption in cell morphology as well AJ (PECAM-1, β-catenin) and TJ (Claudin-5, ZO-1) proteins that demonstrates that prolonged O_2 deprivation severely disrupts cell-cell contacts. In parallel to such dramatic morphological changes hypoxia, and even more near-anoxia, resulted in decreased cell survival through activation of caspase-dependant pro-

apoptotic pathways. In conclusion, O_2 deprivation impairs both barrier and cellular integrity in RBE4 cells.

6.1.2. Pericytes provide better barrier protection than astrocytes during severe O_2 deprivation stress

Astrocytes and more recently pericytes have been shown to induce barrier properties in endothelial cells. Therefore, we firstly assessed the ability of astrocytes and pericytes to induce barrier function in RBE4 cells, in a contact co-culture model. Our study demonstrated that astrocytes and pericytes induced barrier phenotype in endothelial cells against molecules of different size (i.e. sucrose and 40-kDa dextran). Such induction requires presence of these specific cells since substitution with another cell type (i.e. HeLa cells) did not enhance barrier properties in RBE4 cells. Additionally, we challenged the different co-cultures to hypoxic stress in order to demonstrate a positive advantage of astrocyte and pericyte co-cultures over RBE4 monocultures. We observed that astrocyte and pericyte co-cultures partially maintained barrier function during prolonged hypoxia. Moreover, presence of both astrocytes and pericytes resulted in the highest capacity in the maintenance of the barrier function, suggesting a synergistic effect between astrocytes and pericytes.

Surprisingly, under more severe O_2 deprivation stress (near-anoxia) astrocyte co-cultures failed to avoid TJ breakdown in RBE4 monolayers whereas pericytes strongly maintained barrier integrity even after prolonged near-anoxic exposure. Presence of both cells did not show any synergistic effect compared to pericytes co-cultures, suggesting that such protection was mediated by pericytes only. Thus pericytes perform better than astrocytes during near-anoxia.

6.1.3. Maintenance of the barrier function during O_2 deprivation involves caspase and VEGF signaling pathways

As evocated before, hypoxia-induced barrier breakdown involves alteration of both cell junctions and also activation of cell death mechanisms in endothelial cells.

We demonstrated that astrocytes and pericytes were able to partially inhibit caspase-3 activity in RBE4 cells during hypoxic stress. Again, presence of the three cells further inhibited caspase-3 activity. However, during prolonged near-anoxia, pericytes only were able to attenuate caspase activation. Treatment of RBE4 monolayers with ZVAD-fmk provided an advantage only during prolonged O_2 deprivation stress and during reoxygenation phase, suggesting that additional factors are involved in barrier breakdown during acute stress.

VEGF, one of many hypoxia-induced growth factor, has been demonstrated to strongly increase vascular permeability *in vitro* and *in vivo* (Fischer et al., 1999; Minchenko et al., 1994; Schoch et al., 2002). Using SU1498, a VEGF receptor-2 inhibitor, we showed that barrier breakdown initiated by O_2 deprivation was reversed in treated RBE4 cells monolayers. In addition, inhibition of VEGF signaling in astrocytes co-cultures resulted in enhancement of the barrier function during hypoxia and near-anoxia, suggesting that VEGF/VEGF receptor-2 signaling plays an important role in astrocytic response to O_2 deprivation and ultimately to their capacity to maintain barrier function. Pericyte co-cultures showed improvement only under hypoxia, whereas no further enhancement was observed in near-anoxia. Notably, three-cell co-cultures did not show any further increase in barrier function in presence of SU1498, neither in hypoxia nor near-anoxia, suggesting a possible endogenous inhibition of VEGF/VEGF receptor-2 signaling axis.

6.2. 3-dimensional study (Manuscript 2): building up an innovative BBB model on a dish. Design and characterization.

The aim of this second study was to provide an innovative 3-dimensional *in vitro* BBB model that allows proper interactions between the different cell types.

6.2.1. RBE4 cells reproduce in vivo vasculogenesis-angiogenesis

The capacity of endothelial cells to form a vascular network from non-existing structures constitutes the most important criteria in 3-dimensional endothelial cell culture. Therefore, we firstly assessed the ability of RBE4 cells to reproduce *in vivo* vasculogenesis-angiogenesis.

Within the matrix, RBE4 cells reproduced a phenotype similar to *in vivo* situation by formation of cysts from which emanated sprouting processes. These sprouting processes were able to interact with other sprout, to fuse and to form intercystal connections that allow formation connection formation between cysts. Interestingly, intercystal connections changed morphology over time, mainly by showing increased length and diameter, but also by appearance of tube-like structures. These tube-like structures, displaying similar morphology to *in vivo* capillaries, differ from intercystal connections by absence of cysts at their terminus and by formation of collateral branches. In conclusion, our model allows RBE4 cells to reproduce cellular events that naturally occur during *in vivo* vasculogenesis and angiogenesis.

6.2.2. BBB maturation and induction involves astrocytes and pericytes

Formation of BBB *in vivo* involves maturation of the cerebral vasculature followed by induction of BBB phenotype in brain endothelial cells. Thus the second important criteria that need to be assessed in our 3-dimensional model is the ability of astrocytes

and pericytes to induce a maturation and induction processes in RBE4 tube-like structures.

We firstly assessed the ability of astrocytes and pericytes to modulate RBE4 cell angiogenesis. Remarkably, presence of astrocytes and pericytes decreased cell density, cysts and intercystal connections numbers. Interestingly, both astrocytes and pericytes did not influence intercystal connection length and diameter suggesting that vasculogenesis might not be directly regulated by astrocytes and pericytes. However, we observed that astrocytes and pericytes were able to modulate formation of tube-like structures. Astrocytes did not significantly reduced tube-like structures numbers and increased tube length and diameter. At the opposite, pericytes strongly up-regulated formation of tube-like structures but also resulted in decreased diameter. The presence of both astrocytes and pericytes significantly reduced tube-like structures number compared to other groups but significantly increased their length suggesting that astrocytes inhibit pericyte induction. Indeed astrocyte inhibition was even potentiated by presence of pericytes. However, tube-like structure diameters were significantly different from either astrocytes and pericytes groups thus suggesting under such conditions pericytes plays an important role in the regulation of tube-like structure diameter. In conclusion, our model shows that astrocytes and pericytes constitute important modulators of RBE4 cells angiogenesis underlying their importance during brain vasculature development.

Using immunocytochemistry techniques, we demonstrated that tube-like structures formed in RBE4 monocultures displayed an immature phenotype, showing an inability to localize AJ proteins (β-catenin, PECAM-1) at the cellular borders, suggesting an immature and instable vasculature. In addition, we observed similar impairment in protein localization for different BBB phenotype markers such as TJ

proteins (ZO-1, claudin-5) proteins at cellular borders, but also localization of some ABC transporters at the luminal face (P-gp and MRP2). Notably, both astrocytes and pericytes were able to induce BBB phenotype by inducing localization of TJ proteins and ABC transporters at the cell borders and in the luminal face respectively.

In conclusion, we have demonstrated that presence of astrocytes and pericytes were necessary to induce maturation of RBE4 cells vasculature, but also to induce BBB phenotype. These results shows a nice correlation with *in vivo* studies concerning BBB formation and maturation during development and clearly demonstrates the ability of our model to reproduce many *in vivo* features, demonstrating that our model constitute a unique and innovative model compared to actual *in vitro* models and thus we currently validating this model to prove its suitability as a tool to study changes in the BBB during pathological states.

7. DISCUSSION

This thesis focused on the development of innovative BBB models based on 2-dimensional and 3-dimensional cell culture. Our models considered the presence of EC, astrocytes and pericytes, but also the reproduction of 3-dimensional structure and cellular interactions as they occur naturally in the BBB *in vivo*. These two criteria are considered to be essential to understand the BBB physiology and pathophysiology.

The work contained in this thesis was performed using RBE4 cell, an immortalized rat brain EC. In the 3-dimensional model, RBE4 cells were able to form tube-like structures presenting an internal lumen and thus were comparable to *in vivo* capillaries. These tubes showed positive expression of claudin-5 and ZO-1 (two TJ markers) but also expression of P-gp and MRP-2 (two ABC transporters) suggesting that the tubes displayed important BBB phenotype. In the 2-dimensional model, RBE4 cells developed high TEER values, low permeability to water-soluble compounds and localization of claudin-5 and ZO-1, showing presence of a BBB phenotype in RBE4 monolayers. Our results correlated with values classically described in the literature and therefore validate the usage of RBE4 cells as a brain EC (Roux and Couraud, 2005).

It is assumed that that brain vasculature is leaky during development and investiture of the immature vessels by astrocytes end-feet processes results in induction of barrier phenotype in EC. However, recent studies suggest that developing immature cerebral vasculature already expresses TJ proteins (Ballabh et al., 2005) and presents a barrier function against small water-soluble compounds (Ek et al., 2006). Thus presence of a certain barrier phenotype in the developing brain and re-evaluation of the concept of "leaky barrier" may need to be reconsidered. On the other hand, we observed that in our 3-dimensional model, RBE4 cells were not able to induce proper localization of

TJ proteins at the cellular borders and ABC transporters in the luminal compartment. This observation would suggests therefore that brain EC *in vivo* cannot perform a full development of a BBB phenotype on their own and therefore may need interactions with the surrounding cells and ECM. This fact has been largely studied in the literature showing that brain EC-ECM interactions are crucial for EC maturation (del Zoppo and Milner, 2006; Del Zoppo et al., 2006), whereas EC-astrocytes (Abbott, 2002) and EC-pericytes (Balabanov and Dore-Duffy, 1998; Hellstrom et al., 2001) interactions are important role for the stabilization and the induction of the BBB.

The roles of pericytes during BBB development are relatively unclear although some studies showed that pericytes coverage of developing cerebral vasculature were associated with vascular stability (Braun et al., 2007). We showed that pericytes play important roles in our 3-dimensional cell cultures, by increasing angiogenic processes in RBE4 cells, down-regulating RBE4 cell proliferation and by increasing the tube-like structures density compared to RBE4 cells monocultures and astrocytes co-cultures. This observation correlates with other studies performed both *in vitro* (Liu et al., 2008; Saunders et al., 2006) and *in vivo* (Virgintino et al., 2007) showing the importance of pericytes as angiogenesis promoters. An additional important feature observed was the induction of BBB phenotype by pericytes both in our 2-dimensional or 3-dimensional models. Our 3-dimensional pericyte co-culture model was able to reproduce behavior observed *in vivo* and thus shows that our 3-dimensional *in vitro* may be used for studying BBB formation during development.

Our results showed significant up-regulation of barrier function in RBE4 cells in the 2-dimensional model and thus correlates with other studies (Dohgu et al., 2005; Hayashi et al., 2004; Nakagawa et al., 2007), but also to induce proper localization of both TJ and ABC proteins at the cell borders and in the lumen respectively in our 3-

dimensional model. This induction of proper localization would therefore suggest that pericytes might contribute to induce BBB phenotype in EC even before establishment of astrocyte contacts. Therefore this induction would explain the presence of an early BBB phenotype during development supporting our hypothesis and as suggested by others (Ballabh et al., 2005; Virgintino et al., 2008; Virgintino et al., 2004). However, further investigations are necessary and an establishment of micro-perfusion techniques to perfuse our tube-like structures would directly allow us to assess if pericytes induction of proper protein localization correlates with decreased permeability to water-soluble compounds.

Astrocytes are classically described to induce BBB phenotype in brain EC both *in vitro* (Arthur et al., 1987; Dehouck et al., 1990; Rauh et al., 1992) and *in vivo* (Butt et al., 1990). Our two different *in vitro* models show that astrocyte co-cultures were able to up-regulate barrier phenotype in RBE4 and thus make our finding consistent with the literature. In our 2-dimensional model, astrocytes were able to induce significant increase in TEER but also decrease paracellular permeability to water-soluble compounds. As observed with 3-dimensional pericyte co-cultures, astrocytes co-cultures were able to induce appropriate TJ and ABC proteins localization in RBE4 tube-like structures and therefore suggest that astrocytes may induce BBB phenotype. Unlike pericytes, astrocytes did not influenced angiogenesis but played important roles in tube-like structure stabilization and maturation as we observed a decrease in RBE4 cell proliferation and increased tube length and caliber suggesting that astrocytes may play also in important in the maturation process that follow the onset of cerebral vasculature.

The presence of both astrocytes and pericytes do to not occur simultaneously during development, as pericyte-endothelial interactions precede astrocyte-endothelial ones.

Therefore astrocytes and pericytes should coordinate to induce maturation processes that ultimately lead to mature BBB phenotype. In our 2-dimensional study presence of both astrocytes and pericytes resulted in a maximal barrier phenotype induction and therefore the study of Nakagawa and colleagues (Nakagawa et al., 2007) agrees with ours as we both conclude that astrocytes and pericytes have a synergistic effect in induction of barrier phenotype. Previously described in the thesis (see chapters 4.1.6 and 4.1.7) is that astrocytes and pericytes secrete Ang-1 and TGF-β which would explain why we did not obtain only a partial cumulative effect. On the other hand, astrocytes and pericytes may release specific factors that might activate distinct signaling pathways resulting in the partial cumulative effect observed in our 2-dimensional study. However the nature of such factors is still unclear and needs further investigation.

In addition to the synergistic effect demonstrated in the 2-dimensional study, using our 3-dimensional model we saw that both astrocytes and pericytes presence caused a significant decrease in numbers of tube-like structures, as well as a significant increase in average tube length compared to astrocyte and pericyte co-cultures. These results suggest that pericytes might potentiate astrocyte effect instead of inhibiting it. This would further suggest that a cellular cross talk occurs between astrocytes and pericytes that result in such potentiation.

It is interesting to note that the BM delimitating the neurovascular unit physically separates astrocytes and pericytes from each other, therefore direct physical contact between astrocytes and pericytes, but also between EC and astrocytes, appears unlikely. Therefore astrocyte-endothelial interactions may involve secretion of soluble factors, but also a modification of the BM composition may result in induction of BBB phenotype as suggested by different studies made on 2-dimensional *in vitro*

models (Tilling et al., 2002; Tilling et al., 1998). Our two models allowed close contacts between astrocytes and EC, with investiture of RBE4 tube-like structures by astrocytic end-feet processes in our 3-dimensional model. Therefore we can speculate that induction of barrier phenotype in RBE4 tube-like structures might be due to ECM secretion by surrounding end-feet processes in addition to release of soluble factors. Using our 3-dimensional *in vitro* model, we will in future address the possible presence of ECM secretion between EC and astrocytic end-feet process and hopefully determine the nature of these potential secreted matrix proteins.

Pericytes share the same BM as EC and also directly interact with EC by gap junctions, suggesting an additional communication mode between pericytes and EC, besides the release of factors or matrix proteins. Our 3-dimensional model allowed direct interactions between tube-like structures and pericytes and therefore we can speculate that gap junctions may occur between these two cell types.

In our 2-dimensional study, RBE4 cells monolayers maintained an efficient barrier function during acute hypoxic (1% O_2) or near anoxic (0.1% O_2) exposure. However barrier disruption occurred significantly after prolonged exposure with both loss of barrier function and TJ complexes at the cell borders. Our data are in agreement with the literature as we also observed an increase in paracellular permeability (Plateel et al., 1995) and loss in ZO-1 localization (Fischer et al., 2002) and therefore validate the usage of our model to study the effect of astrocytes and pericytes co-cultures. However we showed that drastic alterations in barrier function occurred only after prolonged hypoxic stress, whereas previous studies mostly focused on short-term O_2 deprivation. In addition we demonstrated that disruption of the barrier function in RBE4 monolayers was reversible only during mild hypoxic insult, reoxygenation did not allow recovery after prolonged near-anoxia. Thus our study emphasized that

duration and intensity of the hypoxic insult dictates not only the onset of the barrier breakdown, but also the ability of the BBB to recover adequate barrier function after insult.

HIF-1α stabilization may influence directly or indirectly EC response to hypoxia and therefore raise the role of HIF-1 on the barrier function. Although we did not directly investigate changes in HIF-1α levels during hypoxic stress, we did investigate the role of VEGF signaling, a HIF-1 target gene, in our 2-dimensional cultures. As we have previously discussed (see chapters 4.2.2 & 4.2.3), VEGF constitutes one of the most important HIF-1 target genes. In addition to its important roles pro-angiogenic role, VEGF is known as a factor that induces brain vascular leakage (Fischer et al., 1999; Fischer et al., 2004; Issbrucker et al., 2003; Schoch et al., 2002). Therefore VEGF signaling may play an important role in the onset of the BBB disruption during hypoxia. We observed that VEGF signaling inhibition by blocking VEGFR-2 activation resulted in restoration of the barrier function in RBE4 cell monolayers both in prolonged hypoxia and near-anoxia, correlating with previous studies that showed inhibition of VEGF signaling restored ZO-1 protein localization (Fischer et al., 2004; Fischer et al., 2002) and decreased brain vascular permeability (Kilic et al., 2006; Kumai et al., 2007; Schoch et al., 2002). Therefore we can consider that presence of a VEGF autocrine loop occurs in RBE4 monolayers, leading to pathway signaling activation and ultimately barrier breakdown. Thus excessive VEGF production by prolonged HIF-1α stabilization can cause barrier breakdown.

Astrocyte co-cultures provided partial protection of the barrier function, as we observed better TEER and permeability values compared to RBE4 monocultures during both hypoxia and near-anoxia and agrees with a few studies that have been performed on hypoxic co-cultures (Fischer et al., 2000; Kondo et al., 1996). However

we observed that astrocytes beneficial effect during hypoxia were significantly reduced in near-anoxic co-cultures, such effects were unlikely due to decrease in astrocytes cell survival, as no changes in astrocytes cell survival rate occurred. Therefore the effect might be carried by a hypoxic-inducible factor that elicit barrier breakdown such as VEGF. Our group recently demonstrated that astrocyte VEGF secretion was significantly up-regulated by O_2 and glucose deprivation. Notably, increase in VEGF secretion was proportional to the severity insult, as near-anoxic astrocytes showed a significant increase in VEGF secretion compared to hypoxic astrocytes (Schmid-Brunclik et al., 2008). Therefore, we may consider that the discrepancy in astrocytes protection observed in near-anoxia might be due to exacerbated VEGF secretion levels. Using VEGFR-2 inhibitor we observed that the barrier function was enhanced only in long-term hypoxic and short-term near-anoxic co-cultures suggesting that a gradual increase in VEGF secretion in astrocytes is elicited by decrease in O_2 levels and VEGFR-2 playing again a central role in induction of barrier breakdown in our model. It is interesting to note that both RBE4 and astrocytes showed VEGFR-2 expression, astrocytes VEGFR-2 playing an important role as inducer of astrocytes proliferation (Schmid-Brunclik et al., 2008). However the role of VEGFR-2 signaling in astrocyte VEGF secretion has not been addressed yet, thus further studies would provide useful information concerning a possible negative feedback loop.

Pericytes co-cultures displayed particular response to O_2 deprivation stress as they rapidly lost the ability to maintain appropriate barrier function during acute hypoxic stress and showed protective effects only during long-term hypoxia or during near-anoxia. Our data would agree with a study performed by Hayashi and colleagues (Hayashi et al., 2004), however this study provided only fragmented information on

pericyte co-culture response to hypoxia. We observed that VEGF blockade had an effect in pericyte co-cultures only during hypoxia with no additional increase in barrier function was observed in near-anoxic samples, suggesting that VEGF signaling might be endogenously inhibited.

Different studies have described that VEGF acts as a negative signal for pericyte recruitment as subsequent VEGFR-2 inhibition allowed proper vascular coverage by pericytes (Greenberg et al., 2008). Our study therefore corroborates that VEGF/VEGFR-2 signaling may impair pericyte function and their protective effects towards EC. Nomura and colleagues described that VEGFR-2 was not expressed in pericytes during hypoxia (Nomura et al., 1995). Thus the enhancement of the barrier function observed in presence of VEGFR-2 inhibitor would suggest that activation VEGF/VEGFR-2 autocrine loop in EC during hypoxia might result in release of negative factors compromising protective effects carried on by pericytes. Although we do not know the nature of such negative secreted factors, Ang-2 as a hypoxic-inducible factor secreted by EC might constitute one of these negative factors as Ang-2 results in important impairment in the vasculature and loss of pericyte-induced barrier function in the retina (Feng et al., 2008; Hammes et al., 2004; Maisonpierre et al., 1997; Pichiule et al., 2004).

Our 2-dimensional study showed that presence of both astrocytes and pericytes were necessary to obtain maximal induction of barrier function in RBE4 cells in normoxia. Presence of both astrocytes and pericytes was also necessary to maintain appropriate barrier function during hypoxia showing significant higher values versus two-cell co-cultures. This result again underlines the synergistic effect afforded by presence of both astrocytes and pericytes in the maintenance of the BBB during hypoxic injury. However the synergistic effect was attenuated during near-anoxia despite the

maintenance of the barrier function remaining significantly higher compared to astrocyte co-cultures. Indeed, we observed no significant difference compared to pericyte co-cultures suggesting that pericytes might constitute the cell type that maintains the barrier function in the three-cell co-cultures. Discrepancy of the astrocyte effect might be due to elevated VEGF secretion during near-anoxia. However, we did not observe further improvement of the barrier function in presence of VEGFR-2 inhibitor either in hypoxia or near-anoxia, suggesting that endogenous VEGF signaling inhibition occurs during O_2 deprivation stress, such inhibition likely involves a cross-talk between astrocytes and pericytes. However additional studies needs to be performed to understand how these two cell type coordinate their responses during hypoxia leading to the synergistic effect observed on the barrier function.

Notably although astrocytes and pericytes show relative opposite behavior in regards of endothelial barrier function during O_2 deprivation stress, our current understanding of pericytes response to O_2 deprivation remains still unclear. Therefore further investigation on the pericyte response to hypoxia, focusing on HIF-1α mediated response to hypoxia in pericytes might help to clarify the situation.

In this chapter we mostly discussed of VEGF as a negative factor, inducing BBB breakdown. However it is important to mention that VEGF has non-vascular effects and plays important roles as cell survival factor for EC and other cells during hypoxic stress (Chow et al., 2001; Plaschke et al., 2008; Scheid et al., 2000; Wang et al., 2007; Wang et al., 2005). We have demonstrated that prolonged or severe hypoxic stress induces caspase-3 activation and apoptosis in RBE4 cells; astrocytes and pericytes were able to partially inhibit apoptosis by inhibiting caspase-3 activation. However, other studies have specifically shown that alteration of VEGF signaling occurs in

many neurological diseases (Kilic et al., 2006; Kumai et al., 2007; Sun et al., 2003b; Villar-Cheda et al., 2008; Wang et al., 2007; Yasuhara et al., 2004; Yasuhara et al., 2005) highlighting the importance of VEGF as an important neuroprotective factor. Inhibition of VEGF-mediated barrier breakdown and the maintenance of its neuroprotective role would probably rehabilitate VEGF as a neuroprotective agent (Plaschke et al., 2008; Vogel et al., 2007; Wang et al., 2007; Wang et al., 2005).

The roles of astrocytes and pericytes in the maintenance of the barrier function during hypoxia were investigated extensively using a 2-dimensional model. However, as we have discussed in the beginning of the chapter, this model do not allow optimal physical cellular interactions as they naturally occur *in vivo*. We hypothesized that such contacts between the different components of the BBB might play important roles during cerebral injury.

Data obtained with our 3-dimensional model showed that prolonged hypoxia induced alterations in astrocyte-endothelial interactions. We firstly observed that astrocyte end-feet morphology was altered by prolonged hypoxia, resulting in irregular distribution of the end-feet over the tube and process retraction. This was accompanied by notable changes in tube diameter, suggesting that alteration in astrocyte-endothelial interactions could directly modulate endothelial morphology. Such alterations were more visible in near-anoxic astrocyte co-cultures, showing significant end-feet processes retraction, disruption of the tube-like structure morphology and complete loss of the vascular structure. These results correlate nicely with the data obtained from our 2-dimensional study and therefore may suggest that astrocytes contacts through end-feet processes do indeed play important roles in the maintenance of the barrier function during hypoxic stress. Willis and colleagues (Willis et al., 2004) have suggested the importance of astrocyte-endothelial

interactions for the induction of the BBB, although the experimental approach used in this study remains disputable. In this *in vivo* study, they observed that loss of astrocytes was accompanied by loss of barrier phenotype in the adjacent vasculature and restoration of astrocyte-endothelial interactions was accompanied by restoration of a BBB phenotype in EC.

Although we have not performed yet any study of alterations in endothelial-pericytes interactions in our cultures, recent studies performed by Duz and colleagues (Duz et al., 2007; Gonul et al., 2002) showed that pericytes migration occurs during early onset of cerebral injury, resulting in decreased pericyte coverage of the injured vasculature. Such decrease in pericyte coverage might be associated with destabilization of the blood vessel and subsequent formation of leaky vessels. We are currently investigating the importance of such pericyte movement and migration on barrier integrity using our 3-dimensional model.

In summary this thesis provides a new dynamism within the field by the design of these two innovative *in vitro* models that emphasize the importance of astrocytes and pericytes presence on one hand and the reconstitution of the 3-dimensional cellular interactions as *in vivo* situation on the other. Furthermore these models are easily customizable, allowing addition or removal of different cell types to dissect signaling pathways underlying different cellular interactions, opening these models to a multitude of *in vitro* studies investigating the BBB under physiological or pathological situations. The enthusiasm reflected by the scientific community towards our models and especially to our 3-dimensional model further suggests that there is a need for such new innovations that will be adopted by others. We expect that these models will be frequently used in the near future encouraging the consideration of other integrative approaches during development of *in vitro* models in general.

Despite the fact that our models still remain a simplification of *in vivo* situation, they reach an integrative dimension superior to current *in vitro* models. The two models are not just a simple overlap, but indeed are complimentary as they allow investigation of different features of BBB interactions. We are convinced that these models have a definite place in the field and will contribute to enhance our understanding of the BBB during physiological and pathological situation. Our models, and in particular the 3-dimensional model, define a new step in achievement of the design of integrative *in vitro* models and bring powerful tools to the research field for better understanding of the BBB as a neurovascular unit.

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9. MANUSCRIPTS & ABSTRACTS

Manuscript 1:
A. Al Ahmad, M. Gassmann, O.O. Ogunshola
"Maintaining Blood-Brain Barrier Integrity: Pericytes Perform Better Than Astrocytes During Prolonged Oxygen Deprivation."
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Manuscript 2:
A. Al Ahmad, M. Gassmann, O.O. Ogunshola
"The blood-brain barrier in a dish: designing a innovative 3-dimensional *in vitro* model"
(submitted)

Abstract 1:
Al Ahmad A, Gassmann M, Ogunshola OO.
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Abstract 2:
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Maintaining Blood–Brain Barrier Integrity: Pericytes Perform Better Than Astrocytes During Prolonged Oxygen Deprivation

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The blood–brain barrier (BBB), consisting of specialized endothelial cells surrounded by astrocytes and pericytes, plays a crucial role in brain homeostasis. Many cerebrovascular diseases are associated with BBB breakdown and oxygen (O_2) deprivation constitutes a critical factor that onsets its disruption. We investigated the impact of astrocytes and pericytes on brain endothelial cell permeability and survival during different degrees of O_2 deprivation. Prolonged exposure to 1% O_2 caused barrier breakdown and exposure to 0.1% O_2 dramatically accelerated disruption and induced cell death, mediated at least in part via caspase-3 activation. Reoxygenation allowed only cells exposed to 1% O_2 to re-establish barrier function. Notably co-culture with astrocytes and pericytes substantially enhanced barrier function under normoxic conditions, and produced differential responses during O_2 deprivation. At 1% O_2 astrocytes partially maintained barrier integrity whereas pericytes accelerated its disruption in the short-term, having positive effects only after prolonged exposure. Unexpectedly, at 0.1% O_2 pericytes were more effective than astrocytes in preserving barrier function although the protection afforded by both cells involved inhibition of caspase-3 pathways. Furthermore, cell-specific regulation of auto- and paracrine VEGF signaling pathways were also in part responsible for the differential modulation of barrier function. Our data suggests that cellular cross-talk within the neurovascular unit is crucial for preservation of barrier integrity and that pericytes, not astrocytes, play a significant role during severe and prolonged O_2 deprivation.

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Cerebrovascular diseases constitute the second cause of death worldwide (Lopez et al., 2006) and are defined by an impairment of proper nutrient and O_2 delivery to the cerebral tissue. Hypoxia constitutes one of the critical factors involved in these diseases inducing blood–brain barrier (BBB) breakdown that leads to brain swelling and edema formation in vivo (Ballabh et al., 2004). In vitro studies have demonstrated that hypoxia induces dramatic alterations in the endothelial actin cytoskeleton and tight junction protein localization (Mark and Davis, 2002; Brown and Davis, 2005). Furthermore, in vivo models showed that rodent brain vascular leakage takes place 1 and 24 h after exposure to 6% (Witt et al., 2003) and 8% (Schoch et al., 2002) O_2 concentrations, respectively. These differences suggest that injury time and severity might constitute critical factors to be considered for both rate of breakdown as well as the ability to recover after injury.

The BBB is a neurovascular unit formed by endothelial cells, pericytes, and perivascular astrocytes (Pardridge, 1998). It plays a crucial role in the maintenance of the central nervous system homeostasis by limiting diffusion of soluble compounds from the blood to the brain parenchyma. Astrocytes induce barrier phenotype in brain endothelial cells (that form the blood vessels) through development of end-feet processes and release of soluble factors (Arthur et al., 1987; Rist et al., 1997). In contrast to the wealth of knowledge on astrocyte function, the contribution of pericytes to BBB phenotype remains more obscure. However in vitro (Hayashi et al., 2004; Hori et al., 2004) and in vivo (Hellstrom et al., 2001) studies published recently indicate that pericytes also contribute to barrier function. Furthermore other studies have demonstrated an impairment of astrocyte and pericyte functions in neurological diseases such as Alzheimer's disease (Iadecola, 2004), multiple sclerosis (Argaw et al., 2006) and diabetic retinopathy (Joussen et al., 2003). Clearly O_2 deprivation stress is an important factor linked to induction of vascular leakage and direct effects of hypoxia on the response of astrocytes or pericytes must have significant consequences on BBB function.

Most in vitro studies performed on brain endothelial cells exposed to O_2 deprivation omit the presence of astrocytes and/or pericytes constituting an important limitation of their findings. This study investigates the effects of different O_2 concentrations on endothelial cell barrier function and the putative impact of astrocytes and pericytes on barrier maintenance using transendothelial electrical resistance (TEER) and permeability measurements. We demonstrate that monolayer integrity and cell viability depend on the severity of O_2 deprivation. Moreover astrocytes and pericytes display differential effects during O_2 deprivation, with pericytes performing better than astrocytes during extreme O_2 deprivation.

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Materials and Methods

Cell culture

Rat brain endothelial cell line (RBE4) was used for experiments (Roux et al., 1994). Primary rat astrocytes were isolated from neonatal pups as described previously (Chow et al., 2001) and cultured in 10% FBS and 50 μg/ml gentamycin sulfate. Primary rat brain pericytes were isolated from male adult rat brains following an established protocol (Dore-Duffy, 2003). Briefly, cerebral microvessels were isolated from brains, digested with collagenase/dispase for 2 h at 37°C and plated on uncoated dishes. Freshly isolated pericytes were cultured in DMEM supplemented with 20% FBS, 50 μg/ml gentamycin sulfate and 2.5 μg/ml amphotericin B until near confluency. Subsequent passages were maintained in astrocyte medium. Pericyte cultures were >95% pure as estimated from strong positive α-smooth muscle actin (α-SMA) staining and negative GFAP expression (Supplementary Fig. 1). HeLa cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml streptomycin, 100 μg/ml penicillin, 1% non-essential amino-acids and 1% sodium pyruvate. Rat-tail collagen was isolated using a previously described method (Roll et al., 1980).

O_2 deprivation-reoxygenation experiments

Experiments were performed in a purpose-built hypoxic glove-box chamber (InVivo$_2$ 400, Ruskinn Technologies, Pencoed, UK) maintained at 37°C with 5% CO_2. Cells were exposed to either 1% (hypoxia) or 0.1% (near-anoxia) O_2 for different durations. Using this instrument O_2 concentration is constantly monitored internally by an O_2 sensor and automatically adjusted. Reoxygenation was performed at 21% O_2 for 24 h. Cells were cultured in RBE4 medium during monoculture experiments, or in astrocyte medium during co-culture experiments.

Transendothelial electrical resistance

Transendothelial electrical resistance (TEER) measurements were performed on Transwells™ (Corning, Schiphol, The Netherlands) coated with rat-tail collagen. These inserts allow proper cellular polarization with formation of an apical (upper compartment) and basolateral face (lower compartment) mimicking luminal and abluminal faces (Fig. 3A). RBE4 cells were seeded on the upper side of the insert at a density of 0.5×10^6 cells/insert. TEER was measured using an EVOM chopstick (World Precision Instruments, Sarasota, FL). All resistance measurements were compared to collagen-coated blank filters. Prior to O_2 deprivation, monolayers were cultivated for 8 days (Roux et al., 1994).

In co-culture experiments, RBE4 cells were seeded on the upper side of the filter whereas astrocytes, pericytes or HeLa cells were seeded on the lower side of the filter (Fig. 3A). Each cell type was seeded at a density of 0.5×10^6 cells/insert. Co-cultures were maintained in astrocyte medium for 8 days prior to the start of experiment. TEER measurements during O_2 deprivation were performed in the hypoxic glove-box chamber without reoxygenation.

Permeability assays

Permeability assays were performed on Transwells. Fresh medium containing radiolabelled sucrose (^{14}C-sucrose, 1 μCi/ml, Amersham Biosciences, Otelfingen, Switzerland) or 40 kDa fluorescein isothiocyanate conjugated-dextran (1 mg/ml, Sigma–Aldrich, Buchs, Switzerland) was added to the upper compartment. At 0, 15, 30, 45, and 60 min aliquots were taken from the bottom compartment. Sample radioactivity was determined using a liquid scintillation β-counter (Tricarb 1600TR, Canberra Packard, Schwadorf, Austria) whereas fluorescence was determined with a fluorescence plate reader (FLx800, Biotek Instruments, Winooski, VT). A clearance slope was established from the measurements obtained at the different timepoints and used to calculate permeability coefficient values (Pe) (Rist et al., 1997).

Cell density and viability

Assays were performed on confluent cells grown on 24-well plates. An aliquot of cells were mixed with an equal volume of 0.4% trypan blue solution (Sigma–Aldrich) and cell counting was performed with a glass hematocytometer (Neubauer, Brand, Wertheim, Germany).

Caspase-3 activity assay

Caspase-3 activity assays were performed using a colorimetric activity assay (R&D Systems, Minneapolis, MN). In brief, cells were washed with ice-cold PBS, harvested into lysis buffer and the supernatant assayed for caspase-3 activity. Equal protein amounts were incubated in presence of DEVD peptide conjugated with *p*-nitroaniline (DEVD-*p*NA). Cleavage of DEVD-*p*NA was quantified spectrophotometrically at 405 nm (Multiskan RC, Thermo Labsystems, Zürich, Switzerland). Caspase-3 enzymatic activity in the cell lysate was proportional to optical density. Optical densities were normalized to normoxic values and expressed as fold-change.

Pharmacological inhibition experiments

Caspase inhibition experiments were performed on RBE4 monolayers exposed to RBE4 culture medium supplemented with 100 μM ZVAD-fmk (R&D Systems) or containing 1% DMSO only, prior to hypoxic and near-anoxic incubation.

VEGF signaling inhibition experiments were performed on mono- and co-cultures in medium supplemented with 10 μM SU1498 (Calbiochem, VWR, Dietikon, Switzerland) or containing 0.1% DMSO only, prior to hypoxic and near-anoxic incubation.

Microscopy and immunocytochemistry

Bright-field pictures of confluent RBE4 monolayers were taken using an inverted phase contrast microscope (Axiovert 40 CFL, Zeiss, Feldbach, Switzerland) and CCD digital camera (Cybershot DSC-S85, Sony Corporation, Tokyo, Japan). Micrograph pictures of fluorescence immunocytochemistry for CD31 (mouse, 1:100, Chemicon, Millipore, Zug, Switzerland), β-catenin (mouse, 1:100, BD Biosciences, Allschwil, Switzerland), claudin-5 (rabbit, 1:100, Invitrogen, Basel, Switzerland) and ZO-1 (rabbit, 1:100, Invitrogen) were taken using an inverted fluorescence microscope (Axiovert 200M, Zeiss) coupled to an 8-bit CCD camera (Axiocam HR, Zeiss).

Statistical analysis

All results are expressed as mean ± SD from three or more independent experiments. Statistical significance was assessed by one-way ANOVA for comparison of different timepoints within a group, two-way ANOVA for comparison between different groups. A *P*-value below 0.05 was considered significant.

Results

Barrier function during normoxia

TEER of normoxic RBE4 monolayers was measured using a modified Boyden chamber. Under normoxic conditions a gradual increase in resistance with a peak of 201.13 ± 8.11 Ω cm^2 at day 6 was observed. A period of 8 days was necessary to obtain constant TEER values. Accordingly, all subsequent O_2 experiments were performed after 8 days.

Prolonged O_2 deprivation disrupts RBE4 monolayers

RBE4 monolayers were exposed to either 1% (hypoxia) or 0.1% (near-anoxia) O_2 concentration for up to 48 h. Acute hypoxic exposure (Fig. 1A) caused an initial decrease of resistance that remained constant till 8 h. Monolayers exposed to near-anoxia

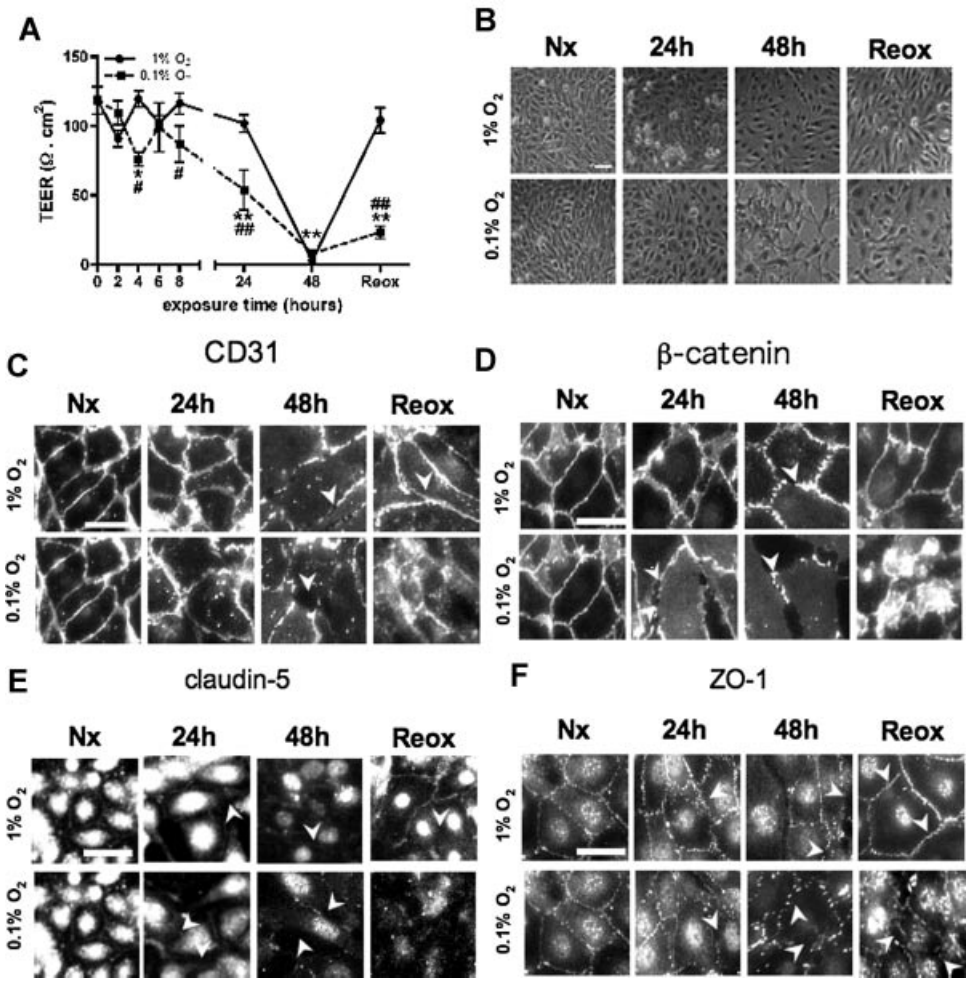

Fig. 1. O_2 deprivation impairs RBE4 barrier function by disruption of cell morphology and cell junctions. RBE4 monolayers were incubated at either 1% or 0.1% O_2 concentrations and TEER was measured for 48 h and reoxygenation was performed for 24 h (A). During short-term experiments hypoxia (circles) did not significantly affect TEER. Long-term hypoxia resulted in complete loss of resistance that was reinstated during reoxygenation. Long-term near-anoxic exposure caused a faster decrease in TEER and significantly worse recovery during reoxygenation. ($^{*}P < 0.05$; $^{**}P < 0.01$ comparison vs. 0 h timepoint, $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ comparison between hypoxic and near-anoxic groups, n = 8 per group). Bright-field microscopy revealed altered RBE4 cell morphology during O_2 deprivation (B). Fluorescence micrographs of CD31 (C), β-catenin (D), claudin-5 (E) and ZO-1 staining (F) show protein delocalization (arrows) during hypoxia and more rapid alterations in near-anoxia. Reoxygenation supported re-localization of junctional proteins only under hypoxic conditions. Scale bar = 25μm.

exhibited an accentuated and sustained decrease in electrical resistance down to $75.85 \pm 4.83\ \Omega\,cm^2$ at 4 h but surprisingly TEER again stayed constant thereafter.

Extended O_2 deprivation completely disrupted barrier function in both hypoxia and near-anoxia (Fig. 1A). Resistance of hypoxic monolayers decreased from 118.79 ± 9.51 to $101.86 \pm 6.50\ \Omega\,cm^2$ at 24 and by 48 h the TEER was just $2.81 \pm 1.96\ \Omega\,cm^2$ indicating complete loss of integrity. Despite this, reoxygenation stimulated resistance recovery indicating a reduction of permeability. Under near-anoxic conditions monolayers showed a more pronounced decrease compared to the hypoxic group with a very significant drop to $54.01 \pm 14.31\ \Omega\,cm^2$ after 24 h. Similar to the hypoxic group 48 h exposure caused complete loss of resistance but reoxygenation did not support a dramatic improvement in resistance levels, with TEER values ($23.43 \pm 4.61\ \Omega\,cm^2$) remaining significantly lower than the hypoxic-reoxygenated group. Notably no significant decrease was observed under normoxic condition during these timepoints (data not shown), showing that loss of barrier function is only due to O_2 deprivation. Thus the degree of O_2 deprivation significantly influences the recovery of barrier properties as well as the rate of barrier breakdown during prolonged hypoxia.

O_2 deprivation alters RBE4 morphology and delocalizes cell junction proteins

Under normoxic conditions RBE4 cells exhibit classical cobblestone morphology (Fig. 1B, first panel) with marked light refraction around the cell borders indicating close and intimate contacts. Hypoxic conditions (1% O_2) changed the light refraction over time and induced cell swelling (Fig. 1B, upper panels). After 24 h approximately 90% of the cells maintained close contacts but by 48 h a clear decrease was observed. Subsequent reoxygenation allowed intimate contacts to be reformed correlating well with previous TEER measurements. Near-anoxia (Fig. 1B, lower panels) caused loss of approximately 50% of intimate contacts already at 24 h. At 48 h

appearance of large gaps was clearly visible and this remained even after reoxygenation.

Notably, immunocytochemistry against CD31 (Fig. 1C), β-catenin (Fig. 1D), claudin-5 (Fig. 1E), or ZO-1 (Fig. 1F) also displayed significant alterations. Under normoxic condition, continuous positive staining of all proteins was observed at cell borders. Hypoxic exposure (Fig. 1C–F, upper panels) resulted in irregular, discontinuous distribution at 24 h that ultimately led to loss of staining at some borders at 48 h (arrows). Remarkably, reoxygenation supported re-localization of these proteins (arrows), again suggesting an improved barrier function. During near-anoxia, a drastic and rapid disruption of cell junction proteins occurred with many regions exhibiting complete loss of staining (Fig. 1C–F, lower panels arrows) that only partially recovered during reoxygenation. Thus severe O_2 deprivation irreversibly disrupts cellular morphology and cell junction protein localization.

Cell death of RBE4 cells correlates with severity of O_2 deprivation

We monitored the effect of O_2 deprivation on cell density and survival (Fig. 2A,B respectively). Cell density during normoxic experiments remained stable. Hypoxia significantly decreased cell density from $100 \pm 6.02\%$ at 0 h to $57.72 \pm 17.75\%$ at 48 h then remained constant during reoxygenation ($61.64 \pm 8.9\%$). Near-anoxia induced an early decrease in cell density to $79.75 \pm 14.47\%$ at 24 h reaching a minimal value of $67.79 \pm 22.41\%$ at 48 h. As in hypoxia reoxygenation did not enhance cell density.

Cell survival was monitored by trypan blue exclusion assay (Fig. 2B). Normoxic cell numbers remained constant while hypoxia decreased cell survival by approximately 30% at 48 h. Reoxygenation attenuated this decrease. Interestingly, near-anoxia accentuated the decrease compared to hypoxic and normoxic groups, showing a value of only $42.03 \pm 16.95\%$ at 48 h. Reoxygenation again arrested cell death.

O_2 deprivation-induced cell death involves caspase-3 activation

We measured activation of caspase-3-dependent pathways during O_2 deprivation-induced cell death (Fig. 2C). In hypoxic monolayers activity increased 2 fold (2.18 ± 0.42) at 24 h compared to normoxic conditions and maximal induction occurred at 48 h (3.11 ± 0.48 fold increase) followed by a decrease during reoxygenation to values similar to control levels. Near-anoxic conditions induced an activity peak of approximately threefold (3.30 ± 0.64) at 24 and 48 h suggesting more rapid induction of cell death during near-anoxia. Caspase-3 levels could not be determined in near-anoxic reoxygenated samples due to extremely low concentrations of protein reflecting the severity of the insult. Thus O_2 deprivation activates caspase-3 dependant pathways in endothelial cells in proportion to insult severity.

Astrocytes and pericytes survive extreme O_2 deprivation

Since astrocytes and pericytes do not grow well in RBE4 medium, we investigated whether astrocyte medium culturing conditions affected RBE4 cell viability (supplementary Fig. 2). Astrocyte medium did not alter survival of RBE4 cells during normoxic, hypoxic or near-anoxic conditions (supplementary Fig. 2A) compared to standard RBE4 medium. O_2 deprivation did not affect astrocyte or pericyte survival (supplementary Fig. 2B and C) even after severe stress conditions (48 h at 0.1% O_2). Thus astrocytes and pericytes can survive extreme O_2 deprivation well.

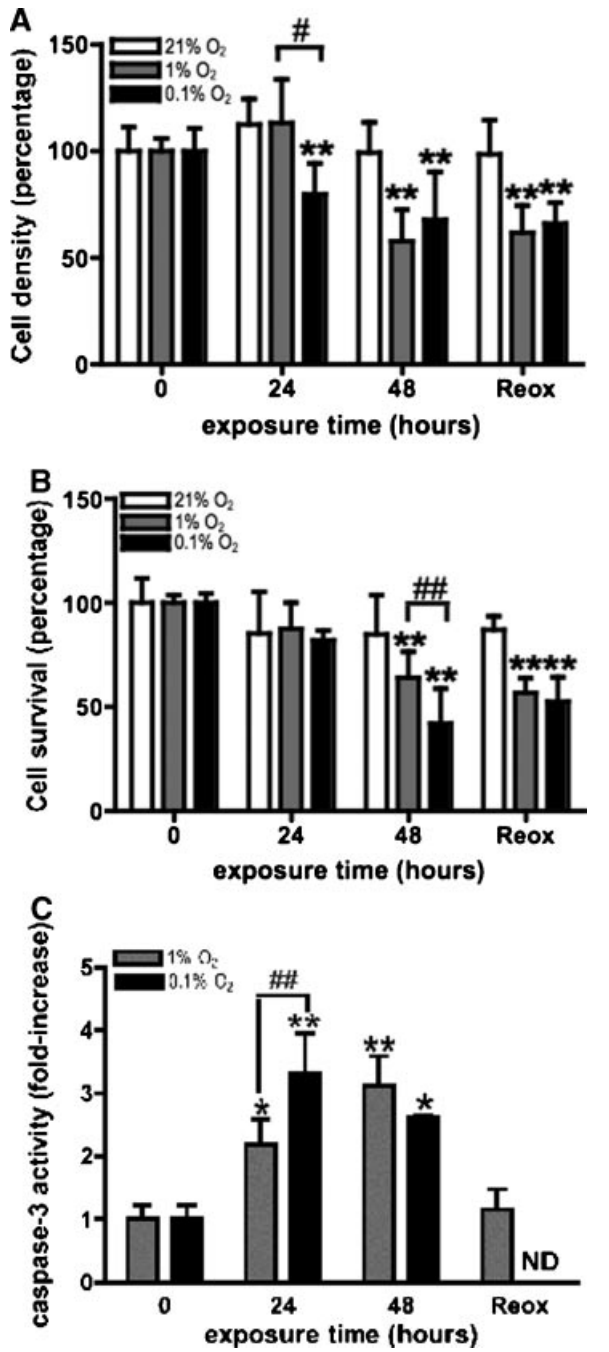

Fig. 2. O_2 deprivation decreased RBE4 cell density and cell viability. Cell density was determined using trypan blue exclusion assay (A). Density of confluent RBE4 monolayers decreased faster in cells exposed to near-anoxia (black bars) than hypoxia (grey bars) and normoxia (open bars). Cell viability was significantly reduced during prolonged O_2 deprivation (B). Caspase-3 activity was up-regulated in RBE4 monolayers during prolonged hypoxia and further induced by near-anoxia (C). ($^{*}P < 0.05$, $^{**}P < 0.01$ compared to the 0 h timepoint; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ comparison between hypoxic and near-anoxic groups, n = 8 per group).

Pericytes and astrocytes augment barrier function during normoxia

Barrier function was measured in RBE4 cells co-cultured in direct contact with astrocytes and/or pericytes as illustrated in

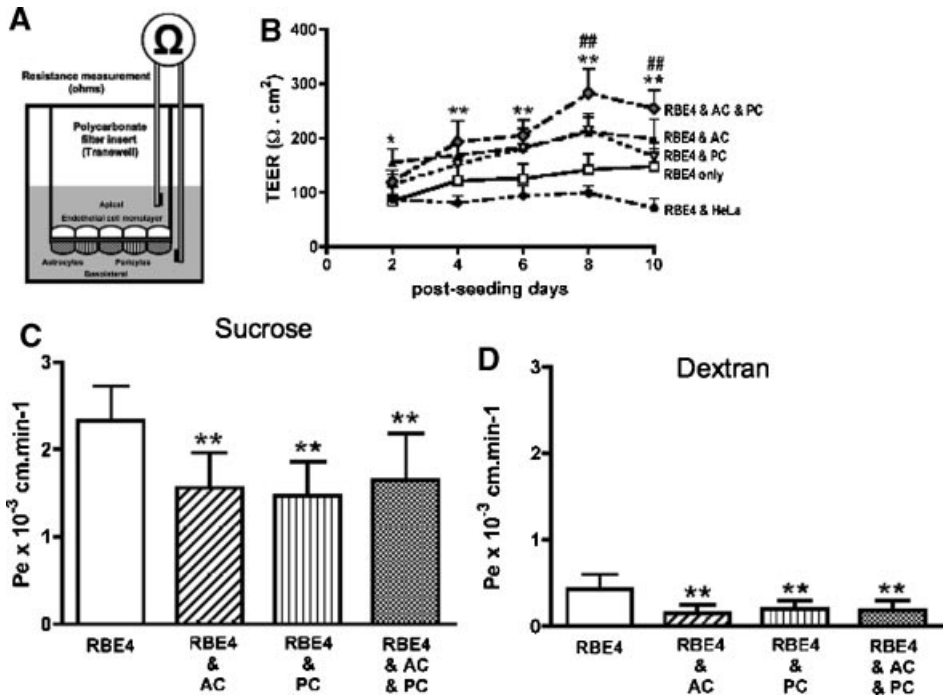

Fig. 3. Astrocytes and pericytes induce barrier properties in RBE4 monolayers. Diagrammatic representation of TEER procedure with RBE4 cells on the topside of the Transwell insert and astrocytes and/or pericytes or HeLa cells on the bottom (A). Astrocytes (AC, solid triangles) and pericytes (PC, open triangles), but not HeLa cells (circles), induce higher TEER values in RBE4 cells compared to monolayers alone (squares; B). Note that TEER increases over time. ($^{*}P < 0.05$; $^{**}P < 0.01$ in comparison between monocultures and co-culture. $^{\#\#}P < 0.01$ for three-cells co-culture vs. two-cells co-cultures, n = 8 per group). Sucrose (C) and dextran (D) permeability assays performed on 8 days cultures demonstrate that both astrocytes and pericytes reduce permeability (Pe) values in RBE4 monolayers. ($^{**}P < 0.01$ in comparison between monocultures and co-cultures, n = 8).

Figure 3A. After 2 days of co-culture with astrocytes (Fig. 3B) RBE4 TEER increased approximately 50% compared to RBE4 cells alone. Interestingly, co-cultures of RBE4 with pericytes, as well as three-cell co-cultures, only increased TEER measurements by ~20% at this time. Subsequently, both astrocytes and pericytes induced the level of resistance by 40% compared to RBE4 monolayers alone. However three-cell co-culture presented the highest TEER of 282.81 ± 45.12 $\Omega\,cm^2$ indicating that astrocytes and pericytes enhance normoxic barrier function and together have synergistic effects. Specificity of astrocyte and pericyte effects was demonstrated by co-culturing with HeLa cells (Fig. 3B). As expected no resistance change was observed confirming that cell-specific interactions and/or release of factors are required to induce endothelial barrier function.

Additionally we performed permeability experiments with radiolabelled sucrose (Fig. 3C) and with FITC-conjugated 40 kDa dextran (Fig. 3D). Monolayers cultured alone always presented highest permeability values. Co-culture with astrocytes, pericytes or both together significantly decreased endothelial monolayer. Notably, higher permeability values were obtained using sucrose due to its small size (360 Da compared to the 40 kDa dextran).

Presence of both astrocytes and pericytes is required to maintain barrier function during prolonged hypoxia

Little is known about the ability of astrocytes and pericytes to maintain endothelial barrier properties during hypoxic stress. We monitored time-dependent TEER changes in RBE4 cells co-cultured with astrocytes and/or pericytes during O_2 deprivation. Short-term hypoxia (Fig. 4A) initially significantly decreased the resistance values of all groups. Co-culture of RBE4 with astrocytes or both astrocytes and pericytes improved TEER values compared to RBE4 cells alone. Unexpectedly, pericyte co-culture exacerbated barrier disruption causing a dramatic decrease (~60%) in resistance within 6 h. Long-term hypoxia also increased permeability of RBE4 monolayers but again co-culture with other vascular-related cells improved barrier function (Fig. 4A). In presence of astrocytes TEER was increased by almost twofold over RBE4 cells alone for up to 48 h and reoxygenation allowed further recovery of electrical resistance. Surprisingly, despite their negative impact in the short-term, pericytes significantly enhanced barrier function to levels comparable with astrocytes during long-term experiments. Notably, presence of all three-cell types was necessary to maintain barrier function at 24 and 48 h wherein TEER values were similar to those at starting timepoints indicating a synergistic effect. As previously shown HeLa did not affect electrical resistance. To confirm the positive role of astrocytes and pericytes in the maintenance of barrier function during O_2 deprivation, we measured alterations in sucrose and dextran permeability (Fig. 4B,C, respectively). RBE4 cell monocultures rapidly increased their permeability to sucrose at 24 h ($4.22 \pm 0.95 \times 10^{-3}$ cm/min) reaching a fivefold increase by 48 h, with a value of $10.60 \pm 1.28 \times 10^{-3}$ cm/min. Notably, presence of astrocytes or pericytes allowed partial maintenance of the barrier function even after 48 h (Fig. 4B). Presence of three cells provided an additional advantage with the lowest increase at 48 h ($2.81 \pm 0.81 \times 10^{-3}$ cm/min). Permeability to dextran remained low throughout the

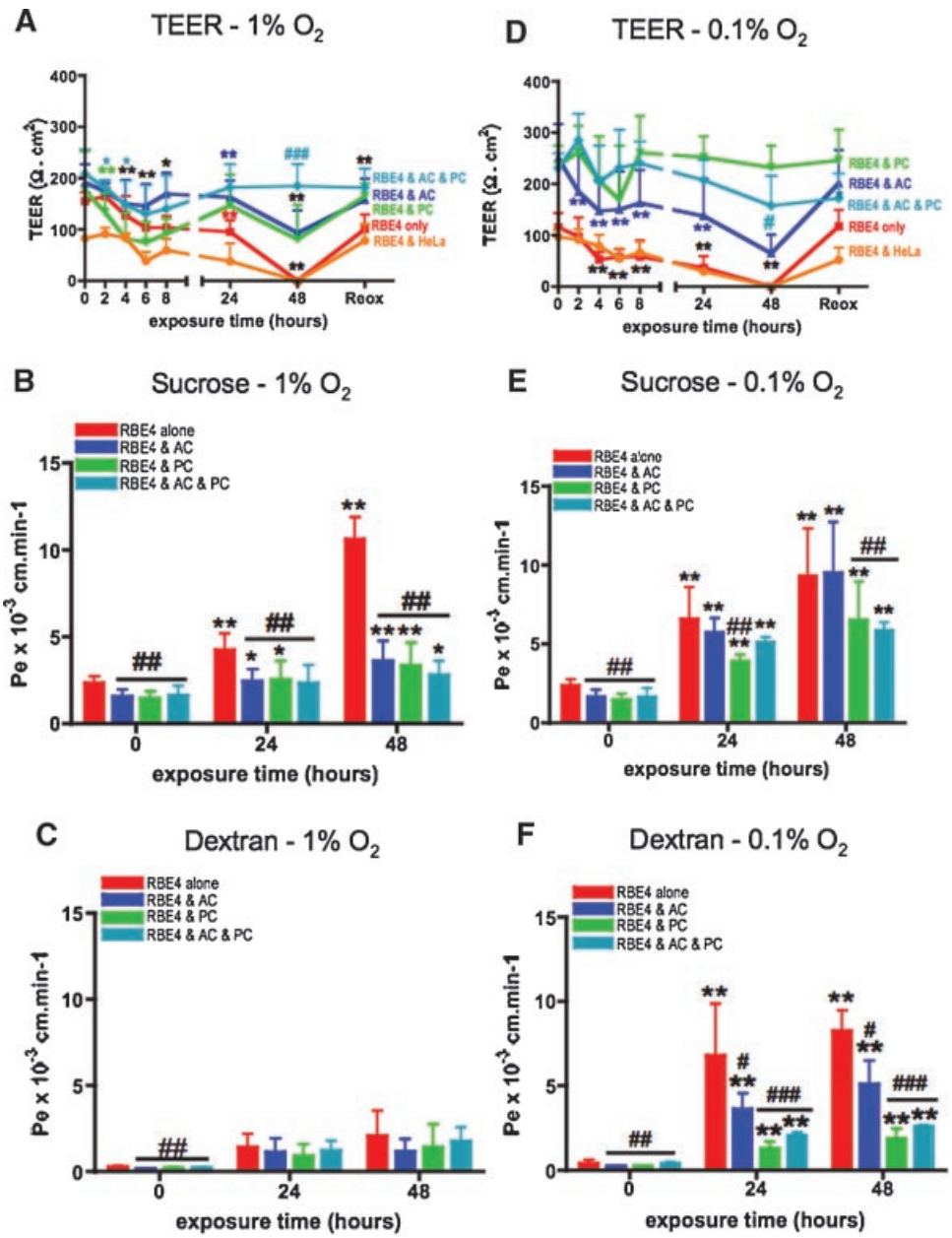

Fig. 4. Astrocytes and pericytes partially maintain barrier function in RBE4 monolayers during O_2 deprivation. (A) Short-term hypoxia significantly decreased TEER in all groups. In particular the pericyte group (RBE4 & PC, green triangles) responded negatively to hypoxia with low TEER values compared to monolayers alone (red squares). Maintenance of barrier function during prolonged hypoxia was partially supported by both astrocyte (RBE4 & AC, blue triangles) and pericyte groups (green triangles) but still remained significantly lower than the initial timepoint. Presence of all three-cells maintained barrier function throughout the experiment. Astrocytes and pericytes both significantly reduced the sucrose permeability increase in RBE4 alone during hypoxia (B), whereas no significant alterations in dextran permeability after hypoxia were observed in any culture group (C). Short-term near-anoxia (D) significantly decreased resistance of RBE4, AC and HeLa groups but not PC and 3 cells groups. Astrocytes were least efficient in maintaining barrier function whereas pericytes performed significantly better. Pericytes and three-cell co-cultures, but not astrocytes, were able to maintain significant lower permeability to sucrose during near-anoxic stress (E). Near-anoxia significantly increased permeability in RBE4 cells but this was attenuated by co-culture with astrocytes and pericytes. Notably at each time point astrocytes performed less well than other co-culture groups (F) (*$P < 0.05$, **$P < 0.01$ in comparison with the initial timepoint; $^{\#\#}P < 0.01$ in comparison between co-cultures and monocultures; n = 5-8 for each timepoint).

experiment, suggesting that hypoxia alone is not sufficient to alter RBE4 permeability to large molecules (Fig. 4C). In conclusion, during chronic hypoxia both astrocytes and pericytes partially maintain barrier function of RBE4 cells, however presence of both cell types confers an additional advantage against hypoxia-induced barrier breakdown.

Pericytes not astrocytes maintain barrier function during near-anoxia

The discrepancy between astrocytes and pericytes in maintaining barrier function was clearly illustrated by severe (0.1%) O_2 deprivation (Fig. 4D). In the short-term RBE4

monolayer TEER was reduced by O_2 deprivation but co-culturing with astrocytes was again advantageous, despite resistance decreasing significantly from 250.44 ± 65.99 to $150.11 \pm 73.85\ \Omega\,cm^2$ at 6 h. Pericyte co-cultures did not prevent a decrease in resistance at 6 h but recovered by 8 h, providing slightly better TEER values over astrocytes. The three-cell group also effectively maintained resistance throughout the experiment similar to results with pericytes.

During prolonged near-anoxia unexpected responses were observed (Fig. 4D). Astrocyte co-culture permeability increased after 48 h ($65.32 \pm 35.89\ \Omega\,cm^2$), although resistance values remained higher than controls. Surprisingly, pericyte co-cultures maintained significantly better TEER values compared to astrocytes both at 24 ($252.34 \pm 40.72\ \Omega\,cm^2$) and 48 h ($232.47 \pm 41.52\ \Omega\,cm^2$) representing a fourfold improvement over controls at all timepoints. The three-cell co-culture displayed intermediate resistance values (between astrocytes and pericytes) with a slight decrease at 24 h ($207.67 \pm 39.16\ \Omega\,cm^2$) followed by a more accentuated reduction at 48 h ($157.45 \pm 58.12\ \Omega\,cm^2$). Additional experiments performed with sucrose (Fig. 4E) showed that RBE4 monolayers exhibited increased permeability to sucrose at 24 and 48 h (6.57 ± 2.03 and 9.32 ± 2.97, respectively). Notably astrocytes did not show any differences compared to RBE4 monocultures, suggesting they do not improve maintenance of the barrier function. Although pericytes co-cultures also showed increased permeability at 24 and 48 h (5.11 ± 0.33 and 5.86 ± 0.50, respectively), the values were significantly lower than monolayers alone or cultured with astrocytes confirming that pericytes and not astrocytes maintain barrier function during prolonged near-anoxia. Similar results were observed with dextran (Fig. 4F). Thus, under severe prolonged O_2 deprivation, astrocytes are less effective than pericytes at preserving barrier function.

Astrocytes and pericytes promote RBE4 cell survival during O_2 deprivation by caspase-3 inhibition

Since activation of caspase-3 correlated with induced cell death in RBE4 during O_2 deprivation, we investigated the possible modulation of capsase-3 activity in endothelial monolayers by astrocytes and pericytes. Notably, hypoxia-mediated activation of caspase-3 in RBE4 cells was abrogated by co-culture with astrocytes after 24 h (Fig. 5A). However when co-cultured with

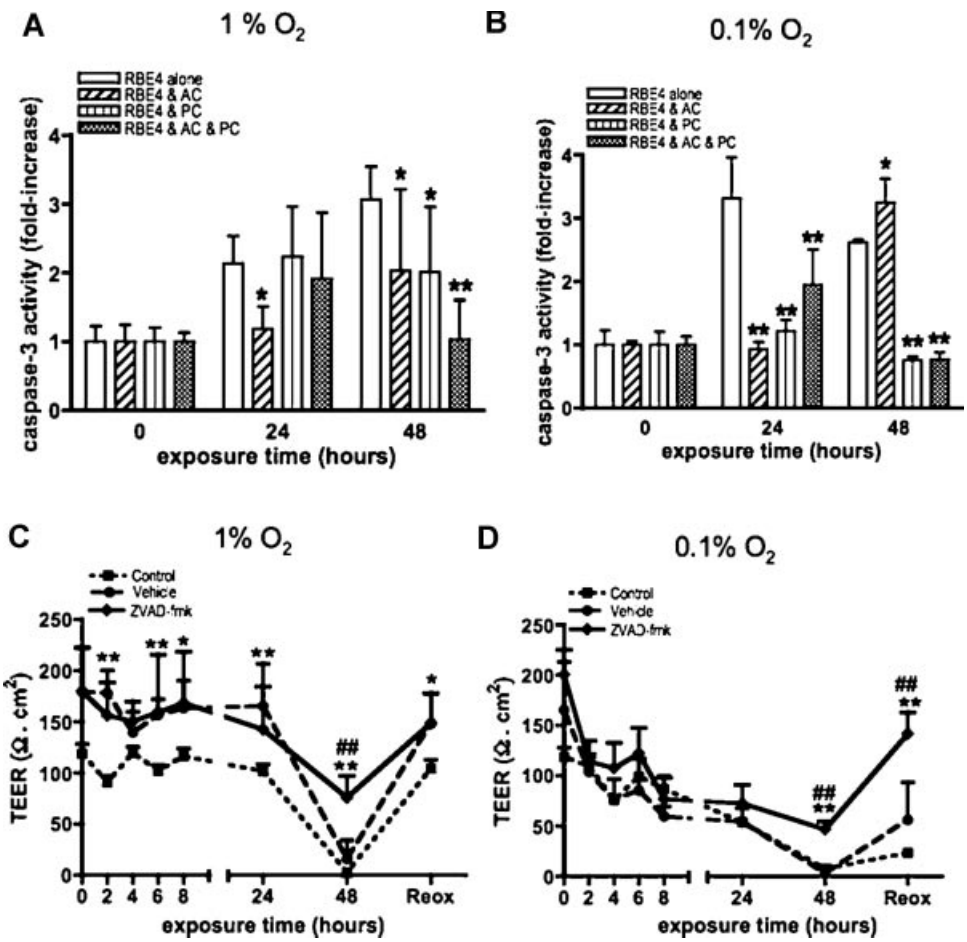

Fig. 5. Caspase-3 inhibition by co-cultures or by pharmacological agent allows better barrier maintenance and recovery during prolonged O_2 deprivation stress. Culture of RBE4 cells with astrocytes and pericytes reduced caspase-3 activation under hypoxia (A). Astrocytes performed better than pericytes at 24 h whereas both cells significantly prevented activation after 48 h. All cells reduced caspase activity after 24 h at 0.1% O_2. Pericytes but not astrocytes maintained low caspase-3 activity during prolonged (48 h) severe oxygen deprivation (B). In RBE4 monocultures caspase inhibition by ZVAD-fmk significantly improved barrier function during prolonged hypoxic stress (C) and during prolonged near-anoxia (D). ($^{*}P < 0.05$; $^{**}P < 0.01$ vs. control; $^{\#\#}P < 0.01$ vs. vehicle; n = 4).

pericytes, RBE4 caspase-3 activity levels remained unchanged at 24 h indicating that pericytes unlike astrocytes do not attenuate early caspase-3 activation (Fig. 5A). Subsequently activation increased in both groups by 48 h but notably remained significantly below that of control cells. The three-cell group, like pericytes, initially displayed higher activity at 24 h (1.91 ± 0.96) but in contrast to other groups completely attenuated activation at 48 h (Fig. 5A).

Similar to hypoxic results, astrocyte co-culture completely prevented caspase-3 activation in near-anoxic endothelial monolayers for up to 24 h (Fig. 5B). However this anti-apoptotic effect was completely lost after 48 h when activity significantly increased (3.24 ± 0.38) compared to even RBE4 alone (2.62 ± 0.04). In contrast, pericyte co-culture attenuated caspase-3 activity in RBE4 cells at both 24 (1.21 ± 0.18) and 48 h (0.76 ± 0.06) again suggesting that pericytes, but not astrocytes, play an important role in endothelial cell survival during prolonged severe O_2 deprivation. Endothelial cells co-cultured with both astrocytes and pericytes showed an increase at 24 h (1.94 ± 0.56) followed by a decrease to basal levels at 48 h (Fig. 5B). To confirm the importance of caspase-3 inhibition on the maintenance of barrier function in RBE4 cells, we exposed monolayers to O_2 deprivation in presence of ZVAD-fmk, a pan caspase inhibitor (Fig. 5C,D). Treatment with the caspase inhibitor did not induce any significant change in TEER compared to vehicle-only treated monolayers during short-term hypoxia (up to 24 h, Fig. 5C). However, after prolonged hypoxic exposure inhibitor treated monolayers maintained better resistance values than vehicle and non-treated samples ($76.19 \pm 21.07\ \Omega\,cm^2$ and $16.07 \pm 18.25\ \Omega\,cm^2$, respectively at 48 h). Notably, reoxygenation reinstated the barrier function in all cases (Fig. 5C).

Presence of the inhibitor provided an advantage only during prolonged near-anoxia (Fig. 5D) by maintaining significantly higher electrical resistance at 48 h timepoint ($3.34 \pm 7.48\ \Omega\,cm^2$ in non-treated vs. $46.89 \pm 7.85\ \Omega\,cm^2$ in treated cells). Importantly, this effect was still evident in the reoxygenation phase with treated-samples recovering more than 70%. Thus inhibition of caspase-dependent pathways provides beneficial during prolonged O_2 deprivation as well as during reoxygenation phase.

Inhibition of VEGF signaling prevents hypoxia-driven increased permeability

Vascular endothelial growth factor (VEGF) constitutes one of the most potent soluble factors described to be induced by O_2 deprivation and to induce hyperpermeability in endothelial cells. To appreciate the importance of paracrine VEGF signaling as an effector of hypoxia-induced barrier breakdown, we treated RBE4 mono- and co-cultures with 10 μM SU1498, a selective VEGF receptor-2 (VEGFR-2) inhibitor and measured changes in TEER (Fig. 6). In RBE4 cell monocultures exposed to hypoxia in presence of inhibitor (Fig. 6A), we observed a significant increase of electrical resistance after 6 h that was maintained throughout hypoxic exposure and the reoxygenation phase. Remarkably, VEGF inhibition during near-anoxia also improved barrier function compared to control and vehicle groups (Fig. 6B). Treatment of astrocyte co-cultures with SU1498 also induced better maintenance of the barrier function after 24 h of hypoxia (Fig. 6C). Again, such positive effects were evident during near-anoxia (Fig. 6D), suggesting an important role of VEGF paracrine signaling between astrocytes and RBE4 cells during hypoxia and near-anoxia. Treatment of pericyte co-cultures with SU1498 also abrogated the breakdown observed during short- and long-term hypoxia (Fig. 6E). Remarkably the inhibitor did not provide any notable difference in pericyte co-cultures during near-anoxia (Fig. 6F), suggesting that inhibition of VEGF/VEGFR-2 signaling might occur endogenously. Interestingly, in the three-cell co-cultures inhibitor treatment had no effect on the barrier function of any of the groups either during hypoxia or during near-anoxia (Fig. 6G,H), pointing to a possible abrogation of VEGF/VEGFR-2 signaling in presence of all three cell types.

Discussion

O_2 deprivation constitutes a critical factor that compromises the integrity of cerebral tissue and is implicated in many cerebrovascular diseases. Remarkably cellular responses that trigger BBB breakdown are only poorly understood. This study directly compares the effects of astrocytes and pericytes on BBB integrity during both mild and severe O_2 deprivation. The investigation of different O_2 levels is very relevant when considering exposure to physiologically occurring hypoxia (as in high altitude exposure) or severe prolonged insult as arises during stroke or other disease states. In particular, this work underscores an important role for pericytes and advocates that astrocytes may not always protect the BBB.

Although endothelial cells maintained barrier integrity during acute severe O_2 deprivation, prolonged O_2 withdrawal severely impaired barrier function in a time-dependent manner. Our data agrees with studies showing that hypoxia decreases ZO-1 levels and increases phosphorylation and protein redistribution (Fischer et al., 2002) as well as other junctional-associated proteins such as occludin (Park et al., 1999), cadherin (Krizbai et al., 2005), and claudin-5 (Koto et al., 2007) in various microvascular cells. Similar to work in human cerebral microvascular endothelial cells (Lee and Lo, 2003) we observed that hypoxia-induced cell death was mediated at least partially through caspase-3 activation. Moreover, preservation of barrier function in RBE4 cells during long-term O_2 deprivation stress by inhibition of caspase-dependent pathways points to direct involvement of cell death mechanisms in barrier breakdown. In contrast to others however, reoxygenation for 24 h led to complete abrogation of caspase-3 activation rather than further induction (Lee and Lo, 2003)—a difference probably due to variation in experimental set-up and degree of O_2 deprivation. Nevertheless this data together with other reports (Chow et al., 2001) underlines the importance of caspase signaling pathways in endothelial cell survival. Thus severity of O_2 deprivation determines not only the rate of loss of barrier function but also reversibility of disruption and thus cerebral tissue recovery, likely involving cytoskeletal changes and deregulation in the balance between cell survival and death.

Both astrocytes (Isobe et al., 1996; Garcia et al., 2004; Haseloff et al., 2005) and pericytes (Balabanov and Dore-Duffy, 1998; Lindahl, 2007) have been implicated in the maintenance and stabilization of the BBB. Like astrocytes, pericytes also secrete factors that upregulate expression of tight junction proteins in vitro (Hayashi et al., 2004; Hori et al., 2004) and in vivo (Hellstrom et al., 2001) and their absence leads to increased brain vascular leakage and edema (Hellstrom et al., 2001). Our data now suggests pericytes are probably as important as astrocytes in BBB maintenance during physiological conditions. This study advocates that O_2 deprivation profoundly and differentially affects BBB cells. Particularly during acute hypoxic exposure, in contrast to astrocytes, pericytes surprisingly exacerbated barrier disruption. Nevertheless in the long-term pericytes performed as well as astrocytes. In contrast to our findings, Hayashi et al. 2004 described pericyte protective effects after 6 h of hypoxia in endothelial/pericytes co-cultures. However their cell isolation methods and experimental paradigm differs significantly from ours. Notably, an O_2-free anaerobic jar was used to obtain hypoxic conditions. Although the O_2 tension

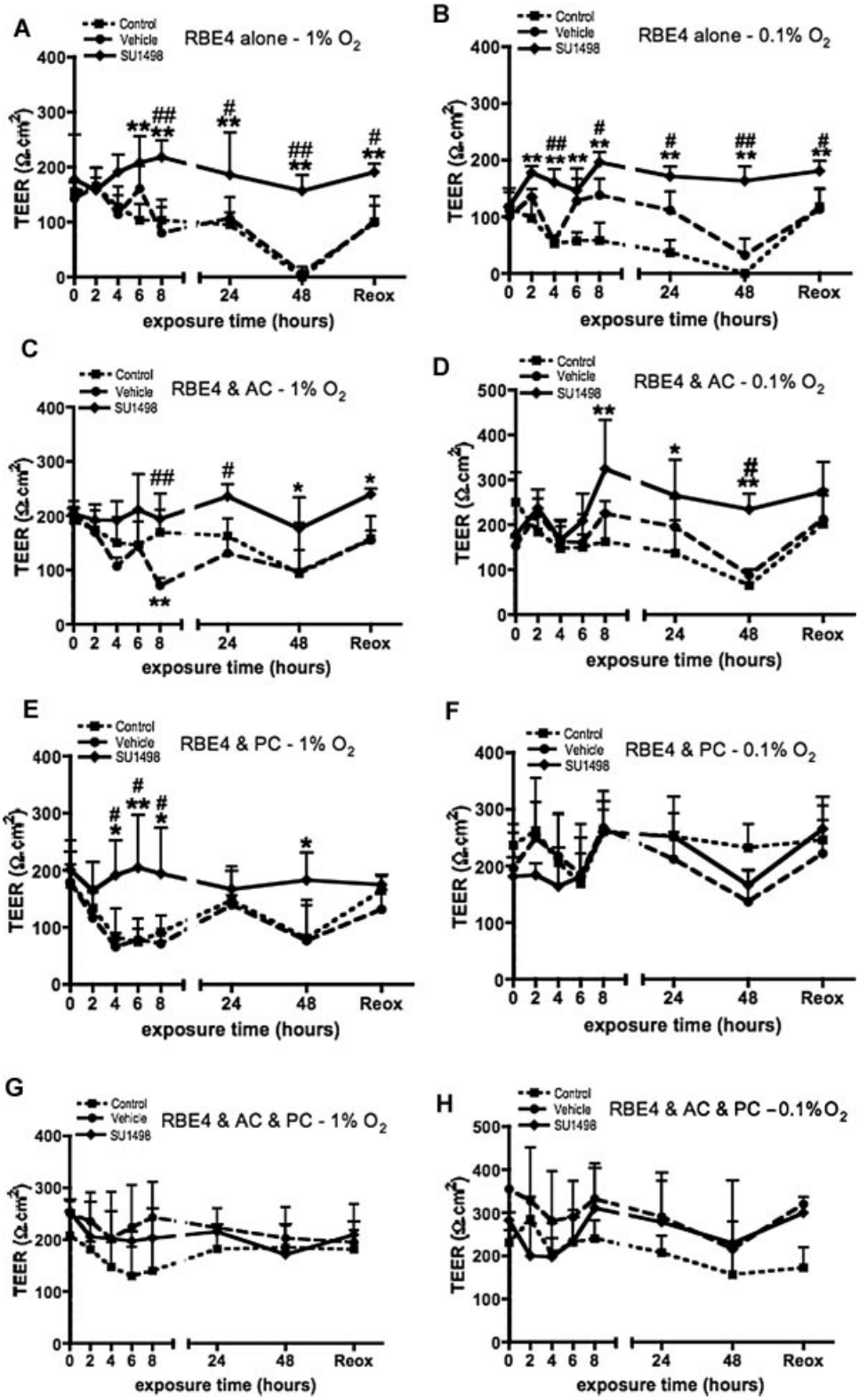

Fig. 6. Inhibition of VEGF signaling improves barrier function during O_2 deprivation. Incubation of RBE4 monolayers with SU1498, a VEGFR-2 inhibitor improved barrier function during hypoxic (A) and near-anoxic stress (B). Interestingly astrocytes (AC) co-cultures showed enhanced TEER values in presence of inhibitor during hypoxia (C) and this beneficial effect was even accentuated during near-anoxia (D). Inhibition of VEGFR-2 improved pericytes (PC) response compared to non-treated groups at almost all timepoints during hypoxia (E). Surprisingly, presence of SU1498 did not provide additional advantage during near-anoxia (F). Presence of inhibitor in the three-cells co-culture did not provide further improvement either during hypoxic (G) or near-anoxic (H) stress, suggesting endogenous inhibition of VEGF/VEGFR-2 paracrine signaling pathway. ($^{*}P < 0.05$; $^{**}P < 0.01$ for significant differences vs. control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ between vehicle and SU1498 groups; n = 3).

measured was approximately 1% after 6 h, such instruments do not allow proper control of O_2 tension over time per se. In our model, O_2 levels were continuously monitored and maintained constant with all measurements performed within the chamber without reoxygenation. Furthermore Hayashi et al. did not obtain significant resistance values for the endothelial culture alone or compare effects of other cells on monolayer permeability during shorter or longer exposure timepoints. Thus, a direct comparison of our results with this study is not possible.

We demonstrate that during long-term hypoxia the presence of both cell types has a synergistic effect that enables re-establishment of barrier function, emphasizing the importance of investigations such as this in addition to single or two cell model. This point is further underscored by effective attenuation of hypoxia-induced caspase-3 activity in endothelial monolayers co-cultured with both cell types to different degrees, inferring the involvement of release of pro-survival factors by both astrocytes and pericytes. The mechanism by which astrocytes and pericytes block activation of caspase-3 and prolong endothelial cell survival remains unclear but may occur through inhibition of p38 signaling preventing caspase activation (Lee and Lo, 2003; Eguchi et al., 2007). Whether co-culture with astrocytes and/or pericytes alters p38 or other signaling pathways, is now under investigation.

Surprisingly, pericytes out-performed astrocytes during severe O_2 deprivation and the three-cell culture did not confer any additional advantage indicating that only pericytes maintain barrier function at this time. Furthermore, the observation that astrocytes cause substantial barrier disruption during extreme O_2 deprivation is also intriguing. As neither astrocytes nor pericytes showed decreased cell survival during extreme O_2 deprivation the discrepancy observed with astrocytes seems attributable to differences in released factors. Astrocytes and pericytes secrete many common factors but VEGF is of particular interest. VEGF is hypoxia-inducible and due to its potent permeability inducing characteristics is frequently associated with BBB disruption in vitro (Kaur et al., 2006; Yeh et al., 2007) and in vivo (Schoch et al., 2002). On the other hand, VEGF is also a survival factor for endothelial cells and is constantly secreted by astrocytes under normal conditions (Ogunshola et al., 2000; Schoch et al., 2002). Since hypoxia-induced VEGF secretion by astrocytes promoted survival of RBE4 cells (Chow et al., 2001), it follows that astrocyte VEGF may also support barrier function by increasing endothelial cell survival. We have shown that VEGF secretion by astrocytes is potentiated by the severity of O_2 deprivation (Schmid-Brunclik et al., 2008). Thus excessive VEGF production and secretion by astrocytes during prolonged near-anoxia may result in high local concentrations that significantly disrupt endothelial cell barrier function. This notion is supported by our findings that VEGFR-2 inhibition reverses barrier breakdown in endothelial cells. Although direct comparison of VEGF expression by astrocytes and pericytes during O_2 deprivation has not been performed so far, it is interesting to note that Nomura and colleagues (Nomura et al. 1995) reported reduced VEGF expression by pericytes. This could contribute to the inability of pericytes to protect against caspase-3 induction and increased permeability during early hypoxic exposure due to reduced levels of protective VEGF. During chronic insult however lower VEGF concentrations may be advantageous and cytoprotective. Indeed, co-culture with pericytes alone was effective during severe O_2 deprivation.

VEGF/VEGFR-2 signaling by astrocytes and pericytes plays an important role in hypoxia-driven BBB breakdown. Receptor inhibition promoted recovery of barrier function in RBE4 monolayers either during hypoxia or near-anoxia, likely by inhibition of VEGF autocrine loop. In astrocyte co-cultures inhibition of VEGFR-2 signaling supported the notion that elevated VEGF levels constitute a negative factor for the barrier function during O_2 deprivation stress. Surprisingly, pericytes co-culture had differential responses to SU1498 treatment during hypoxia and near-anoxia. Although the nature of VEGF signaling between endothelial cells and pericytes remains unclear, it was shown that pericytes express VEGFR-1 only whereas endothelial cells express both VEGFR-1 and VEGFR-2 (Nomura et al., 1995). Thus, the positive effect of VEGFR-2 inhibition observed during hypoxia likely targets the endothelial cell and raises the hypothesis that O_2 deprivation-induced endothelial VEGF signaling results in secretion of negative factors towards pericytes. Such a candidate might be angiopoietin-2 that, like VEGF, is hypoxically up-regulated in endothelial cells (Pichiule et al., 2004) and astrocytes (Beck et al., 2000) and can induce pericyte loss and vessel destabilization (Hammes et al., 2004). Notably, no enhancement of TEER values in our three-cell co-cultures under any condition suggests that cell-specific synergy during O_2 deprivation prevents VEGFR-2 activity and contributes to improved barrier integrity.

Another important factor secreted by pericytes that reduces permeability and has been demonstrated to be important in blood retinal barrier (Wang et al., 2007) and BBB (Hori et al., 2004) stabilization is angiopoietin-1. Indeed many growth factors secreted by both astrocytes and pericytes are also likely to modulate permeability of the BBB although current evidence is very limited. Reports of secretion of TGF-β (Garcia et al., 2004; Dohgu et al., 2005), erythropoietin (Chavez et al., 2006; Chi et al., 2008), glial derived neurotrophic factor and neurotrophin (Lin et al., 2006) by astrocytes and/or pericytes during cerebral hypoxia/ischemia reveals the diversity of hypoxically regulated factors that might promote barrier maintenance and cell survival in endothelial cells.

Surprisingly, presence of DMSO also modulated TEER values in endothelial cells and astrocyte co-cultures but not in pericyte and three-cell co-cultures. DMSO is a non-specific free radical scavenger and a positive effect of DMSO treatment during reperfusion injury in cerebral ischemia by partially decreasing lesion size and by partial inhibition of matrix metalloproteinase-2 (MMP-2) and MMP-9 activities was demonstrated (Nagel et al., 2007). Indeed, ROS production might constitute an additional factor that elicits barrier breakdown in endothelial cells during O_2 deprivation, raising questions concerning possible differences between astrocyte and pericyte ROS production or antioxidant roles during such conditions.

Nevertheless, our observations clearly demonstrate that unique responses of astrocytes and pericytes to O_2 deprivation have major consequences for vascular regulation and cell survival. These observations are of vital importance considering various conditions and disease states that lead to systemic and/or local hypoxia in brain regions and other vascular beds. Indeed modulation of barrier function under physiological and pathological conditions constitutes a complex mechanism involving important molecular and cellular interactions between the vascular endothelium and surrounding cells. Notably, pericytes seem to have an essential role in maintaining BBB integrity particularly during severe O_2 deprivation. To be able to identify suitable molecular and cellular therapeutic targets to combat BBB disruption, future investigations must focus not only on understanding how the individual components of the neurovascular compartment function, but also as a unit in its entirety.

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The blood-brain barrier on a dish: designing an innovative 3-dimensional in vitro model

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ABSTRACT

Formation of the vascular tree constitutes a crucial event in vertebrate development. The cerebral vasculature differs from other vascular beds by the presence of the blood-brain barrier (BBB). The BBB, consisting of specialized endothelial cells surrounded by astrocytes and pericytes, plays a crucial role in cerebral homeostasis. Despites BBB breakdown is associated with many neurological diseases, the availability of an accurate *in vitro* BBB model that mimic *in vivo* situation do not yet exist. In this study, we attempted the design of an innovative 3-dimensional *in vitro* BBB model and performed its characterization. In our model, brain endothelial cells were able to form tube-like structures by vasculogenesis and angiogenesis. Remarkably, astrocytes and pericytes were able to provide cellular interactions with such tube-like structures similar to *in vivo* situation. Such interactions correlated with the ability of astrocytes and pericytes to modulate angiogenesis and tube-like structure morphology. In addition, presence of astrocytes and/or pericytes was necessary to induce appropriate TJ complexes formation and lumen polarization.

In conclusion, we designed and characterized an innovative 3-dimensional model that allows reproduction of many features described *in vivo,* but also proper interactions between the different cells of the neurovascular unit. Such model will hopefully become an useful tool to foster our understanding of the BBB as a neurovascular unit during physiological and pathological situations.

INTRODUCTION

The blood-brain barrier (BBB) is a neurovascular unit formed by specialized endothelial cells (ECs), pericytes and perivascular astrocytes [4,5]. ECs present at the BBB display a barrier phenotype characterized by presence of tight junctions (TJ) between endothelial junctions [6] thus limiting diffusion of water-soluble compounds from the blood to the cerebral tissue. In addition, such barrier acts as a selective filter towards lipophilic molecules by expression of ATP-binding cassette transporters in their luminal and abluminal side [7]. Astrocytes and more recently pericytes have been shown to induce barrier phenotype in brain ECs using both *in vitro* [8,9] and *in vivo* [10-12] models. Despites the induction of barrier phenotype involves secretion of soluble factors by astrocytes and pericytes the nature of most of these factors remains unknown. In addition, different studies highlighted the importance of contacts between the different cell types in order to obtain optimal induction; *in vitro* models of the BBB (relying on the usage of semi-porous membranes) dramatically restrict appropriate cellular interactions between the different cells of the neurovascular unit and thus require the usage of innovative cell culturing conditions.

The aim of this study was to design an innovative *in vitro* model of the BBB based on collagen, a scaffold matrix widely used to study vasculogenesis and angiogenesis in vitro [18-20]. This study provides an exhaustive characterization of our newly developed innovative *in vitro* BBB model. Our model was developed using the RBE4 cell line, a brain rat brain EC line that maintains a certain barrier phenotype, coupled with primary rat brain astrocytes and pericytes. Our 3-dimensional cell culture system remarkably allowed brain ECs to form a tube-like structure network sharing many similarities with *in vivo* capillary structures as observed by the presence of a 3-dimensional network and the presence of a lumen within the tubes. In addition, co-

cultures of these tubes with astrocytes resulted in formation of astrocytic end-feet processes overlapping RBE4 tube-like structures. In the other hand, pericytes directly overlapped tubes with their cellular bodies.

RESULTS

Collagen matrix initiates vasculogenesis in RBE4 cells

We primarily focused on the ability of a 3-dimensional collagen matrix to induce tube formation in RBE4 cells (Fig.1b) cultured alone. Remarkably, RBE4 cells were able to form cyst structures. These cysts were clearly visible after 1 day of culture, singularly contrasting from single cell suspension observed during the first hour of culture (Fig.1a). A staining of such structure by fluorescence-based immunocytochemistry (Fig.1b, lower panel) revealed that indeed cyst formation appeared as a multicellular complex formed by ZO-1 positive cells. Cysts evolved over time, as observed by formation of sporuting processes after 2 days and clearly visible for up to 4 days. These sprouting processes by which originated the intercystal connection (Fig.1b, upper panel) appeared to form a primary endothelial network. PI staining (Fig.1b, lower panel) suggests that such intercystal connection may be made from single ECs. Interestingly, further cultivation of RBE4 monocultures allowed evolution of these intercystal connections to a more elaborated structure, a “tube-like” structure that distinguished from intercystal connections by the absence of cysts in both extremities but also by the presence of swollen areas within these tubes (see arrow). The immunocytochemistry demonstrated that these tube-like structures were indeed multinucleated and therefore suggest that such tube may be a multicellular complex. It is interesting to note that such structures were clearly absent from astrocytes and pericytes monocultures (Supplemental fig.1) demonstrating not only

the purity of our primary cell cultures but also that the unique phenotype observed with ECs may represent the hallmark of vasculogenesis and angiogenesis processes.

The collagen matrix allows BBB cells to form a 3-dimensional cellular network

Although most of the cellular structures observed in the matrix were exclusively visible in RBE4 monocultures, we observed that astrocytes and pericytes were able to organize a cellular network between 4 and 6 days of cultures (Supplementary fig.1). Therefore we investigated if the different cell types of the BBB were able to form a three-dimensional network. In order to clearly observe such cellular network we stained each cell types with specific markers (α-SMA, GFAP and PECAM-1 for pericytes, astrocytes and RBE4 cells respectively) and subsequently performed a z-stack image reconstruction (Fig.1c-e). RBE4 cells 3-dimensional network (Fig.1c) distinguished from the other monocultures by the presence of reminiscent cysts. It also shows that RBE4 cells monocultures appear as a heterogeneous network as we can observe cysts (white arrow), intercystal connections (white dot) and tube-like structures (white lozenge). In contrast, both astrocytes (Fig.1d) and pericytes (Fig.1e) monocultures appeared more homogenous as observed as a 3-dimensional branched cellular network. Again, these results highlight the singularity observed with RBE4 cells monocultures and therefore we will focus mostly on variations of RBE4 cell morphogenesis during co-culturing conditions in our further experiments.

RBE4 tube-like structures form a lumen

In order to assess the similarity between the tube-like structures observed and *in vivo* capillaries, we investigated RBE4 tube-like structures by electron microscopy (Fig.1f & 1g). Tube-like structures clearly distinguish from collagen fibers of the

extracellular matrix (ecm) by its rectilinear shape and by its average size, but also presence of endothelial cell organelles (Fig.1f). In some sections that medially cut these tube-like structures, we can clearly observe presence of a hollow structure that appears as a lumen (lum) delimited by two endothelial cell layers (endo) that clearly makes the separation with the extracellular matrix (ecm). It is also interesting to note the presence of a nucleus (nuc) facing the lumen, suggesting that these tube-like structures share many similarities with *in vivo* capillaries including the presence of a lumen.

Collagen down-regulated increase in astrocyte and pericyte cell densities

We firstly quantified cell density in our different cultures (Fig.2a) to understand if the formation of a 3-dimensional network by each cell types was associated with an increased cell population. Interestingly, RBE4 cells cell density remained low after 2 days of cultures (1.74 ± 0.26) and showed significant increase only after 4 and 6 days (6.52 ± 1.70 and 13.30 ± 2.20 fold-increase respectively). Whereas pericyte monocultures also showed an increase cell density over time, the cellular proliferation rate appeared much modest than RBE4 cells, reaching a maximal value of only 4.50 ± 0.51 after 6 days. Astrocyte monocultures remarkably differed from the two other monocultures by a constant cell density over time, showing an average value of 1.36 ± 0.54 after 4 days. These results suggests that formation of a 3-dimensional network does not necessarily requires an increase in cell population over time but also suggests that the collagen extracellular matrix may acts differently on the proliferation of each cell type. Thus it is important to consider such parameter for the establishment of co-cultures that respect cell ratios obtained *in vivo*.

Quantification of RBE4-associated vasculogenesis and angiogenesis

Characterization of RBE4-associated cellular features constitutes an important step for the standardization of our model. Therefore we quantified the different features that chronologically appeared in our model and monitored changes over time.

We firstly quantified cysts number per droplet over time (Fig.2b) to observe a possible correlation between increased cell density and cysts number. Notably cyst numbers remained stable over time, with a minimal value at day 1 (137.2 ± 33.01 cysts/droplet) and a maximal value at day 2 (166.7 ± 34.90), suggesting that increase in cell density unlikely correlates with cysts number over time. Moreover, we quantified intercystal connections formed over time in RBE4 monocultures and normalized against cysts number (Fig.2c). Despites intercystal connections number was low even after 2 days of cultures (0.08 ± 0.01 intercystal connection/cyst), their number rapidly increased reaching a maximal value of 0.59 ± 0.11 after 6 days. Furthermore, such increase in intercystal connections was accompanied by an increase in intercystal connections length (Fig.2d). We observed that the median length significantly increased from 70.79μm at day 2 to 83.25μm at day 4 and finally reached a maximal value of 146.6μm at day 6. In parallel, increased length correlated with increased diameter (Fig.2e) as we observed an increase from 9.91μm at day 2 to 21.47 μm at day 6. These results suggests that intercystal connections formation constitute a dynamic process over time but also demonstrate that these formed intercystal connections undergo a certain maturation process since we observed a continuous increase in both length and diameter.

Tube-like structures formation translates an angiogenic activity

In order to quantify tube-like structures from intercystal connections, we performed a series of immunocytochemistry in 6-days old cultures; obtained values were directly compared to intercystal connections (Fig.2f-h). Notably we observed 4-times more tubes than intercystal connections in our experimental setup (Fig.2f), showing values of 1.93 ± 0.02 versus 0.59 ± 0.11 features/cyst respectively. Despites median length (Fig.2g) appeared similar between intercystal connections and tube-like structures (146.6 and 154.3µm respectively), the heterogeneity observed in the tube-like structure group resulted in significant difference in population distribution between the two groups. Minimal values obtained for intercystal connections and tube-like structures were 86.8 and 68.6µm respectively, maximal values were 202.5 and 290.4µm. At the opposite, diameter comparison (Fig.2h) outlined that tube-like structure diameter was significantly smaller than intercystal connections, showing median values of 16.44 and 21.47µm respectively. In conclusion, tube-like structures appeared as a dynamic process involving *de novo* formation but also a morphological evolution of pre-existing intercystal connections and thus reflect the presence of an angiogenic activity over the intercystal connection mediated-vasculogenesis.

Astrocyte and pericyte co-cultures do not impair RBE4 vasculogenesis, but down-regulate RBE4 cell proliferation.

As addition of astrocytes and pericytes cannot be performed after tube like structure onset, we simultaneously co-cultivated RBE4 cells with astrocytes and/or pericytes and monitored possible impairment in RBE4 vasculogenesis/angiogenesis (Supplemental Fig.2a). Decrease in initial RBE4 cell density from 500.000 cells/mL to 250.000 cells/mL as well as presence of astrocytes and/or pericytes did not interfere with RBE4 morphogenesis as we observed cysts, intercystal connections and

ultimately tube-like structures formation in the different co-cultures. Furthermore, we quantified cell density in our different culturing conditions (Fig.3a). Decrease in initial RBE4 cell density did not affected cell density increase over time as observed in our previous culturing condition (Fig.2a), reaching a maximal value of 6.78 ± 2.18 fold-increase after 6 days of culture. Interestingly, presence of astrocytes at a ratio of 5:1 (astrocytes/RBE4) resulted in absence of significant increase over time, showing a maximal value of 1.14 ± 0.15 at 6 days. Similar observation were performed in RBE4 cells co-cultured at a ratio of 1:1 displaying a value of 1.17 ± 0.46 fold-increase after 6 days of culture. Presence of both astrocytes and pericytes resulted in a value of 1.52 ± 0.85 in 6 days-old co-cultures. These results suggests that astrocytes and pericytes may down-regulate RBE4 cells proliferation, thus may result in angiogenesis modulation.

Astrocytes and pericytes down-regulate both RBE4 cysts and intercystal connections number

We further investigate whether cell density maintenance over time was associated with a decrease in cysts and intercystal connections number. We firstly investigated the possible cysts number modulation by astrocytes and pericytes (Fig.3b). Astrocyte co-cultures showed significant decrease after 6 days compared to RBE4 monocultures (79.19 ± 26.89 cysts/droplet). Outstandingly, pericyte co-cultures outlined other cultures by showing significant lower values than RBE4 monocultures already after 2 days of cultures, reaching the lowest value of all groups after 6 days (31.42 ± 6.85 respectively). However, it is important to denote that presence of both astrocytes and pericytes resulted to similar effect than astrocyte co-cultures (79.00 ± 22.49 cysts/droplet at 6 days). These results suggest that the blunted cell density

increase observed in our co-cultures correlates with decrease cysts formation, but also highlighted that pericytes may have a distinct effect compared to astrocytes on RBE4 morphogenesis.

Thus, we further investigated intercystal connections formation in our co-cultures (Fig.3c). As expected, intercystal connections number significantly increased from 2 days (0.087 ± 0.010) to 6 days (0.59 ± 0.11) in RBE4 cells monocultures. Astrocyte co-cultures attenuated such increase reaching a value of 0.031±0.008 after 6 days whereas pericyte co-cultures showed a value of 0.157±0.161 intercystal connections/cyst. Presence of both astrocytes and pericytes resulted in an intermediate value of 0.096 ± 0.064. These results suggest to us that astrocytes and pericytes down-regulate formation of intercystal connections ad thus may modulate RBE4-mediated vasculogenesis. However, neither astrocytes nor pericytes significantly induced changes in intercystal connections length or diameter (Supplemental Fig.2b & 2c) and therefore may suggest that the impact of astrocytes and pericytes on vasculogenesis may be limited to certain aspects.

Astrocytes and pericytes oppositely regulates RBE4 angiogenesis

As our previous results suggested a limited contribution to RBE4 vasculogenesis, we investigated whether astrocytes and pericytes were able to modulate tube-like structures and subsequently to play a role in angiogenesis. We firstly counted the number of tube-like structures present in our different cultures and normalized it to the number of cysts (Fig.3d). Interestingly, astrocyte co-cultures did not display significant differences compared to RBE4 monocultures (1.80 ± 0.15 and 1.92 ± 0.02 tubes/cyst) whereas pericyte co-cultures outperformed all other groups by showing a value of 13.55 ± 1.05 tubes/cyst demonstrating the important pro-angiogenic effects

carried on by pericytes. Surprisingly, presence of both astrocytes and pericytes abolished the pro-angiogenic effect carried on by pericytes as we measured an average value of 0.97 ± 0.43 tubes/cyst. Again, such results comfort our hypothesis that astrocytes and pericytes may have differential effects on RBE4 morphogenesis as observed in Fig.3b. Despite astrocyte co-culture had limited effect on the number of tube-like structure formed, it significantly increased tube length (Fig.3e) compared to RBE4 monocultures (median values of 208.7μm and 154.3μm respectively). Presence of pericytes did not significantly induced longer tubes, with a median value of 173.3μm. Surprisingly, presence of both astrocytes and pericytes resulted to the longest tubes, showing a median value of 227.0 μm.

Notably, the increased tube length observed in astrocyte co-cultures was accompanied by an increased tube diameter (Fig.3f) compared to RBE4 monocultures (20.53μm versus 16.55μm respectively), whereas pericyte co-cultures presented tubes with the smallest diameter of all groups (11.88μm). Surprisingly, presence of both astrocytes and pericytes resulted not only in slightly smaller tube diameter than RBE4 monocultures (15.40μm), but also to a diameter significantly different than the other co-culture groups.

In conclusion, our results clearly show that pericytes have differential effects. In our model, pericytes played an important pro-angiogenic role whereas astrocytes rather played a role in tube maturation. However, we observed that presence of both astrocytes and pericytes did not only blunted the pericyte pro-angiogenic effect, but potentiated the astrocytic effect, suggesting a synergistic function between these two cell types in favor to astrocytes.

The 3-dimensional BBB model reproduces astrocyte-endothelial and pericyte-endothelial *in vivo* interactions.

In this experiment, we assessed that our model allows suitable cellular interactions between RBE4 cells, astrocytes and pericytes. Thus, we analyzed cellular interactions by immunocytochemistry using cell specific markers (Fig.4a-d). Endothelial-astrocytes interactions (Fig.4a) were marked by presence of GFAP-positive astrocyte end-feet processes investing Tie-2 positive RBE4 tube-like structures, reproducing nicely the interactions classically described in the literature. In the other hand, endothelial-pericyte interactions (Fig.4b) differed by the absence of branching processes, replaced by the recruitment and tube-like structure overlap by pericytes cellular bodies. Remarkably, presence of the three cells (Fig. 4c & 4d) maintained distinct cellular interactions between astrocytes and RBE4 on one hand, pericytes and RBE4 on the other hand. These results show that in our model, both astrocytes and pericytes interact with RBE4 cells through processes that naturally occur *in vivo*.

Co-culture with astrocytes or pericytes is necessary to induce BBB phenotype.

Maturation of newly formed blood vessels is characterized by formation of cell-cell junctions. In order to appreciate the maturity levels of RBE4 tube-like structures we examined adherens junction (AJ) and tight junction (TJ) complexes protein localization in our different cultures (Fig.4e-4t). Interestingly, tube-like structures formed in RBE4 monocultures showed adequate β-catenin (Fig.4e) localization suggesting AJ complexes formation. However PECAM-1 staining (Fig.4i) as well as claudin-5 (Fig.4m) and ZO-1 (Fig.4q) appeared diffused inside the whole structure, suggesting that despites RBE4 cell tube-like structures were able to form cohesive cell-cell contacts by AJ, absence of TJ complexes highlight an absence of BBB

phenotype in these tubes. Presence of astrocytes in the co-cultures resulted in an important segregation of all these four cell junction proteins studied (Fig.4e; Fig.4j; Fig.4n & Fig.4r) at the cell borders forming appropriate AJ and TJ complexes. Interestingly, pericyte co-cultures appeared to have an immunolocalization profile between RBE4 monocultures and astrocyte co-cultures. Whereas β-catenin (Fig.4g) and PECAM-1 (Fig.4k) appeared more diffuse than astrocyte co-cultures, presence of TJ complexes was much visible, both for claudin-5 (Fig.4o) and ZO-1 (Fig.4s) suggesting the presence of a barrier phenotype. Presence of both astrocytes and pericytes resulted in similar results that observed with pericyte co-cultures, as observed with β-catenin (Fig.4h), PECAM-1 (Fig.4l), claudin-5 (Fig.4p) and ZO-1 (Fig.4t). Our results thus suggest that RBE4 cells by their own cannot induces TJ complexes formation and requires the presence of either astrocytes or pericytes. In addition, our data suggest that the differences observed between astrocytes and pericytes may reflect the importance of the cellular contacts in cell junctions formation and localization.

RBE4 lumen polarization requires presence of astrocytes or pericytes

As our previous experiment suggested the importance of astrocytes and pericytes in induction of TJ complexes, we therefore investigated their possible involvement in induction of tube polarity by z-stack image reconstruction obtained from immunostained tube-like structures (Fig.5).

We firstly investigated abluminal side polarization using utrophin, a cytoskeleton protein localized at the abluminal face in endothelial cells (Fig.5a). RBE4 monocultures tube section showed a heterogeneous utrophin localization. Interestingly, tube sections obtained from astrocyte co-cultures (Fig.5b) displayed a

more restricted utrophin staining suggesting certain localization. However, pericytes co-cultures tube sections (Fig.5c) clearly showed an utrophin positive staining on the tube periphery suggesting an appropriate utrophin staining localization compared to the lumen. Such localization also appeared in three-cell co-cultures (Fig.5d), overlaid by the PECAM-1 staining. These results may suggests that pericytes and in lesser extent astrocytes may play crucial roles in the polarization of the abluminal face.

We further investigated lumen side polarization by investigating P-gp (Fig.5e-h) and MRP2 (Fig.5i-l), two important ABC proteins localized in the luminal face. As observed with utrophin, RBE4 cells monocultures tubes were not able to localize either P-gp (Fig.5e) or MRP2 (Fig.5i) inside the lumen as the staining appears on the outer side of the tubes. Interestingly, astrocyte co-cultures showed localization of both proteins (Fig.5f-5j) at the luminal face as suggested the staining facing the lumen. Similar observations were visible for pericyte co-cultures (Fig.5g-5k) and three-cell co-cultures (Fig.5h-5l). In conclusion, our data suggests that as observed with TJ complexes, tube polarization requires the presence of astrocytes or pericytes and thus confirm their importance for proper tube maturation.

Validation of the 3-dimensional model: the O_2 deprivation insult test

In order to validate our 3-dimensional *in vitro* model, we challenged our co-cultures to prolonged O_2 deprivation insult (Fig.6) and compared the ability of astrocytes and pericytes to maintain TJ complexes according to our previous study performed on an established 2-dimensional *in vitro* data [8]. We firstly investigated changes in claudin-5 localization (Fig.6a-l). RBE4 cells monocultures were not able to induce TJ complexes under normal conditions, as seen for both claudin-5 (Fig.6a) and ZO-1 (Fig.6m), whereas presence of astrocytes (Fig.6b; Fig.6n), pericytes (Fig.6c; Fig.6o)

or both cells (Fig.6d; Fig.6p) resulted in localization of both claudin-5 and ZO-1 at the cell borders. Exposure to 48h at 1% O_2 induced a certain claudin-5 distribution in the middle of the tube (Fig.6e) but also showed presence of gaps inside (white arrows), whereas more severe O_2 deprivation stress (Fig.6i) resulted in tube dislocation. Notably, similar observation were obtained for ZO-1 either at 1% O_2 (Fig.6q) or 0.1% O_2 (Fig.6u) suggesting that O2 deprivation stress did not induce discrepancy in TJ protein localization and subsequently loss of barrier phenotype. Interestingly, astrocyte co-cultures exposed to 1% O_2 concentration induced a certain loss in claudin-5 (Fig.6f) and ZO-1 (Fig.6r) delocalization from the cell borders to the cytoplasm but still partially displayed presence of TJ complexes. However, exposure to more severe O_2 deprivation stress resulted in complete loss of claudin-5 (Fig.6j) and ZO-1 (Fig.6v) localization at the cell borders and therefore let us suggests complete abrogation of the barrier phenotype observed under normal conditions (Fig.6b & 6n). Pericyte co-cultures presented the most interesting results from all groups. Whereas prolonged exposure to 1% O_2 resulted in loss of claudin-5 expression (Fig.6g), more severe conditions (0.1% O_2) allowed indeed reinforcement of existing TJ complexes as observed with both claudin-5 (Fig.6k) and ZO-1 (Fig.6w) and clearly outperformed astrocyte co-cultures and thus agrees with our results found in our previous study. Presence of both astrocytes and pericytes resulted in more contrasted results. We observed that both cell types were necessary to obtain a complete maintenance of TJ complexes during prolonged exposure at 1% O_2 as observed with claudin-5 staining (Fig.6h) whereas exposure at 0.1% O_2 (Fig.6l) showed a diffuse claudin-5 staining along the tubes. ZO-1 staining appeared diffuse during prolonged exposure at 1% O_2 (Fig.6t) whereas it clearly appeared decreased in 0.1% O_2 (Fig.6x).

In conclusion, our model response to O_2 deprivation stress nicely fitted with our study performed on an established 2-dimensional *in vitro* model and thus constitutes an argument in favor of its validation and its suitability as a model.

DISCUSSION

The BBB (a neurovascular unit formed by endothelial cells, astrocytes and pericytes) plays a crucial role in the brain homeostasis. Despites BBB breakdown was classically associated with cerebral hypoxia/ischemia [8,21,22], recent studies demonstrates indeed that loss f barrier function occurs in many neurological diseases that including Alzheimer's disease [13,23,24], multiple sclerosis [14,25,26], neuroinflammation [16,27], neuroAIDS [15] or Parkinson's disease [13,28]. It appears therefore crucial that maintenance of the barrier function constitute an important therapeutical target to fight such diseases. Although our knowledge on the cellular and molecular mechanisms underlying BBB physiology and pathophysiology is constantly increasing, the capacity to translate our *in vitro* findings to *in vivo* situation remains sub-optimal.

A major drawback encountered with current *in vitro* models concerns the absence of appropriate cellular interactions as observed *in vivo*. In this study, we designed a novel *in vitro* BBB model based on a 3-dimensional matrix containing three important cell types of the BBB: endothelial cells, astrocytes and pericytes. Our extracellular matrix is based on collagen I, a scaffold protein widely used in 3-dimensional *in vitro* models of vasculogenesis and angiogenesis [18,29-34]. In our model, RBE4 cells presented many multicellular structures such as cysts, intercystal connections and tube-like structures that were comparable to those observed during vascular development in vertebrates such as blood islands, primary vascular plexus and

angiogenic blood vessels [35,36]. Although our study do not constitute the first that demonstrate the ability of cerebral endothelial cells to form a 3-dimensional vascular network *in vitro* [29,32], we are the first group to provide to provide a quantitative approach to such phenomenon, but also to pinpoint the roles played by astrocytes and pericytes during cerebral angiogenesis *in vitro*. Our study showed that presence of astrocyte in the co-cultures moderately on vasculogenesis, showing a down-regulation of intercystal connections formed compared to RBE4 monocultures and rather played important roles on angiogenesis by increasing average tube length and diameter. However, pericytes showed the most interesting results. Presence of pericytes significantly decreased cysts and intercystal connections on one hand and showed the highest tube-like structure numbers on the other hand. These results demonstrate that pericytes act definitively as a crucial pro-angiogenic factor towards RBE4 cells and agrees with previous studies highlighting the pro-angiogenic effects of pericytes [37,38]. However we demonstrated that presence of astrocytes and pericytes resulted in abolishment of the pro-angiogenic stimulus induced by pericytes. The nature of such inhibition is not known yet and we do not yet if such inhibiting mechanisms act on pericytes or rather involve a competition between astrocytes and pericytes signaling.

Pericytes appear to be the first cell type to interact with cerebral microvessels during early stages of embryogenesis [11,12], whereas astrocyte-endothelial interactions are initiated only in late developmental stages, when maturation of the BBB occurs [39-41]. We can therefore speculate that pericytes might promote angiogenesis during early development to provide the developing central nervous system with optimal O_2 and nutrient supply. Once neurogenesis start to decline and intitate neuronal differentiation, differentiated astrocytes may therefore inhibit the pro-angiogenic effect of pericytes and act synergistically to induce BBB maturation. Our group has

recently demonstrated that astrocytes and pericytes were able to synergistically act on endothelial barrier function during physiological and during O_2 deprivation stress [8], demonstrating the presence of a cellular cross-talk between these two cell types. We can therefore imagine that similar cross-talk might occur in our model, however the nature of such signaling remains unclear and requires further investigations.

It is interesting to note the ability of RBE4 cells to induce the formation of a complete vascular network from only single cells, suggesting an important disruption inside the endothelial cell phenotype that might result in endothelial de-differentiation and subsequently loss of barrier function. Our model showed an absence of TJ strands as observed by absence of proper claudin-5 and ZO-1 localization in newly formed tube-like structures. Our study therefore agrees with other observation denoting that barrier function loss in prolonged primary cell cultures [42,43] and support therefore that the maintenance of the barrier function in cultured brain endothelial cells requires the presence of other components of the neurovascular unit. Our study shows that either presence of astrocytes or pericytes is required to induce adequate both TJ complexes formation and endothelial cell tube polarization. Such observation demonstrates the active functions played by astrocytes and pericytes to RBE4 tube-like structures maturation and subsequently establishment of a barrier phenotype and thus agree with two-dimensional *in vitro* models [8,9]. Despites the establishment of a validation procedure for our model is limited by its innovative approach, the ability of our co-cultures to modulate TJ complexes localization under O_2 deprivation stress reflect the ability of astrocytes and pericytes to modulate barrier function in 2-dimensional *in vitro* models [8] under similar stress conditions. Subsequently, we can consider the ability of our model to reproduce similar behavior than established *in vitro* models as an argument to favor its validation as a research tool. Although our model supersedes

existing *in vitro* models by many features, it constitutes an excellent complementary model rather than a replacement of those well-established models.

Our model highlighted the important functions played by astrocytes and pericytes during the BBB maturation processes and clearly raise questions about the cellular and molecular mechanisms underlying such phenomenon. The nature by which astrocytes and pericytes induce the barrier function remains unclear, however it has been that different factors such as angiopoietin-1 [44], basic fibroblast growth factor [45], transforming growth factor-β [46,47] and vascular endothelial growth factor [48,49] constitute some of the important factors secreted by both cells. However, secreted factors may represent only a partial mechanism by astrocytes and pericytes exert induction of BBB phenotype. Presence of both secreted basement membrane matrices and direct cellular contacts may also provide an important contribution in barrier induction [50,51]. We are convinced that our model opens therefore the crucial keys to attempt the answers of such questions.

In conclusion, our model demonstrated the ability to develop an innovative *in vitro* BBB model outlining existing models by its integrative approach. We hope that the usage of such model will contribute to foster a better understanding of the BBB during physiological and pathological states.

MATERIAL AND METHODS

Cell culture

Rat brain endothelial cell line (RBE4, [52]) from passages 45 to 55 were used for experiments and cultured on gelatin-coated dishes (Corning Inc, Corning, NY). RBE4 cells were maintained in MEM-α/Ham's Nutrient F-10 Mix (1:1) (GIBCO, Invitrogen, Basel, Switzerland) supplemented with 10% fetal bovine serum,

100µg/mL penicillin, 100U/mL streptomycin and 1 ng/mL basic fibroblast growth factor (Roche Diagnostics, Rotkreuz, Switzerland). Primary rat astrocytes were isolated from neonatal pups as described previously [29]. Astrocytes were cultured on gelatin-coated dishes and maintained with DMEM supplemented with 10% FBS and 50mg/mL gentamycin sulfate. Primary rat brain pericytes were obtained from adult rat brains and isolated following the protocol established by Dore-Duffy [53]. Isolated pericytes were cultured in DMEM supplemented with 20% FBS, 50 mg/mL gentamycin sulfate and 2,5mg/mL amphotericin B. After the first passage, pericytes were cultivated in DMEM supplemented with 10% FBS and 50mg/mL gentamycin sulfate. Pericytes purity was assessed by α-smooth muscle actin (α-SMA) and glial fibrillary acidic protein (GFAP). Pericytes cultures showed over 95% of α-SMA positive cells whereas GFAP-positive cells were less than 1%.

Collagen matrix

Rat tail collagen was extracted following a method established by Bell and colleagues [54]. Briefly, rat-tail tendrils were sectioned, minced and solubilized overnight at 4°C in 15mmol/L acetic acid solution (Sigma-Aldrich, Buchs, Switzerland). Collagen solution was purified by ultracentrifugation during 1h, 20.000g at 4°C (RC 5C Plus, Sorvall Products, Newton, CT). Supernatant was lyophilized using a vacuum desiccator (RC 10.10, Jouan, France) and stored at -20°C upon usage. Collagen I solution was prepared at a concentration of 2.5mg/mL (dry weight) dissolved in 15mmol/L acetic acid solution and stored at 4°C upon usage.

Cell density determination

Cells grown on Petri dishes were routinely dissociated using 0.25% Trypsin/EDTA solution (GIBCO). Collagen matrix was dissociated in collagen digestion buffer (1mg/mL collagenase, 0.1mmol/L $CaCl_2$, 1mmol/L $MgCl_2$) incubated for 20 minutes at 37°C. Cells were centrifuged for 5 minutes at 1000rpm and resuspended in 100 µL of medium. An aliquot of cell suspension was mixed with equal volume of 0.4% Trypan Blue (Sigma-Aldrich) prepared in PBS. Cell density was determined using a glass hematocytometer (Neubauer, Brand, Wertheim, Germany).

Three-dimensional cell culture

The detailled technical procedure concerning the establishment of 3-dimensional cell culture is schematized in Fig.1a. In brief, collagen solution was mixed with $1/10^{th}$ volume of 10X Earle's Buffered Saline Solution (Sigma-Aldrich) containing phenol red. Acidity was neutralized by addition of 1N NaOH solution (Sigma-Aldrich). Cell suspension was added at a density of 5×10^5 cells/mL in monoculture experiments. In co-culture experiments, RBE4 cells and pericytes were added at a density of 2.5×10^5 cells/mL each. Astrocytes and pericytes were added at a density of 1.25×10^6 and 2.5×10^5 cells/mL respectively, in accordance with the literature [55] [56]. Cell suspension was disposed as droplets onto 60mm Petri dishes (Nunc, Roskilde, Denmark), incubated 10 minutes at 37°C to allow gel formation. Collagen droplets were overlaid with 5mL of RBE4 medium and incubated at 37°C with 5% CO_2. Medium was replaced after 3 days of culture.

Morphological analysis

Droplets were observed using inverted fluorescence microscope (Axiovert 200M, Carl Zeiss, Feldbach, Switzerland) set in brightfield mode. Micrographs pictures were

acquired in 10X magnification field using an 8-bit CCD camera (Axiocam HRm, Carl Zeiss) and processed using Axiovision 4.6 software (Carl Zeiss). Features observed were determined in four droplets per experiment. Length and diameter features were determined using the measure tool included in Axiovision software package.

Immunocytochemistry

After 5 days of culture, droplets were washed with cold PBS and fixed with 4% paraformaldehyde solution. Cells were permeabilized with 0.1% Triton X-100 solution and blocked with 10% normal goat serum during 4h at room temperature. Droplets were incubated overnight at 4°C with primary antibodies raised against platelet-endothelial cell adhesion molecule-1 (PECAM-1, mouse, 1:100, Chemicon, Millipore, Zug, Switzerland); glial fibrillary acidic protein (GFAP, mouse, 1:100, Sigma-Aldrich), α-smooth muscle actin isoform (α-SMA, mouse, 1:100, Sigma-Aldrich), ZO-1 (rabbit, 1:100. Invitrogen), claudin-5 (rabbit, 1:100, Invitrogen), β-catenin (mouse, 1:100, Chemicon), Tie2 (rabbit, 1:100, Santa Cruz Biotechnology, Heidelberg, Germany), and P-glycoprotein (C219 clone, mouse, 1:100, Calbiochem, VWR, Dietikon, Switzerland). Utrophin and MRP2 rabbit polyclonal antibodies were a gift from J.M. Frtischy [7]. Nuclei counterstaining was obtained by incubation with propidium iodide (PI, Calbiochem).Detection was performed using either Alexa-488 or Alexa-546 conjugated antibodies (Invitrogen). Samples were observed using a Leica SP2 confocal microscope (Leica Microsystems, Heerbrugg, Switzerland) and acquired with Leica Confocal Software. Obtained micrographs pictures and subsequent 3-dimensional and z-stack image reconstructions were performed by Imaris 6.0 software (Bitplane, Zurich, Switzerland). In the observation of the three cell types, pericytes were labeled with DiD lipophilic dye (Invitrogen) diluted at

1:200, following the manufacturer instructions for the dye incorporation. Pericytes were stained before incorporation inside the collagen matrix.

Transmitted electron microscopy

5 days old droplets were fixated with 3% glutaraldehyde buffer and postfixed with 2% OsO_4 buffer and dehydrated with 70-100% ethanol-containing solutions. Dehydrated samples were embedded into Epon 813 resin (Epon, Hexion, Rotterdam, The Netherlands). Slices were cut at a thickness of 70nm using a diamond knife ultramicrotome. Samples were observed using a transmission electron microscope (CM-100, Philips Research, Eindhoven, The Netherlands), micrograph pictures were obtained using a 12-megapixel digital camera (Gatan GmbH, München, Germany) and acquired by Digital Micrograph software (Gatan GmbH). Images were processed using ImageJ 1.41 (ImageJ, NIH, Bethesda, MD).

O_2 deprivation stress experiments

Experiments were performed in a purpose-built hypoxic glove-box chamber (InVivo$_2$ 400, Ruskinn Technologies, Pencoed, UK) maintained at 37°C with 5% CO_2. Cells were exposed to either 1% (hypoxia) or 0.1% (near-anoxia) O_2 for 48h. Using this instrument O_2 concentration is constantly monitored internally by an O_2 sensor and automatically adjusted.

Statistical analysis

Data are expressed as mean ± SD of minimum three experiments. n-way ANOVA were used for comparison inside and between groups, *t*-tests in the comparison between intercystal connections and tube-like structures.

ACKNOWLEDGMENTS

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FIGURE LEGENDS

Figure 1: Collagen matrix induces 3-dimensional cell morphogenesis.

(a) Schematic representation of the 3-dimensional cell culture model. The micrograph picture shows the single cell suspension within the matrix (b) RBE4 cells form cysts, intercystal connections and tube-like structures. Upper panels: Brightfield micrograph pictures. Lower panels: Immunocytochemistry performed against ZO-1. Nuclei counterstained with PI. Scale bar = 50μm. (c-f) 3-dimensional image reconstruction obtained from RBE4, astrocytes and pericytes monocultures stained against PECAM-1, GFAP and α-SMA respectively. Scale bar = 100μm. (f) Tube-like structure sagittal electron micrograph picture. ecm = extracellular matrix, endo = endothelial cell. (g) Medial electron micrograph picture. ecm = extracellular matrix, endo = endothelial cell, lum = lumen, nuc = nucleus.

Figure 2: Quantification of RBE4 cell mediated-vasculogenesis and angiogenesis.

(a) Cell density increased in RBE4 and pericytes monocultures only. ** denotes $P < 0.01$ compared to 0 day timepoint, ## $P < 0.01$ versus RBE4 cells group. (b) Cysts number per droplet remained constant in RBE4 cells monocultures. n=5 experiments. (c) Number of intercystal connections increased over time. Intercystal connections were normalized to cysts number at the defined timepoint. ** = $P < 0.01$, n=5 experiments. (d-e) Intercystal connection length and diameter box-plots. Note the widened distribution for both length and diameter in 6 days-old droplets. * $P<0.05$, ** $P < 0.01$ versus day 2 timepoint, n=40-60 intercystal connections counted in 5 experiments. (f) Tube-like structures (TLS) outnumber intercystal connections (ICC). TLS were normalized against cysts number counted in 6 days-old droplets and compared to ICC counted for the same timepoint. ** $P < 0.01$, n=5 experiments. (g-h) TLS and ICC length and diameter comparative box-plots. Data were collected from 6 days-old cultures. * $P < 0.05$, n=40-60 collected from 5 experiments.

Figure 3: Astrocytes and pericytes modulate RBE4 morphogenesis.

(a) Astrocyte (AC) and pericyte (PC) co-cultures resulted in cell density maintenance over time. Cell ratios were 5:1, 1:1 and 5:1:1 for astrocyte, pericyte and three cells co-cultures respectively. ** $P<0.01$ versus day 0 timepoint, ## $P<0.01$ between mono- and co-cultures, n=5/group. (b) PC and long-term AC co-cultures down-regulate cyst

numbers. ** P<0.01 versus day 0 timepoint, ## P<0.01 between mono- and co-cultures, n=5 experiments/group. (c) Co-cultures reduced formation of intercystal connections. Intercystal connections were normalized against cyst numbers. ** P<0.01 versus day 0 timepoint. ## = P<0.01 between mono- and co-cultures, n=5 experiments/groups. (d) PC strongly up-regulated tube-like structures number compared to RBE4 monocultures and astrocyte co-cultures. Values were obtained from 6 days-old cultures and normalized against cyst numbers. ** P<0.01 versus RBE4 monocultures, n=5 experiments/group. (e) Tube-like structure length box-plot ** P<0.01 versus RBE4 monocultures, n=25-70. (f) Tube-like structure diamater box-plot ** P<0.01 versus RBE4 monocultures, ## = P<0.01 between co-cultures. n=25-70.

Figure 4: Astrocyte and pericytes interactions correlate with barrier maturation phenotype. (a) Astrocyte co-cultures show tube investiture by astrocytes end-feet processes. Astrocytes stained with GFAP (green), RBE4 tubes stained with Tie2 (red). (b) Pericytes overlap tube-like structures. Pericytes are stained with DiD (blue), RBE4 cells with Tie2 (red). (c-d) Representative three-cells confocal micrograph pictures. Astrocytes are stained with GFAP (green), pericytes with DiD (blue) and RBE4 tubes with Tie2 (red). (e-h) β-catenin staining micrograph pictures. AC denotes astrocytes, PC denotes pericytes. Scale bar = 50μm. (i-l) PECAM-1 staining micrograph pictures. Note the clear discrepancy between RBE4 monocultures and astrocytes co-cultures. Claudin-5 (m-p) and ZO-1 (q-t) staining micrograph pictures demonstrates the inability of RBE4 monocultures to obtain adequate TJ complexes.

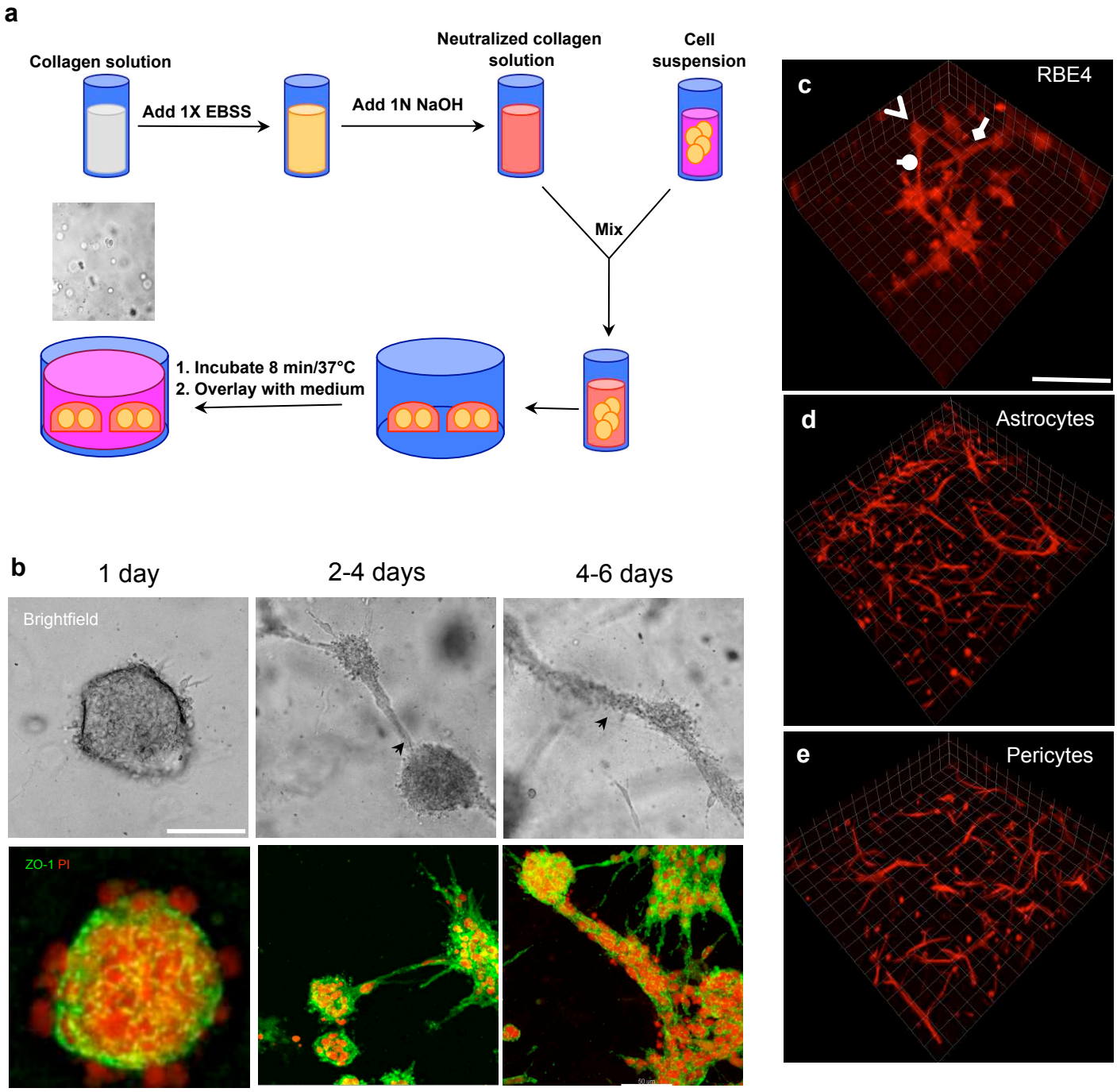
Figure 5: Astrocytes and pericytes are necessary to induce tube polarity. *z*-stack image reconstruction obtained from a *z*-step of 500nm. RBE4 cell monocultures are unable to locate utrophin (a) on the abluminal side as well as to locate P-gp and MRP2 in luminal side (e & I respectively). Despite astrocytes did not show adequate utrophin localization (b), it clearly localize P-gp (f) and MRP2 (j) in the luminal side. Presence of pericytes was necessary to induce utrophin localization at the abluminal side (c). Pericyte co-cultures also induced proper P-gp (g) and MRP2 (k) localization at the luminal side. Presence of both astrocytes and pericytes allowed optimal utrophin (d), P-gp (h) and MRP2 (l) localization. Scale bar = 10 μm.

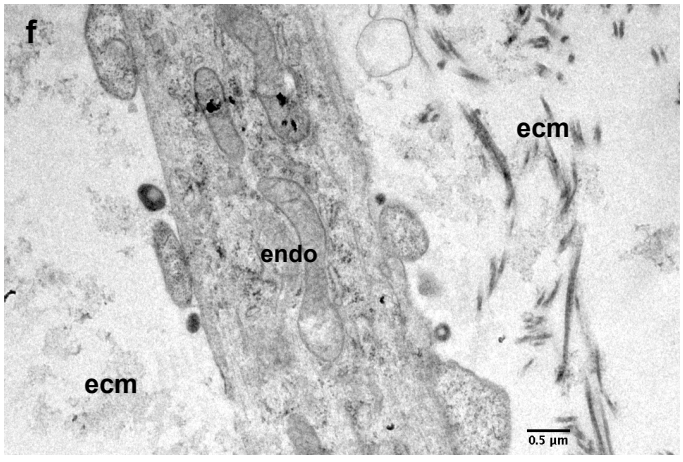
Figure 6: Co-cultures partially maintain TJ complexes during O_2 deprivation insult. Claudin-5 staining was performed on cultures exposed 48h at 21% (a-d), 1% (e-h) or 0.1% (i-l) O_2 concentrations. ZO-1 staining was performed on cultures exposed 48h at 21% (m-p), 1% (q-t) or 0.1% (u-x) O_2 concentrations. AC denotes astrocytes, PC denotes pericytes. Scale bar = 50μm.

Supplemental figure 1: Astrocytes and pericytes do not reproduce RBE4 morphogenesis. Representative brightfield micrograph pictures. Scale bar = 100μm.

Supplemental figure 2: Astrocytes & pericytes do not alter RBE4 morphogenesis. (a) Representative brightfield micrograph pictures. AC denotes astrocytes, PC denotes pericytes. Scale bar = 100μm. (b-c) intercystal connections length & diameter box-plots obtained from co-cultures. * $P<0.05$; **<0.01 versus day 2. n=20-60 from 5 experiments

Figure 1


a
Collagen solution
Add 1X EBSS
Add 1N NaOH
Neutralized collagen solution
Cell suspension
Mix
1. Incubate 8 min/37°C
2. Overlay with medium
b
1 day
2-4 days
4-6 days
Brightfield
ZO-1 PI
c
RBE4
d
Astrocytes
e
Pericytes


f
ecm
endo
ecm
0.5 µm

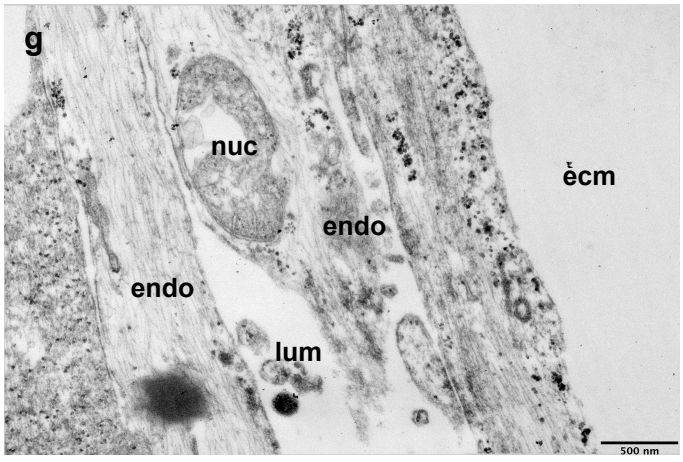

g
nuc
ecm
endo
endo
lum
500 nm

Figure 2

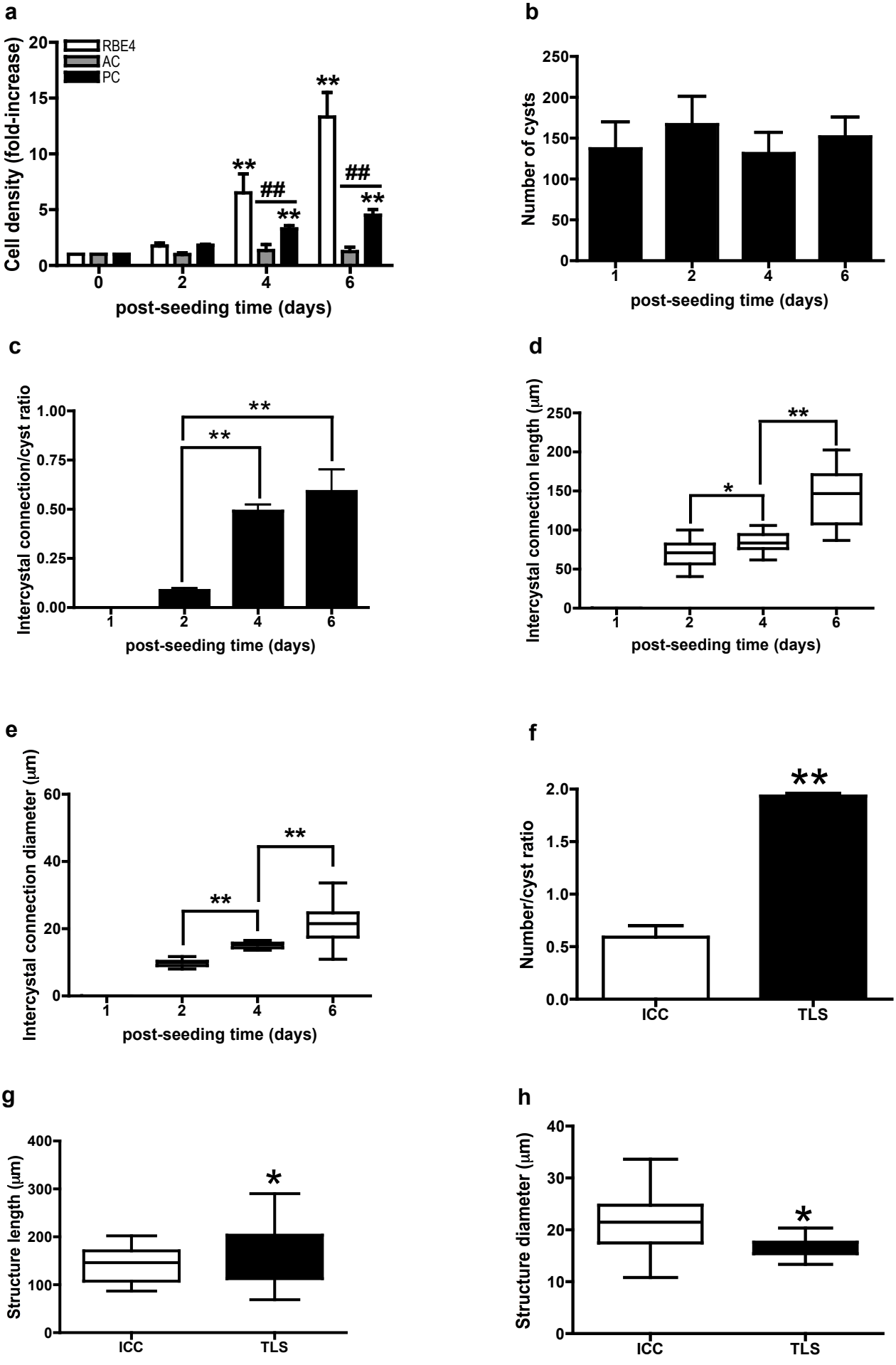

a
RBE4
AC
PC
Cell density (fold-increase)
post-seeding time (days)
b
Number of cysts
post-seeding time (days)
c
Intercystal connection/cyst ratio
post-seeding time (days)
d
Intercystal connection length (μm)
post-seeding time (days)
e
Intercystal connection diameter (μm)
post-seeding time (days)
f
Number/cyst ratio
ICC
TLS
g
Structure length (μm)
ICC
TLS
h
Structure diameter (μm)
ICC
TLS

Figure 3

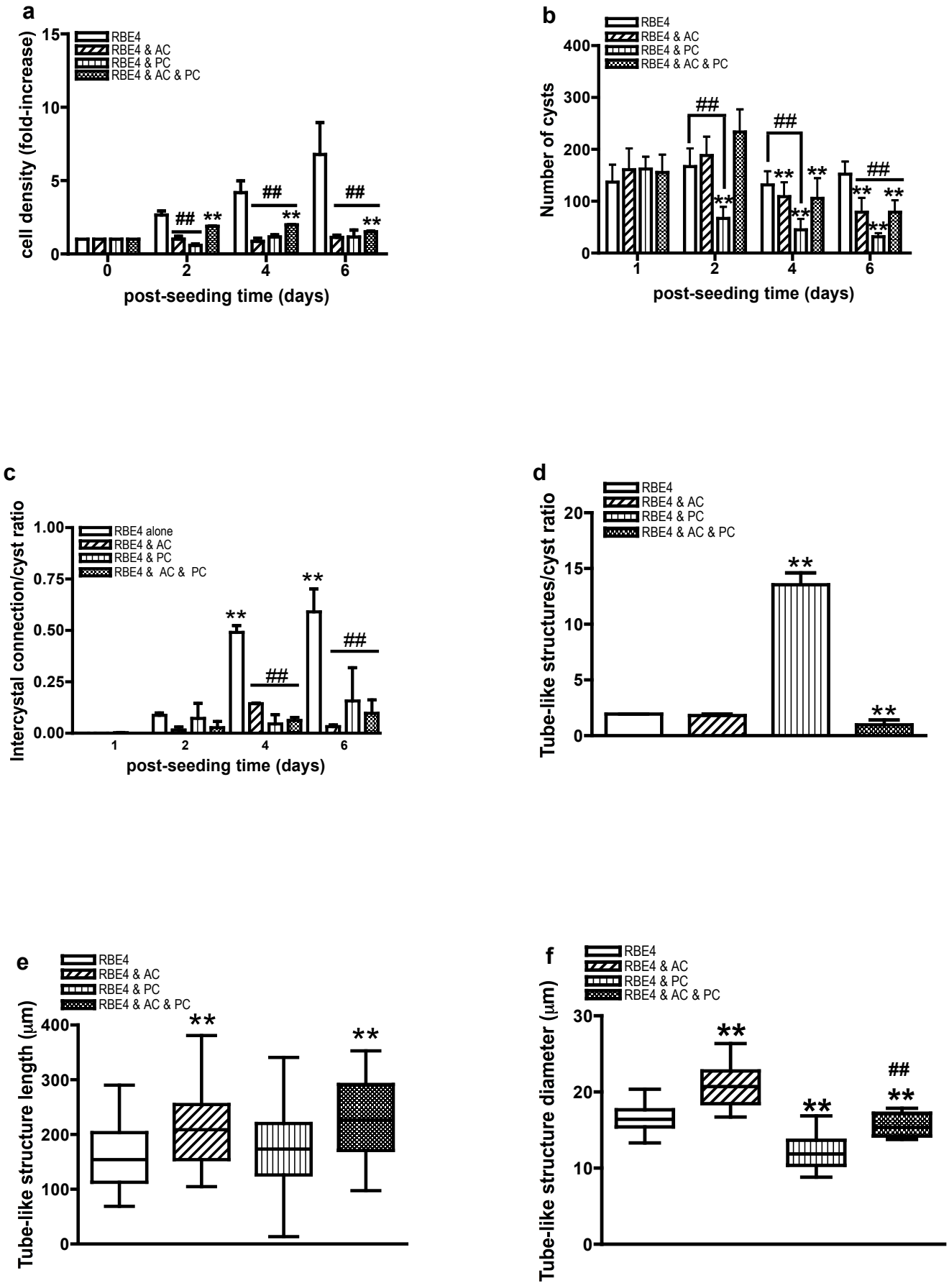

a
cell density (fold-increase)
post-seeding time (days)
RBE4
RBE4 & AC
RBE4 & PC
RBE4 & AC & PC
b
Number of cysts
post-seeding time (days)
c
Intercystal connection/cyst ratio
RBE4 alone
d
Tube-like structures/cyst ratio
e
Tube-like structure length (μm)
f
Tube-like structure diameter (μm)

Figure 4

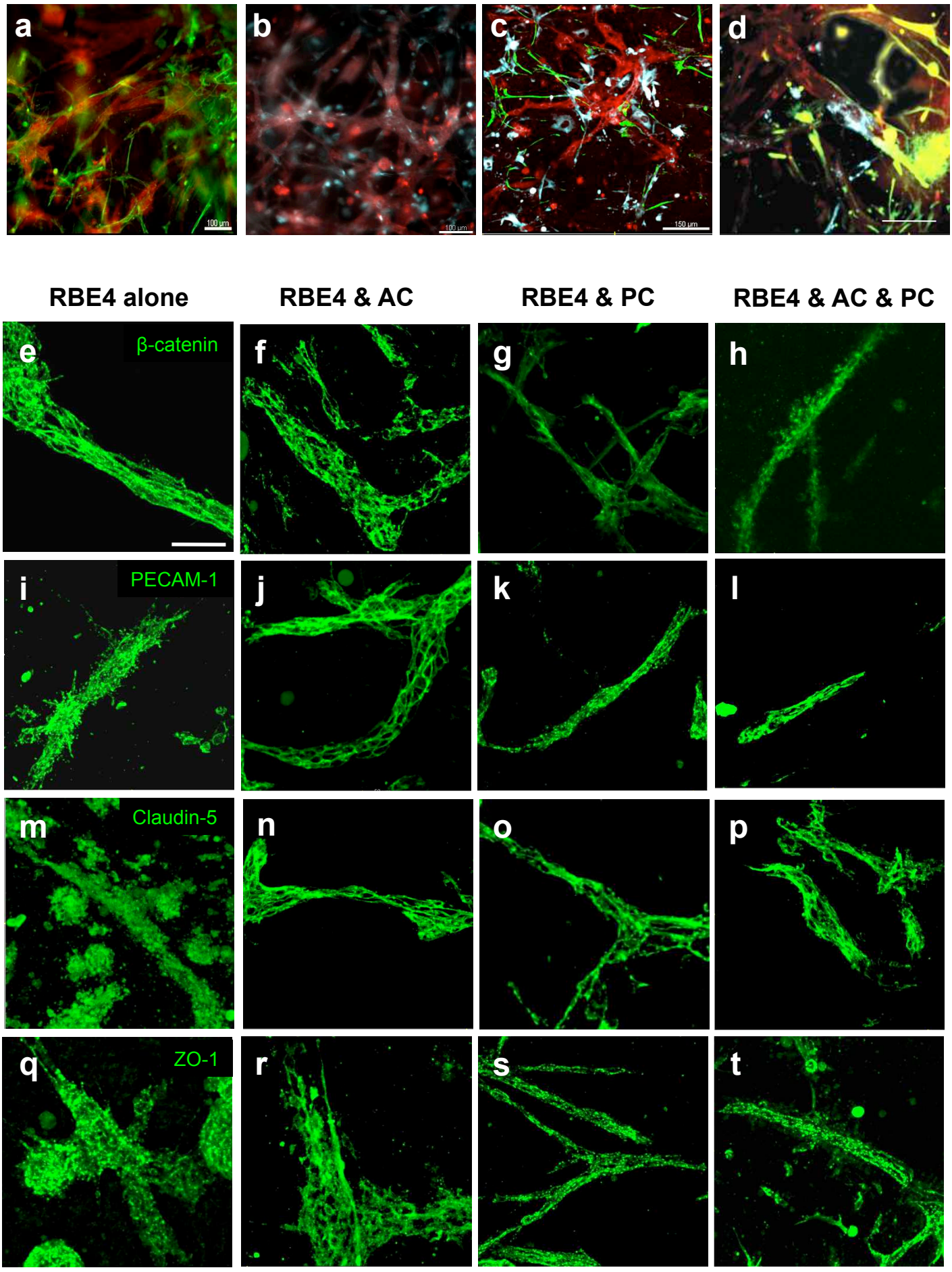
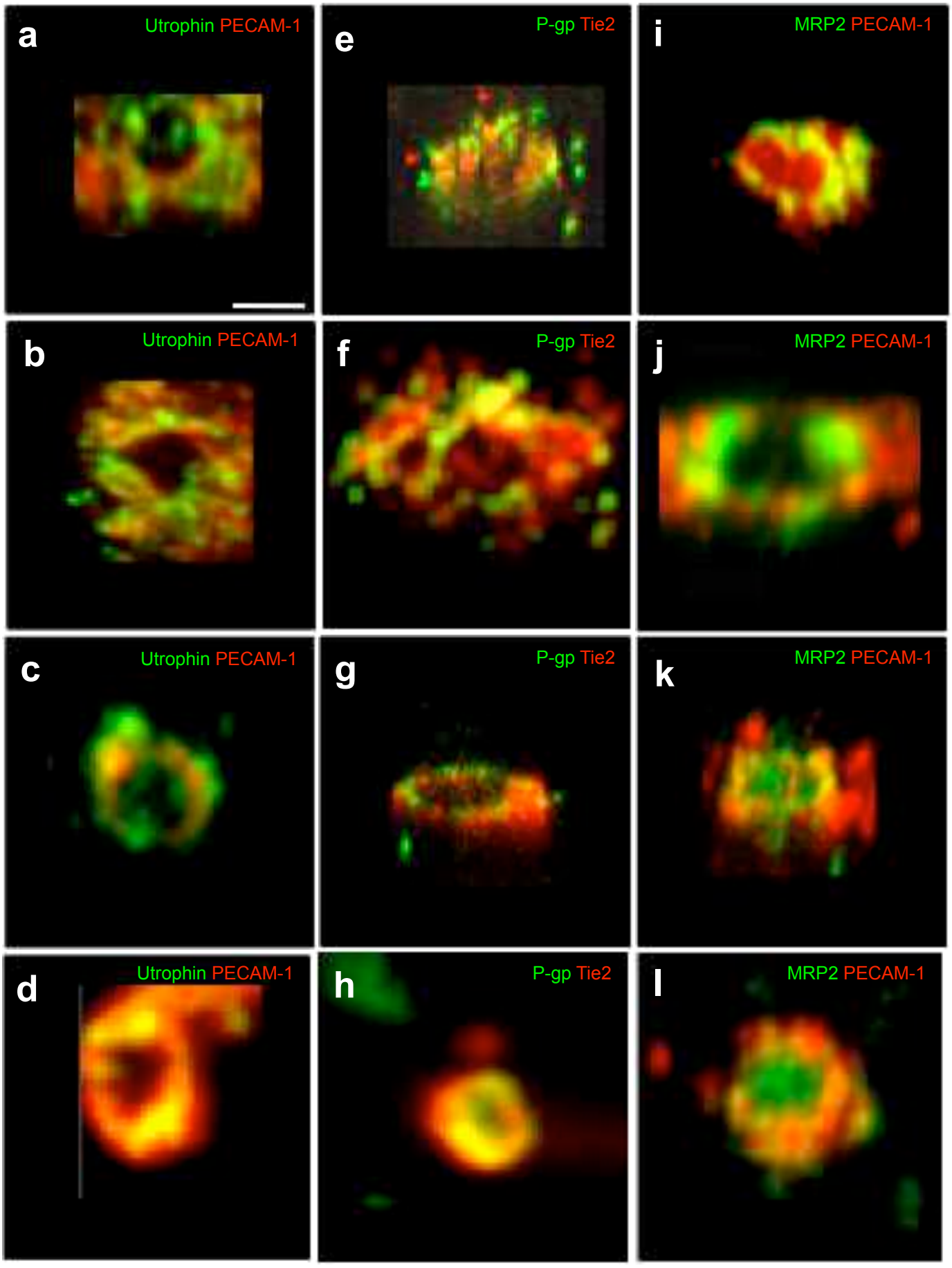

a
b
c
d
RBE4 alone
RBE4 & AC
RBE4 & PC
RBE4 & AC & PC
e
β-catenin
f
g
h
i
PECAM-1
j
k
l
m
Claudin-5
n
o
p
q
ZO-1
r
s
t

Figure 5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Utrophin PECAM-1
e
P-gp Tie2
i
MRP2 PECAM-1
b
Utrophin PECAM-1
f
P-gp Tie2
j
MRP2 PECAM-1
c
Utrophin PECAM-1
g
P-gp Tie2
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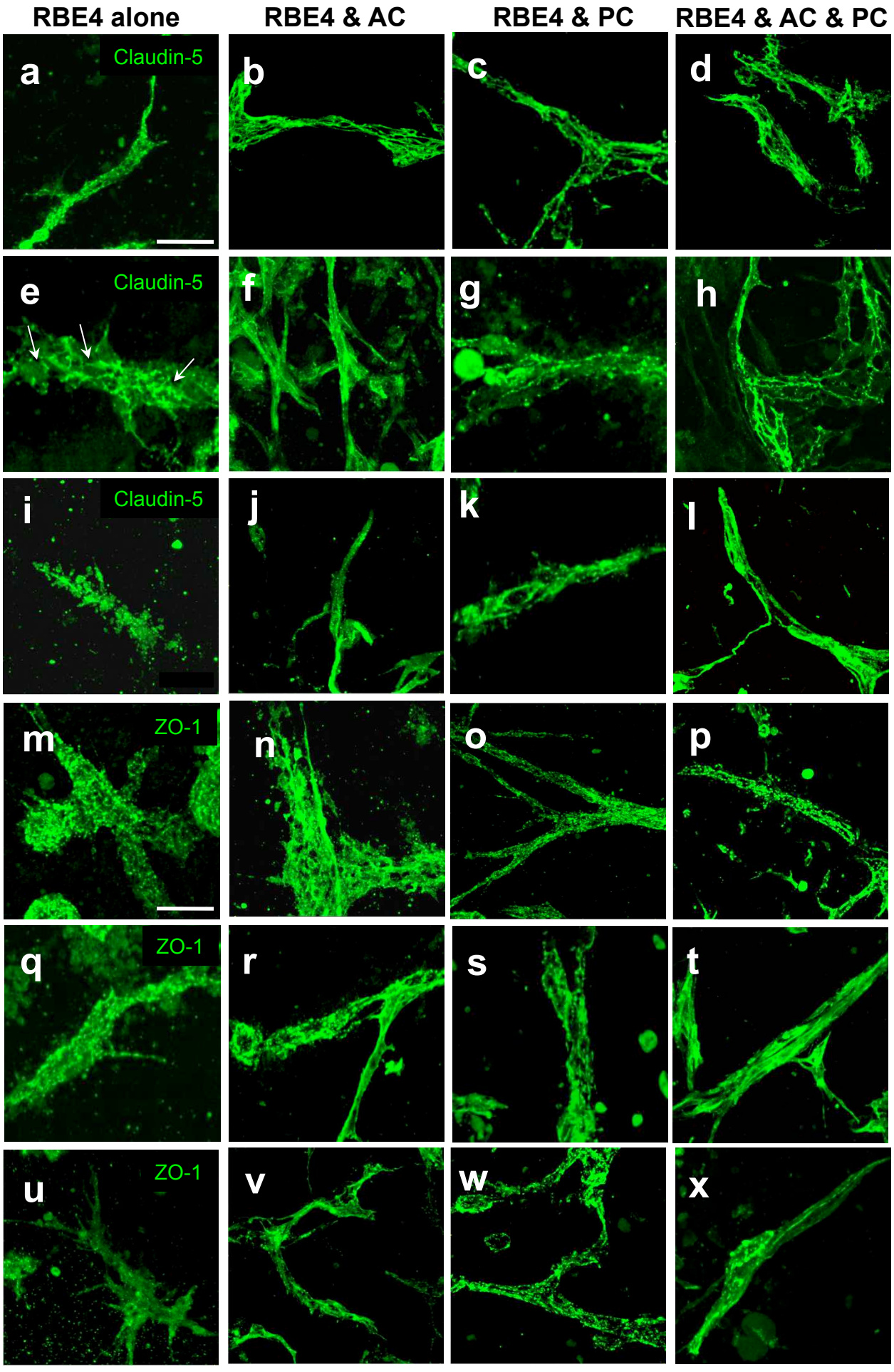
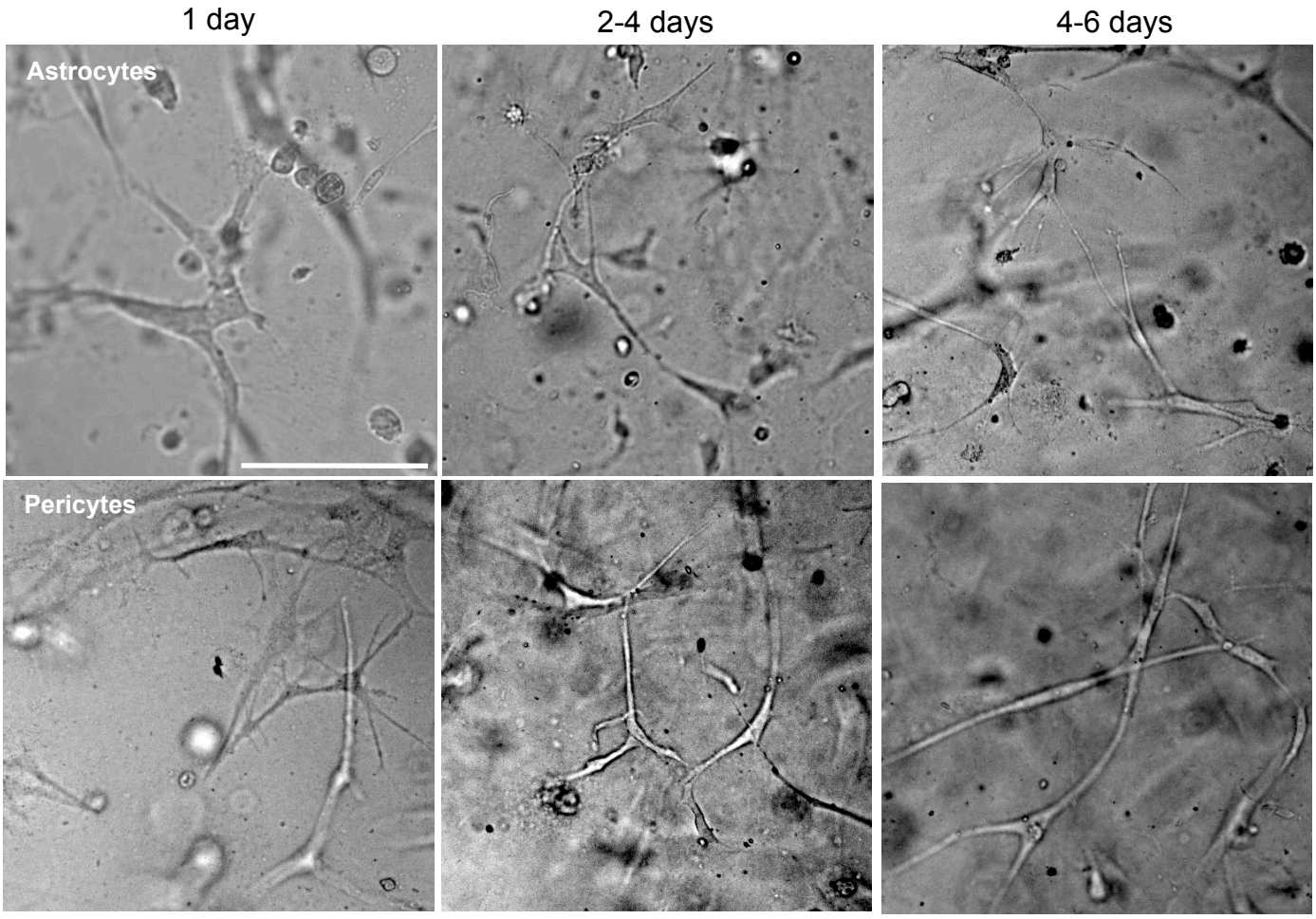
d
Utrophin PECAM-1
h
P-gp Tie2
l
MRP2 PECAM-1

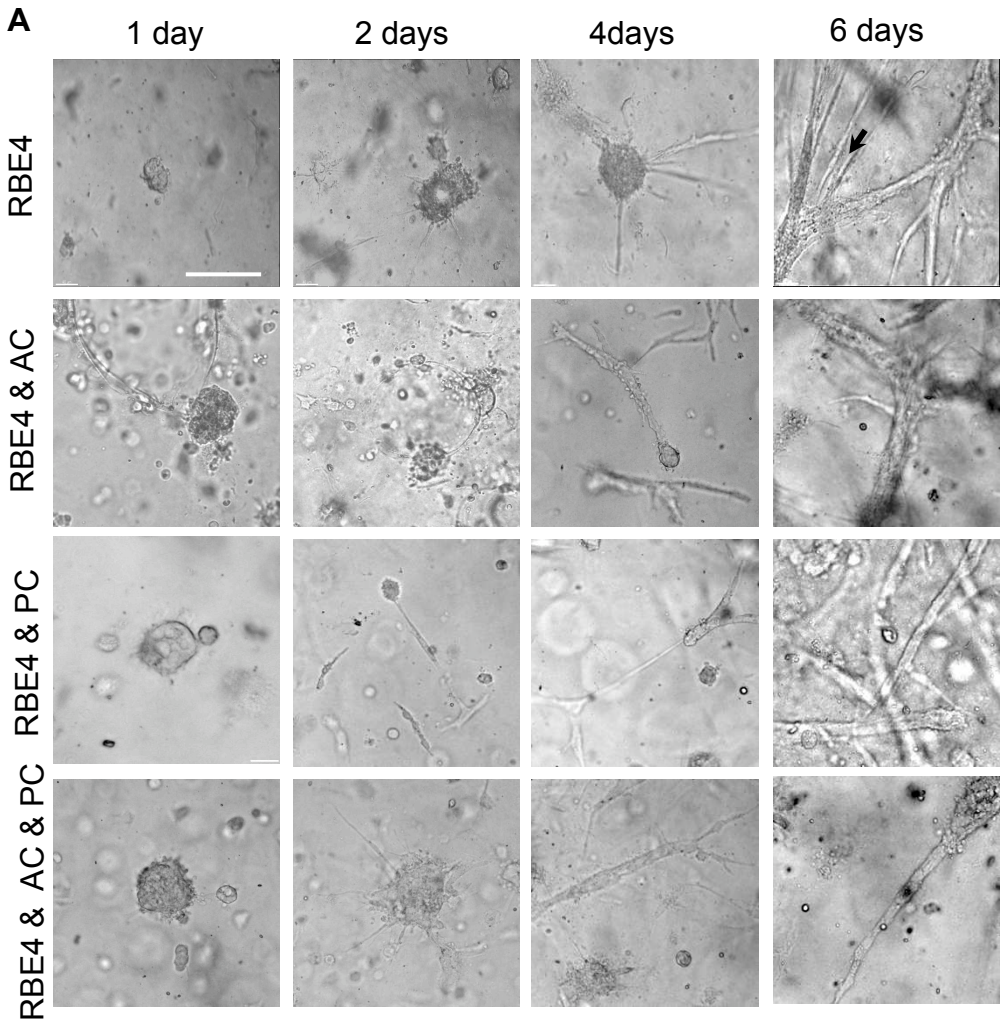
Figure 6

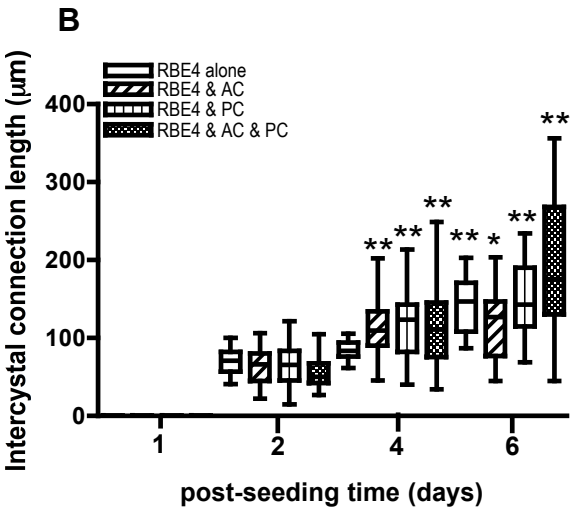

RBE4 alone
RBE4 & AC
RBE4 & PC
RBE4 & AC & PC
a
Claudin-5
b
c
d
e
Claudin-5
f
g
h
i
Claudin-5
j
k
l
m
ZO-1
n
o
p
q
ZO-1
r
s
t
u
ZO-1
v
w
x

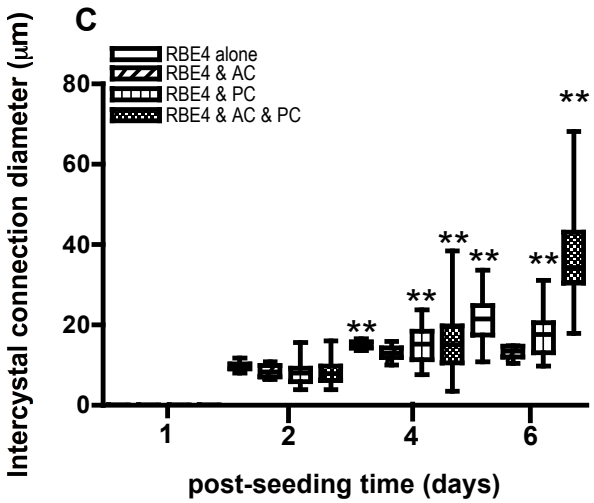
Supplemental Figure 1



Supplemental Figure 2







Abstract 1:
Al Ahmad A, Gassmann M, Ogunshola OO.
Maintaining blood-brain barrier integrity: pericytes perform better than astrocytes during prolonged oxygen deprivation. FENS Abstracts (2008) 4; A051.01.

The blood-brain barrier (BBB), consisting of specialized endothelial cells surrounded by astrocytes and pericytes, plays a crucial role in maintaining cerebral homeostasis. Many neurological diseases are associated with BBB breakdown and hypoxia constitutes one of the critical factors that onsets its disruption. We investigated the effect of astrocytes and/or pericytes on endothelial cell permeability and survival during different degrees of O2 deprivation. Prolonged exposure to 1% O2 was necessary to induce barrier breakdown and exposure to 0.1% O2 dramatically accelerated disruption. Of note, reoxygenation allowed cells exposed to 1% O2 to reestablish barrier function whereas 0.1% O2 deprivation resulted in significant cell death mediated at least in part via caspase 3 activation. Under normoxic conditions, co-culture with astrocytes and pericytes substantially induced barrier function. Interestingly, although O2 deprivation caused barrier breakdown in all situations, we observed differential hypoxic responses with astrocytes and pericytes. At 1% O2 astrocytes partially maintained tight junctions whereas pericytes accelerated disruption of the barrier in the short term, having positive effects only after prolonged exposure. Unexpectedly, at 0.1% O2 pericytes displayed a marked protective effect whereas astrocytes did not perform as well. Both astrocytes and pericytes appear to protect against monolayer disruption at least in part by preventing activation of caspase-3. Our data suggests that pericytes, but not astrocytes, play a significant role in maintaining barrier function during severe and prolonged oxygen deprivation.

Abstract 2:
Al Ahmad A, Gassmann M, Ogunshola OO.
Designing an accurate three-dimensional blood-brain barrier model - fact or fiction? FASEB J. 2007 21:749.1

The blood-brain barrier (BBB) is an anatomical barrier formed by specialized brain endothelial cells surrounded by astrocytes and pericytes - cells described to induce this BBB phenotype in endothelial cells. Specific cellular interactions are crucial for a proper BBB induction and their disruption during cerebral vascular diseases leads to barrier disruption. Most BBB in vitro models developed so far are limited by the 2-dimensional nature of the model, use of only 2 of the three cell types that form the barrier as well as the absence of normal cellular interactions. In this study, we have developed a novel in vitro three-dimensional BBB model. Brain endothelial cells form tube-like structures in three-dimensional matrices and astrocytes co-cultured with the endothelial cells interact through astrocytic end-feet contact as described in vivo. Recently we completed the model with pericytes. This model system will provide information on specific cellular interactions and signals that promote induction of BBB formation during development and can be readily subjected to different insults to understand BBB disruption.

Supported by 3R Research Foundation Switzerland, Project number 93/04 to O.O.

10. CURRICULUM VITAE

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Education

- **University Of Zürich, Zürich, Switzerland.**
  2004-Present: PhD student in Integrative Molecular Medicine PhD program. Thesis supervised by Dr. 'Lara Ogunshola (Institute Of Veterinary Physiology).
- **University Louis Pasteur, Strasbourg, France.**
  2004: Master in Physiology & Pharmacology. Master Thesis supervised by Pr. Genevieve Ubeaud-Sequier (Dept of Pharmacokinetic & Metabolism, Faculty Of Pharmacy) (2.2 honors).
  2002: BS in Biochemistry (2.2 honors).
  Lycee Jean Rostand, Strasbourg, France
  1997: Technical Baccalaureat (A-level) in Biochemistry-Biological Engineering (2.2 honors).

Work accomplishment

- Establishment of a 2-dimensional *in vitro* blood-brain barrier model based on Transwell© technology, suitable for barrier function studies under normal and pathological (i.e. O_2 deprivation stress) conditions.
- Establishment and characterization of a innovative 3-dimensional *in vitro* blood-brain barrier model (poster presentation award at the Gordon Research Conference – Barriers of the CNS – 2008)

Technical skills

- Cell culture of epithelial (Caco-2, HeLa) and endothelial (RBE4, EAhy.926) cell lines. Murine cerebral microvessels isolation, cerebral astrocytes and pericytes primary cultures.
- Assessment of barrier function in cell monolayers by physical (TEER) and biochemical assays (radiolabelled and fluorescent paracellular flux markers).
- Fluorescent immunocytochemistry and microscopy techniques (wide-field, confocal and TEM microscopy). Additional knowledge in live imaging, 3-D image reconstruction (Imaris) and image deconvolution (Huygens).
- Liquid chromatography (HPLC-UV)
- Western-blot techniques (ECL detection).

Publication list

- Al Ahmad A, Gassmann M, Ogunshola OO. The blood-brain barrier in a dish: designing a innovative 3-dimensional *in vitro* model (Manuscript submitted).

- Al Ahmad A, Gassmann M, Ogunshola OO. Maintaining blood-brain barrier integrity: pericytes perform better than astrocytes during prolonged oxygen deprivation. J Cell Physiol. 2009 Mar;218(3):612-22.
- Al Ahmad A, Gassmann M, Ogunshola OO. Maintaining blood-brain barrier integrity: pericytes perform better than astrocytes during prolonged oxygen deprivation. FENS Abstracts (2008) 4; A051.01.
- Al Ahmad A, Gassmann M, Ogunshola OO. Designing an accurate three-dimensional blood-brain barrier model - fact or fiction? FASEB J. 2007 21:749.1
- Milane HA, Al Ahmad A, Naitchabane M, Vandamme TF, Jung L, Ubeaud G (2007). Transport of quercetin di-sodium salt in the human intestinal epithelial Caco-2 cell monolayer 139. Eur J Drug Metab Pharmacokinet: 32(3):139-47.

Travel grants, awards

- Poster presentation award at the Gordon Research Conference 2008 entitled "Barriers of the CNS"
- Travel grant award from the Swiss Physiological Society in 2007 and 2008.
- Grant extension from our current project sponsor (appreciation of draft manuscript of our 3-dimensional model) (http://www.forschung3r.ch/en/projects/pr_93_04.html)

Languages

Native French speaker
Fluent in English and German
Basic knowledge in Near-Eastern Arabic.

References

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Miscalleneous

Experience in PC (MSDOS/Windows) and Macintosh (OS9/OSX) environments (IT support). Computer enthusiatstic (retrocomputing). Science-fiction, fantasy books & movies.
Music (Soundtrack, Metal, Pop-Rock)

11. ACKNOWLEDGEMENTS

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12. APPENDIX

* Reference letter (Gutachten) from Pr. Dr. Max Gassmann

* Reference letter (Gutachten) from Pr. Dr. Hugo H. Marti

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Zürich, 24. Februar 2009

Gutachten zur Dissertationsschrift von Herr Abraham Al Ahmad

Dear colleagues,

As the doctoral father of Abraham Al Ahmad's thesis committee, I have followed Abraham's PhD work summarized in the thesis manuscript entitled *"Building The Blood-Brain Barrier In a Dish: Design of Two Innovative In Vitro Models"*.

Abraham has done remarkable work on the design of these two innovative *in vitro* blood-brain barrier models and their development as new tools for fundamental research. In the first published scientific article (see Manuscript 1), Abraham has demonstrated differential effects of astrocytes and pericytes in the induction and maintenance of the blood-brain barrier during oxygen deprivation insult. This work implies that studies such as this may in future provide new avenues for targeting therapeutical agents for treating cerebrovascular diseases.

In the second submitted paper (see Manuscript 2), Abraham has assisted the development and characterization of a novel 3-dimensional *in vitro* blood-brain barrier model. Importantly this model exhibits many morphological features comparable to the *in vivo* situation. This study has also highlighted the important roles played by astrocytes and pericytes during blood-brain barrier development and maturation. It is worth mentioning that both models have been positively accepted by the blood-brain barrier scientific community and Abraham received an award for his poster communication on the 3 dimensional model (**see his curriculum vitae**). This success emphasizes the pertinence of his work.

Abraham Al Ahmad is a very dedicated young scientist who works very hard hours in the lab. He has matured to an innovative, critical and well-organized researcher who is never afraid to learn new technologies to answer scientific questions.

I have positively reviewed Abraham Al Ahmad's thesis manuscript that is very well written and consider it ready for submission. I therefore consent to the submission of his application for the doctorate examination.

With best regards,

Max Gassmann

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24.02.2009 HHM /

Betrifft: Abraham Al Ahmad, Gutachten

Gutachten
Zur Dissertation von Herrn Abraham Al Ahmad, geb. 12.01.1979 in Schiltigheim (F)
Building the blood-brain barrier in a dish: Design of two innovative in vitro models

Herr Al Ahmad beschäftigt sich in seiner Arbeit mit den Komponenten, welche *in vitro* die Blut-Hirn-Schranke (BHS) ausmachen. Es ist bekannt, dass das Zusammenspiel von Gehirn-Endothelzellen, Astrozyten und Perizyten in vivo für eine dichte BHS verantwortlich ist. Bisher ist es aber nur unvollständig gelungen, die BHS in vitro nachzubilden. Hier setzt die Arbeit von Herrn Al Ahmad an.

Herr Al Ahmad entwickelt in seiner Arbeit zwei neue Modelle für die BHS in vitro. Mit aufwändigen zellbiologischen, physiologischen, molekularbiologischen und biochemischen Methoden hat Abraham Al Ahmad *in vitro* mit 2- und 3-dimensionalen Modellen die Interaktion von Endothelzellen, Astrozyten und Perizyten der *in vivo* Situation der BHS nachgebildet und analysiert. Er konnte zeigen, dass neben Astrozyten unter starker Hypoxie vor allem Perizyten eine wichtige Rolle für die Barrierefunktion spielen.

Diese wichtigen Befunde von Herrn Al Ahmad eröffnen neue Untersuchungsmöglichkeiten zur Beeinflussung der BHS, welche in Zukunft zu neuen therapeutischen Ansätzen bei der Behandlung des Hirnödems, wie es bei Tumoren, Schädel-Hirn-Verletzungen, ischämischen Erkrankungen oder in der Höhe auftritt, führen könnten.

Die Promotionsschrift ist klar gegliedert und verständlich formuliert Die sehr lange Einleitung gibt den aktuellen wissenschaftlichen Stand wieder mit einer sehr ausführlichen Darstellung der Mechanismen der BHS und einem Überblick über die Effekte von Sauerstoffmangel (Hypoxie) auf die BHS-Funktion. An die Einleitung schliesst logisch die Fragestellung an, wie die BHS in 2- und 3-dimensionalen Modellen *in vitro* nachgebildet werden kann. Die angewandten zellbiologischen Methoden sind anspruchsvoll. Die Ergebnisse sind übersichtlich gegliedert. Zunächst wird die veröffentlichte Arbeit, welche das 2-D-Modell

beschreibt, dargestellt, dann folgt die Beschreibung des 3-D-Modells als Manuskript, das zur Einreichung vorgesehen scheint. Zwischen Einleitung und Manuskripten werden die Ergebnisse kurz zusammengefasst und unter Berücksichtigung der aktuellen Literatur diskutiert.

Den ersten Teil seiner Ergebnisse (2-D-Modell) hat Herr Al Ahmad Ende 2008 als Erstautor im Journal of Cellular Physiology (Impact Factor: 3.642; Ranking: 22 von 78 in der Kategorie: Physiology) publiziert. Der zweite Teil der Ergebnisse (3-D-Modell) liegt als Manuskript vor, wobei Herr Al Ahmad ebenfalls Erstautor ist. Dieses Manuskript hat zurzeit einen überwiegend beschreibenden Charakter, enthält aber wenig funktionelle Daten zur Beschaffenheit der BHS und somit zur Tauglichkeit als BHS-Modell. Es ist nicht klar, ob das Manuskript in seiner jetzigen Form eingereicht werden soll, oder ob noch funktionelle Daten hinzugefügt werden. Es ist aber davon auszugehen, dass auch dieses zweite Manuskript zu einer Publikation führen wird.
Herr Al Ahmad hat also aus seiner Doktorarbeit bisher eine Veröffentlichung generiert und eine zweite wird höchstwahrscheinlich folgen. Damit ist die Publikationsleistung als durchschnittlich anzusehen. Mit seiner Dissertationsschrift hat Herr Al Ahmad zweifelsfrei beweisen, dass er zu selbständigem wissenschaftlichen Arbeiten in der Lage ist.

Aus all diesen Gründen empfehle ich der Mathematisch-Naturwissenschaftlichen Fakultät der Universität Zürich, die vorliegende Dissertationsschrift von Herrn Al Ahmad anzunehmen.

Freundliche Grüsse

Prof. Dr. med. Hugo H. Marti