**Msx1** is expressed in retina endothelial cells at artery branching sites

Miguel Lopes*‡, Olivier Goupille*‡§, Cécile Saint Clément and Benoît Robert†

Institut Pasteur, Génétique moléculaire de la Morphogenèse & CNRS URA 2578, 25 Rue du Docteur Roux, 75015 Paris, France

*These authors contributed equally to this work
‡Present address: IPSEN Innovation, Scientific affairs department, 5 Avenue du Canada, 91966 Les Ulis Cedex, France
§Present address: CEA, DSI/IMETI/ST/LEPDS & INSERM U962, 18 Route du Panorama, 92265 Fontenay aux Roses Cedex, France

†Author for correspondence (benoit.robert@pasteur.fr)

Biology Open 1, 378–384
doi: 10.1242/bio.2012017

**Summary**

Msx1 and Msx2 encode homeodomain transcription factors that play a role in several embryonic developmental processes. Previously, we have shown that in the adult mouse, Msx1lacZ is expressed in vascular smooth muscle cells (VSMCs) and pericytes, and that Msx2lacZ is also expressed in VSMCs as well as in a few endothelial cells (ECs). The mouse retina and choroid are two highly vascularized tissues. Vessel alterations in the retina are associated with several human diseases and the choroid has been intensely used for angiogenesis studies, whereas the retina has been intensely used for angiogenesis studies, whereas the choroid has been much less investigated. Using the reporter alleles, we observed that Msx2 is not expressed in the eye vascular tree in contrast to Msx1, for which we establish the spatial and temporal expression pattern in these tissues. In the retina, expression of Msx1 takes place from P3, and by P10, it becomes confined to a subpopulation of ECs at branching points of superficial arterioles. These branching sites are characterized by a subpopulation of mural cells that also show specific expression programs. Specific Msx gene inactivation in the endothelium, using Msx1 and Msx2 conditional mutant alleles together with a Tie2-Cre transgene, did not lead to conspicuous structural defects in the retinal vascular network. Expression of Msx1 at branching sites might therefore be linked to vessel physiology. The retinal blood flow is autonomously regulated and perfusion of capillaries has been proposed to depend on arteriolar precapillary structures that might be the sites for Msx1 expression. On the other hand, branching sites are subject to shear stress that might induce Msx1 expression. In the choroid vascular layer Msx1lacZ is expressed more broadly and dynamically. At birth Msx1lacZ expression takes place in the endothelium but at P21 its expression has shifted towards the mural layer. We discuss the possible functions of Msx1 in the eye vasculature.

© 2012. Published by The Company of Biologists Ltd. This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial Share Alike License (http://creativecommons.org/licenses/by-nc-sa/3.0).

Key words: Retina, Choroid, Endothelium, VSMC, Homeobox genes, Conditional mutation

**Introduction**

Angiogenesis is the process by which pre-existent vessels rearrange in order to give rise to new vascular beds (Jain, 2003). The mouse retina is one of the best characterized models for *in vivo* vessel formation as it displays all of the morphological hallmarks of angiogenesis (i.e. sprouting, branching, fusion, remodelling and maturation) after birth (Fruttiger, 2002, 2007). In contrast to human, murine newborns do not possess a developed retinal vascular plexus (Gyllensten and Hellstrom, 1954). In these, the superficial retinal vascular plexus forms during the first week after birth by radial outgrowth of vessels from the optic nerve entry point to the periphery (Fruttiger, 2007; Stahl et al., 2010). These superficial vessels reach the retinal edges at approximately post-natal day (P) 9 and from this stage onwards, vertical sprouting forms the deep and then intermediate vascular beds, that reach the retinal periphery at approximately P12 and P15, respectively. The three retina vascular beds become fully mature and interconnected at the end of the third postnatal week (Stahl et al., 2010). At this stage, the retinal superficial vasculature is composed of six major arteries and veins that form primary branches into arterioles and venules. Arterioles and venules branch into a capillary network that is in direct contact with deep and intermediate layers. This network is covered with pericytes, providing retina with the densest coverage of pericytes in the whole vasculature (Shepro and Morel, 1993). The choroidal vascular system, which resides between the retina and the sclera, is fully developed before birth and supplies oxygen and nutrients to the avascular retina (Campochiaro, 2000). At early stages, the blood supply of the eye is also provided by the hyaloid vasculature that originates from the central hyaloid artery in the optic nerve and extends through the primitive vitreous toward the anterior segment (Saint-Geniez and D’Amore, 2004). The hyaloid vasculature regresses when ocular development proceeds in order to leave a transparent visual axis (Mitchell and Gingras, 1998; Brown et al., 2005).

Blood vessels are mainly composed of an inner endothelial cell (EC) layer externally covered by mural cells, namely vascular smooth muscle cells (VSMCs) in arteries and veins, and pericytes in capillaries (Jain, 2003). In the mature vascular plexus, mural cells contribute to endothelial tube stabilization, maintenance of vascular permeability and regulation of the blood flow. In small arterioles and capillaries, most of these activities are performed
by pericytes (Orlidge and D’Amore, 1987; Sato and Rifkin, 1989; Diaz-Flores et al., 2009; Armulik et al., 2011). In the central nervous system, not all the pericytes contain the muscle-specific actin isoform, only those in the pre- and postcapillary regions (i.e. on arterioles and venules) (Nehls and Drenckhahn, 1991; Rucker et al., 2000). This led to the proposition that blood flow is regulated mainly at the level of precapillary arterioles (Anderson and McIntosh, 1967; Baez, 1977). However, time-lapse and functional analyses have demonstrated that capillaries are capable of regulating blood flow along their whole length (Mettea and Newman, 2006; Peppiatt et al., 2006). In the eye, vasocostriction and vasodilatation via the pericytes are evoked by light-induced stimulation of perivascular astrocytes (Mettea and Newman, 2006).

Msx genes encode homeodomain transcription factors and are essential for normal craniofacial and limb development, nervous system patterning, eye formation, as assessed by phenotypic abnormalities in knock-out mice (Bach et al., 2003; Wu et al., 2003; Ishii et al., 2005; Lallemand et al., 2005, 2009). In the cardiovascular system, Msx genes have previously been shown to be important for outflow tract patterning (Ishii et al., 2005; Chen et al., 2003), head vessel maturation (Lopes et al., 2011) and adult vessel calcification (Towler et al., 2006). In adult mice, Msx2lacZ is mainly expressed in a subset of VSMCs in peripheral arteries and veins (brachial, femoral and caudal), as well as a few ECs of the aorta. Msx1lacZ is expressed to a lesser extent by VSMCs of peripheral arterial trunks, but is highly expressed in mural cells of arteries and capillaries that irrigate the tissues (Goupille et al., 2008).

In this paper, we observe that Msx2lacZ expression is not detectable in eye vessels, and describe Msx1 expression patterns in the retina and choroid vasculature. In the choroid, Msx1lacZ is expressed in a broad dynamic pattern, initially in the endothelium. Expression is observed later on in VSMCs, similar to Msx1 general behaviour in the peripheral vasculature. In contrast, in the retina, Msx1lacZ expression is restricted to ECs of the arteriolar branching points in the superficial vascular network. At these branch points, we further characterised a subpopulation of pericytes, which express high levels of NG2, α-SMA and desmin. We thus demonstrate specific properties of a population of pericytes, which express high levels of NG2, in the peripheral edge of the retina, the capillary bed has begun to develop into the deeper layers and circulation has started (Brown et al., 2005; Fruttiger, 2007). Strikingly, at this stage Msx1lacZ expression became restricted to primary branching points in arterioles (Fig. 1C, arrowheads). Restricted expression was not yet completed by P12, since a few cells were still expressing Msx1 along the length of arteries (Fig. 2A, arrowheads), but was achieved at P14 (Fig. 1D, arrowheads). In most cases, more than one nucleus was labelled (Fig. 1F, Fig. 3D–F, Fig. 4A,C). At the branching points, we further observed strong Ib4 concentration that co-localized with Msx1-expressing cells (Fig. 1C, arrowheads). Ib4 is known to bind the basement membranes (specifically, the versican protein) and thus can label not only ECs but also microglial cells and probably other cell types including some vascular mural cells. On trypsin-digested retinas, Ib4 was unambiguously associated with vessels (not shown). Maximum Msx1 expression occurred between P14 and P23 (Fig. 1D–E), but expression was maintained at high levels up to P480 (Fig. 1F) and probably lasts over the lifetime of the animal. From P14, we observed that Msx1 also labelled secondary branches in the arteriolar network (Fig. 1F, Fig. 4D). Noticeably, at all stages, Msx1 expression was observed only in rather mature arteries that were covered by a SMA-positive mural cell coating suggesting that Msx1 expression in the endothelium requires EC-VSMC interactions. Msx1-labeled branching sites were quantitated throughout development (Fig. 2). There was a large and steady increase between P9 and P23 and thereafter the number plateaued. Therefore, restriction of the expression to branching sites lagged behind the formation of the vascular superficial bed, for which arteries reach the retinal periphery around P8 (Fruttiger, 2007). This was not achieved before the onset of blood flow, which in the mouse retina takes place between P3 and P4 (Brown et al., 2005). From P23, we observed Msx1 expression at all primary, and some secondary, branching points along the main retinal arteries.

Arteries and veins were identified in flat mounts by virtue of their distinct morphologies and differential coating by mural cells. We did not observe Msx1lacZ labelling of venules in the retina (data not shown), as in any other veins (Goupille et al., 2008). Only the superficial vascular network was labelled. This is in keeping with the fact that deeper beds are primarily formed by sprouting from veins (Fruttiger, 2007). Xgal staining reflected the specific expression of Msx1 in retinal vessels since no staining was observed in normal mice (data not shown). In contrast to what was observed in peripheral arteries (Goupille et al., 2008), Msx2lacZ could not be detected in the hyaloid, retina or choroid vessels at any stage of their development (data not shown).

Msx1lacZ is expressed specifically in ECs
Xgal staining is useful for a broad analysis of expression patterns. However, it does not provide sufficiently high resolution for precise co-localization studies using optical microscopy. In order to identify in which cell type Msx1lacZ is expressed, we used an anti β-gal antibody together with endothelial-specific (anti-CD31) and mural-specific (anti-α-SMA) antibodies, to perform

Results
Kinetics of Msx1lacZ expression in vessels of the developing retina
Outgrowth of vessels in the retina occurs postnatally from the central optical disc. This results in the formation of a stereotyped vascular network (Fruttiger, 2007). Analysis of Msx1lacZ mice at P0 revealed that the primitive plexus was formed of ECs and did neither express smooth muscle actin, nor Msx1 (Fig. 1A, arrowheads). The hyaloid vasculature, which is present during prenatal development and regresses shortly after birth, expressed strong levels of Msx1lacZ as revealed by Xgal staining, and was covered with α-SMA-expressing mural cells (Fig. 1A, arrows). Considering the morphology of the β-galactosidase (β-gal)-positive nuclei and their position relative to isolectin B4 (IB4) and α-SMA domains, Msx1-expressing cells were likely ECs. At P3, retinal arteries have started to form from the primitive plexus. These arteries, which were part of the newly-formed vascular plexus (Fig. 1B; data not shown), were covered with α-SMA-positive mural cells. In these arteries, Msx1 expression was detected in cells with elongated nuclei, suggesting they belonged to the endothelium (Fig. 1B, arrowheads). At this stage, no Msx1 expression could be detected in less mature vessels not covered by mural cells (Fig. 1B, arrowheads). At P10, the superficial vessels have reached the peripheral edge of the retina, the capillary bed has begun to develop into the deeper layers and circulation has started (Brown et al., 2005; Fruttiger, 2007). Strikingly, at this stage Msx1lacZ expression became restricted to primary branching points in arterioles (Fig. 1C, arrowheads). Restricted expression was not yet completed by P12, since a few cells were still expressing Msx1 along the length of arteries (Fig. 2A, arrowheads), but was achieved at P14 (Fig. 1D, arrowheads). In most cases, more than one nucleus was labelled (Fig. 1F, Fig. 3D–F, Fig. 4A,C). At the branching points, we further observed strong Ib4 concentration that co-localized with Msx1-expressing cells (Fig. 1C, arrowheads). Ib4 is known to bind the basement membranes (specifically, the versican protein) and thus can label not only ECs but also microglial cells and probably other cell types including some vascular mural cells. On trypsin-digested retinas, Ib4 was unambiguously associated with vessels (not shown). Maximum Msx1 expression occurred between P14 and P23 (Fig. 1D–E), but expression was maintained at high levels up to P480 (Fig. 1F) and probably lasts over the lifetime of the animal. From P14, we observed that Msx1 also labelled secondary branches in the arteriolar network (Fig. 1F, Fig. 4D). Noticeably, at all stages, Msx1 expression was observed only in rather mature arteries that were covered by a SMA-positive mural cell coating suggesting that Msx1 expression in the endothelium requires EC-VSMC interactions. Msx1-labeled branching sites were quantitated throughout development (Fig. 2). There was a large and steady increase between P9 and P23 and thereafter the number plateaued. Therefore, restriction of the expression to branching sites lagged behind the formation of the vascular superficial bed, for which arteries reach the retinal periphery around P8 (Fruttiger, 2007). This was not achieved before the onset of blood flow, which in the mouse retina takes place between P3 and P4 (Brown et al., 2005). From P23, we observed Msx1 expression at all primary, and some secondary, branching points along the main retinal arteries.

Arteries and veins were identified in flat mounts by virtue of their distinct morphologies and differential coating by mural cells. We did not observe Msx1lacZ labelling of venules in the retina (data not shown), as in any other veins (Goupille et al., 2008). Only the superficial vascular network was labelled. This is in keeping with the fact that deeper beds are primarily formed by sprouting from veins (Fruttiger, 2007). Xgal staining reflected the specific expression of Msx1 in retinal vessels since no staining was observed in normal mice (data not shown). In contrast to what was observed in peripheral arteries (Goupille et al., 2008), Msx2lacZ could not be detected in the hyaloid, retina or choroid vessels at any stage of their development (data not shown).
**Fig. 1. Kinetics of Msx1lacZ expression in the retina.** Flat-mount retinas from P0, P3, P10, P14, P23 and P480 Msx1lacZ+/− mice were analysed. (A–F) show light microscopy views in which Xgal staining (blue, arrows) labels the nucleus of Msx1-expressing cells. (A–F') show the corresponding fluorescence fields for ECs (IB4, green) and mural cells (α-SMA, red). At P0 (A, A'), the superficial plexus that is just beginning to develop around the optic nerve does not express Msx1lacZ (arrowheads). At the same stage the hyaloid vessels are covered with α-SMA-positive cells and are labelled with Xgal (arrows). At P3 (B, B'), Msx1lacZ is expressed in elongated nuclei along the first formed retinal arteries (arrows) that are covered with α-SMA-positive cells. Note that these arteries are in continuity with the vascular immature, endothelial plexus that does not express Msx1lacZ (arrowheads). At around P10 (C, C'), Xgal staining starts to concentrate at the primary branching sites of the superficial retinal arteries (arrows). Note that some branching sites express Msx1 very weakly at this stage (arrowheads). Expression is observed in all primary branch points at later stages, such as P23 (E, E') and P480 (F, F'), and even at some secondary branches (F, F'). Xgal staining was most intense between P14 and P23 (D, E). In all panels, scale bar = 50 μm.

**Fig. 2. Quantification of Msx1-expressing branching sites during retinal vessel development.** At P12 (A), some expression is still observed along the vessels (arrowheads) in addition to branch sites (arrows). From P23 to P480 (B,C), expression is restricted to branch sites (arrows). In A–C, artery layout is depicted for legibility. (D) shows the total number of branch points along a single main artery that express Msx1 at each stage analysed. At P0 and P3, no Msx1-positive branching points were observed. They then increased significantly in number from P9 to P23. From this stage, this number remained stable. n=6, (*) P<0.05, (**) P<0.01, (***) P<0.001. In panels A–C, scale bar = 50 μm.
Confocal microscopy on flat mount retinas. This showed that the Msx1 reporter gene is expressed in ECs (Fig. 3B, E, arrowheads) and not in mural cells (Fig. 3A, D, arrows). Indeed, the nuclear β-gal signal was completely surrounded by CD31 protein and was observed in a luminal position relative to the mural marker α-SMA (Fig. 3C, F). As expected, β-gal colocalized with nuclear Hoechst staining, since the lacZ gene in this transgenic mouse is associated with a nuclear localization sequence (not shown).

Correlation between expression of Msx1 and branch point-characteristic proteins
To get insight into the role of Msx1 at arteriolar branching points, we investigated possible correlations with accumulation of other proteins. We co-stained the retina from Msx1lacZ animals for β-gal together with α-SMA (Fig. 4A, A′), desmin (Fig. 4B, B′) and NG2 (Fig. 4C, C′). In the rat retina, NG2 and desmin are expressed in immature mural cells around birth, but NG2 is expressed in pericytes in the adult and weakly in arteriole and vein VSMCs, whereas desmin remains at a high level in pericytes and VSMCs. On the contrary, α-SMA, which is also expressed in immature mural cells, becomes highly expressed in VSMCs and weakly in pericytes after birth (Hughes and Chan-Ling, 2004). According to our data, the primary branching structures were supported by a sub-population of mural cells that expressed α-SMA (Fig. 4A′), desmin (Fig. 4B′) and NG2 (Fig. 4C′). Of note, calponin is expressed primarily in VSMCs, although it has been described in pericytes of aged animals (Hughes and Chan-Ling, 2004; Hughes et al., 2006). Calponin was expressed in a fraction of branching structures, and always in cells on the distal side relative to the arteriole (not shown). Thus, at the branch sites, the sub-population of mural cells differs from others by its capacity to express simultaneously VSMC-associated proteins, such as α-SMA and calponin, together with pericyte markers like NG2 and desmin. Of note, α-SMA, desmin and calponin are proteins that participate in contraction, making this structure a candidate for specific contractile properties. Furthermore, according to Iba1 (Fig. 1C′–F′) and laminin (Fig. 4D′, arrows) expression levels, the density of the basement membrane at these branching points appeared high.
Msx1 is broadly expressed in choroid vessels

To verify whether this branch site-restricted expression pattern is more widespread in the eye vasculature, we investigated Msx1 expression in the choroid. In the mouse, choroidal vasculature is fully developed before birth and supplies oxygen and nutrients to the anterior region of the retina (Campochiaro, 2000). Similar to the retinal vasculature, it develops according to a stereotyped pattern, constituted of major arteries that grow from the optic nerve entry point toward the periphery. Msx1lacZ expression was detectable as early E16.5 and became conspicuous at E17.5. At these stages, expression took place in arterioles covered with α-SMA-expressing mural cells and also in mural cell-free capillaries (data not shown). At P0, Msx1lacZ was broadly expressed in choroidal vessels, at a high level (Fig. 6A, A'). On flat mounts, expression was observed in internal, longitudinal cells, which matched lb4 labelling, suggesting it took place in ECs. Similar to prenatal stages, expression was observed in mural cell-covered (Fig. 6A, A', arrows) as well as mural cell-free (Fig. 6A, A', arrowheads) arteries. Surprisingly, at P14 and later, Msx1lacZ expression was detected in cells resembling mural cells, since the β-gal-positive nuclei exhibited a rounder shape and a more external position (Fig. 6B, B'). Furthermore, expression was now restricted to mural cells-covered arteries. We analysed Msx1lacZ expression until P150 and the location and intensity of Xgal staining did not change with age (Fig. 6C, C').

To confirm the shift in expression of Msx1lacZ from endothelial to mural cells, we performed a confocal analysis of choroid transverse sections. At P0, the nuclear β-gal protein was observed in most CD31-positive cells of the major arterioles (Fig. 7A, arrows). Clearly, α-SMA-positive cells did not accumulate β-gal protein (Fig. 7B, arrowheads). At P21 the localization of β-gal had changed dramatically: CD31-surrounded nuclei appeared completely devoid of β-gal protein (Fig. 7C, arrows) whereas α-SMA-positive cells expressed high levels of this protein (Fig. 7D, arrowheads). This change in pattern was observed in the whole choroid vascular tree as shown in Fig. 6.

Discussion

Studying the different structures that compose the retina and choroidal vasculature may give insight into disease processes such as diabetic or hypertensive retinopathy, hypertensive choroidopathy and macular degeneration. Taking advantage of Msx1lacZ and Msx2lacZ knock-in mice, we have established that Msx1, but not Msx2, is expressed in the mouse retinal and choroidal vasculature. One of the most striking results we obtained is the specific expression of Msx1 in a cluster of ECs at primary, and sometimes secondary, arteriolar branching sites in the retina. This property might be more widespread in the peripheral vasculature. We previously reported that Msx1 expression is more intense in arterioles of the thigh muscle at branching sites (Goupille et al., 2008). Our data suggested that expression took place in VSMCs, but the situation should be revisited using confocal microscopy at these sites. The expression pattern we observe in retina raises questions about the function Msx1 may play and the mechanisms that might activate it at branching sites.

Msx1 is unlikely to be associated with formation or stabilization of retinal arteriole branches, since disruption of the Msx1 gene specifically in the endothelium does not lead to defects in branching frequency or branch stability. A function related to vessel physiology is therefore more likely. In any case, Msx1 may be responding to specific signalling molecules at these sites. The Notch pathway is of particular relevance to this issue. The initial pattern of Jag1 expression in the retina is different from that of Msx1, but at P15, Jag1-positive cells are concentrated at branch points in arteries, in both endothelial and mural cells, in a pattern strikingly similar to Msx1 (Hofmann and Luisa Iruela-Arispe, 2007). However, Jag1 null mice die by E11.5 as a result of a lack of vascular remodelling (Xue et al., 2007).
a phenotype very different from the one observed in \textit{Msx1} single or \textit{Msx1; Msx2} double null mutant (Satokata et al., 2000; Lallemand et al., 2005). Furthermore, Notch signalling plays a major role in controlling sprouting and at P6, in endothelium-specific \textit{Jag1} mutants, the density of newly formed vessels at the periphery of the retina is clearly reduced (Benedito et al., 2009). This phenotype was not observed in the endothelium-specific mutation of \textit{Msx1} (Fig. 5C,F). Altogether, these considerations imply that, if \textit{Msx1} is involved in the Notch pathway, it relays only a fraction of Notch activity.

\textit{Tie1}, an endothelial-specific receptor of the Tie receptor tyrosine kinase family with unknown ligand, is also expressed in the retinal vessels, and further concentrated at branching sites (Porat et al., 2004). Other signalling pathways, not yet characterized in the retinal vasculature, may play a role in \textit{Msx1} expression.

Conspicuously, branching sites in retinal arterioles form specific structures, which have been designated as arteriolar annuli (Henkind and De Oliveira, 1968; Simoens et al., 1992). These are characterized by hypercellularity and specific or enhanced expression of a number of genes. Among these are $\alpha$-Sma, Vimentin (Bandopadhyay et al., 2001) and Jag1 (Hofmann and Luisa Iruela-Arispe, 2007). Our own data confirm and extend these reports; in particular, they demonstrate that mural cells at the branching sites express NG2, the most characteristic marker of pericytes to date (Ozerdem et al., 2001), and simultaneously, $\alpha$-Sma and calponin, which, in mature retinal vasculature, are essentially restricted to VSMCs (Hughes and Chan-Ling, 2004).

The basement membrane, which is formed by a synergistic process between endothelial and mural cells, is also modified at \textit{Msx1}-expressing bifurcation points. A higher density of basement membrane proteins such as versican (which was detected by Ib4) and laminin is observed. Noticeably, \textit{Msx1} does not seem to play a role in endothelial secretion of basement membrane proteins since Ib4 and laminin staining in \textit{Msx1 flox/flox Msx2 flox/flox Tie2-Cre} retinas is completely normal (data not shown). Altogether, our data show that, in addition to mural cells, ECs at branching sites also exhibit specific expression programs as assessed by \textit{Msx1} expression.

---

\textbf{Fig. 5.} There is no evident phenotypic alterations in the \textit{Msx1 flox/flox Msx2 flox/flox Tie2-Cre} mouse retinas.

Retinas from heterozygous (A–C) or mutant (D–F) P7 mice were stained for CD31 (green, A,D,C,F), $\alpha$-SMA (red, B,E); similarly, retinas from 2 months old mice were stained for CD31 (green, G,J), $\alpha$-SMA (red, H,K) and desmin (red, LL). At P7, the overall structure of retina vasculature does not look changed (A,D) in mutants. Close up images of the angiogenic front do not show differences in vascular density or in the morphological characteristics and number of tip cells (C,F). At this stage, only the main arterial branches are covered by VSMCs in both the mutant (E) and the control (B). In retinas from 2 month-old mice, the density of branches along the endothelial tube appears unchanged in the mutant according to CD31 staining (G,J). The intensity and number of cells expressing $\alpha$-SMA is similar between the \textit{Msx1 flox/flox Msx2 flox/flox Tie2-Cre} mutant (K) and the \textit{Msx1 flox/+/Msx2 flox/+/Tie2-Cre} control (H). The desmin-positive population of pericytes does not look affected by the endothelial-specific Msx gene inactivation (L). Notice that, in D, the centre of the retina was accidentally lost during dissection. In panels A,B and D,E, scale bar is 400 $\mu$m, in panels G,H and J,K, 40 $\mu$m, in panels C, F and I, 100 $\mu$m and in panel L, 50 $\mu$m, respectively.
Hyperaemia (i.e. the increase in blood perfusion associated with neural activity), which is at the basis of functional neuroimaging (Magistretti and Pellerin, 1999), implies mechanisms to regulate blood flow. These have been proposed to take place at the precapillary level in the arteriole (Anderson and McIntosh, 1967). However, the existence of a precapillary sphincter in the retina at the junction between arterioles and capillaries is controversial, has been poorly documented in recent years and the sphincter itself not always rigorously defined (Friedman et al., 1964; Anderson and McIntosh, 1967; Wiedeman et al., 1976; Baez, 1977). From structural analyses, some investigators reported its existence (Benjamin et al., 1998; Ikebe et al., 2001), while other could not detect it (Pannarale et al., 1996). Furthermore, theoretical and experimental studies have shown that regulation of blood flow at the precapillary level is unlikely to play a major role in the capillary filtration coefficient (Bentzer et al., 2001; Boas et al., 2008). Functional analyses indicate that flow regulation takes place over the whole capillary network, via a coupling between astrocytes and blood vessels (Zonta et al., 2003; Cauli et al., 2004; Metea and Newman, 2006; Peppiatt et al., 2006). However, a more recent functional study, based on laser speckle flowmetry, suggests that in the retina, activity-dependent changes in blood flow are controlled largely by arterioles and that capillaries contribute little to them, without documenting specific structures responsible for this control (Srienc et al., 2010). Msx1 expression in the retina is restricted to primary and secondary branching sites between arteries and arterioles. At these sites, contraction-associated proteins are concentrated, and it is plausible that Msx1 may play a role at the arteriolar level in controlling blood flow.

Arteriolar annuli, and also Msx1 expression, could be linked to mechanical constraints at branching sites. Vascular bifurcations are associated with local modifications in rate and pattern of blood flow, including low wall and high oscillatory shear stress (Zarins et al., 1983). Noticeably, the ECs are primarily affected by shear stress, as they are in direct contact with blood flow. Indeed, genes in the endothelium are activated in response to shear stress (Andersson et al., 2005; Ni et al., 2010) via specific DNA responsive elements (Boon and Horrevoets, 2009). In addition, increased hemodynamic stresses in vivo enhance smooth muscle cell coverage of microvessels (Van Gieson et al., 2003). Shear stress is not negligible in retina arteries and arterioles (Ganesan et al., 2010), thus Msx1 expression may be linked to blood flow at branching sites. Noticeably, restriction of Msx1 expression at these sites correlates with the onset of blood flow in the deeper vascular beds of the retina (Stahl et al., 2010), which might substantially change shear stress at bifurcations. It would be necessary to perform arterial obstructive lesions in the retina to evaluate the relation between blood flow and Msx1 expression.

**Fig. 6. Kinetics of Msx1<sup>lacZ</sup> expression in the mouse choroid.** Flat-mounted chorioids at P0, P14 and P150 were stained with Xgal (blue), anti-α-SMA antibody (red) and Ib4 (green), demonstrating a broad expression of Msx1<sup>lacZ</sup> in choroidal vessels. At P0 (A,A’), Msx1-expressing cells seem to be in a luminal position relative to α-SMA and to coincide with Ib4 labelling. At this stage, they are observed in vessels either covered with α-SMA-positive cells (arrows) or not (arrowheads). At P14 (B,B’), Msx1<sup>lacZ</sup> expression is still strong in the arterioles and major capillaries, but the β-gal-positive nuclei look rounder and their position appears more to the exterior. At P150 (C,C’), this pattern of expression is maintained. In all panels, scale bar = 50 μm.

**Fig. 7. Dynamic expression of Msx1<sup>lacZ</sup> in the choroidal vessels.** Immunofluorescence was performed on transverse sections of mouse chorioids. VSMCs were labelled for α-SMA (red), ECs for CD31 (green) and Msx1<sup>lacZ</sup> expression was revealed using anti-β-gal antibodies (yellow). At P0, the β-gal protein can be observed in CD31-expressing endothelial cells (A, arrows), α-SMA expressing cells are completely negative for β-gal (B, arrowheads). At P21, the endothelial CD31-positive cells do not contain β-gal (C, arrows), in contrast to VSMCs that are strongly labelled for β-gal (D, arrowheads). These results reflect an EC-to-VSMC shift of Msx1<sup>lacZ</sup> expression during the first three weeks after birth. In all panels, scale bar = 10 μm.
Another intriguing result that we have obtained concerns the Msx1

expression in the choroid, which shifts from endothelial to mural cells in the first three weeks of life (Fig. 7). Previously, we published that Msx1 is expressed in adult mouse VSMCs and pericytes and, in the embryo, in ECs and VSMCs (Goupille et al., 2008). However, we have never observed expression of Msx1 in endothelial and mural cell populations in the same vascular bed, either simultaneously or consecutively. Both cell types are regulated by distinct mechanisms and Msx1 has been associated with a number of different cell types in distinct developmental contexts. Therefore, we think that Msx1 plays different and independent roles in the endothelial and mural lineages. In addition, we should stress that, after P21, the Msx1 pattern of expression observed in the choroid is quite similar to the pattern observed in other peripheral arterioles and capillaries (Goupille et al., 2008).

Materials and Methods

Mice

Generation of Msx1flox/flox and Msx2flox/flox mutant mice has been described previously (Houzelstein et al., 1997; Lallemand et al., 2005; Bensoussan et al., 2008). The Msx1flox conditional mutant (Fu et al., 2007) was a generous gift from Dr. Robert Maxson (Los Angeles, California, USA), the Tie2-Cre transgenic mouse (Kisaka et al., 2001), from Dr. Masashi Yanagisawa (Dallas, Texas, USA). The γ-Sm22Cre (Holtwick et al., 2002) and Rosa26lacZ reporter mouse (Muzumdar et al., 2007) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). All strains were maintained on an NMRI outbred background. Genotyping primers were previously described (Lopes et al., 2011). Phenotypic analyses were conducted with mutant embryos using !termates as controls. Animals were housed in the Institut Pasteur animal facilities accredited by the French Ministry of Agriculture to perform experiments on live mice (accreditation # B 75 15-05, issued on May 22, 2008), in accordance with the French and European regulations on care and protection of the Laboratory Animals (EC Directive 86/ 609, French Law 2001–486 issued on June 6, 2001). Protocols were approved by the veterinary staff of the Institut Pasteur animal facility and were performed in compliance with the NIH Animal Welfare Assurance #A5476-01 issued on 02/07/2007.

Processing of eye tissues

Eyes were collected at postnatal days 0 to 480 (P0–P480). Eyes were enucleated and immediately fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) pH 7.2 (Sigma) for 5 to 30 min depending on mouse age. The cornea, lens, sclera, and vitreous were excised by limbal incision under a dissecting microscope. Radial incisions were made towards their edges. Sclera, and vitreous were excised by limbal incision under a dissecting microscope. After staining, tissues were post-fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS, followed by incubation with 2.5 × 1000. Retinas were washed again three times for 5 min with 0.1% Tween-20 in PBS). The same blocking solution was used for incubation with primary antibodies overnight at 4 °C under gentle agitation. Primary antibodies are listed in Table 1. The retinas were washed four times for 5 min with 0.1% Tween-20 in PBS. The same blocking solution was used for incubation with primary antibodies overnight at 4 °C under gentle agitation. Primary antibodies are listed in Table 1. The retinas were washed four times for 5 min with 0.1% Tween-20 in PBS. Washing of eye tissues

Xgal staining

Msx1flox/flox and Msx2flox/flox genes expression was visualized by Xgal staining as described by (Houzelstein et al., 1997). After staining, tissues were post-fixed in 4% PFA for 1 h at room temperature, then washed 3 times in PBS before immunohistochemistry.

Immunohistochemistry

Retinas were permeabilised with PBS containing 0.2% Triton X-100 and 30 mM NH4Cl for 30 min. Tissues were washed 3 times in PBS then treated for 1 h with blocking buffer (1 mM MgCl2, 1 mM CaCl2, 10% goat serum and 0.5% Tween-20 in PBS). The same blocking solution was used for incubation with primary antibodies overnight at 4 °C under gentle agitation. Primary antibodies are listed in Table 1. The retinas were washed four times for 5 min with 0.1% Tween-20 in PBS, incubated for 1 h with secondary antibodies diluted in blocking buffer. Secondary antibodies (Invitrogen) were Alexa Fluor 488 goat anti-mouse and goat anti-rabbit, Alexa Fluor 568 goat anti-rabbit, Alexa Fluor 647 goat anti-rabbit, Alexa Fluor 635 goat anti-rabbit at 1:300 and Alexa Fluor 488 streptavidin at 1/1000. Retinas were washed again three times for 5 min with 0.1% Tween-20 in PBS, followed by incubation with 2.5 μg/ml of Hoechst 33342 (bisBenzimidazole trihydrochloride, Sigma) in PBS for 15 min to label nuclei. They were finally washed in PBS and two times in water and flat mounted with Dako mounting medium.

Microscopy of retinal and choroid whole-mounts

Whole-mount retinas were primarily observed under a Zeiss Axioskop fluorescence microscope, and a Zeiss Axioplan equipped with an Apotome, and analysed with the Axiovision software (Carl Zeiss, Jena, Germany). Co-localizations were performed with a confocal microscope Zeiss LSM 700 equipped with the Zen software (Carl Zeiss, Jena, Germany). All captured images were assembled using Adobe Photoshop or Adobe Illustrator (Adobe Systems, San Jose, CA, USA). For quantitative analyses, one-way ANOVA was used to compare independent experiments. Comparison between data groups was performed with the non-parametric Dunnett test.

Acknowledgements

We are very grateful to Dr. Colin Crist for critical reading of the manuscript, to Dr. Robert Maxson and Dr. Masashi Yanagisawa for generously sharing mouse strains. This work was supported by the Institut Pasteur, the CNRS and grants from the French Association pour la Recherche sur le Cancer (ARC) and Ligue contre le Cancer (LCC). Miguel Lopes was the recipient of a fellowship from the Portuguesa Fundação Ciência e Tecnologia (FCT).

Competing Interests

The authors declare no competing interests.

References

Anderson, B., Jr and McIntosh, H. D. (1967). Retinal circulation. Ann. Rev. Med. 18, 15–26.
Anderson, M., Karlsson, L., Svensson, P. A., Ulfhammer, E., Ekman, M., Jernas, M., Carlsson, L. M. and Jern, S. (2005). Differential global gene expression response patterns of human endothelium exposed to shear stress and intraluminal pressure. J. Vasc. Res. 42, 441–452.
Armulik, A., Genove, G. and Betsholtz, C. (2011). Pericytes: developmental, physiological, and pathological perspectives, problems, and promises. Dev. Cell 21, 193–215.
Bach, A., Lallemand, Y., Nicola, M.-A., Ramos, C., Mathis, L., Maurras, M. and Robert, B. (2003). Msx1 is required for dorsal diencephalon patterning. Development 130, 4025–4036.
Baez, S. (1977). Microcirculation. Ann. Rev. Physiol. 39, 391–415.
Bandopadhyay, R., Orte, C., Lawrenson, J. G., Reid, A. R., De Silva, S. and Allt, G. (2001). Contractile proteins in pericytes at the blood-brain and blood-retinal barriers. J. Neurocytol. 30, 35–44.
Benedetti, R., Rocca, C., Sorensen, L., Adams, S., Gossler, A., Fruttiger, M. and Adams, R. H. (2009). The notch ligands Dll4 and Jagged1 have opposing effects on angiogenesis. Cell 137, 1124–1135.
Benjamin, L. E., Hemo, I. and Keshet, E. (1998). A plasticity window for blood vessel remodelling is defined by pericyte coverage of the preformed endothelial network and regulated by PDGF-B and VEGF. Development 125, 1591-1598.
Bensoussan, V., Lallemand, Y., Moreau, J., Saint Clément, C., Langa, F. and Robert, B. (2008). Generation of an Msx2flox conditional null allele. Genesis 46, 276–282.
Breitfuss, P., Kogstad, L. and Grande, P. O. (2001). Capillary filtration coefficient is independent of number of perfused capillaries in cat skeletal muscle. Am. J. Physiol. Heart Circ. Physiol. 280, H2697-H2706.
Boas, D. A., Jones, S. R., Devor, A., Huppert, T. J. and Dale, A. M. (2008). A vascular anatomical network model of the spatio-temporal response to brain activation. NeuroImage 40, 1116–1129.
Biology Open

Magistretti, P. J. and Pellerin, L. (1999). Cellular mechanisms of brain energy metabolism and their relevance to functional brain imaging. Philos. Trans. R. Soc. Biol. Sci. 364, 999-969.

Friedman, E., Smith, T. R. and Kwabarara, T. (1964). Retinal Microcirculation in Vivo. Invest. Ophthalmol. 3, 217-226.

Fruttiger, M. (2002). Development of the mouse retinal vasculature: angiogenesis and vasculature maturation. Blood vessel formation is controlled by a genetic program for retinal vascularization. Invest. Ophthalmol. Vis. Sci. 43, 461-470.

Hughes, S. and Chan-Ling, T. (2004). Characterization of smooth muscle cell and pericyte differentiation in the retina in vivo. Invest. Ophthalmol. Vis. Sci. 45, 2795-2806.

Hughes, S., Gardiner, T., Hu, P., Baxter, L., Rosinova, E. and Chan-Ling, T. (2006). Altered pericyte-endothelial relations in the retina during aging: implications for vessel stability. Neurobiol. Aging 27, 1833-1847.

Ikebe, T., Shimada, T., Ina, K., Kitamura, H. and Nakatsuika, K. (2001). The three-dimensional architecture of retinal blood vessels in KR mice, with special reference to the smooth muscle cells and pericytes. J. Electron Microsc. (Tokyo) 50, 125-132.

Ishii, H., Han, J., Yen, H. Y., Sucov, H. M., Gridley, T. et al. (2002). Smooth muscle-selective deletion of Msx1 and Msx2 are expressed in sub-populations of vascular smooth muscle cells. Dev. Dyn. 227, 2187-2194.

Gyllensten, L. J. and Hellstrom, B. E. (2000). Cellular mechanisms of brain energy metabolism and their relevance to functional brain imaging. Philos. Trans. R. Soc. Biol. Sci. 364, 1455-1462.

Mitchell, D. E. and Gingrich, G. (1998). Visual recovery after monocular deprivation is driven by absolute, rather than relative, visually evoked activity levels. Curr. Biol. 8, R897.

Zuurbier, M. D., Tasic, B., Miyamichi, K., Li, L. and Luo, L. (2007). A global dynamic-fluorescence genetic reporter screen. Nature 450, 594-599.

Nehls, V. and Drenckhahn, D. (1991). Heterogeneity of microvascular pericytes for smooth muscle type alpha-actin. J. Cell Biol. 113, 147-154.

Liu, C. W., Qiu, H. and Ho, H. (2010). MicroRNA-663 upregulated by oscillatory shear stress plays a role in inflammation of endothelial cells. Am. J. Physiol. Heart Circ. Physiol. 300, H1762-H1769.

Orlidge, A. and D’Amore, P. A. (1987). Inhibition of capillary endothelial cell growth by pericytes and smooth muscle cells. J. Cell Biol. 105, 1457-1462.

Ozderem, U., Grako, K. A., Dahlbin-Huppe, K., Monosov, E. and Stallcup, W. B. (2001). NG2 proteoglycan is expressed exclusively by mural cells during vascular morphogenesis. Dev. Dyn. 222, 218-227.

Pannarale, L., Onori, P., Ripani, M. and Gaudio, E. (1996). Precapillary and perrervascular cells in the retinal microvasculature. A scanning electron microscope study. J. Anat. 188, 693-703.

Peppiatt, C. M., Howarth, C., Mobbs, P. and Attewell, D. (2006). Bidirectional control of CNS capillary diameter by pericytes. Nature 443, 700-704.

Porat, R. M., Grunevald, M., Glöborner, A., Hlin, A., Barshbin, G., Albonen, L., Alitalo, K. and Kesel, E. (2004). Specific induction of tiel promoter by disturbed flow in atherosclerosis-prone vascular niches and flow-obstructing pathologies. Circ. Res. 94, 394-401.

Rucker, H. K., Wynder, H. J. and Thomas, W. E. (2000). Cellular mechanisms of CNS pericytes. Brain Res. Bull. 51, 363-369.

Kamieniecki, J. M., and D’Amore, P. A. (2004). Development and pathology of the hyaloid, choroidal and retinal vasculature. In: Developmental Biology: A Functional Approach, 2nd ed. (eds. D. E. Lewis and J. F. Enders), pp. 128-141.

Stahl, A., Connor, K. M., Sapica, P., Chen, J., Dennon, R. J., Krah, N. M., Seaward, M. R., Willert, K. L., Aderman, C. M., Guerin, K. I. et al. (2010). The mouse retina as an angiogenesis model. Invest. Ophthalmol. Vis. Sci. 51, 2813-2826.

Sweet, D. T., Chen, Z., Wiley, D. M., Bautch, V. L. and Tzima, E. (2011). The adaptor protein Shc integrates growth factor and ECM signaling during postnatal angiogenesis. Blood [Epub ahead of print] doi: 10.1182/blood-2011-10-384560.

Towler, D. A., Shan, J. S., Cheng, S. L., Finkinger, J. M. and Loewy, A. P. (2006). Osteogenic regulation of vascular calcification. Ann. New York Acad. Sci. 1068, 327-333.