Msx genes define a population of mural cell precursors required for head blood vessel maturation

Miguel Lopes[1], Olivier Goupille[1§], Cécile Saint Cloment[1], Yvan Lallemand[1], Ana Cumano[2] and Benoît Robert[1]*

[1] Institut Pasteur, Génétique Moléculaire de la Morphogenèse, CNRS URA 2578, F-75015 Paris, France
[2] Institut Pasteur, Dévelopment des Lymphocytes, INSERM U668, F-75015 Paris, France

[*] Correspondence to Benoît Robert, Institut Pasteur, Génétique Moléculaire de la Morphogenèse, 25 rue du Dr Roux, 75724 Paris Cedex15, France
E-mail: benoit.robert@pasteur.fr
Telephone: +33(0)145688965
Fax: +33(0)145688963

[§]Present address: Commissariat à l'Energie Atomique, 18 route du Panorama BP6 92265 Fontenay aux Roses cedex

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ABSTRACT

Vessels are primarily formed of an inner endothelial layer, secondarily covered by mural cells, namely vascular smooth muscle cells (VSMCs) in arteries and veins, and pericytes in capillaries and veinules. We previously showed that, in the mouse embryo, *Msx1*^{Lacz} and *Msx2*^{Lacz} genes are expressed in mural and in a few endothelial cells. To unravel the role of Msx genes in vascular development, we have undertaken to inactivate the two Msx genes specifically in mural cells by combining the *Msx1*^{lacz}, *Msx2*^{lox} and *α-Sm22Cre* alleles. Optical projection tomography (OPT) demonstrated abnormal branching of the cephalic vessels in E11.5 mutant embryos. The carotid and vertebral arteries showed an increase in caliber, related to reduced vascular smooth muscle coverage. Taking advantage of a newly constructed *Msx1*^{CreERT2} allele, we could demonstrate by lineage tracing that the primary defect lies in a population of VSMC precursor cells. The abnormal phenotype that ensues is a consequence of impaired BMP signaling in the VSMC precursors that leads to downregulation of the metalloprotease-2 (*Mmp2*) and metalloprotease-9 (*Mmp9*) genes. These two genes are essential for cell migration and integration into the mural layer. Improper coverage by VSMCs secondarily leads to incomplete maturation of the endothelial layer.

INTRODUCTION

The cardiovascular system is composed of the heart, arteries, arterioles, capillaries, veinules and veins. Two distinct layers constitute the blood vessels: the inner endothelium and the external mural layer. Endothelial cell (EC) assembly takes place first. Within the mouse embryo, mesodermal progenitors give rise to the angioblast, an endothelial precursor. The angioblasts start to aggregate as early as embryonic day (E) 7.5, forming the ventral and dorsal aortas as well as the vitelline vascular tree. The expansion and specialization of this initial basic system is achieved by angiogenesis, involving endothelial cell sprouting, vessel branching and intussusception from existing blood vessels (Risau, 1997).

Vascular maturation takes place secondly and confers the existing endothelial tube contractility and resistance (Jain, 2003). Its primary actors are the mural cells that form a multi-layered vascular smooth muscle around arteries and veins, whereas, in capillaries and veinules, the mural layer is composed of sparse pericytes. These mural cells originate from different sources depending on their position within the body. Most vascular smooth muscle cells (VSMCs) of the head and aortic arch have a neural crest origin (Jiang et al., 2000; Etchevers et al., 2001; Korn et al., 2002; reviewed in Majesky, 2007) whereas those of the trunk primarily derive from the somitic and lateral plate mesoderm (Esner et al., 2006;

Majesky, 2007; Santoro et al., 2009; Wiegreffe et al., 2009). Vascular maturation further involves the development of an elastic lamina between endothelial and vascular smooth muscle cells and of an extracellular matrix (ECM) that embeds both layers (Jain, 2003).

Vascular maturation involves proliferation, migration and differentiation of VSMCs. These are orchestrated by multiple signaling pathways, including: PDGF-*ß* / PDGF-R*ß* (Hellstrom et al., 1999), TGF-ß (Majack, 1987; Battegay et al., 1990; Nishishita and Lin, 2004), Angiopoetin1-Tie2 (Fukuhara et al., 2008; Saharinen et al., 2008), BMP (El-Bizri et al., 2008; Spiekerkoetter et al., 2009; Bai et al., 2010).

In the mouse, the Msx gene family is composed of three homeodomain transcription factors. *Msx1* and *Msx2* play important and often overlapping functions in the development of craniofacial structures, neural tube and limb (Alappat et al., 2003; Bach et al., 2003; Lallemand et al., 2009). In humans, *MSX1* mutations lead to cleft palate and lips, as well as tooth agenesis (Vastardis et al., 1996; Kapadia et al., 2007). *MSX2* has been involved in craniosynostosis (Wilkie, 1997). *Msx3* is absent from the human genome (Finnerty et al., 2009), and in the mouse its expression is restricted to the dorsal aspect of the neural tube (see Ramos and Robert, 2005).

Recently, we have shown that in the adult mouse, both *Msx1* and *Msx2* genes are expressed in a subset of peripheral artery VSMCs. Furthermore, *Msx1* is expressed in pericytes of capillaries and *Msx2* in endothelial cells of the dorsal aorta. In the embryo, both *Msx1* and *Msx2* were detected in some endothelial cells of the aorta from E14.5 and *Msx1* additionally in mural cells of the intersomitic arteries, from E10.5 (Goupille et al., 2008). We have undertaken the present study to clarify the role of Msx genes in vascular development. Phenotypic analyses and gene expression assays demonstrate that VSMC coverage is reduced in the *Msx1*$^{-/-}$*; Msx2*$^{lox/-}$*; α-Sm22Cre* double mutant (hereafter referred to as *Sm22Cre Msx1/2* mutant), resulting in an increase in vessel diameter and impairment of endothelium maturation. By genetic lineage tracing, we show that the primary defect lies in a population of VSMC precursor cells. BMP signaling and its targets *Mmp2* and *Mmp9* are downregulated in *Msx1; Msx2* mutant VSMCs, leading to an impaired incorporation of *Msx1*-expressing vascular smooth muscle precursors in the mural layer.

MATERIAL AND METHODS

Mice

We previously reported the generation of *Msx1* null (*Msx1*^{LacZ}), *Msx2* null (*Msx2*^{GFP}, *Msx2*^{LacZ}) and conditional (*Msx2*^{lox}) mutant alleles (Houzelstein et al., 1997; Lallemand et al.,

2005; Bensoussan et al., 2008). The *Msx1*^{lox} conditional mutant allele (Fu et al., 2007) was a generous gift from Dr. Robert Maxson (Los Angeles, California, USA), the *Tie2-Cre* transgenic mouse (Kisanuki et al., 2001), from Dr. Masashi Yanagisawa (Dallas, Texas, USA). The α-*Sm22Cre* (Zhang et al., 2006) and *Rosa*$^{mT/mG}$ (Muzumdar et al., 2007) engineered mice were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA). The *Msx1*^{CreERT2} mouse was generated by introducing the CreERT2 coding sequence (Feil et al., 1996; a kind gift of Pierre Chambon, Illkirch, France) at the initiator ATG site of *Msx1* by homologous recombination in ES cells. *CreERT2* interrupts the *Msx1* coding sequence, thus creating a null allele. After Southern blot selection, recombinant cells were injected into blastocysts and these reimplanted using standard protocols (Y. Lallemand and B. Robert, unpublished). All mice were maintained on an NMRI outbred background. Genotyping primers are listed in Supplementary Table S1. All studies were conducted using mutant embryos with littermates as controls.

Administration of Tamoxifen to *Msx1*^{CreERT2} mice

Tamoxifen (Sigma) was dissolved in ethanol, emulsified in sunflower oil (Sigma) and then sonicated 3 times for 5 seconds at a final concentration of 10 mg/ml. Tamoxifen was intraperitoneally injected either three times at 3.5 mg per injection per pregnant female (weight = 30 g), or two times at 2.5 mg per injection. Successive injections were always performed with 12 hours intervals.

Optical Projection Tomography

Embryos were fixed overnight in 4% paraformaldehyde and non-specific epitopes blocked over night in 0.1% sodium azide, 1 mM Mg/Ca, 1% BSA, 10% Goat Serum, 0.5%triton and 0.5% tween. A one-week incubation with rat anti-mouse CD31 (BD pharmigen) antibody was performed followed by a 5-day incubation with a secondary anti-rat antibody (Alexa 546, Invitrogen). Acquisition and treatment of images using Optical Projection Tomography was achieved by Bioptonics MRC Technology, Crewe Road South Edinburgh EH4 2SP Scotland according to published protocols (Sharpe et al., 2002).

Quantitative Real Time PCR (qRT-PCR) on dissected embryonic cephalic tissues

RNA was extracted with RNeasy mini extraction kit (Qiagen). Real time PCR was performed with a Step One Plus machine (Applied Biosystems, Warrington, UK) using Sybr PCR Mastermix (Applied Biosciences). Primers are listed in Table S4 in Supplementary material.

The *Gapdh* gene was used as a reference. PCR efficiency was in the range of 98% to 100% for all assays. PCR cycle parameters were: 10 minutes at 95°C initial incubation, followed by 15 seconds at 95°C; and 1 minute at 60°C for 40 cycles. For each gene studied, multiple RNA samples were analyzed, n=5 for simple *Msx1* and simple *Msx2* mutants, n=4 for *Sm22Cre Msx1/2* mutants, each in duplicate. Data are expressed as fold changes ($2^{-(\Delta ctgene-\Delta ctref)}$) using $Msx1^{+/-}$; $Msx2^{lox/+}$; a-$Sm22^{cre}$ triple heterozygotes as a reference.

***In situ* hybridization**

E11.5 embryos were fixed for 2 hours in 4% paraformaldehyde (Sigma-Aldrich), immersed in 15% sucrose and O.C.T Compound (TissUE-Tek) before being frozen in liquid N_2 and cryostat sectioned at 20 µm. The *Bmp4* probe was a gift from Dr. B. Hogan. Automated in situ hybridizations were performed with an InsituPro VSi apparatus (Intavis bioanalytical instruments AG, Köln, Germany).

Immunohistochemistry

Immunohistochemistry was performed as in Goupille et al. (2008) except that Xgal staining was omitted. Antibodies are listed in Supplementary Tables S2 and S3. A Zeiss Axioplan equipped with an Apotome and Axiovision software (Carl Zeiss, Jena, Germany) was used for vessel section surface measurements. All images were assembled in Adobe Photoshop and Adobe Illustrator (Adobe Systems, San Jose, CA, USA).

Flow Cytometry

E12.5 embryos used were either heterozygous or homozygous at the a-*Sm22Cre*; $Rosa^{mT/mG}$; $Msx1^{LacZ}$; $Msx2^{LacZ}$ loci. They were genotyped before cell dissociation. Cells were dissociated mechanically with a 1ml 26 GA syringe and resuspended in Dulbecco's Modified Eagle Medium (Invitrogen) containing 2% Fetal Bovine Serum (Invitrogen). VSMCs were sorted on the basis of *Gfp* expression. For ECs, the cell suspension was labeled with Phycoerythrin-conjugated anti-CD31 antibodies (BD-Biosciences). Cells were then washed with PBS and fluorescence was quantified as relative fluorescence units on a MoFlo High Performance Cell Sorter (Beckman Culture, Krefeld, Germany). Values are reported as mean fluorescence units.

In vitro assays

VSMCs were FACS-sorted as described above and maintained in DMEM supplemented with 10% fetal bovine serum and streptomycin on collagen I (BD Biosciences) coated plates. The

medium was changed every 48 hours and cells were passed for a maximum of 6 times (i.e. up to 20 days) using accutase digestion (GIBCO). Recombinant mouse Bmp4 (Roche) was added to *Msx1*$^{-/-}$; *Msx2*$^{-/-}$ cells and these were incubated for a further 48 hours, then cells were lysed and RNA extracted with RNeasy mini extraction kit (Qiagen). *Msx1*$^{+/-}$; *Msx2*$^{+/-}$ cells were transfected with *Msx1* and *Msx2* siRNA (siGENOME SMART pool, Dharmakon) and with siGLO Risc-Free control siRNA (Dharmakon) as a control of transfection efficiency. DharmaFECT transfection reagent (Dharmakon) was used according to the manufacturer's instructions. After 48 hours, cells were lysed and RNA extracted as described above.

Statistical analysis

For results from qRT-PCR, diameter measurements and cell number quantification, means ± SEM were calculated. For qRT-PCR experiments, results from Msx mutant analysis were expressed as fold change relative to Msx double heterozygous littermates or as absolute values. Cell counts and area measurements were performed with the ImageJ version 1.43g software. One-way ANOVA was used to compare independent experiments. Comparison between data groups was performed using non-parametric Dunnett test. All the statistical analyses were performed using GraphPad Prism version 5.0 for Apple, GraphPad software, San Diego, CA, USA).

RESULTS

***Msx1* and *Msx2* gene expression in the mouse embryo head vessels and mutation strategy**

We previously demonstrated that, in the adult mouse, *Msx1* and *Msx2* are expressed in the VSMCs of peripheral arteries (Goupille et al., 2008). In E11.5 embryos, *Msx1*^{Lacz} was detected in a few endothelial cells of the carotid artery (CA) (Fig.1 A). *Msx1*^{Lacz} expression was also observed in mesenchymal cells outside the CA, some of which co-expressed *Msx2*^{GFP} (Fig.1 C). *Msx2*^{GFP} was mainly expressed in differentiated VSMCs of the CA (Fig. 1 B). At E12.5, *Msx1*^{Lacz} was detected mainly in the endothelial layer of the CA and vertebral artery (VA) but not in veins (Supplementary Fig. S1). Expression of *Msx1*^{Lacz} was also observed in a few cells of the most external VSMC layer, and again in mesenchymal cells further away from the CA (Supplementary Fig. S1), *Msx2*^{GFP} expression was maintained in the VSMCs at this stage.

To inactivate both genes in blood vessels, we combined a null (Lallemand et al. 2005) and a floxed (Bensoussan et al., 2008) *Msx2* allele and the *α-Sm22Cre* transgene (Zhang et al.,

2006), together with *Msx1*^{lacZ} null alleles. Using this strategy *Msx1* is inactivated in the two layers of the blood vessel whereas *Msx2* is inactivated only in the VSMCs. The specific *α-Sm22Cre* transgene we used was chosen to inactivate *Msx2*^{lox} because of its early activation in mural cells (El-Bizri et al., 2008). The *Rosa*$^{mT/mG}$ allele ubiquitously produces a membrane-bound Tomato-red protein, which is replaced by a membrane-bound GFP protein after Cre-mediated recombination (Muzumdar et al., 2007). When associated with this allele, the *α-Sm22Cre* transgene drives expression of the GFP in VSMCs, at embryonic stages, before they integrate into the carotid artery (CA) and consequently, before they express differentiation markers such as alpha-smooth muscle actin (*α-Sma*) (Supplementary Fig. S2). *Sm22Cre Msx1/2* mutants die a few hours after birth due to *Msx1* deficiency (Houzelstein et al., 1997), in contrast to constitutive double mutants that die at E14.5 (Lallemand et al., 2005).

Inactivation of *Msx1* and *Msx2* in the VSMC lineage, but not the endothelium, leads to defects in head vascularization

By Optical Projection Tomography (OPT), we first observed over-branching of the CA at E11.5 in the *Sm22Cre Msx1/2* mutant (Fig.1 D-G; supplementary movies S1A, S1B) but neither in the *Msx1* nor *Sm22Cre-Msx2* single mutants (data not shown). The number of primary and secondary CA branches was found to increase by about 1.4 fold in the mutant (Fig.1 H). No branching defect was detected in capillaries by immunofluorescence on sections (data not shown). Furthermore, a two-fold increase in arterial caliber was observed in the *Sm22Cre Msx1/2* mutant. At E11.5, the CA section surface raised from ca. 1,400 μm^2 in the control to ca. 2,900 μm^2 in the mutant (Fig. 1I-L). The vertebral artery (VA) caliber was also increased (data not shown). No change was observed in single *Msx1* or *Sm22Cre-Msx2* mutants. Contrary to the CA, no significant change was observed in dorsal aorta sections of the double mutant (ca. 13,200 μm^2) relative to control (ca. 12,800 μm^2) (Fig. 1M-P).

Strikingly, quantification of α-SMA-positive cells in the CA demonstrated a two-fold reduction in the number of VSMCs in the double mutant (Fig. 2A,D) as compared to control (Fig. 2G). RNA was extracted from heads of E11.5 embryos (Fig. 2H) and the expression level of a set of genes measured by qRT-PCR. In the *Sm22Cre Msx1/2* mutant, VSMC-specific transcripts were decreased correlatively to the reduced coverage of the vessels (Supp. Fig. S3). The number of ECs was not reduced (Fig. 2G), and accordingly, expression of *CD31* and Jagged1 (*Jag1*), two genes expressed early in the endothelium, was not affected by the mutation (Fig. 2B,E,I). Unexpectedly, the VE-cadherin level appeared markedly decreased on sections (Fig. 2C,F). According to qRT-PCR (Fig. 2I), *VE-cadherin* transcripts were reduced

by 26% in the *Msx1* simple mutant and 57% in the *Sm22Cre Msx1/2* double mutant. Similarly, von Willebrand's Factor (*vWF*) transcripts were reduced by 25% and 39%, respectively. Noticeably, *VE-cadherin* and *vWF* are among the genes expressed late in the endothelium (Dejana et al., 1989; Navarro et al., 1995), suggesting impairment in maturation. The basal lamina, analyzed using anti-collagen IV, laminin 1 and fibronectin antibodies, appeared normal in the mutant (Supp. Fig. S4).

At birth, vascular anomalies were observed in the head of *Sm22Cre Msx1/2* mutants. The superficial temporal artery showed more branching than in the control; aneurysms and hemorrhages were frequently observed (Supp. Fig. S5). No major branching defect or hemorrhage was observed in either *Msx1* or *Sm22Cre-Msx2*$^{lox/-}$ single mutants.

To identify in which cell layer *Msx1* is required, we took advantage of a conditional *Msx1* allele (Fu et al., 2007) to selectively inactivate *Msx1* in the endothelium and in the VSMC lineages (Supp. Fig. S6). This showed that *Sm22Cre*-driven inactivation of *Msx1* and *Msx2* results in the same phenotype as the *Sm22Cre Msx1/2* mutation, i.e. increase in the CA section surface and reduction in VSMC coverage (Supp. Fig. S6A,B,D). On the other hand, *Tie2Cre*-driven *Msx1* and *Msx2* inactivation did not affect the CA diameter or VSMC coverage (Supp. Fig. S6B,C,D). Therefore, *Msx1* must be required in cells of the smooth muscle lineage, and no role for Msx genes in the endothelium could be evidenced.

Msx1 and *Msx2* are expressed in VSMCs progenitors

According to our results, Msx1 and Msx2 transcription factors are essential for proper formation of the mural cell layer. However, the weak expression of *Msx1*^{Lacz} in this layer precludes a role for Msx1 in mature VSMCs. An alternative explanation is that Msx genes would be required in VSMC precursors before they are recruited to the vessel wall. To investigate this hypothesis, we constructed a Tamoxifen-inducible *Msx1*^{CreERT2} allele (Lallemand et al., in preparation). Properties of this allele are briefly reported in the Supp. Fig. S7. *Msx1*^{CreERT2} was used in conjunction with the *Rosa*$^{mT/mG}$ allele (Muzumdar et al., 2007).

Pregnant dams were injected three times with Tamoxifen at E8.0, E8.5 and E9.0. Embryos were thereafter analyzed either at E10.5, E11.5 or E12.5. At E10.5 and E11.5, we observed a number of GFP-positive mesenchymal cells in the region between the neural crest-derived root ganglion and the carotid artery (Fig. 3A,B). Furthermore, from E10.5 to E12.5, the VSMC layer was progressively invested by GFP-positive cells. Many of them co-expressed *α-Sma*, demonstrating that they had differentiated into bona fide VSMCs (Fig. 3C-

F). The CA is just forming at E9.0 (Walls et al., 2008), and is unlikely to be covered by mural cells. Therefore, the GFP-positive cells we observed in the mural layer at E12.5 after Msx1^{CreERT2} activation at E8.0-E9.0 are unlikely to derive from pre-existing mural cells proliferating in situ.

Using *Msx1*^{CreERT2}; *Rosa*$^{mT//mG}$, we further analyzed covering of blood vessels by GFP-positive cells in an Msx null context, taking advantage of the fact that *Msx1*^{CreERT2} is an *Msx1* null allele (Fig. 3G-J). Tamoxifen was injected at E8.0 and E8.5 and embryos analyzed at E11.5 or E12.5. At E11.5, when at least one functional allele for *Msx1* and *Msx2* remained, we observed GFP-positive cells in close proximity to the CA, and a population of cells that had reached the mural layer (Fig. 3G, arrows). When both *Msx1* and *Msx2* were inactivated, GFP-positive cells similarly migrated to the CA region, but very few were found close to or in the mural layer (Fig. 3H, arrow), to which they failed to attach. At E12.5, significantly fewer GFP-positive cells were observed immediately adjacent to the CA, and even less in the mural layer, in the mutant (Fig. 3I-J).

Msx1-positive precursors were observed to express also *Msx2*^{Lacz} before they integrate the mural layer (Supp. Fig. 8A). Furthermore, we used the *Msx1*^{CreERT2} allele to inactivate *Msx2*, in an *Msx1* mutant context (*Msx1*$^{CreERT2/-}$; *Msx2*$^{lox/lox}$). Tamoxifen was injected at E8.0, E8.5 and E9.0. At E11.5, the CA exhibited the same abnormal phenotype as in *Sm22Cre Msx1/2* mutants, i.e. increase in vessel diameter and depletion in mural cells (Supp. Fig. 8B, C). We conclude that *Msx2* was inactivated by *Msx1*^{CreERT2} in *Msx1*-expressing precursors before they reached the mural layer.

Msx genes are essential for proper *Mmp2* and *Mmp9* expression

Migration, survival and proliferation of VSMCs have been shown to critically depend on the matrix metalloproteinases Mmp2 and Mmp9 (reviewed in Newby, 2006). We therefore evaluated the level of Mmp2 protein by immunofluorescence on transverse sections from E12.5 embryos, in which *Msx1*-expressing precursors had been labeled with GFP at E8.0-E9.0 using *Msx1*^{CreERT2} and *Rosa*$^{mT//mG}$ alleles (Fig. 4A-F). Whereas, in the control, many GFP-positive cells accumulated Mmp2 and integrated in the mural layer, very few cells did so in the mutant (compare Fig. 4A,C,E and B,D,F). Mmp reduction in the mutant was confirmed by qRT-PCR on cephalic VSMCs, FACS-sorted from E12.5 embryo heads using the *α-Sm22Cre* transgene together with Rosa$^{mT/mG}$ (Supp. Fig. S9). In *Msx1*$^{-/-}$; *Msx2*$^{-/-}$ mutant VSMCs, we observed a severe reduction in expression of both *Mmp2* (52% of control) and *Mmp9* (40%) (Fig. 4G). *Mmp2* expression was also lower in the *Msx1*$^{-/-}$ mutant (56% of

control) but in this genotype, this was compensated by an increase of *Mmp9*. No reduction in expression was observed in the *Msx2*$^{-/-}$ mutant.

Mmp2 and Mmp9 are critical factors for survival and proliferation of VSMCs (Newby, 2006). However, we observed no change in apoptosis, using lysotracker staining (Supp. Fig. S10A,B), or in proliferation rate, using an anti-phospho-Histone H3 antibody (Supp. Fig. S10C,D), between controls and *Sm22Cre Msx1/2* mutants. Phospho-Histone H3-positive cells represented 2.7% of total cells in either genotype (Supp. Fig. S10E).

The BMP pathway is affected in *Msx1*$^{-/-}$; *Msx2*$^{-/-}$ VSMCs

Mmp2 and *Mmp9* have previously been characterized as targets of the BMP signaling pathway in mural cells (El-Bizri et al., 2008). Msx genes are involved in BMP signaling at several sites during development. We therefore analyzed the expression levels of a number of BMP ligands and receptors in VSMCs, FACS-sorted as previously described (Fig 5.A). *Bmp4* showed the most significant downregulation in *Sm22Cre Msx1/2* mutant cells. *Bmp2, Bmp6* and *Bmp7* transcripts were also reduced, albeit to a lesser extent. *Bmp2* expression level proved low and its variation is not expected to account for a reduction in the overall BMP signaling. On the other hand, *Bmp7* is expressed at a higher level than *Bmp4* and may contribute to this reduction in the mutant. No significant change in expression was observed for the Bmp receptor genes, namely *Bmpr1a*, *Bmpr1b* and *Bmpr2*.

Reduction of *Bmp4* expression around the CA was confirmed by *in situ* hybridization on sections from E11.5 *Sm22Cre Msx1/2* embryos (Fig. 5Bb''). The reduction in BMP signaling was further demonstrated by analyzing Smad1/5/8 phosphorylation by immuno-histochemistry at E11.5. The number of phospho-Smad positive cells was conspicuously reduced in the double mutant (Fig. 5C). Quantification of phospho-Smad-positive cells on sections showed that, in control embryos, 22% of the cells stained with the anti-phospho-Smad antibody versus 13% in the double mutant, corresponding to a 40% decrease (Fig. 5D).

To further demonstrate that, in the VSMC lineage, Msx genes act upstream of BMPs, VSMCs possessing one active allele for *Msx1* and *Msx2* were sorted using *a-Sm22*-activated *Rosa*$^{mT/mG}$, and Msx gene inactivation was mimicked in culture using Msx-targeted siRNAs. VSMCs retained their phenotype when cultured, based on *α-Sma* expression and morphological characteristics (Fig. 6A). Transfection efficiency was assessed using a fluorescent non-targeted siRNA (NT siRNA) (Fig. 6A), which did not interfere with *Msx1* or *Msx2* expression (Fig. 6B). Increasing amounts of Msx-specific siRNAs progressively reduced *Msx1* and *Msx2* transcript levels, reaching maximal efficiency at 25 ng/ml (Fig. 6 B).

Under these conditions, 88% of *Msx1* and *Msx2* transcripts were lost. *Bmp4* and *Bmp7* transcripts were correlatively reduced, and similarly *Mmp2* (Fig. 6 C). To get further confirmation, VSMC precursors were sorted in the same way from *Msx1; Msx2* double null mutants and after a few days in culture, demonstrated to express low levels of *Mmp2* and insignificant levels of *Mmp9* transcripts. After 48 hours in culture with increasing amounts of exogenous Bmp4, the cells did not show phenotypic changes (Fig. 6D) but the *Mmp2* expression levels raised significantly in a dose-dependent manner (Fig. 6E). Altogether, these results confirm that Msx genes act upstream of *Bmp4* and *Bmp7* in VSMC progenitors and that, as previously demonstrated (El Bizri et al., 2008), BMP signaling is required for *Mmp2* expression. In culture, *Mmp9* expression decreased dramatically and no conclusion could be drawn for this gene.

Lower VSMC coverage leads to reduction in mural to endothelial cell signaling and impairs endothelium maturation

As mentioned above, whereas the number of ECs and expression of early endothelial markers were not decreased in the *Sm22Cre Msx1/2* mutant, late-expressed endothelial markers were downregulated (Fig. 2G). We verified that this was not correlated with a change in arterio-venous identity of the CA using antibodies against neuropilin-1, an artery-specific marker (Klagsbrun et al., 2002) (Supp. Fig.11). Downregulation of endothelial markers suggested that lower VSMC coverage leads to impairment in signaling between the two layers, resulting in defects in endothelium maturation. By qRT-PCR, we did not observe a significant difference in expression for ligands produced by the endothelium, such as *Tgfb1* or *Pdgfb*, in single or double Msx mutants as compared to controls (Fig. 7A). In contrast, mRNAs for mural cell-secreted factors were detected at lower levels in the *Sm22Cre Msx1/2* mutants. *VEGF-A* and *Angpt1* levels were decreased by 37% and 36% respectively (Fig. 7B). This can be readily explained by the reduction in mural cell coverage, which would impact on the endothelium. It has recently been demonstrated that, in culture Tie2 is concentrated at the cell membrane by its ligand, Angpt1 (Fukuhara et al., 2008; Saharinen et al., 2008). In keeping with these results, we observed, in normal embryos, a high concentration of Tie2 at the endothelial cell membrane on the external side, facing the mural cells, and on the lateral side adjacent to neighboring endothelial cells (Fig. 7C,C',D). On the contrary, Tie2 was diffusely distributed over the EC membrane in the *Sm22Cre Msx1/2* mutant (Fig. 7E,E',F) correlating with the decreased Angpt1 secretion by the thinner mural layer.

To confirm these results, we sorted ECs by FACS (using a CD31 antibody) from E12.5 heads (Supp. Fig. S9). In ECs, Kruppel-like factor 2 (*Klf2)* expression is dependent on Tie2 activation (Sako et al., 2009). Accordingly, we detected a 50% decrease in *Klf2* expression in the constitutive *Msx1; Msx2* double mutant ECs (Fig. 7G). Fig. 7H schematically represents the consequences for the endothelium of the reduction in mural cell coverage associated with Msx deficiency.

DISCUSSION

An essential role for Msx genes in head vasculature

We report for the first time that combined *Msx1* and *Msx2* gene deficiencies lead to major defects in blood vessels, namely an excess of branching and increase in caliber of major head arteries such as the CA, hemorrhages and aneurysms. None of these defects is observed in *Msx1* or *Msx2* single mutants. Vascular defects are related to a reduction in mural cell coverage of head arteries.

Similar to the cardiac outflow tract (Rentschler et al., 2010), VSMCs in most head vessels derive from neural crest cells (NCCs), whereas the endothelium is of mesodermal origin (Jiang et al., 2000; Etchevers et al., 2001; Korn et al., 2002). In particular, in the mouse embryos, contribution of NCCs has been demonstrated for the CAs, whereas neural crest-derived VSMCs could not be detected at any stage in the dorsal aorta (Jiang et al. 2000). In the latter, VSMCs have been shown to share a lineage with somitic mesoderm (Esner et al., 2006; Wiegreffe et al., 2009). *Msx1* and *Msx2* are strongly expressed in NCCs (reviewed in Bendall and Abate-Shen, 2000; Ramos and Robert, 2005). Contrary to Xenopus, where *Msx1* is mandatory for neural crest formation (Monsoro-Burq et al., 2005), combined deficiencies of *Msx1* and *Msx2* do not preclude neural crest formation or migration in the mouse. However, they severely affect neural crest subpopulations, resulting in pleiotropic defects in NCC derivatives (Ishii et al., 2005). Thus, the *Msx1; Msx2* double mutation impairs differentiation, but not migration, of cranial NCCs that form frontal bones (Han et al., 2007). In the outflow tract of the heart, Msx genes are required to inhibit excessive proliferation of post-migratory NCCs (Chen et al., 2007). In contrast, Msx genes do not seem to play a role in proliferation of the NC-derived cells that contribute to the cranial ganglia and the first pharyngeal arch, but in preventing their apoptosis (Ishii et al., 2005). We propose that the head smooth muscle defects reported here are linked to a specific neural crest subpopulation that depends on functional Msx genes to give rise to smooth muscle progenitor cells. Indeed, some images (Fig. 3A,B) strongly suggest that the *Msx1*-expressing VSMC precursors

migrate from dorsal regions. Of note, some VSMCs form in Msx mutant head vessels, confirming that only a subpopulation of precursors is affected. This is in agreement with the heterogeneity of origin for VSMCs (reviewed in Majesky, 2007). In addition, we have not observed branching defects in the intersomitic vessels (data not shown) or a reduction in the mural coverage of the dorsal aorta (data not shown). Thus, Msx deficiency does not seem to affect non-NC derived VSMCs and the vessels they cover.

***Msx1* is expressed in VSMC precursors that require Msx genes to migrate to the CA**

At E12.5, *Msx1* is predominantly expressed in the endothelium. Defects in the double mutant might therefore result from the conjunction of *Msx1* deficiency in the endothelium and *Msx2* in the VSMCs. However, the same abnormal vascular phenotype was observed in *α-Sm22Cre*; $Msx1^{lox/lox}$; $Msx2^{lox/lox}$ mutants, whereas specific mutation of *Msx1* and *Msx2* in the endothelium did not lead to vascular defects. Our data show that the primary defect in the *Sm22Cre Msx1/2* mutant lies in mural cell precursors before they reach the blood vessels. Noticeably, the *Sm22Cre* transgene we used is expressed early in the mural lineage (Fig. S2), allowing inactivation of *Msx1* and *Msx2* before VSMCs attach to the mural layer. Taking advantage of the $Msx1^{CreERT2}$ allele and an inducible reporter, we could trace back cells that once expressed *Msx1*. Tamoxifen injections were performed at stages (E8.0-E9.0) when mural cells have not yet differentiated in the head vessels (Walls et al., 2008). Under these conditions many GFP-positive cells were observed at E11.5 and even more at E12.5 in the mural layer. This implies that these cells derive from VSMC precursors that express *Msx1*, which migrate to progressively populate the mural layer. VSMCs derived from *Msx1*-expressing precursors do not express *Msx1* any more after differentiation. Similarly, switch-off of *Msx1* expression before differentiation has been reported for VSMC adventitial progenitors in the aorta (Passman et al., 2008).

Cell lineage studies clearly demonstrate that cells derived from *Msx1*-expressing precursors accumulate in normal amounts in the region around the CA, in a context of Msx deficiency. However, most of these cells fail to reach the artery and to integrate in the mural layer. This suggests late migration defects of the VSMC precursors, linked to reduction in *Mmp2* and *Mmp9* expression in the mutant. Mmp2 and Mmp9 are metalloproteinases known for freeing smooth muscle cells from the cell-matrix contacts that normally restrict their migration (Kenagy et al., 1997; Newby, 2006). The majority of *Msx1*-expressing VSMC precursors that fail to reach the CA do not accumulate detectable amounts of Mmp2, and may therefore be impeded in making their way through the vessel ECM. Of note, some cells

manage to integrate the mural layer while not expressing Mmp2. These possibly express other proteinases, in keeping with the phenotypic heterogeneity of VSMCs (Majesky, 2007).

Proper BMP signaling in head VSMCs depends on Msx genes

Msx genes have been previously associated with BMP signaling at several sites during development. Functional analysis of tooth or palate formation further demonstrated that these genes can act either upstream or downstream of *Bmp4* (Chen et al., 1996; Zhang et al., 2002). *Sm22Cre Msx1/2* double mutants exhibit a severe downregulation of the BMP pathway in the VSMC lineage. Noticeably, Bmp7 is the prevalent ligand of this family in the head VSMCs and its depletion, together with that of Bmp4, should result in a strong reduction in global BMP signaling in mutant VSMCs. The BMP pathway has pleiotropic effects on the vasculature, as discussed by Abe (2006). Manipulations reducing BMP levels usually result in impairment of VSMC coverage and a correlative dilation of the vessel [e.g. *flk1*-driven deletion of *BmpR1a* (Park et al., 2006); *a-Sm22*-driven deletion of *Bmpr1a* (El-Bizri et al., 2008); knock-down of *Bmpr2* (Liu et al., 2007); mutation of *Smad5* (Yang et al., 1999)]. These phenotypes are strikingly similar to those we describe in the head vessels of the *Sm22Cre Msx1/2* mutant. Interestingly, the BMP pathway plays a major role in *Mmp2* and *Mmp9* expression. *a-Sm22Cre*-driven inactivation of *Bmpr1a* severely affects their expression in VSMCs, which leads to a reduction in mural cell coverage and consequent vessel dilation (El-Bizri et al. 2008). In head VSMCs, Msx genes seem to act upstream of Bmp expression and consequently before BMP receptor activation, since the level of Smad phosphorylation is reduced in the double mutant. Indeed, we show that *Mmp2* expression can be rescued in mutant VSMCs by exogenous Bmp4. Furthermore, Roybal et al. (2010) have shown that *Bmp4* expression is similarly decreased in NCC-derived osteogenic cells upon inactivation of both *Msx1* and *Msx2* by a *Wnt1-Cre* transgene, although the overall level of BMP signaling is enhanced. Altogether, these results suggest an autocrine or paracrine mechanism amongst VSMCs producing their own BMPs. This is in keeping with the observation that gut smooth muscle precursor cells also produce concurrently BMPs and their receptors (Torihashi et al., 2009).

Impaired VSMC coverage indirectly affects the endothelium

In the *Sm22Cre Msx1/2* mutant, markers expressed late in the endothelium (*vWF*, *VE-Cadherin*) are donwnregulated. This suggests that impairment of the endothelium maturation process derives from defects in mural cells. Indeed, reduction in the *Angpt1* expression level

correlates well with reduction in the number of VSMCs, which predominantly secrete this factor. Decrease in *Angpt1* expression leads to a change in membrane localization of Tie2 in endothelial cells, which was demonstrated in cell culture (Fukuhara et al., 2008; Saharinen et al., 2008) and which we confirm *in vivo*. Furthermore, the expression of *Klf2*, which is induced by activation of Angpt1 (De Val and Black, 2009; Sako et al., 2009), is significantly reduced in mutant endothelial cells. Our interpretation is that reduced coverage by mural cells leads to a reduction in secretion of mural factors, such as Angpt1. Consequently, there is an incomplete activation of endothelial receptors (such as Tie2), resulting in impairment of endothelial cell maturation.

Conclusion

Our *in vivo* data show that the Msx family of homeodomain transcription factors plays a major role in head vascular maturation and that their mutation leads to mural layer defects. We demonstrate *Msx1* expression in VSMC precursors, which require both *Msx1* and *Msx2* to integrate the mural layer. From these data, we may propose a mechanism (**Fig. 8)**: in head VSMCs, Msx act upstream of Bmp expression that controls the expression of *Mmp2* and *Mmp9*. Deficiency in these metalloproteinases prevent VSMCs to reach the mural layer, thus leading to reduced mural coverage, vessel dilation and impairment of endothelium maturation.

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REFERENCES

Abe, J. (2006). Bone morphogenetic protein (BMP) family, SMAD signaling and Id helix-loop-helix proteins in the vasculature: the continuous mystery of BMPs pleotropic effects. *J Mol Cell Cardiol* **41**, 4-7.

Alappat, S., Zhang, Z. Y. and Chen, Y. P. (2003). Msx homeobox gene family and craniofacial development. *Cell Res* **13**, 429-42.

Bach, A., Lallemand, Y., Nicola, M. A., Ramos, C., Mathis, L., Maufras, M. and Robert, B. (2003). Msx1 is required for dorsal diencephalon patterning. *Development* **130**, 4025-36.

Bai, H., Gao, Y., Arzigian, M., Wojchowski, D. M., Wu, W. S. and Wang, Z. Z. (2010). BMP4 regulates vascular progenitor development in human embryonic stem cells through a smad-dependent pathway. *J Cell Biochem* **109**, 363-74.

Battegay, E. J., Raines, E. W., Seifert, R. A., Bowen-Pope, D. F. and Ross, R. (1990). TGF-beta induces bimodal proliferation of connective tissue cells via complex control of an autocrine PDGF loop. *Cell* **63**, 515-24.

Bendall, A. J. and Abate-Shen, C. (2000). Roles for Msx and Dlx homeoproteins in vertebrate development. *Gene* **247**, 17-31.

Bensoussan, V., Lallemand, Y., Moreau, J., Clement, C. S., Langa, F. and Robert, B. (2008). Generation of an Msx2-GFP conditional null allele. *Genesis* **46**, 276-82.

Chen, Y., Bei, M., Woo, I., Satokata, I. and Maas, R. (1996). Msx1 controls inductive signaling in mammalian tooth morphogenesis. *Development* **122**, 3035-44.

Chen, Y. H., Ishii, M., Sun, J., Sucov, H. M. and Maxson, R. E., Jr. (2007). Msx1 and Msx2 regulate survival of secondary heart field precursors and post-migratory proliferation of cardiac neural crest in the outflow tract. *Dev Biol* **308**, 421-37.

De Val, S. and Black, B. L. (2009). Transcriptional control of endothelial cell development. *Dev Cell* **16**, 180-95.

Dejana, E., Lampugnani, M. G., Giorgi, M., Gaboli, M., Federici, A. B., Ruggeri, Z. M. and Marchisio, P. C. (1989). Von Willebrand factor promotes endothelial cell adhesion via an Arg-Gly-Asp-dependent mechanism. *J Cell Biol* **109**, 367-75.

El-Bizri, N., Guignabert, C., Wang, L., Cheng, A., Stankunas, K., Chang, C. P., Mishina, Y. and Rabinovitch, M. (2008). SM22alpha-targeted deletion of bone morphogenetic protein receptor 1A in mice impairs cardiac and vascular development, and influences organogenesis. *Development* **135**, 2981-91.

Esner, M., Meilhac, S. M., Relaix, F., Nicolas, J. F., Cossu, G. and Buckingham, M. E. (2006). Smooth muscle of the dorsal aorta shares a common clonal origin with skeletal muscle of the myotome. *Development* **133**, 737-49.

Etchevers, H. C., Vincent, C., Le Douarin, N. M. and Couly, G. F. (2001). The cephalic neural crest provides pericytes and smooth muscle cells to all blood vessels of the face and forebrain. *Development* **128**, 1059-68.

Feil, R., Brocard, J., Mascrez, B., LeMeur, M., Metzger, D. and Chambon, P. (1996). Ligand-activated site-specific recombination in mice. *Proc Natl Acad Sci U S A* **93**, 10887-90.

Finnerty, J. R., Mazza, M. E. and Jezewski, P. A. (2009). Domain duplication, divergence, and loss events in vertebrate Msx paralogs reveal phylogenomically informed disease markers. *BMC Evol Biol* **9**, 18.

Fu, H., Ishii, M., Gu, Y. and Maxson, R. (2007). Conditional alleles of Msx1 and Msx2. *Genesis* **45**, 477-81.

Fukuhara, S., Sako, K., Minami, T., Noda, K., Kim, H. Z., Kodama, T., Shibuya, M., Takakura, N., Koh, G. Y. and Mochizuki, N. (2008). Differential function of Tie2 at cell-cell contacts and cell-substratum contacts regulated by angiopoietin-1. *Nat Cell Biol* **10**, 513-26.

Goupille, O., Saint Cloment, C., Lopes, M., Montarras, D. and Robert, B. (2008). Msx1 and Msx2 are expressed in sub-populations of vascular smooth muscle cells. *Dev Dyn* **237**, 2187-94.

Han, J., Ishii, M., Bringas, P., Jr., Maas, R. L., Maxson, R. E., Jr. and Chai, Y. (2007). Concerted action of Msx1 and Msx2 in regulating cranial neural crest cell differentiation during frontal bone development. *Mech Dev* **124**, 729-45.

Hellstrom, M., Kalen, M., Lindahl, P., Abramsson, A. and Betsholtz, C. (1999). Role of PDGF-B and PDGFR-beta in recruitment of vascular smooth muscle cells and pericytes during embryonic blood vessel formation in the mouse. *Development* **126**, 3047-55.

Houzelstein, D., Cohen, A., Buckingham, M. E. and Robert, B. (1997). Insertional mutation of the mouse Msx1 homeobox gene by an nlacZ reporter gene. *Mech Dev* **65**, 123-33.

Ishii, M., Han, J., Yen, H. Y., Sucov, H. M., Chai, Y. and Maxson, R. E., Jr. (2005). Combined deficiencies of Msx1 and Msx2 cause impaired patterning and survival of the cranial neural crest. *Development* **132**, 4937-50.

Jain, R. K. (2003). Molecular regulation of vessel maturation. *Nat Med* **9**, 685-93.

Jiang, X., Rowitch, D. H., Soriano, P., McMahon, A. P. and Sucov, H. M. (2000). Fate of the mammalian cardiac neural crest. *Development* **127**, 1607-16.

Kapadia, H., Mues, G. and D'Souza, R. (2007). Genes affecting tooth morphogenesis. *Orthod Craniofac Res* **10**, 105-13.

Kenagy, R. D., Hart, C. E., Stetler-Stevenson, W. G. and Clowes, A. W. (1997). Primate smooth muscle cell migration from aortic explants is mediated by endogenous platelet-derived growth factor and basic fibroblast growth factor acting through matrix metalloproteinases 2 and 9. *Circulation* **96**, 3555-60.

Kisanuki, Y. Y., Hammer, R. E., Miyazaki, J., Williams, S. C., Richardson, J. A. and Yanagisawa, M. (2001). Tie2-Cre transgenic mice: a new model for endothelial cell-lineage analysis in vivo. *Dev Biol* **230**, 230-42.

Klagsbrun, M., Takashima, S. and Mamluk, R. (2002). The role of neuropilin in vascular and tumor biology. *Adv Exp Med Biol* **515**, 33-48.

Korn, J., Christ, B. and Kurz, H. (2002). Neuroectodermal origin of brain pericytes and vascular smooth muscle cells. *J Comp Neurol* **442**, 78-88.

Lallemand, Y., Bensoussan, V., Cloment, C. S. and Robert, B. (2009). Msx genes are important apoptosis effectors downstream of the Shh/Gli3 pathway in the limb. *Dev Biol* **331**, 189-98.

Lallemand, Y., Nicola, M. A., Ramos, C., Bach, A., Cloment, C. S. and Robert, B. (2005). Analysis of Msx1; Msx2 double mutants reveals multiple roles for Msx genes in limb development. *Development* **132**, 3003-14.

Liu, D., Wang, J., Kinzel, B., Mueller, M., Mao, X., Valdez, R., Liu, Y. and Li, E. (2007). Dosage-dependent requirement of BMP type II receptor for maintenance of vascular integrity. *Blood* **110**, 1502-10.

Majack, R. A. (1987). Beta-type transforming growth factor specifies organizational behavior in vascular smooth muscle cell cultures. *J Cell Biol* **105**, 465-71.

Majesky, M. W. (2007). Developmental basis of vascular smooth muscle diversity. *Arterioscler Thromb Vasc Biol* **27**, 1248-58.

Monsoro-Burq, A. H., Wang, E. and Harland, R. (2005). Msx1 and Pax3 cooperate to mediate FGF8 and WNT signals during Xenopus neural crest induction. *Dev Cell* **8**, 167-78.

Muzumdar, M. D., Tasic, B., Miyamichi, K., Li, L. and Luo, L. (2007). A global double-fluorescent Cre reporter mouse. *Genesis* **45**, 593-605.

Navarro, P., Caveda, L., Breviario, F., Mandoteanu, I., Lampugnani, M. G. and Dejana, E. (1995). Catenin-dependent and -independent functions of vascular endothelial cadherin. *J Biol Chem* **270**, 30965-72.

Newby, A. C. (2006). Matrix metalloproteinases regulate migration, proliferation, and death of vascular smooth muscle cells by degrading matrix and non-matrix substrates. *Cardiovasc Res* **69**, 614-24.

Nishishita, T. and Lin, P. C. (2004). Angiopoietin 1, PDGF-B, and TGF-beta gene regulation in endothelial cell and smooth muscle cell interaction. *J Cell Biochem* **91**, 584-93.

Park, C., Lavine, K., Mishina, Y., Deng, C. X., Ornitz, D. M. and Choi, K. (2006). Bone morphogenetic protein receptor 1A signaling is dispensable for hematopoietic development but essential for vessel and atrioventricular endocardial cushion formation. *Development* **133**, 3473-84.

Passman, J. N., Dong, X. R., Wu, S. P., Maguire, C. T., Hogan, K. A., Bautch, V. L. and Majesky, M. W. (2008). A sonic hedgehog signaling domain in the arterial adventitia supports resident Sca1+ smooth muscle progenitor cells. *Proc Natl Acad Sci U S A* **105**, 9349-54.

Ramos, C. and Robert, B. (2005). msh/Msx gene family in neural development. *Trends Genet* **21**, 624-32.

Rentschler, S., Jain, R. and Epstein, J. A. (2010). Tissue-tissue interactions during morphogenesis of the outflow tract. *Pediatr Cardiol* **31**, 408-13.

Risau, W. (1997). Mechanisms of angiogenesis. *Nature* **386**, 671-4.

Roybal, P. G., Wu, N. L., Sun, J., Ting, M. C., Schafer, C. A. and Maxson, R. E. (2010). Inactivation of Msx1 and Msx2 in neural crest reveals an unexpected role in suppressing heterotopic bone formation in the head. *Dev Biol* **343**, 28-39.

Saharinen, P., Eklund, L., Miettinen, J., Wirkkala, R., Anisimov, A., Winderlich, M., Nottebaum, A., Vestweber, D., Deutsch, U., Koh, G. Y. et al. (2008). Angiopoietins assemble distinct Tie2 signalling complexes in endothelial cell-cell and cell-matrix contacts. *Nat Cell Biol* **10**, 527-37.

Sako, K., Fukuhara, S., Minami, T., Hamakubo, T., Song, H., Kodama, T., Fukamizu, A., Gutkind, J. S., Koh, G. Y. and Mochizuki, N. (2009). Angiopoietin-1 induces Kruppel-like factor 2 expression through a phosphoinositide 3-kinase/AKT-dependent activation of myocyte enhancer factor 2. *J Biol Chem* **284**, 5592-601.

Santoro, M. M., Pesce, G. and Stainier, D. Y. (2009). Characterization of vascular mural cells during zebrafish development. *Mech Dev* **126**, 638-49.

Sharpe, J., Ahlgren, U., Perry, P., Hill, B., Ross, A., Hecksher-Sorensen, J., Baldock, R. and Davidson, D. (2002). Optical projection tomography as a tool for 3D microscopy and gene expression studies. *Science* **296**, 541-5.

Spiekerkoetter, E., Guignabert, C., de Jesus Perez, V., Alastalo, T. P., Powers, J. M., Wang, L., Lawrie, A., Ambartsumian, N., Schmidt, A. M., Berryman, M. et al. (2009). S100A4 and bone morphogenetic protein-2 codependently induce vascular smooth muscle cell migration via phospho-extracellular signal-regulated kinase and chloride intracellular channel 4. *Circ Res* **105**, 639-47.

Torihashi, S., Hattori, T., Hasegawa, H., Kurahashi, M., Ogaeri, T. and Fujimoto, T. (2009). The expression and crucial roles of BMP signaling in development of smooth muscle progenitor cells in the mouse embryonic gut. *Differentiation* **77**, 277-89.

Vastardis, H., Karimbux, N., Guthua, S. W., Seidman, J. G. and Seidman, C. E. (1996). A human MSX1 homeodomain missense mutation causes selective tooth agenesis. *Nat Genet* **13**, 417-21.

Walls, J. R., Coultas, L., Rossant, J. and Henkelman, R. M. (2008). Three-dimensional analysis of vascular development in the mouse embryo. *PLoS One* **3**, e2853.

Wiegreffe, C., Christ, B., Huang, R. and Scaal, M. (2009). Remodeling of aortic smooth muscle during avian embryonic development. *Dev Dyn* **238**, 624-31.

Wilkie, A. O. (1997). Craniosynostosis: genes and mechanisms. *Hum Mol Genet* **6**, 1647-56.

Yang, X., Castilla, L. H., Xu, X., Li, C., Gotay, J., Weinstein, M., Liu, P. P. and Deng, C. X. (1999). Angiogenesis defects and mesenchymal apoptosis in mice lacking SMAD5. *Development* **126**, 1571-80.

Zhang, J., Zhong, W., Cui, T., Yang, M., Hu, X., Xu, K., Xie, C., Xue, C., Gibbons, G. H., Liu, C. et al. (2006). Generation of an adult smooth muscle cell-targeted Cre recombinase mouse model. *Arterioscler Thromb Vasc Biol* **26**, e23-4.

Zhang, Z., Song, Y., Zhao, X., Zhang, X., Fermin, C. and Chen, Y. (2002). Rescue of cleft palate in Msx1-deficient mice by transgenic Bmp4 reveals a network of BMP and Shh signaling in the regulation of mammalian palatogenesis. *Development* **129**, 4135-46.

LEGENDS TO FIGURES

Fig. 1 – Over-branching and caliber increase of the carotid artery. (A-C) Transverse sections of E11.5 embryo heads reveal expression of *Msx1*^{lacZ} in inner endothelial cells of the carotid artery (CA) (**A, arrowheads**) as well as in external mesenchymal cells **(A, arrows)**. *Msx2*^{GFP} is expressed in VSMCs **(B, arrowheads)** as well as in some external mesenchymal cells **(B, arrows)** that may also express *Msx1*^{lacZ} **(C, arrows)**. **(D-G)** Extra-branching of the CA was identified by OPT. At a low magnification, where the whole embryonic vasculature is visible, the *Sm22Cre Msx1/2* mutant embryos **(F)** show major vascular alterations in the head as compared to controls **(D)**. Close up images confirm increase in branching density from the CA in the *Sm22Cre Msx1/2* mutant (**G**) as compared to the control (**E**). The CA and its major branches are highlighted in green. **(H)** Primary and secondary branch quantification, from the right and left CA of the embryos in **(D-G)**. For each genotype n=3, (***) P<0.001. **(I-P)** Measure of the section surface of the CA **(I-L)** and dorsal aorta (DA) **(M-P)**, both visualized by the endothelial marker CD31 (green) on transverse sections at E11.5. **(I)** and (**M)** show the section planes for the CA and DA, respectively. In the *Sm22Cre Msx1/2* mutant, the CA **(K)** section surface is increased when compared to the control **(J)**. In contrast, the DA surface is not significantly different in the mutant **(O)** and the control **(N)**. Average area quantification is represented in **(L)** for the CA and **(P)** for the DA, respectively. For each genotype, n=8, (**) P<0.01.

Fig. 2 - *Msx* genes are crucial for proper VSMC coverage and endothelial maturation. **(A-F)** Immunofluorescence analyses of transverse sections from E11.5 embryos show a strong decrease in the number of α-SMA-positive cells around the CA in the *Sm22Cre Msx1/2* mutant **(D)** as compared to the control **(A).** A reduction in the number of cells positive for VE-cadherin **(F)**, but not CD31 **(E)**, is also observed in mutants as compared to controls **(C and B respectively)**. **(G)** The number of nuclei surrounded with α-SMA or CD31 was quantified in different genotypes**.** For each, n=5. **(I)** Reduction in expression of late-expressed endothelial genes was confirmed by qRT-PCR using RNA from cephalic tissues dissected as described in **(H)** (red-outlined area). Expression of *vWF* and *VE-cadherin* was reduced in the *Msx1*, and even more in the *Sm22Cre Msx1/2* mutant. In contrast, *Jag1* and *CD31* showed no significant change. (*) P<0.05, (**) P<0.01 and (***) P<0.001.

Fig. 3 - *Msx1* and *Msx2* are essential in VSMC precursors. (A-F) Head transverse sections at E10.5 **(A, C)**, E11.5 **(B, D)** and E12.5 **(E, F)**. *Msx1*^{CreERT2} *Rosa*$^{mT/mG}$ gravid mice were injected with tamoxifen at E8.0-E9.0. Cells expressing the Cre-activated GFP show up in green while α-SMA is revealed in red. **(A-B)** Low magnification images reveal GFP-positive mesenchymal cells between the dorsal root ganglion (R.G) and the CA, suggesting a migration route for neural-crest cells to the CA. **(C-F)** On higher magnification images, no GFP-positive cell can be detected in the mural layer of the CA at E10.5 **(C)**. At E11.5 **(D),** some GPP-expressing cells are conspicuous close to the artery. At E12.5 many more GFP-positive cells are present in the mural layer **(E)**, and most of them have started co-expressing *α-Sma* (**F**, yellow). **(G-J)** Similar analysis on Msx double heterozygous **(G, I)** or double null **(H, J)** embryos matched from the same litters. Tamoxifen was injected at E8.0 and E8.5. In *Msx1*$^{-/-}$, *Msx2*$^{-/-}$ double mutant embryos at E11.5 **(H)** and E12.5 **(J),** the global number of GFP-positive cells does not look reduced as compared to controls (**G** and **I**, respectively). However, the numbers of GFP-positive cells reaching the artery and expressing *α-Sma* is significantly lower in the mutant (arrows in **G-J**).

Fig. 4 - Migration is altered in the Msx double mutants. (A, C, E) Head transverse section of an Msx1$^{+/-}$; Msx2$^{+/-}$ double heterozygous embryo at E12.5. **(B, D, F)** Section of a stage-matched *Msx1*$^{-/-}$; *Msx2*$^{-/-}$ double homozygous mutant embryo. *Msx1* early expressing cells were labeled by GFP (green) at E8.0 and E8.5 using the Msx1^{creERT2} allele, activated by Tamoxifen, together with the *Rosa*$^{mT/mG}$ reporter allele. In an *Msx1*$^{+/-}$ *Msx2*$^{+/-}$ context **(A, C, E)** we observe cells expressing Mmp2 (red) (**A**) and GFP (green) (**C**) in the CA mural layer as well as in surrounding regions. Overlapping of both labels (yellow) is extensive **(E)**. In the *Msx1*; *Msx2* mutant **(B, D, F),** the Mmp2 protein (red) level is dramatically reduced in the CA mural layer, although the number of GFP-positive (green) or Mmp2-positive cells outside the CA does not seem to be changed. **(G)** *Mmp2* and *Mmp9* expression levels were measured on FACS-sorted VSMCs by qRT-PCR. In the single *Msx1* mutant, *Mmp2* shows a severe decrease in expression that is in part balanced by over expression of *Mmp9*. In the *Msx1*$^{-/-}$; *Msx2*$^{-/-}$ double mutant, a severe decrease in expression for both *Mmp2* and *Mmp9* is observed. Fold changes are represented relative to the level of expression in the *Msx1*$^{+/-}$; *Msx2*$^{+/-}$ double heterozygotes (dotted line).

Fig. 5 - Mmp2 and Mmp9 reduction is due to impairment in BMP signaling. (A) qRT-PCR analysis of FACS-sorted VSMCs. *Bmp4* expression decreases dramatically in double

Msx1; Msx2 mutant cells as compared to single *Msx1*$^{+/-}$ or *Msx2*$^{+/-}$ mutant cells. *Bmp7* is the most highly expressed Bmp gene in the control cell population and its expression is reduced by 45% in the double homozygous mutant. *Bmp2* and *Bmp6* are weakly expressed in control cells and almost undetectable in *Msx1*$^{-/-}$; *Msx2*$^{-/-}$ mutant cells. *Bmpr1a* and *Bmpr2* are highly expressed in the VSMCs at E12.5. Their expression is not significantly affected in the *Msx1*$^{-/-}$*; Msx2*$^{-/-}$ cells. **(B)** In situ hybridization of head transverse sections of E11.5 embryos shows *Bmp4* mRNA accumulation mainly in the mural layer of the CA and around **(b')**. No significant *Bmp4* expression was observed in the endothelium. In the double Msx mutant, the level of *Bmp4* mRNA is strongly reduced in cells of the mural layer and in close vicinity to the CA **(b'', arrowheads)**. In contrast, *Bmp4* expression remains elevated close to the pharyngeal region of foregut (PRF). **(C)** To confirm the role of Msx genes in the BMP pathway, we performed an immunofluorescence assay against the phosphorylated forms of Smad 1/5/8. In the double *Sm22Cre Msx1/2* mutant (**c"**), there is a reduction in the number of phospho-Smad-positive cells relative to the control (**c'**). This reduction was quantified as the ratio between the number of phospho-Smad labeled nuclei and the total number of Hoechst-positive nuclei **(D)**. For both mutant and control, n=5. (*) $P<0.05$.

Fig. 6 - *Ex vivo* analyses confirm that *Bmp4* is downstream of Msx genes in VSMCs. (A, D) After four passages, FACS-sorted VSMCs maintain α-SMA expression (red) and a smooth muscle cell morphological aspect. **(A)** Non-targeted fluorescent siRNA was transfected successfully and is localized in the cell cytoplasm. **(B, C)** *Msx1*$^{+/-}$*; Msx2*$^{+/-}$ cells were transfected with increasing concentrations of *Msx1* + *Msx2* specific siRNAs. 88% (i.e. maximal) inactivation of *Msx1* and *Msx2* was reached using 25 ng/ml of siRNA **(B)**. Downregulation of Msx gene expression had a significant impact on *Bmp4* and *Bmp7* expression (57% and 43%, respectively) and a slighter impact on *Mmp2* (35%) **(C)**. **(D, E)** To confirm an effect of Bmp4 on *Mmp2* and *Mmp9* expression, we added increasing amounts of exogenous Bmp4 to *Msx1*$^{-/-}$ *Msx2*$^{-/-}$ VSMCs. This did not affect cell viability or phenotype **(D)**. *Mmp2* expression increases proportionally to Bmp4 concentration **(E)**. *Mmp2* endogenous expression level in *Msx1*$^{+/-}$ *Msx2*$^{+/-}$ cells is represented by the dotted horizontal line. *Mmp9* expression in cultured VSMCs decreased to insignificant levels and consequently no conclusion could be drawn for this gene.

Fig. 7 - Improper VSMC coverage leads to impairment in endothelium maturation. (A) qRT-PCR of cephalic tissue RNA (see Fig. 2H) shows no significant decrease in the level of

endothelial-to-mural signaling factors, such as *PDGFß* and *Tgf-ß1*, in the *Sm22Cre Msx1/2* mutant. **(B)** In contrast, expression of *Angpt1* and *VEGF-A,* two genes associated with mural-to-endothelial cell communication, is significantly decreased in the mutant. (**) P<0.01 and (***) P<0.001. **(C-E)** Transversal sections of carotid arteries from E11.5 control **(C, C')** or *Sm22Cre Msx1/2* mutant **(E, E')** embryos were labeled for Tie2 (green) and α-SMA (red). Nuclear Hoechst is in blue. Tie2 in the endothelium appears localized close to the VSMC layer (α-Sma-positive) in normal **(C, C', D)**, but more diffuse over the whole endothelial cells membrane in mutant **(E, E', F)** embryo vessels. This is more conspicuous in higher magnifications images **(D, F)**. Arrowheads point to endothelial nuclei that are on the luminal side of the artery relative to Tie2 in the control **(D)**, but look embedded in the Tie2 signal in the mutant **(F)**. This is further exemplified in optical stacks along the Z axis of the same sections **(C', E')**. Optical section planes for C' and E' are drawn in C and E**,** respectively **(**thin lines). **(G)** qRT-PCR analysis of FACS-sorted CD31-positive cells shows that *Klf2* expression is reduced in the double $Msx1^{-/-}; Msx2^{-/-}$ mutant as compared to either single $Msx1^{-/-}$ or $Msx2^{-/-}$ mutant. Changes in expression are expressed as fold changes relative to control (dotted line). **(H)** Schematic interpretation of the results. VSMCs are represented in red, Angpt1 secreted protein in yellow, Tie2 receptor in green and Klf2 protein in black. Reduction in VSMCs leads to lower Angpt1 secretion, which result in impaired concentration of Tie2 at the mural side of the endothelial cell membrane and reduction in *Klf2* expression.

Fig8 – The role of Msx genes in a sub-Population of VSMCs.

Using the $Msx1^{CreERT2}$ and the $Rosa^{mT/mG}$ alleles, we labeled *Msx1*-expressing cells at E8.0-8.5. At this stage Msx1 is strongly expressed in the neural crest, from which head VSMCs originate. At E10.5, we observed GFP-positive cells close to the CA but not in contact with it. Additionally we observed a significant number of GFP-positive cells in the dorsal root ganglia and in the region comprised between the latter and the CA. Last, we have shown that Msx genes are essential for late migration of a subpopulation of VSMC precursors via the control of signaling by different BMPs and of *Mmp2/Mmp9* expression. Expression of *Mmp2* and *Mmp9* correlates with phosphorylation of Smad1, 5 and 8.