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# Requirement of the MADS-box transcription factor MEF2C for vascular development

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## SUMMARY

**The embryonic vasculature develops from endothelial cells that form a primitive vascular plexus which recruits smooth muscle cells to form the arterial and venous systems. The MADS-box transcription factor MEF2C is expressed in developing endothelial cells and smooth muscle cells (SMCs), as well as in surrounding mesenchyme, during embryogenesis. Targeted deletion of the mouse *MEF2C* gene resulted in severe vascular abnormalities and lethality in homozygous mutants by embryonic day 9.5. Endothelial cells were present and were able to differentiate, but failed to organize normally into a vascular plexus, and smooth muscle cells did not differentiate in *MEF2C* mutant embryos. These vascular defects resemble those in mice lacking the vascular-specific endothelial cell growth factor VEGF or its receptor Flt-1, both of which are expressed in *MEF2C* mutant embryos. These results reveal multiple roles for MEF2C in vascular development and suggest that MEF2-dependent target genes mediate endothelial cell organization and SMC differentiation.**

Key words: MADS-box,Transcription factor, MEF2C, Vascular defect, Mouse

## INTRODUCTION

Formation of the vascular system is an essential early event in embryogenesis as well as wound healing and pathological processes associated with neovascularization, such as tumor angiogenesis (reviewed in Risau, 1995; Folkman and D'Amore, 1996). The process of vessel formation begins when angioblasts, which are derived from embryonic and extraembryonic mesoderm, become committed to an endothelial cell fate and organize into a primitive vascular plexus (Baldwin, 1995). This phase of vessel formation, referred to as vasculogenesis, requires endothelial cell migration and cell-cell interactions, lumen formation, and precise patterning of the vascular template. Smooth muscle cells (SMCs), which arise from multiple types of progenitors including neural crest cells, mesenchymal cells and endothelial cells, are then recruited to the endothelial cell network where they differentiate and form the contractile vessel walls (Owens, 1995; Kirby and Waldo, 1995). Subsequent proliferation and migration of endothelial cells within the primary vessels results in sprouting and remodeling of the vasculature, a process known as angiogenesis. Vessel formation also occurs within the embryonic yolk sac, however, in contrast to the vasculature of the embryo proper, the yolk sac vasculature does not contain SMCs and is composed solely of endothelial cells derived from the blood islands.

Recent studies have revealed important roles for several peptide growth factors and their cell surface receptors in vasculogenesis and angiogenesis. The endothelial cell-specific vascular endothelial growth factor (VEGF) can promote angiogenesis in vivo and in vitro (Breier and Risau, 1996) and mouse embryos lacking VEGF show delayed endothelial cell differentiation and impaired blood vessel development (Carmeliet et al., 1996a; Ferrara et al., 1996). Remarkably, mouse embryos heterozygous for a *VEGF* null allele also die during mid-gestation due to impaired blood island formation and angiogenesis, demonstrating the exquisite sensitivity of endothelial cells to extracellular concentrations of VEGF (Carmeliet et al., 1996a; Ferrara et al., 1996). The VEGF tyrosine kinase receptors, Flt-l and Flk-1, are also required for the formation of blood islands and vessels, but the defects seen in mutant mouse embryos lacking each receptor are different, suggesting that these receptors play distinct roles in mediating the actions of VEGF. In Flk-1 mutant embryos, endothelial cells fail to differentiate and vasculogenesis is disrupted (Shalaby et al., 1995, 1997). In contrast, endothelial cells can differentiate in Flt-1 mutant embryos, but they are improperly assembled into

vessels (Fong et al., 1995). The tyrosine kinase receptor, Tie-1, and the related receptor Tie-2/Tek, which binds the angiogenic growth factor angiopoietin-1, are also expressed specifically in vascular endothelial cells (Dumont et al., 1992; Partanen et al., 1992; Sato et al., 1993; Davis et al., 1996). In mouse mutants lacking Tie-1, blood vessel integrity is impaired and in mice lacking Tie-2 angiogenesis and vascular remodeling fail to occur (Dumont et al., 1994; Sato et al., 1995). *Angiopoietin-1* mutant embryos also have an abnormal vasculature architecture due to the failure of endothelial cells to recruit SMCs and pericyte precursors to the developing vessel wall (Suri et al., 1996; Maisonpierre et al., 1997). These phenotypes have led to the conclusion that the angiopoietin signaling system regulates chemotaxis and proliferation of mesenchymal cells and their differentiation into SMCs. Based on their temporal patterns of expression and the phenotypes of mutant mice, VEGF and its receptors are thought to act at an earlier step in vascular development than angiopoietin and its receptors. In the final steps of vascular development, vessels are remodeled and stabilized. Platelet-derived growth factor (PDGF), transforming growth factor-β and tissue factor have been shown to play important roles in these processes (Leveen et al., 1994; Dickson et al., 1995; Oshima et al., 1996; Carmeliet et al., 1996b).

While much has been learned about the roles of peptide growth factors and their receptors in vasculogenesis and angiogenesis, little is known of the transcription factors that regulate the formation, differentiation and patterning of endothelial cells or SMCs during vessel formation. Members of the myocyte enhancer factor-2 (MEF2) family of MADS (MCM1, Agamous, Deficiens, Serum response factor)-box transcription factors are expressed in developing skeletal, cardiac and smooth muscle cells (Edmondson et al., 1994; Subramanian and Nadal-Ginard, 1996; Ticho et al., 1996), as well as in endothelial cells and surrounding mesenchyme during embryogenesis (this study). There are four vertebrate *MEF2* genes, *MEF2A, MEF2B, MEF2C* and *MEF2D,* whose products bind as homodimers and heterodimers to an A/T-rich DNA sequence in the control regions of numerous of muscle-specific genes (reviewed in Black and Olson, 1998). The single *MEF2* gene in *Drosophila* is also expressed in developing muscle cell lineages during embryogenesis and is essential for myogenesis and morphogenesis of all muscle cell types in the embryo (Bour et al., 1995; Lilly et al., 1995; Ranganayakulu et al., 1995).

We recently reported that MEF2C was required for cardiac myogenesis and morphogenesis in the mouse. In mouse embryos lacking MEF2C, the future right ventricle failed to form, there was a lack of ventricular trabeculation and a subset of cardiac muscle genes was not expressed (Lin et al., 1997). Given the importance of MEF2 for differentiation of diverse muscle cell types in the *Drosophila* embryo, we investigated in this study whether *MEF2C* mutant embryos might exhibit defects in smooth muscle development. We show that mouse embryos lacking MEF2C fail to form a functional vascular system and exhibit major defects in vasculogenesis that result in embryonic death. Endothelial cells are specified in *MEF2C* mutant embryos, but they fail to organize into a primitive vascular network and SMCs do not differentiate. The vascular defects in *MEF2C* mutant embryos resemble those in mouse mutants lacking VEGF and Flt-1 and suggest that MEF2C is required either directly or indirectly for VEGF signaling during vasculogenesis. These results demonstrate that MEF2C is required for appropriate intercellular interactions between endothelial cells and mesenchymal cells during formation of the vasculature.

## MATERIALS AND METHODS

### *MEF2C* mutant mice and genotyping

The *MEF2C* gene was targeted by homologous recombination as described (Lin et al., 1997). The targeted allele lacks the region encoding the DNA-binding and dimerization domains of MEF2C and acts as a null allele. The targeted allele was maintained in a C57BL/6 background. Tail and yolk sac DNA was analyzed by Southern blot analysis for the targeted *MEF2C* locus, as described (Lin et al., 1997).

### In situ hybridization and histology

To analyze the phenotype of *MEF2C* null embryos, timed pregnancies were set up and embryos were harvested at the indicated days, with the morning after mating being considered as E 0.5. Embryos were fixed in 4% paraformaldehyde immediately after harvesting. In situ hybridization on paraffin sections was performed as described in Wilkinson et al. (1987), with modifications described by Frohman et al. (1990). Following prehybridization, embryo sections were hybridized at 55°C with sense and antisense riboprobes, $7\times10^5$ cpm per slide. Following overnight incubation, unhybridized probe was removed through stringent washes and treatment with RNase A. Slides were subsequently coated with K.5 emulsion (Ilford, UK) and exposed at 4°C for 21 days. The slides were developed, counter-stained with hematoxylin and examined using bright- and dark-field optics.

### PECAM staining

Whole-mount staining for PECAM was performed using anti-mouse PECAM monoclonal antibody MEC13.3 (Pharmingen). Embryos were fixed in 4% paraformaldehyde/PBS overnight at 4°C and bleached with 5% $H_2O_2$ for 4 hours to block endogenous peroxidase. Embryos were then incubated in PBSMT (3% instant skim milk, 0.1% Triton X-100 in PBS) for 2 hours at room temperature, then with 10 μg/ml anti-PECAM-1 antibody overnight at 4°C, followed by 5 washes for 1 hour each in PBSMT and incubation with 1:100 goat anti-rat HRP-coupled antibody overnight at 4°C and an additional wash. The color reaction was done in PBS containing 0.3 mg/ml 3,3′-diaminobenzidine (Sigma), 0.5% $NiCl_2$, 0.03% $H_2O_2$.

### Staining for *lacZ* expression

Staining of transgenic embryos for *lacZ* expression was performed as described (Cheng et al., 1993). Briefly, embryos were fixed in 2% paraformaldehyde-0.2% glutaraldehyde for up to 1 hour, after which they were rinsed in PBS and incubated in PBS containing 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6{:}3H_2O$, 2 mM $MgCl_2$ and 1 mg/ml X-gal overnight. Reactions were stopped by rinsing embryos with PBS, followed by further fixation in 4% paraformaldehyde. The *SM22-lacZ* transgene contained the 1343 bp *SM22* promoter linked to *lacZ* (Li et al., 1996b). Expression of *lacZ* under control of the *Flk-1* promoter was obtained using a targeted *Flk-1* allele, as described (Shalaby et al., 1995). Both *lacZ* markers were introduced into the *MEF2C* mutant background by interbreeding the appropriate strains of mice.

### RT-PCR

Total RNA was isolated from wild-type and mutant embryos or yolk sacs on the days indicated in the figures, treated with ribonuclease-

free deoxyribonuclease I and resuspended in 20 μl water. First-strand cDNA synthesis was performed as described (Lin et al., 1997), using Moloney murine leukemia virus reverse transcriptase (BRL) and random primers. PCR amplification was performed with 0.5 μl of the cDNA, 0.1 μCi of [$^{32}$P]deoxycytidine triphosphate (dCTP) and gene-specific primers under conditions of linearity for each individual primer set. Most oligonucleotide primers spanned introns to potentially detect genomic DNA contamination. Duplicate PCR reactions were also performed in the absence of reverse transcriptase to confirm the absence of genomic DNA. All PCR products corresponded to the size predicted for the corresponding transcript. PCR cycles were as follows: 99°C for 2 minutes then 18 to 25 cycles of 96°C for 30 seconds, 58°C for 30 seconds and 72°C for 45 seconds. PCR products were separated by 6% polyacrylamide gel electrophoresis and quantified by analysis on a phosphorimager. Sequences of primers are available upon request.

## RESULTS

### Absence of the yolk sac vasculature in *MEF2C* mutant embryos

The mouse *MEF2C* gene was inactivated by introduction of a deletion mutation that removed the region of the gene encoding the DNA-binding and dimerization domains (Lin et al., 1997). Mice heterozygous for this mutation are phenotypically normal, whereas homozygous mutant embryos begin to show growth retardation at about E9.0 and die between E9.5 and E10. In the course of analyzing *MEF2C* mutant embryos, we observed an obvious abnormality in appearance of the yolk sac, which suggested defects in the yolk sac vasculature.

The embryonic circulatory system is composed of an extraembryonic (yolk sac) and embryonic vasculature. The yolk sac vasculature, which lacks a smooth muscle component, forms from endothelial cells that establish a complex vascular network between E8.5 and E9.5 (Risau, 1991). In contrast to the extensive vascular network in the yolk sac of wild-type embryos at E9.0, there was no evidence of vascularization in the yolk sacs of *MEF2C* mutant embryos (Fig. 1, compare A,B with D,E). Instead, the mutant yolk sacs exhibited a rough appearance and were pale compared to those of wild-type embryos. Histological analysis of wild-type yolk sacs revealed endothelial vessels containing red blood cells between the mesothelial and endodermal cell layers (Fig. 1B,C). In the mutant, the two cellular layers of the yolk sac were poorly adherent and there was an absence of discreet vascular channels (Fig. 1F). Blood islands, which form as discreet structures containing hematopoietic cells, are normally present throughout the yolk sac. In the mutant, red blood cells were detected in the yolk sac, indicating that hematopoiesis was able to occur.

### Cardiovascular defects in *MEF2C* mutant embryos

To determine whether MEF2C was also required for formation of the vasculature within the embryo, we analyzed histological sections of wild-type and mutant embryos. The dorsal aorta, the first vascular structure to form in the embryo, develops along both sides of the notochord beginning at about E7.75. In wild-type embryos at E9.0, the bilateral dorsal aortae and anterior cardinal veins can be seen on either side of the neural tube (Fig. 2A,C). In contrast, these and other major vessels did not form normally in the rostral regions of *MEF2C* mutant embryos (Fig. 2B,D). In the regions where these vessels should have been located, there were spaces containing disorganized cells. There were traces of the branchial arch arteries in the mutant, but when they were detectable, they were obviously abnormal, with thin walls and an overall lack of integrity. The severity of the vascular phenotype was variable and ranged from the complete absence of major vessels to the presence of abnormally formed vessels with obvious ruptures and lack of integrity. The vascular phenotype was most severe in the rostral region of the embryo. In the caudal region of the mutants, the dorsal aorta could be seen, but it was grossly dilated (Fig. 2, compare E and F). Similarly, in those mutants in which the sinus venosa were present, they showed extensive dilatation (Fig. 2F). Red blood cells were present in the caudal dorsal aorta of the mutant, indicating that there was some circulation.

At the linear heart tube stage (E8.0), the hearts of the mutants appear normal and initiate contractions, but fail to undergo normal looping and the future right ventricular region does not form (Lin et al., 1997). The ventricular wall of the mutant heart is also thinner than normal, due to a defect in trabeculation. Cardiac defects in the mutant are clearly evident in transverse histological sections (Fig. 2A,B). Whereas the bulbus cordis (future right ventricle), left ventricle and atrium are clearly demarkated in wild-type embryos at E9.5, mutants have a single left-sided ventricular chamber. The endocardium was present in the heart of the mutant (Fig. 2B), but it appeared disorganized.

### Expression of MEF2C in angioblasts, endothelial cells and SMCs

Previous studies of MEF2C expression focused primarily on skeletal and cardiac muscle and neurons (Edmondson et al., 1994; Lyons et al., 1995; Subramanian and Nadal-Ginard, 1996; Ticho et al., 1996). The expression pattern of MEF2C in the developing vasculature has not been previously examined in detail. We therefore performed in situ hybridization on embryo and yolk sac sections to determine whether MEF2C expression correlated with the vascular defects seen in *MEF2C* mutant embryos. MEF2C transcripts were detected in mesenchymal cells that give rise to endothelial cell-derived vessels of the yolk sac at E8.5 (Fig. 3A,B). In the embryo at E9.0, MEF2C transcripts were expressed at the highest levels in the heart and somite myotome, but expression was also detected in mesenchyme and pericytes adjacent to the developing vasculature (Fig. 3C). Expression of MEF2C at this stage was also particularly pronounced in neural crest cells within the developing branchial arches (Fig. 3C,D), which are essential for formation and patterning of the aortic arch arteries and cardiac outflow tract (Bergwerff et al., 1998). While there appeared to be a low level of MEF2C expression in developing endothelial cells in the embryo at E9.5, endothelial cell expression of MEF2C did not become pronounced until E10.5, when it was also observed at high levels in surrounding SMCs (Fig. 3E-G). This vascular expression was especially apparent in the walls of the dorsal aorta, seen in sagittal section (Fig. 3F,G). Expression in heart, myotomes and the vascular plexus of developing meninges was also seen at E10.5 (Fig. 3G).

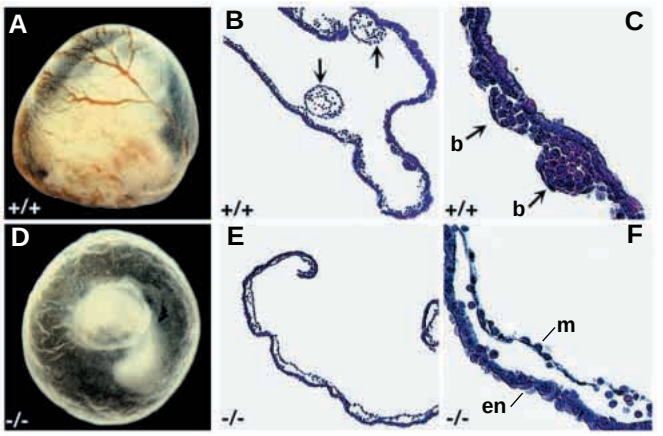


**Fig. 1.** Defects in the yolk sac vasculature in MEF2C mutants. Yolk sacs from wild-type (A-C) and mutant (D-F) embryos at E9.5 are shown. (B,C,E,F) Sections stained with H and E. Note the normal blood vessels (arrows in B) in wild-type and their absence in mutant yolk sac. b, blood islands; en, endodermal layer; m, mesodermal layer.

We also assayed for the presence of MEF2C transcripts in the developing yolk sac by semiquantitative RT-PCR. At E8.5, 9.5 and 10.5, MEF2C transcripts were readily detectable (Fig. 4). The ubiquitously expressed transcript L7 was measured as a positive control. Together, these results confirm that MEF2C is expressed at the appropriate time and place in the embryo and yolk sac to participate in vascular development.

### Lack of endothelial cell organization in *MEF2C* mutant embryos

Because the vasculature forms from an endothelial cell template that recruits SMCs to form the vessel walls, we investigated whether the vascular defects in *MEF2C* mutant embryos might be attributable to the absence of endothelial cells. The cell adhesion molecule PECAM (platelet endothelial cell adhesion molecule) is a marker for endothelial cells (Baldwin et al., 1994) and has been shown to be essential for aggregation of endothelial cells as they form the vascular plexus (Baldwin et al., 1994; Baldwin, 1997). Immunostaining of wild-type embryos at E9.0 with an anti-PECAM antibody showed the highly intricate endothelial cell network throughout the embryo (Fig. 5A,C). Staining was particularly evident in the intersomitic and elaborate cranial vasculature, the forming aortic arch arteries, and endocardium of the heart. Mutant embryos also showed PECAM staining, but it was diffuse and disorganized, lacking an obvious network, and there appeared to be fewer PECAM-positive cells than normal (Fig. 5B,D). The presence of PECAM-positive cells demonstrates that endothelial cells were able to be specified and differentiate, but they were unable to become organized into a vascular network in *MEF2C* mutant embryos.

To further characterize endothelial cell defects in *MEF2C* mutant embryos, we examined expression of Flk-1 using a targeted *Flk-1* null allele containing *lacZ* under transcriptional control of the endogenous *Flk-1* promoter (Shalaby et al., 1995). This transgene is expressed in endothelial cells throughout the embryonic vasculature (Fig. 5E) and yolk sac (not shown) at E9.5. In *MEF2C* mutant embryos, *Flk1-lacZ* was expressed, but the expression pattern was disorganized in vessel-forming regions and the heart, mirroring PECAM expression (Fig. 5F).

We also examined, by in situ hybridization, expression of Tie-2 transcripts, which mark developing endothelial cells in wild-type embryos. In contrast to the organized expression pattern demarkating the vasculature of wild-type embryos, in the mutants, Tie-2 transcripts were seen in a diffuse pattern (Fig. 5I,J).

Together, these different endothelial cell markers show that MEF2C is required for proper organization of endothelial cells into a vascular network, but not for endothelial cell differentiation.

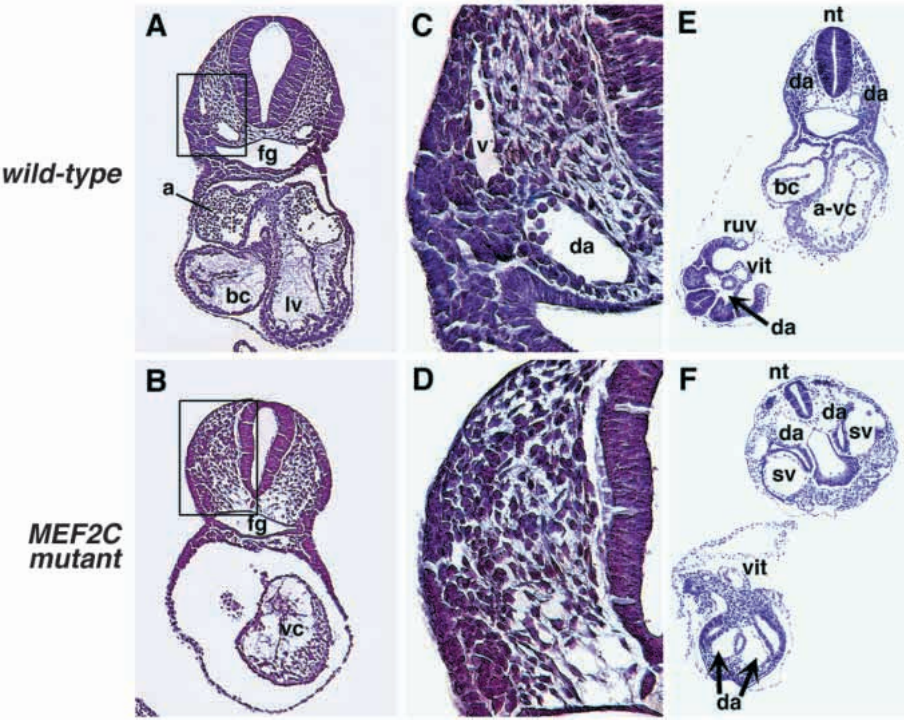


**Fig. 2.** Vascular defects in *MEF2C* mutant embryos. Transverse sections of E9.5 wild-type (A,C,E) and mutant (B,D,F) embryos were stained with H and E. Note the absence of the dorsal aorta (da) and anterior cardinal vein (v) in the mutant (B,D). The mutant also has only a single ventricular chamber (vc), located on the left side, whereas the bulbus cordis (bc), left ventricle (lv) and atrium (a) are clearly evident in the wild-type embryo (compare A and B). The caudal dorsal aorta is dilated in the mutant (compare E and F). The upper embryo section in F is caudal to the heart and passes through the sinus venosus (sv). A-vc, atrioventricular canal; fg, foregut; nt, neural tube; ruv, right umbilical vein; vit, vitelline vein.

### Expression of angiogenic growth factors and receptors in *MEF2C* mutant embryos

The vascular defects in *MEF2C* mutant embryos were reminiscent of those in embryos lacking VEGF or its receptor Flt-1 (Carmeliet et al., 1996a; Ferrara et al., 1996; Fong et al., 1995). We therefore used semi-quantitative RT-PCR to examine expression of transcripts encoding these and other vascular growth factors and receptors. Transcripts for VEGF and its receptors, Flt-1 and Flk-1, were detected at similar levels in wild-type and mutant embryos at E9.0 (Fig. 6). Similarly, levels of mRNA encoding Ang-1 and its receptors, Tie-1 and Tie-2, as well as tissue factor and the bHLH transcription factor ARNT, which have been shown to be required for normal vascular development (Carmeliet et al., 1996b; Maltepe et al., 1997; Kozak et al., 1997) were unaffected in *MEF2C* mutants. The constitutively expressed transcript L7 was used as an internal control. While these results do not rule out the possibility of subtle changes in expression of these genes in specific regions of mutant embryos, together with the results in Fig. 5, they suggest that the vascular defects in *MEF2C* mutant embryos do not arise from downregulation of these vascular signaling molecules.

### Defects in SMC differentiation in *MEF2C* mutant embryos detected with an *SM22-lacZ* transgene

We next investigated whether differentiated SMCs were present in *MEF2C* mutant embryos. As a marker for SMCs, we used a *lacZ* transgene linked to the promoter of the *SM22* gene, which encodes a calponin-related protein expressed in all SMCs throughout embryogenesis and adulthood, as well as in the looping heart tube and in skeletal myocytes within the somites (Li et al., 1996a). As described previously (Li et al., 1996b), the *SM22-lacZ* transgene was expressed in the developing heart, somites and arterial system of wild-type embryos (Fig. 7A,C). In *MEF2C* mutant embryos, the *SM22-lacZ* transgene was not expressed in the vasculature or myotomes of the somites (Fig. 7B,D). Because SM22 expression is restricted to differentiated muscle cells, these results suggest that MEF2C is required for differentiation of SMCs within the forming vasculature, as well as skeletal muscle cells within the somites.

Expression of *SM22-lacZ* also revealed an interesting defect in atrioventricular gene expression in mutant embryos. Whereas *SM22-lacZ* expression was restricted to the conotruncus and bulbus cordis and was not detected in the atrium of wild-type embryos (Fig. 7A,C), in the mutants, transgene expression was restricted predominantly to the atrial chamber and was undetectable in the developing ventricular chamber (Fig. 7B,D). Thus, there appears to be a specific alteration in chamber-specific gene expression in the mutant.

## DISCUSSION

Formation of the vascular system requires reciprocal intercellular signaling and interactions between endothelial cells, SMCs and pericytes. Whereas numerous studies have documented the roles of peptide growth factors and their receptors in vascular development, little is known of the underlying transcriptional mechanisms that control endothelial or SMC differentiation or patterning. The phenotype of *MEF2C* mutant embryos demonstrates that MEF2C plays multiple roles in formation of the vascular system by controlling patterning of endothelial cells into a primitive vascular plexus, as well as differentiation of SMCs. In the absence of MEF2C, endothelial cells are specified, but they fail to become organized into a vascular network and SMCs do not differentiate. These defects resemble those of mutants lacking VEGF or its receptor Flt-1 and suggest that VEGF and MEF2C may function in a common developmental pathway for vascular development.

### Reciprocal signaling between endothelial and mesenchymal cells

Formation of the vasculature involves organization of endothelial cells into a vascular network (vasculogenesis), recruitment of SMCs and pericytes from surrounding mesenchyme and, ultimately, sprouting and remodeling of the

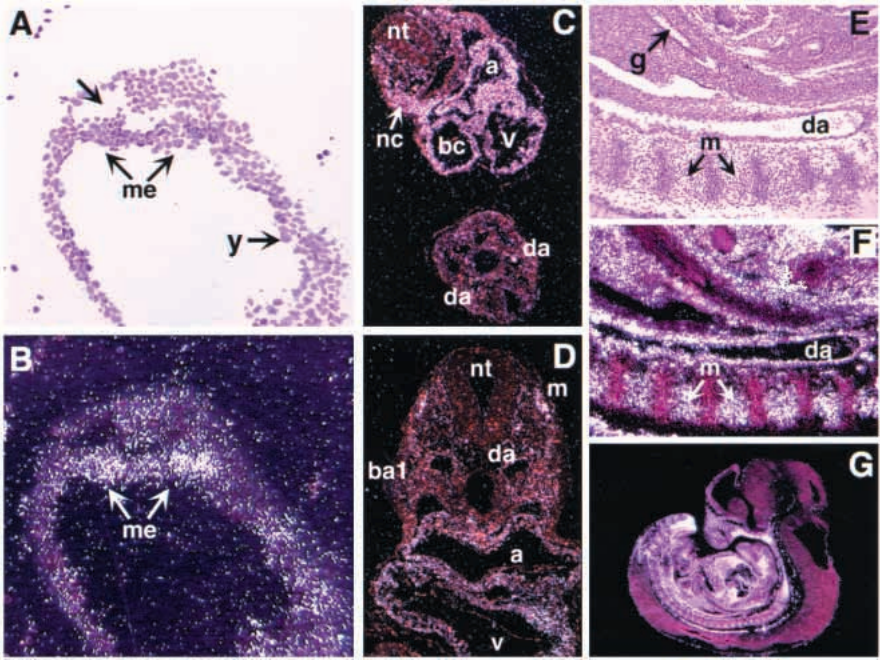


**Fig. 3.** Expression of MEF2C in endothelial cells, mesenchyme and SMCs. *MEF2C* transcripts were detected by in situ hybridization to embryo or yolk sac sections, as indicated. (A,B) Bright-field and dark-field micrographs, respectively, of *MEF2C* expression in E8.5 yolk sac (y). Arrow in A points to a blood vessel. me, yolk sac mesenchyme. (C,D) Transverse sections showing *MEF2C* expression in E9.0 and 9.5 embryos, respectively. (E,F) Bright-field and dark-field micrographs, respectively, of *MEF2C* expression in a sagittal section of an E11.5 embryo. Sections show enlarged regions of the dorsal aorta and somites from the embryo shown in G. a, atrium; ba, branchial arch 1; bc, bulbus cordis; da, dorsal aorta; g, foregut; m, myotomes; nc, neural crest cells; nt, neural tube; v, ventricle. Hybridization using sense probe showed no specific signals (not shown).

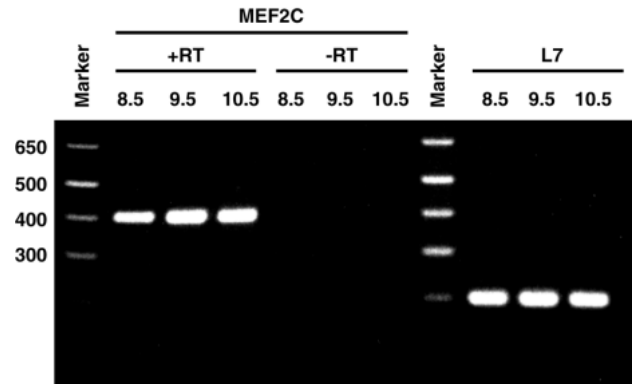


**Fig. 4.** Detection of MEF2C transcripts in yolk sac by RT-PCR. RNA isolated from yolk sacs at E8.5, E9.5 and E10.5 was used for semiquantitative RT-PCR reactions to detect the presence of MEF2C and L7 mRNA, as indicated. RT-PCR products for MEF2C and L7 are 401 and 202 bp, respectively, and were visualized by staining with ethidium bromide.

primary vasculature to give rise to a complex vascular network (angiogenesis). Each step in this process is dependent on growth factor-receptor interactions between endothelial and mesenchymal cells. The early steps in vasculogenesis are exquisitely sensitive to VEGF concentrations, as illustrated by the severe and lethal defects in vasculogenesis and angiogenesis in mice heterozygous for a *VEGF* null mutation (Carmeliet et al., 1996a; Ferrara et al., 1996). This phenotype is exacerbated in *VEGF* null embryos, which die at E8.5-9.0. The VEGF receptors, Flk-1 and Flt-1, are required for endothelial cell differentiation and assembly of endothelial cells into a functional vascular plexus, respectively (Shalaby et al., 1995; Fong et al., 1995). *Flk-l* mutant embryos die at E8.5-9.5 due to failure of endothelial cells to differentiate. The phenotype of *Flk-1* mutants is more severe than that of *VEGF* mutants, suggesting that VEGF is not the only ligand for this receptor. In *Flt-1* mutant embryos, endothelial cells can differentiate, but later aspects of vasculogenesis are disrupted, resulting in thin-walled vessels, which are often larger in diameter than normal. Angiopoietins, expressed by mesenchymal cells and their receptors, Tie1 and Tie2, expressed by endothelial cells, act subsequent to VEGF-dependent signaling steps to recruit mesenchymal cells to the primary endothelial cell network (Dumont et al., 1994; Sato et al., 1995; Davis et al., 1996; Suri et al., 1996). TGF-β is also required for vasculogenesis (Dickson et al., 1995; Oshima et al., 1996) and PDGF is essential for formation of pericytes (Leveen et al., 1994). Mice lacking tissue factor TF also die from vascular abnormalities at E8.5 due to a defect in recruitment of SMCs/pericytes to the endothelial plexus (Carmeliet et al., 1996b). Interestingly, the severity of the *TF* null mutation varies depending on genetic background. Whereas TF is essential for vasculogenesis in a 129/Sv background, in a 129/Sv × C57 BL/6 background a significant percentage of mutants survive until birth (Toomey et al., 1997). We have not yet examined for possible strain variations of the *MEF2C* phenotype.

A summary of cardiovascular defects in *MEF2C* mutant embryos is shown in Table 1. All of these defects can be ascribed to cells that express MEF2C, though some could arise secondarily from abnormal cell-cell interactions or functional abnormalities in the cardiovascular system.

What might be the molecular basis for the vascular defects in *MEF2C* mutants? Our results show that MEF2C is expressed in the endothelial cell-derived vascular plexus and surrounding mesenchyme in the yolk sac by E8.5. MEF2C is also expressed in endothelial cells, surrounding mesenchyme and smooth muscle cells of the developing vasculature of the embryo proper, as well as within neural crest cells in the branchial arches, which give rise to the branchial arch arteries and cardiac outflow tract (Bergwerff et al., 1998). In the absence of MEF2C, the endothelial cell plexus is not stabilized and SMCs do not differentiate. Therefore, we propose that MEF2C is likely to play multiple roles in vascular development, being required for endothelial cell interactions and in mesenchymal cells surrounding the endothelial network for their responsiveness to endothelial cell signaling, their migration or their differentiation. The apparent failure of endothelial cells to interact properly to form a vascular plexus in *MEF2C* mutant embryos could reflect an underlying defect in cell adhesion. Of note, in *Drosophila*, MEF2 has been shown to regulate expression of αPS2 integrin (Ranganayakulu et al., 1995) required for visceral muscle morphogenesis.

Although the vascular phenotype of *MEF2C* mutants resembles that of *VEGF* and *Flt-1* mutants, VEGF and its receptors are expressed in *MEF2C* mutant embryos. Therefore, we believe it is unlikely that reduced expression of these genes accounts for the vascular phenotype of *MEF2C* mutants, though we cannot rule out the possibility that subtle changes in expression of these genes in localized regions of the embryo could contribute to the phenotype. We think a more likely possibility is that MEF2C may function within the VEGF signaling pathway or may control the expression of components of this pathway required for VEGF signaling. It is interesting in this regard that MEF2C has been shown to mediate changes in gene expression in response to growth factor signaling in other cell types (reviewed in Black and Olson, 1998). Activation of the MAP kinase pathway by serum and epidermal growth factor in fibroblasts and HeLa cells leads to phosphorylation of MEF2C and enhanced

**Table 1. Cardiovascular defects in *MEF2C* mutant embryos**

| Embryonic compartment | Phenotype |
|---|---|
| Yolk sac | Disorganized assembly of endothelial cells<br>Absent vasculature |
| Vasculature | Absent or malformed rostral dorsal aortae<br>Absent or dilated sinus venosus<br>Dilated caudal dorsal aortae<br>Disorganized assembly of endothelial cells |
| Heart | Absent future right ventricle<br>Looping abnormality<br>Non-elongated atrioventricular canal<br>Disorganized and hypoplastic trabeculae<br>Reduced expression of contractile protein genes<br>Absent cardiac jelly<br>Absent endocardial cushions<br>Disorganized endocardium |

expression of MEF2-dependent genes (Han and Prywes, 1995; Coso et al., 1997; Han et al., 1997; Kato et al., 1997). Because VEGF has been shown to activate the MAP kinase pathway (D'Angelo et al., 1995), it is conceivable that MEF2C acts downstream in the VEGF signaling pathway to control expression of target genes required for vascular development.

Abnormalities in vascular development have also been observed in mouse embryos lacking the zinc finger transcription factor LKLF (Kuo et al., 1997). However, in LKLF mutant mice, vasculogenesis and angiogenesis occur normally, but vessel walls are unstable. The bHLH transcription factors ARNT and HIF1α which control gene expression in response to hypoxia are also required for development of the embryonic and yolk sac vasculature (Maltepe et al., 1997; Kozak et al., 1997; Iyer et al., 1998). The vascular defects observed in *ARNT* and *HIF1α* mutant mice are similar to those in *MEF2C* mutants, but the functions of these factors are different from those of MEF2C.

### Cardiac defects in *MEF2C* mutant embryos

Previously, we described several defects in cardiac morphogenesis and myogenesis in *MEF2C* mutant embryos (Table 1; Lin et al., 1997). In addition to abnormalities in the myocyte component of the heart, the endocardium is disorganized and hypoplastic in the mutant. There is an interdependency between endocardial and myocardial development in which reciprocal signaling between these two cell types is required for their differentiation (reviewed in Fishman and Chien, 1997). In *MEF2C* mutants, the ventricular wall is hypoplastic and lacks trabeculation. This cardiac defect is similar to that of mouse mutants lacking neuregulins or their receptors (Meyer and Birchmeier, 1995; Lee et al., 1995; Gassmann et al., 1995) and suggests that certain myocardial defects may also arise from abnormal signaling from the endocardial layer.

Because *MEF2C* mutant embryos exhibit severe defects in cardiac morphogenesis, which result in reduced cardiac function (Lin et al., 1997), it is reasonable to wonder whether any of the vascular defects in these mutants might arise as a secondary consequence of abnormal blood flow in the developing vasculature. While we cannot formally rule out this possibility, there are several reasons why we believe the vascular phenotype of *MEF2C* mutants cannot be attributed solely to cardiac defects. First, vascular defects are observed in the yolk sac vasculature by E8.5, which is prior to obvious cardiac defects. Second, the types of vascular defects seen in *MEF2C* mutants are not seen in other mouse mutants, which also exhibit lethal cardiac defects. Third, *MEF2C* is expressed at the correct time and place to function in vascular development. Finally, recent results show that VEGF signaling stimulates transcriptional activity of the MEF2C transcription activation domain and is therefore likely to be important for VEGF-dependent steps in vasculogenesis (J. L. and E. O., unpublished).

### Defects in muscle differentiation in *MEF2C* mutants

In the absence of MEF2C, SMC differentiation was arrested, based on the lack of expression of *SM22-lacZ*. Whether the failure of SMCs to differentiate reflects a primary role for MEF2C in activation of smooth-muscle-specific genes or whether SMCs fail to develop because of a defect in signaling between endothelial cells and pericytes is unclear. Previous studies have shown that the *SM22* promoter is activated by serum response factor and does not contain an essential MEF2-binding site (Li et al., 1997; Kim et al., 1997). Therefore, the apparent dependence of the *SM22* promoter on MEF2C suggests the existence of an indirect pathway for MEF2C-dependent activation of *SM22* transcription. In principle, this could occur through protein-protein interactions between MEF2C and other promoter-bound factors, as has been shown to occur for myogenic bHLH proteins and MEF2C (Molkentin et al., 1995). Alternatively, MEF2C could regulate the expression or activity of another factor (SRF perhaps?) that is essential for *SM22* transcription.

MEF2C is required for expression of a subset of cardiac muscle genes and for formation of the future right ventricular chamber (Lin et al., 1997). Analysis of *SM22-lacZ* expression in *MEF2C* mutant embryos revealed an intriguing distortion in gene expression in the developing heart. Whereas this transgene is normally expressed in the developing right ventricle between E9.0 and E13.5 (Li et al., 1996b), in mutant embryos, *SM22-lacZ* expression was observed in the atrium, rather than the ventricle. The same alteration in expression is observed by in situ hybridization of endogenous SM22 transcripts (data not shown). This alteration in atrio-ventricular gene expression is not a general characteristic of the mutant because other atrial- and ventricular-specific genes, such as *MLC2V* and *MLC2A*, are expressed in the correct chambers of the mutant heart (Lin et al., 1997). It is unclear why the spatial pattern of SM22 expression is specifically altered in the heart of the mutant. One possibility is that the dynamic changes in temporospatial expression of contractile protein genes within the developing heart tube are partially disrupted in the mutant. SM22, which normally shows a broad expression pattern in the heart tube before becoming resticted to the bulbus cordis, may fail to be appropriately downregulated in atrial precursors in the mutant.

The *SM22-lacZ* transgene also revealed a defect in skeletal muscle differentiation within the somites of *MEF2C* mutant embryos. Expression of the myogenic regulatory gene *myogenin* in the somites is also reduced in the mutants (data not shown). MEF2C is expressed in the myotomal compartment of the somites beginning at about E8.5 (Edmondson et al., 1994; Subramanian et al., 1997) and a MEF2-binding site in the *myogenin* promoter has previously been shown to be important for amplification of *myogenin* expression in the developing myotome (Cheng et al., 1993; Yee et al., 1993). Thus, MEF2C appears to be required for activation of the skeletal muscle differentiation program in vivo.

In skeletal muscle, MEF2 factors cooperate with members of the MyoD family to activate skeletal muscle gene expression (Kaushal et al., 1994; Molkentin et al., 1995) and, in *Drosophila*, MEF2 is required for differentiation of all muscle cell types (Lilly et al., 1995; Bour et al., 1995; Ranganayakulu et al., 1995). Based on these genetic and biochemical findings, we have proposed that MEF2 establishes combinatorial codes for muscle gene activation by interacting with cofactors unique to each muscle cell type

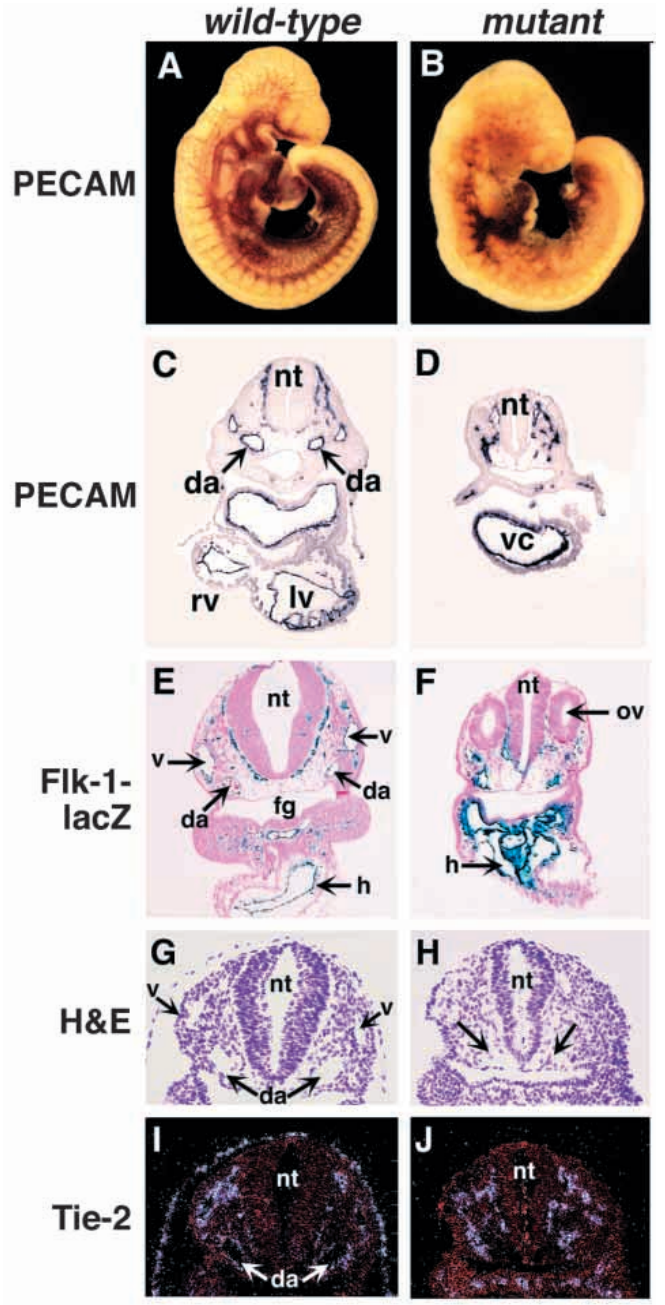


**Fig. 5.** Expression of endothelial cell markers in wild-type and MEF2C mutant embryos. Wild-type (A,C,E,G,I) and mutant (B,D,F,H,J) embryos at E9.5 were stained for PECAM, Flk1-lacZ, or Tie-2, as described in Materials and Methods. (C,D) Sections of PECAM-stained embryos in A and B, respectively. (G,H) H&E-stained sections corresponding to the dark-field micrographs in I and J. All endothelial cell markers are detected in the mutant, but they are expressed in a disorganized pattern. da, dorsal aorta; fg; foregut; h, heart; lv, left ventricle; nt, neural tube; ov, otic vesicle; rv, right ventricle; v, anterior cardinal vein;vc, ventricular chamber.

(Molkentin and Olson, 1996). The potential partners for MEF2 factors in the smooth muscle and endothelial cell lineages remain to be identified. Of note, the bHLH transcription factors dHAND and eHAND are expressed in the

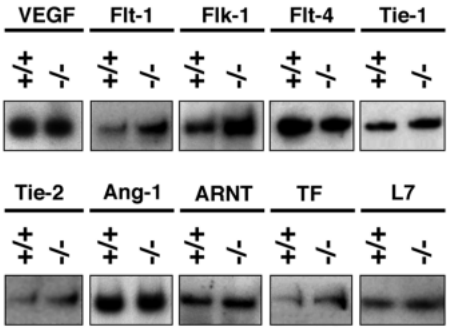


**Fig. 6.** Detection of endothelial cell growth factor receptor transcripts by RT-PCR. RNA was isolated from wild-type (+/+) and mutant (–/–) embryos at E9.5 and the indicated transcripts were detected by semiquantitative RT-PCR using gene-specific primers as described in Materials and Methods. PCR products were labeled with $^{32}P$ and only the region of the gel containing each product is shown No reproducible differences in expression of any of the transcripts shown were observed between wild-type and *MEF2C* mutant embryos.

developing vasculature of the embryo and yolk sac (Cserjesi et al., 1995; Srivastava et al., 1995; Firulli et al., 1996). In the absence of either gene, vascular development is disrupted (Srivastava et al., 1997; Firulli et al., 1998).

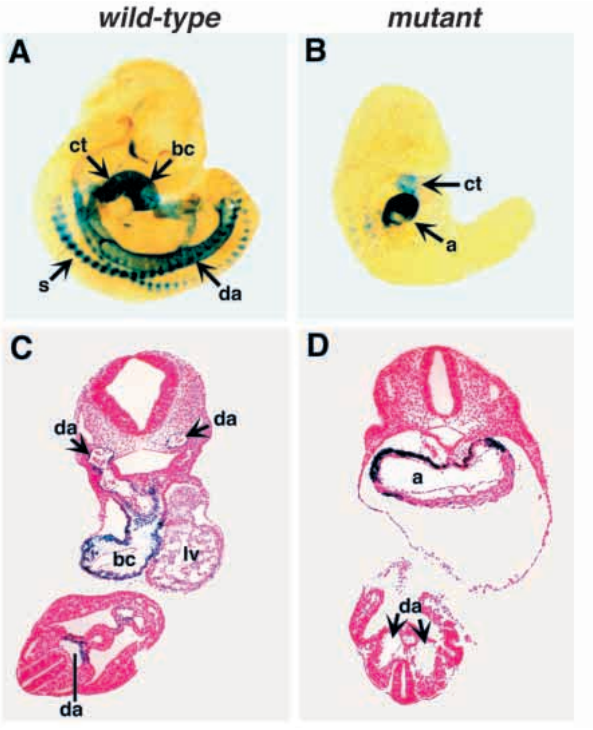


**Fig. 7.** Expression of *SM22-lacZ* transgene in wild-type and *MEF2C* mutant embryos. A *SM22-lacZ* transgene was introduced into wild-type (A) and mutant (B) backgrounds by breeding. Embryos were stained at E9.5. (C,D) H&E-stained sections of the embryos in A and B, respectively. Staining of the bulbus cordis (bc), conotruncus (ct), right ventricle (rv), somites (so) and dorsal aorta (da) is readily detectable in wild-type, whereas staining is absent from the somites and dorsal aorta of the mutant and expression in the heart is shifted primarily to the atrial chamber (a).

## Diverse functions for MEF2 in vascular growth and development

The results of this study reveal multiple roles for MEF2C in formation of the vascular system. The expression patterns of MEF2A, MEF2B and MEF2D overlap with, but are distinct from, that of MEF2C (Edmondson et al., 1994; Subramanian et al., 1996; Ticho et al., 1996; Molkentin et al., 1996) and the functions of the four MEF2 factors are similar in most assays. Nevertheless, the phenotype of *MEF2C* mutant embryos demonstrates that MEF2C possesses functions in vascular development distinct from those of the other factors.

In addition to the role of MEFC in vascular development, we and others have shown that MEF2 factors are upregulated in adult SMCs in response to vascular injury (Suzuki et al., 1995; Firulli et al., 1996). Given the importance of SMC proliferation and angiogenesis in numerous pathological processes, it will be of interest to determine whether MEF2C functions in aspects of vessel growth and development in the adult.

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