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Cardiovascular development and survival require *Mef2c* function in the myocardial but not the endothelial lineage

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Abstract

MEF2C is a member of the highly conserved MEF2 family of transcription factors and is a key regulator of cardiovascular development. In mice, *Mef2c* is expressed in the developing heart and vasculature, including the endothelium. Loss of *Mef2c* function in germline knockout mice leads to early embryonic demise and profound developmental abnormalities in the cardiovascular system. Previous attempts to uncover the cause of embryonic lethality by specifically disrupting *Mef2c* function in the heart or vasculature failed to recapitulate the global *Mef2c* knockout phenotype and instead resulted in relatively minor defects that did not compromise viability or result in significant cardiovascular defects. However, previous studies examined the requirement of *Mef2c* in the myocardial and endothelial lineages using Cre lines that begin to be expressed after the expression of *Mef2c* has already commenced. Here, we tested the requirement of *Mef2c* in the myocardial and endothelial lineages using conditional knockout approaches in mice with Cre lines that deleted *Mef2c* prior to onset of its expression in embryonic development. We found that deletion of *Mef2c* in the early myocardial lineage using *Nkx2–5*^{Cre} resulted in cardiac and vascular abnormalities that were indistinguishable from the defects in the global *Mef2c* knockout. In contrast, early deletion of *Mef2c* in the vascular endothelium using an *Etv2::Cre* line active prior to the onset of *Mef2c* expression resulted in viable offspring that were indistinguishable from wild type controls with no overt defects in vascular development, despite nearly complete early deletion of *Mef2c* in the vascular endothelium. Thus, these studies support the idea that the

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Author contributions

S.C.M. and T.S. designed, interpreted, performed experiments, and helped write the manuscript. K.L.V.B. and R.M.B. designed, interpreted, and performed experiments. B.L.B. designed and interpreted experiments and wrote the manuscript. All authors read, edited, and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

requirement of MEF2C for vascular development is secondary to its requirement in the heart and suggest that the observed failure in vascular remodeling in *Mef2c* knockout mice results from defective heart function.

Keywords

MEF2C; heart development; vascular development; endothelial cells; endothelium; vascular remodeling; morphogenesis; mouse

1. Introduction

MEF2C is a member of the myocyte enhancer factor 2 family of transcription factors. MEF2 proteins are defined by the presence of amino terminal MADS and MEF2 domains through which they dimerize and bind DNA in a sequence specific manner (Black and Olson, 1998). Like other MEF2 proteins, MEF2C functions as a signal-responsive transcriptional regulator and has extensive interactions with other transcription factors, co-activators, and co-repressors. It can either activate or repress transcription depending on posttranslational modifications and co-factor interactions (Black and Cripps, 2010; Dong et al., 2017; McKinsey et al., 2002). MEF2C is widely appreciated for its role in cardiovascular development (Black and Cripps, 2010; Potthoff and Olson, 2007). Global disruption of *Mef2c* function in mice leads to profound defects in the heart and vasculature and results in embryonic lethality between E9.5 and E10.5 (Lin et al., 1997). In *Mef2c*-null mutants, cardiomyocytes are specified, but the heart tube does not elongate sufficiently for proper looping, leading to the formation of only one common ventricle (Lin et al., 1997). Similarly, vascular endothelial cells are specified in *Mef2c* mutants and form a primitive vascular network, but the vasculature fails to remodel properly leading to severe defects (Bi et al., 1999; Lin et al., 1998; Lin et al., 1997).

Mef2c is expressed in myocardial progenitors within the cardiac crescent by embryonic day (E) 7.5 and is detectable in vascular endothelial cells by E8.5 (Dodou et al., 2004; Edmondson et al., 1994). Interestingly, numerous, discrete enhancers in the *Mef2c* locus control its transcription in the heart and the vasculature (Agarwal et al., 2011; De Val et al., 2004; De Val et al., 2008; Dodou et al., 2004; von Both et al., 2004). Detailed studies of those enhancers demonstrate that *Mef2c* activation is controlled by different upstream regulatory pathways in the myocardial and endothelial lineages and suggest that *Mef2c* has autonomous requirements in cardiomyocytes and endothelial cells (De Val et al., 2004; De Val et al., 2008; Dodou et al., 2004; von Both et al., 2004). However, no study designed to test cell autonomous requirements has recapitulated the germline phenotype. Conditional knockout of *Mef2c* in cardiomyocytes caused only mild or no deficiencies in the embryonic heart (Vong et al., 2005). Deletion of *Mef2c* from the endothelial lineage has been reported to result in defective branching in embryonic development and postnatally, but animals survive and have no overt defects (Sacilotto et al., 2016; Xu et al., 2012). One potential explanation for why these prior studies did not recapitulate the germline knockout phenotype is that *Mef2c* was deleted in those studies using Cre lines driven by enhancers of cardiac or vascular differentiation genes that are activated after the initiation of *Mef2c* expression

(Agah et al., 1997; Chen et al., 1998; Dodou et al., 2004; Edmondson et al., 1994; Koni et al., 2001; Moses et al., 2001; Wang et al., 2010). Expression of *Mef2c* prior to Cre-mediated deletion may thus limit the severity of the observed phenotypes by allowing initiation of MEF2C-dependent gene regulatory networks.

Here, we tested whether *Mef2c* function is required in the heart and vasculature by creating lineage specific knockouts prior to the onset of *Mef2c* transcription in either tissue. When *Mef2c* was deleted specifically in early stage cardiac precursors using *Nkx2–5*^{Cre}, we found that embryos recapitulate the germline knockout phenotype, including vascular deficiencies, and die around E9.5. In contrast, *Mef2c* deletion in the endothelium using an *Etv2::Cre* line, which is active prior to the onset of *Mef2c* expression, resulted in viable offspring with no overt defects in early vascular development despite complete excision of *Mef2c* from the endothelium prior to E8.5. These data strongly support the idea that the vascular defects, which occur as a result of loss of *Mef2c* function, are secondary to the loss of *Mef2c* in the heart and are a likely consequence of failed vascular remodeling due to defective cardiac development and function.

2. Results and discussion

2.1 Early deletion of Mef2c in the heart causes embryonic demise at midgestation

To delete *Mef2c* specifically in the cardiac lineage prior to onset of *Mef2c* expression, we utilized the well-characterized *Nkx2–5*^{Cre} line (Moses et al., 2001). In this knock-in line, the expression of Cre accurately mirrors the temporospatial pattern of expression of the *Nkx2*-5 gene, one of the earliest lineage markers of cardiac fate (Lints et al., 1993). Importantly, *Nkx2–5* expression commences prior to assembly of the linear heart tube at around E7, which precedes *Mef2c* expression. The *Nkx2–5*^{Cre} expression domain encompasses all cardiac progenitors of the first and second heart field, including the cells of the myocardium and endocardium (Ma et al., 2008; Moses et al., 2001).

In crosses of *Nkx2–5*$^{Cre/+}$;*Mef2c*$^{+/-}$ with *Mef2c*$^{flox/flox}$ mice, the *Nkx2–5*^{Cre} cardiac conditional knockout mice (*Mef2c*$^{flox/-}$; *Nkx2–5*$^{Cre/+}$) died between E9.5 and E10.5, just as *Mef2c* germline KO animals (Fig. 1). At E9.5, *Mef2c*$^{flox/-}$; *Nkx2–5*$^{Cre/+}$ embryos were clearly smaller than their control littermates and exhibited an overt phenotype that is essentially identical to that of germline *Mef2c*$^{-/-}$ knockout mice (Fig. 1C and G). *Nkx2–5*^{Cre} cardiac conditional knockout embryos were not detected at E14.5, but control littermates of all other genotypes were present in expected ratios (Table 1A). This is in contrast to previous cardiac-specific deletions of *Mef2c* with *MLC-2v*^{Cre} [*Myl2*$^{tm1(cre)Krc}$; (Chen et al., 1998)] or α*MyHC-Cre* [*Tg(Myh6-cre)*^{2182Mds}; (Agah et al., 1997)], which did not adversely affect embryonic development (Vong et al., 2005). Cre recombinase activity in *MLC-2v*^{Cre} and α*MyHC-Cre* mice begins significantly later than in *Nkx2–5*^{Cre} mice and after the onset of *Mef2c* expression in cardiac progenitors (Agah et al., 1997; Chen et al., 1998; Dodou et al., 2004; Edmondson et al., 1994; Moses et al., 2001). We interpret the recapitulation of the germline *Mef2c* knockout phenotype in *Nkx2–5*^{Cre} cardiac conditional knockout mice to be due to the earlier excision of *Mef2c* in cardiac progenitors.

2.2 Early deletion of Mef2c in the heart leads to cardiac and vascular defects

The first step of cardiac morphogenesis is the formation of a linear heart tube. In *Mef2c* germline knockout mice, the linear heart tube is hypoplastic compared to controls and fails to undergo rightward looping, effectively abolishing the formation of the right ventricle (Lin et al., 1997; Vong et al., 2006); (Fig. 1, compare panels B and D). Similarly, conditional deletion of *Mef2c* with *Nkx2–5*^{Cre} resulted in an unlooped, hypoplastic heart tube at E9.5 (Fig. 1H), essentially mimicking the phenotype observed in global knockout mice (Fig. 1, compare panels D and H). The *Nkx2–5*^{Cre} line was generated by insertion of the *Cre* sequence into the *Nkx2–5* locus, thereby disrupting *Nkx2*-5 function from the targeted allele (Moses et al., 2001). However, *Mef2c*$^{flox/+}$; *Nkx2–5*$^{Cre/+}$ littermates have normal heart morphology (Fig. 1, compare panels A and B to panels E and F), confirming that disruption of one functional *Nkx2–5* allele and one functional *Mef2c* allele did not result in a structural heart phenotype. Additionally, *Mef2c*$^{flox/+}$; *Nkx2–5*$^{Cre/+}$ mice were born at predicted Mendelian frequency (Table 1A), further supporting the idea that the loss of one *Nkx2–5* allele did not contribute to the observed *Mef2c* phenotype in *Mef2c* conditional knockout mice.

There are profound deficiencies in the anatomy of the blood vasculature in *Mef2c* germline knockout mice (Bi et al., 1999; Lin et al., 1998; Lin et al., 1997; Fig. 1D, K, and L). At E9.5, the major blood vessels of the trunk, the dorsal aorta and cardinal vein, had clearly formed in controls, but were not visible in transverse sections of mutant embryos (Fig. 1B and D). *Nkx2–5*^{Cre} is not expressed in vascular endothelium (Moses et al., 2001), thus sparing the *Mef2c* locus from excision in endothelial cells of the vasculature in *Mef2c*$^{flox/-}$; *Nkx2–5*$^{Cre/+}$ conditional knockout embryos. Nevertheless, profound vascular defects were still present in *Mef2c*$^{flox/-}$; *Nkx2–5*$^{Cre/+}$ conditional knockout embryos. In the embryo proper, this included absence of the dorsal aorta and cardinal vein and disrupted or absent intersomitic vessels (Fig. 1H and O). In the yolk sac, the vascular plexus, which was clearly remodeled to an arborized structure in control embryos, failed to properly remodel in cardiac-restricted conditional knockouts (Fig. 1N and P). Importantly, these defects in vascular development and remodeling were essentially identical to those observed in global *Mef2c* knockout embryos (Fig. 1D, K, and L).

CD31 (PECAM-1) is a marker of endothelial cells (Garlanda and Dejana, 1997). The presence of CD31 expression in both germline and *Nkx2–5*^{Cre} cardiac conditional knockout embryos suggests that endothelial cells are properly specified in the absence of MEF2C and that the vascular defects in *Mef2c* germline knockout mice are secondary to primary defects in heart development and function. The absence of major blood vessels in the embryo and the failure of the vasculature to remodel is likely due to the loss of cardiac contraction and the resultant hemodynamic forces, which are required for vessel remodeling and stabilization (Lucitti et al., 2007; Udan et al., 2013).

2.3 Etv2::Cre results in early recombination in endothelial and hematopoietic lineages and derivatives

Etv2 is an Ets transcription factor and one of the earliest lineage determinants of endothelial and blood cells in embryonic development. In *Etv2* mutants, no endothelial or blood cells

are specified, and embryos die by E9.5 due to severe vascular and hematopoietic defects (Ferdous et al., 2009; Lee et al., 2008). Etv2 binds directly to an early endothelial-specific enhancer in the *Mef2c* locus to activate *Mef2c* expression *in vivo* (De Val et al., 2008). Because Etv2 is upstream of *Mef2c*, we reasoned that inactivation of *Mef2c* by Cre expression under the control of early regulatory elements from the *Etv2* gene would allow excision of *Mef2c* prior to its expression. Therefore, we cloned a 3.3 kb-fragment of the upstream control region of *Etv2*, including the endogenous promoter and transcription start site, into a Cre expression vector and generated stable transgenic mouse lines. To define the lineage marked by the activity of this *Etv2* promoter and enhancer, we crossed this *Etv2::Cre* transgenic line to $Rosa26^{LacZ/LacZ}$ Cre-dependent *lacZ* reporter mice (Soriano, 1999). Cre-mediated recombination occurred in endothelial and hematopoietic progenitor cells in the yolk sac and embryo proper prior to E7 (Fig. 2A). By E8.5, the entire endothelial lineage was marked by the activity of the *Etv2::Cre* transgene (Fig. 2B), and robust X-gal staining at E9.5 was observed in endothelial and blood cells throughout the developing embryo and in the extra-embryonic vasculature (Fig. 2C-J). Although the entire endothelium was marked by the activity of the *Etv2::Cre* transgene, no ß-galactosidase activity or expression was observed in the myocardium (Fig. 2D and J), indicating that little or no excision was occurring in cardiac myocytes. Importantly, Cre activity from this *Etv2::Cre* transgenic line precedes *Mef2c* expression (Dodou et al., 2004; Edmondson et al., 1994), allowing excision prior to any expression of *Mef2c* in the vasculature. In a previously published study, Rasmussen *et al.* described a very similar *Etv2::Cre* transgenic line (*Er71*-Cre) made with a 3.9-kb *Etv2* proximal fragment, including the minimal promoter and transcriptional start site (Rasmussen et al., 2011). That study reported essentially identical early activity of that independently-generated *Etv2::Cre* line when crossed to $Rosa26^{LacZ/LacZ}$ reporter mice to the Cre activity observed here (Rasmussen et al., 2011), supporting our findings that *Etv2::Cre* acts very early in embryonic development with activity throughout all endothelium and blood.

2.4 Early deletion of Mef2c in the endothelium does not result in an overt phenotype and does not negatively affect viability

In crosses of $Etv2::Cre^{Tg/0};Mef2c^{+/-}$ with $Mef2c^{flox/flox}$ mice, the endothelial-specific conditional knockout mice ($Etv2::Cre^{Tg/0};Mef2c^{flox/-}$) were born and survived at Mendelian-predicted frequencies (Table 1B) and were viable and fertile with no overt defects. During embryonic development, *Etv2::Cre* endothelial-specific conditional knockouts were indistinguishable from controls at all stages examined (Fig. 3A-H). The hearts of endothelial-specific conditional knockout embryos looped normally and had normal morphology (Fig. 3A and B). Likewise, vascular development appeared normal: dorsal aortae and cardinal veins were properly assembled and patent, and all other vessels in the conditional knockout animals appeared normal and functional (Fig. 3).

To ensure that *Mef2c* exon 2 was deleted in vascular endothelial cells, we isolated $CD31^{+}$ cells from endothelial-specific conditional knockout and control embryos at E8.5 by FACS sorting and determined the prevalence of *Mef2c* coding exons 2 and 3 in genomic DNA (Fig. 3I). Coding exon 2 is the floxed exon in $Mef2c^{flox/flox}$ mice (Vong et al., 2005); exon 3 serves as an internal control in these analyses. Deletion of exon 2 still allows a *Mef2c*

transcript (lacking the deleted exon) to be produced, but it does not encode a functional protein (Barnes et al., 2016; Vong et al., 2005). Importantly, *Etv2::Cre* expression led to a nearly complete deletion of *Mef2c* exon 2 by E8.5 (Fig. 3I). Consistent with complete deletion of *Mef2c* from endothelial cell genomic DNA by E8.5, expression of exon 2 was undetectable in *Mef2c* transcripts in blood vessels and endocardium of $Mef2c^{flox/-}$;$Etv2::Cre^{Tg/0}$ knockout embryos by RNA *in situ* hybridization under conditions in which it was easily detectable in control embryos (Fig. 3J and K). *Mef2c* exon 2-containing transcript was readily detectable in the myocardium of conditional knockout embryos (Fig. 3J and K). To quantify *Mef2c* expression in endothelial cells specifically, we again used FACS sorting to isolate $CD31^+$ cells and assessed the prevalence of exon 2 and exon 3 in *Mef2c* transcripts by quantitative reverse transcriptase-PCR (Fig. 3L). We observed a 97% decrease in exon 2-containing, functional *Mef2c* transcripts isolated from *Etv2::Cre* conditional knockouts compared to controls (Fig. 3L), indicating essentially complete loss of *Mef2c* in endothelial cells with no evident phenotype.

Previous studies of *Mef2c* global knockout mice suggested a possible cell autonomous requirement for *Mef2c* in endothelial cells to support proper vascular development (Lin et al., 1998). However, the lack of an apparent phenotype in endothelial-specific knockout embryos using *Etv2::Cre*, an early and efficient Cre line, suggests instead that there is not an absolute cell-autonomous requirement for *Mef2c* in endothelial cells for embryonic development and viability. Rather, our data strongly suggest that the observed early vascular defects are secondary to impaired heart function and are a consequence of the loss of *Mef2c* in the cardiac lineage. Interestingly, other studies deleting *Mef2c* specifically in endothelial cells have revealed later, postnatal roles for MEF2C in angiogenesis and in the interplay of endothelial cells with surrounding vascular smooth muscle cells (Lu et al., 2017; Maiti et al., 2008; Sacilotto et al., 2016; Xu et al., 2012). The lack of an early developmental vascular phenotype in the absence of *Mef2c* specifically in the vascular endothelium may reflect the fact that MEF2 proteins may act redundantly (Desjardins and Naya, 2016). Indeed, this notion is supported by a recent report of impaired angiogenic sprouting in embryonic development that required the simultaneous knockout of *Mef2c* and *Mef2a* in the vasculature (Sacilotto et al., 2016).

Mef2c is widely appreciated as a marker of early endothelial cells, where its expression is controlled by multiple, deeply conserved endothelial-specific enhancers (De Val et al., 2004; De Val et al., 2008). The observation that *Mef2c* is not required cell autonomously for vascular development raises interesting questions about why *Mef2c* is expressed in endothelial cells during early development and why early, highly-specific, conserved endothelial enhancers of *Mef2c* exist. It will be interesting in future studies to determine if early-acting *Mef2c* endothelial enhancers are required for later expression of *Mef2c*, when it is involved in postnatal endothelial cell functions.

3. Materials and Methods

3.1 Transgenic and mutant mice

$Mef2c^{flox/flox}$,[$Mef2c^{tm1Jjs}$; MGI:3603182], $Mef2c^{+/-}$ [$Mef2c^{tm1Eno}$; MGI:1857491], $Rosa26^{LacZ/LacZ}$ [$Gt(ROSA)26Sor^{tm1Sor}$; MGI:1861932]; $Rosa26^{mTmG/mTmG}$

[*Gt(ROSA)26Sor*$^{tm4(ACTB-tdTomato,-EGFP)Luo}$; MGI:3716464], and *Nkx2–5*$^{Cre/+}$ [*Nkx2–5*$^{tm1(cre)Rjs}$; MGI:2654594] mice have each been described previously (Lin et al., 1997; Moses et al., 2001; Muzumdar et al., 2007; Soriano, 1999; Vong et al., 2005). Cre-dependent recombination of the *Mef2c*^{flox} allele leads to removal of *Mef2c* coding exon 2, corresponding to amino acids 18–86, resulting in a null allele but leaving the remainder of the *Mef2c* transcript intact (Vong et al., 2005). To generate *Etv2::Cre* transgenic mice a 3,309-bb fragment of the *Etv2* upstream control region (Mm10, chr7:30636190–30639498), including the endogenous transcription start site, was amplified from mouse genomic and cloned into a promoterless Cre transgenic vector described previously (Verzi et al., 2005), followed by purification of the transgene fragment and generation of transgenic mice by oocyte microinjection as described previously (Anderson et al., 2004). Genotyping was performed on DNA isolated from yolk sacs or from tail biopsies by Southern blot or PCR. All mouse lines used in these studies were maintained on an outbred CD-1/FVB mixed background for 10+ generations; no variable penetrance was observed. All experiments using animals were approved by the UCSF Institutional Animal Care and Use Committee and complied with federal and institutional guidelines.

3.2 Histology, immunofluorescence, immunohistochemistry, and in situ hybridization

Section and whole-mount RNA *in situ* hybridization and whole-mount X-gal or Salmon-gal staining to detect ß-galactosidase activity was performed as previously described (Anderson et al., 2004; Kishigami et al., 2006; Rojas et al., 2005). For *Mef2c* in situ hybridization, we used a 208-bp probe that corresponds to coding exon 2 as described elsewhere (Anderson et al., 2015). Lineage analysis using *Etv2::Cre* and *Rosa26*$^{LacZ/LacZ}$ mice was performed as described previously (Verzi et al., 2005). For whole mount immunohistochemistry, rat anti-mouse CD31 (1:250, BD Biosciences, #553370) and horse radish peroxidase (HRP)-conjugated goat anti-rat secondary antibody (1:250, Abcam, ab7097) were used and detected with the DAB substrate kit (Vector, SK-4100), as described previously (Barnes et al., 2010). Immunofluorescence detection of ß-galactosidase, CD31, and myosin heavy chain (MF20) was performed as described previously (Schachterle et al., 2012). The following primary antibodies were used at a 1:100 dilution: rat anti- mouse CD31 (BD Biosciences, #553370), mouse anti-chicken MYH1E (DSHB, MF20), and chicken anti-ß-galactosidase (Abcam, ab9361). The following secondary antibodies were used at a 1:300 dilution: goat anti-rat AF488 (Abcam, ab150157), goat anti-mouse AF488 (Abcam, ab150113), and goat anti-chicken AF594 (Abcam, ab150172).

3.3 Flow cytometry and quantitative PCR

For analysis of recombination efficiency, *Etv2*::*Cre*$^{Tg/0}$; *Mef2c*$^{+/-}$ mice were crossed to *Rosa26*$^{mTmG/mTmG}$; *Mef2c*$^{flox/flox}$ mice, and embryos were collected at E8.5 or E9.5 and dissociated to create a single cell suspension for fluorescent-activated cell sorting (FACS), as described previously (Hu et al., 2015). At E8.5, cells were for sorted for GFP; at E9.5 embryos were co-stained with an APC-conjugated anti-CD31 antibody (BD Pharmingen, clone #553370) and sorted for GFP followed by CD31 sorting (Lizama et al., 2015). DNA or RNA of individual embryos was isolated from sorted cells with the Qiagen Allprep Micro Kit according to the manufacturer's instructions. Reverse transcription was conducted with the BioRad iScript reagent and quantitative PCR was performed as described previously

(Anderson et al., 2017). For quantification of excision, we used primers against targeted exon 2 (E2-F, 5′-gtgctgtgcgactgtgagat-3′; E2-R, 5′-tctgagtttgtccggctctc-3′) or exon 3 (E3-F, 5′-ttgccttccctgtccatacc-3′; E3-R, 5′-acccttgcctgcttacttca-3′) that is not excised and serves as the control. Genotypes were confirmed by PCR using individual DNA samples.

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Highlights

- MEF2C is an essential transcriptional regulator of cardiovascular development
- Deletion of *Mef2c* in early cardiac progenitors causes heart and vascular defects
- Deletion of *Mef2c* in early endothelial progenitors results in no overt phenotype
- Early vascular defects in *Mef2c*-null mice are secondary to heart defects

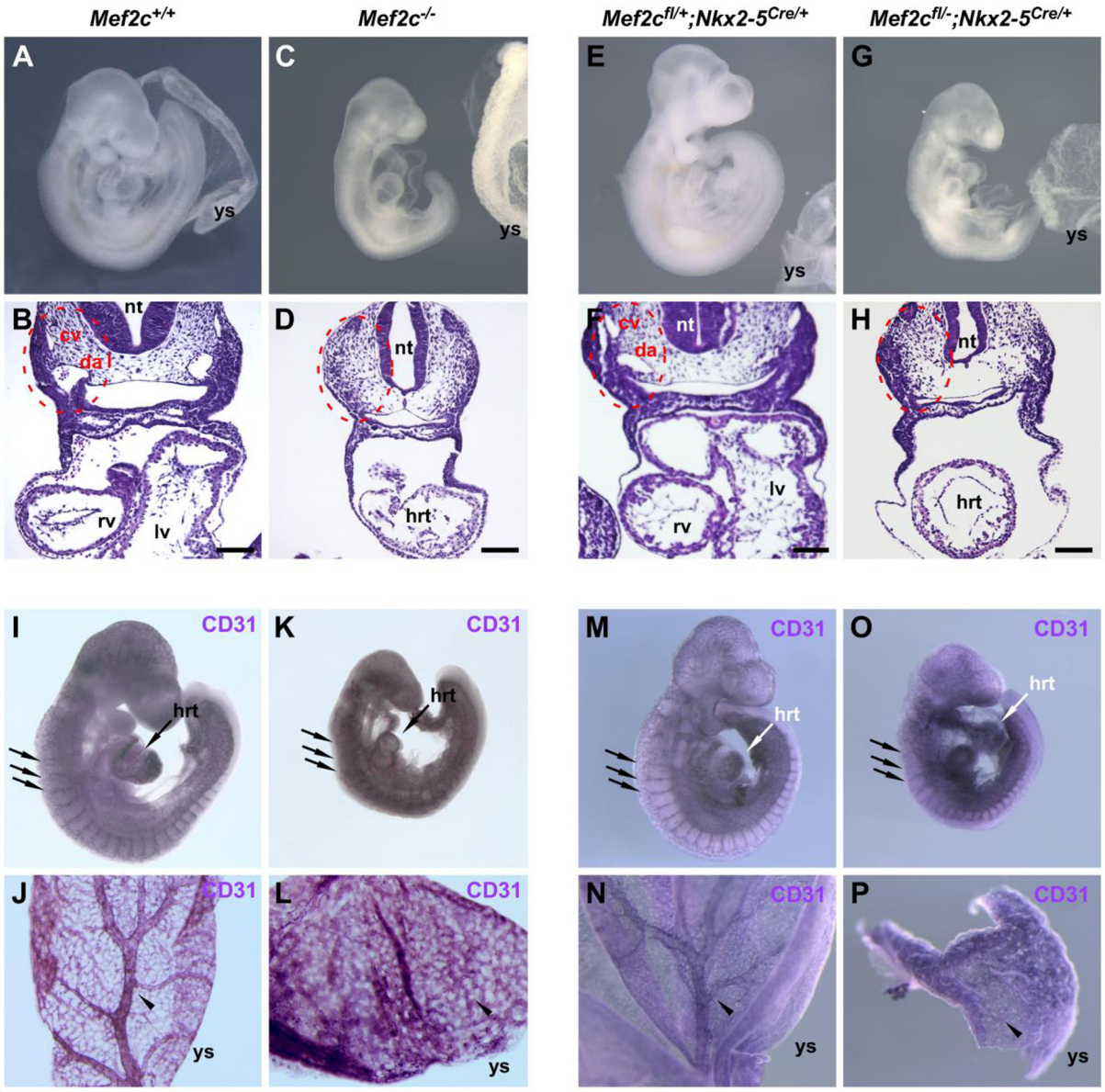

Fig. 1.

Cardiac-specific deletion of *Mef2c*, using *Nkx2–5*$^{Cre/+}$, recapitulates the global *Mef2c* knockout phenotype. Embryos were harvested at embryonic day 9.5, inspected for gross appearance (A, C, E, G) or sectioned and hematoxylin and eosin (H&E) stained (B, D, F, H). Embryos with global or cardiacspecific loss of *Mef2c* function (C, G) were noticeably smaller than control littermates (A, E). The main blood vessels in the trunk were present in cross sections of control embryos (red dashed circles in B, F), but absent in global or conditional knockouts (red dashed circles in D, H). Immunohistochemical staining with anti-CD31 antibody in whole mount embryos (I, K, M, O) or yolk sacs (J, L, N, P) shows that the blood vasculature initially forms in all embryos (unlabeled arrows in I, K, M, O indicate intersomitic vessels), but fails to remodel in global and *Nkx2–5*^{Cre} cardiac-specific knockout embryos. Yolk sacs of control embryos show arborization (arrowheads in J, N), but it remained as an unremodeled plexus in global and *Nkx2–5*^{Cre} conditional knockouts

(arrowheads in L, P). cv, cardinal vein; da, dorsal aorta; hrt, heart; lv, left ventricle; nt, neural tube; rv, right ventricle; ys, yolk sac. Scale bars, 100 μm.

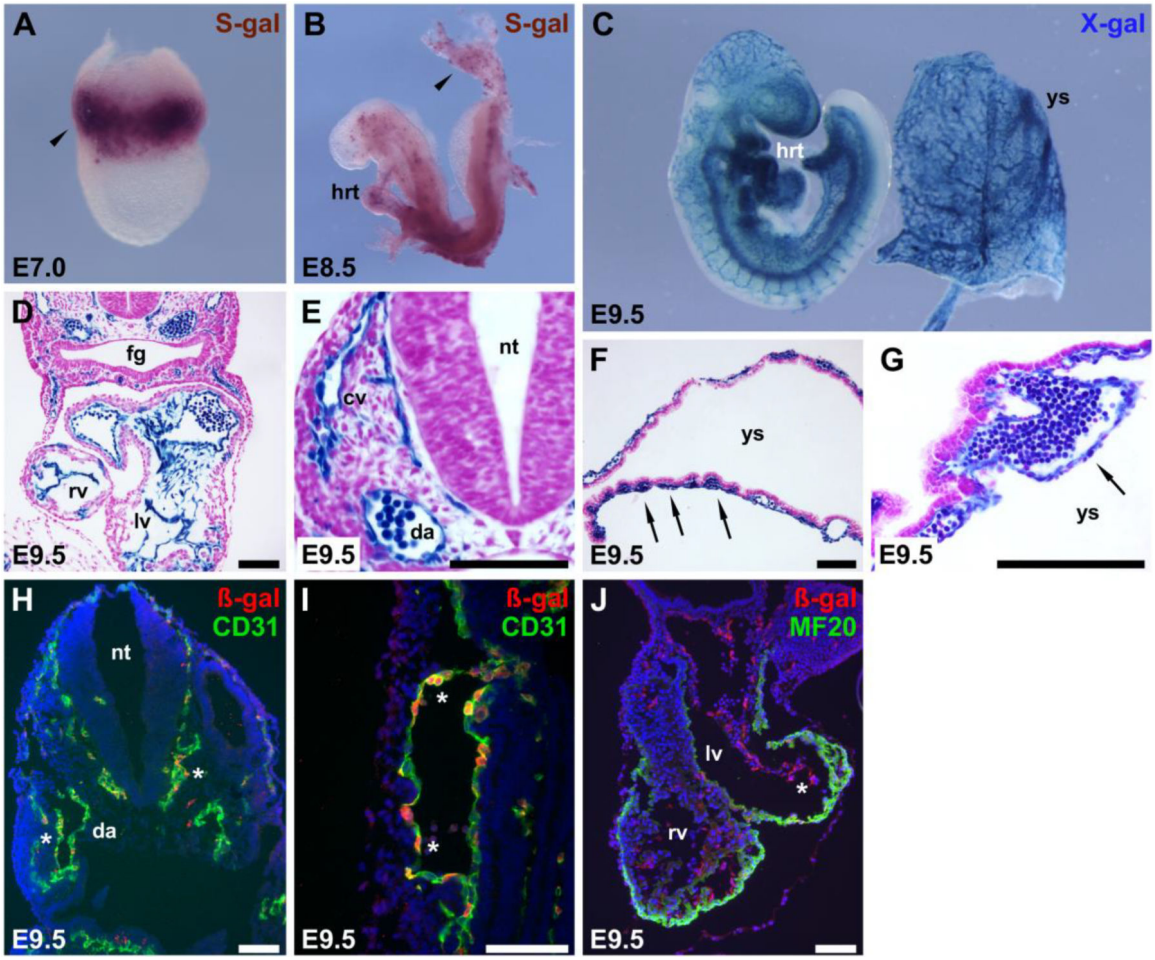

Fig. 2.

Genetic fate mapping of *Etv2::Cre* shows that Cre activity is restricted to hematopoietic and endothelial cells and/or their precursors. *Etv2::Cre*$^{Tg/0}$ mice were crossed to *Rosa26*$^{lacZ/lacZ}$ mice, and embryos were collected at E7.0 (A), E8.5 (B), or E 9.5 (C–G), and stained with either Salmon-gal (S-gal) or X-gal to detect ß-galactosidase activity from the *Rosa26*$^{lacZ/lacZ}$ reporter. Embryos and yolk sacs were analyzed either as whole mounts (A–C) or sectioned and counterstained with neutral fast red (D– G). Arrowheads in (A, B) indicate staining in extra-embryonic blood and blood vessel-forming regions. At E9.5, the reporter was active exclusively in endocardium (D), vascular endothelial cells of the embryo and yolk sac (arrows in F, G), and blood cells contained within patent vessels. (H–J) ßgalactosidase expression (red) overlapped with CD31 expression (green) in vascular endothelium (asterisks in H, I). (J) MF20 antibody detects myosin heavy chain (Myosin 4), expressed in cardiomyocytes (red) and did not colocalize with endocardial reporter expression (green). Asterisk in (J) marks *Etv2::Cre* ß-gal activity in the endocardium. cv, cardinal vein; da, dorsal aorta; ec, endocardium; fg, foregut; hrt, heart; lv, left ventricle; nt, neural tube; rv, right ventricle; ys, yolk sac. Scale bars, 100 μm.

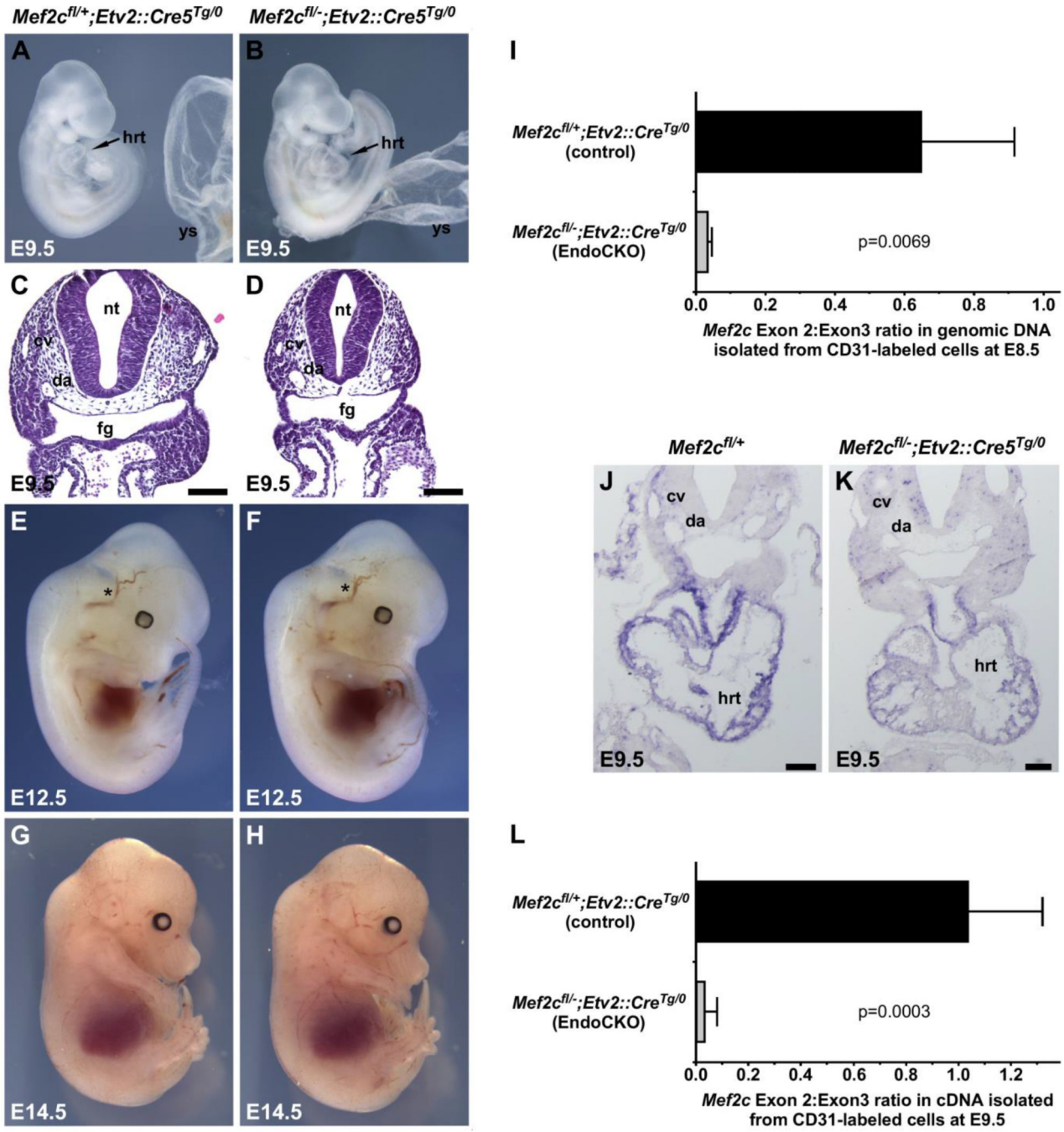

Fig. 3.

Complete endothelium-specific deletion of *Mef2c* prior to its expression in the vasculature does not impair formation of the cardiovascular system. (A-H, J, K) Wild type or *Etv2::Cre* endothelial-specific *Mef2c* knockout embryos were harvested at E9.5 (A–D, J, K), E12.5 (E, F), or E14.5 (G, H) and examined for gross appearance or sectioned and H&E stained (C, D). In all cases and at all time points *Etv2::Cre* endothelial-specific *Mef2c* knockout embryos were indistinguishable from control littermates. Asterisks mark the carotid artery at E12.5 (E, F). (I) Quantification of exons 2 and 3 in genomic DNA by quantitative PCR

revealed efficient excision of exon 2 in endothelial cells by E8.5 in *Mef2c*$^{flox/-}$; *Etv2*::*Cre*$^{Tg/0}$ conditional knockout embryos. P-values were calculated by Student's t-test; p=0.0069; n=5. (J, K) *In situ* hybridization for exon 2 in the *Mef2c* transcript shows loss of detectable *Mef2c* transcript in the vasculature at E9.5 in *Etv2::Cre* endothelial-specific *Mef2c* knockout embryos; transcripts were still clearly evident in the heart. (L) Exon 2-containing *Mef2c* transcripts were reduced by 97% at E9.5 in *Etv2::Cre* endothelial-specific *Mef2c* knockout embryos compared to controls. Pvalues were calculated by Student's t-test; p=0.0003; n=6. cv, cardinal vein; da, dorsal aorta; fg, foregut; hrt, heart; nt, neural tube; ys, yolk sac. Scale bars, 100 μm.

Table 1.

Genotype frequencies of progeny from crosses to generate (A) cardiac or (B) endothelial-specific *Mef2c* knockout mice. Expected and observed progeny of each genotype from (A) cardiac *Nkx2–5*$^{Cre/+}$; *Mef2c*$^{+/-}$ × *Mef2c*$^{flox/flox}$ crosses, collected at E14.5; or (B) *Etv2::Cre*$^{Tg/0}$; *Mef2c*$^{+/-}$ × *Mef2c*$^{flox/flox}$ crosses, collected at P10. CKO, conditional knockout; df, degrees of freedom in χ^2 test.

A	Genotype	% Expected	# Observed
	Mef2c$^{flox/+}$ (wt)	25	8
	Mef2c$^{flox/+}$; *Nkx2–5*$^{Cre/+}$ (het)	25	13
	Mef2c$^{flox/-}$	25	9
	Mef2c$^{flox/-}$;*Nkx2–5*$^{Cre/+}$ (CKO)	25	0
	χ^2=11.867, 3 df; P=0.0079		
B	Genotype	% Expected	# Observed
	Mef2c$^{flox/+}$ (wt)	25	23
	Mef2c$^{flox/+}$;*Etv2::Cre*$^{Tg/0}$ (het)	25	18
	Mef2c$^{flox/-}$	25	14
	Mef2c$^{flox/-}$;*Etv2::Cre*$^{Tg/0}$ (CKO)	25	16
	χ^2=2.521, 3 df; P=0.4715 (not signif.)		