

# Endothelial $\alpha 5$ and $\alpha v$ integrins cooperate in remodeling of the vasculature during development

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## SUMMARY

Integrin cell adhesion receptors and fibronectin, one of their extracellular matrix ligands, have been demonstrated to be important for angiogenesis using functional perturbation studies and complete knockout mouse models. Here, we report on the roles of the  $\alpha 5$  and  $\alpha v$  integrins, which are the major endothelial fibronectin receptors, in developmental angiogenesis. We generated an integrin  $\alpha 5$ -floxed mouse line and ablated  $\alpha 5$  integrin in endothelial cells. Unexpectedly, endothelial-specific knockout of integrin  $\alpha 5$  has no obvious effect on developmental angiogenesis. We provide evidence for genetic interaction between mutations in integrin  $\alpha 5$  and  $\alpha v$  and for overlapping functions and compensation between these integrins and perhaps others. Nonetheless, in embryos lacking both  $\alpha 5$  and  $\alpha v$  integrins in their endothelial cells, initial vasculogenesis and angiogenesis proceed normally, at least up to E11.5, including the formation of apparently normal embryonic vasculature and development of the branchial arches. However, in the absence of endothelial  $\alpha 5$  and  $\alpha v$  integrins, but not of either alone, there are extensive defects in remodeling of the great vessels and heart resulting in death at  $\sim$ E14.5. We also found that fibronectin assembly is somewhat affected in integrin  $\alpha 5$  knockout endothelial cells and markedly reduced in integrin  $\alpha 5/\alpha v$  double-knockout endothelial cell lines. Therefore, neither  $\alpha 5$  nor  $\alpha v$  integrins are required in endothelial cells for initial vasculogenesis and angiogenesis, although they are required for remodeling of the heart and great vessels. These integrins on other cells, and/or other integrins on endothelial cells, might contribute to fibronectin assembly and vascular development.

**KEY WORDS:** Aortic arch remodeling defect, Compensation, Fibronectin, Integrin, Mouse, Tie2-Cre

## INTRODUCTION

Vascular and congenital heart diseases are major causes of lethality (Bruneau, 2008) and much effort has been directed towards developing anti-angiogenic drugs (Ferrara and Kerbel, 2005). Therefore, there is considerable interest in understanding the roles of molecules that drive and control vascular development.

One important participant in developmental and pathological angiogenesis is fibronectin (Astrof and Hynes, 2009), a secreted glycoprotein that assembles into a fibrillar extracellular matrix. Fibronectin-null mice show lethality at embryonic day (E) 9.5 and have severe defects in the development of vasculature and somites (Francis et al., 2002; George et al., 1997; George et al., 1993). Integrins are heterodimeric cell adhesion receptors that mediate cell-matrix and cell-cell interactions (Hynes, 2002b; van der Flier and Sonnenberg, 2001).  $\alpha 5\beta 1$  integrin binds to the Arg-Gly-Asp (RGD) tri-peptide motif in fibronectin. This binding site is also recognized by  $\alpha v$  integrins (i.e.  $\alpha v\beta 1$ ,  $\alpha v\beta 3$ ,  $\alpha v\beta 5$ ,  $\alpha v\beta 6$  and  $\alpha v\beta 8$ ) (Pankov and Yamada, 2002; Leiss et al., 2008).  $\alpha 5$  integrin-null embryos show a similar phenotype to the fibronectin nulls, but die 1 day later, at  $\sim$ E10.5 (Francis et al., 2002; Goh et al., 1997; Yang et al., 1993). Similarly, targeted inactivation of the RGD site in fibronectin (Fn<sup>RGE</sup>) results in placental and cardiovascular

defects and lethality at E10.5 (Takahashi et al., 2007; Takahashi et al., 2009). All these data point to a key role for the  $\alpha 5\beta 1$  integrin-fibronectin interaction in angiogenesis.

Complete knockout of all  $\beta 1$  integrins is lethal at the preimplantation stage (Fassler and Meyer, 1995; Stephens et al., 1995). Recently, several studies have shown that endothelial-specific knockout of  $\beta 1$  integrins results in severe vascular defects and lethality at E10 (Carlson et al., 2008; Lei et al., 2008; Tanjore et al., 2008; Zovein et al., 2010). This raises the question of which  $\alpha$  subunit is involved in developmental angiogenesis. Deletion of  $\alpha 4$  integrins is embryonic lethal (E10/11.5), with placental and cardiac defects (Yang et al., 1995). However,  $\alpha 4$  endothelial-specific knockout mice are viable, with hematopoietic defects but no vascular phenotype (Priestley et al., 2007).  $\alpha 9$  integrin-null mice have lymphatic defects and die of chylothorax soon after birth (Bazigou et al., 2009; Huang et al., 2000b), but lack other major vascular defects. No major vascular developmental defects have been reported for knockouts of the other ten  $\alpha$  integrin subunits, including those of the laminin- and collagen-binding families.

The complete ablation of all five  $\alpha v$  integrins does not block angiogenesis, but 80% of the embryos die at E11.5 due to placental defects and the remaining embryos succumb at birth with brain hemorrhage and cleft palate (Bader et al., 1998). The brain hemorrhage has been linked to defective activation of TGF $\beta$  by  $\alpha v\beta 8$  on glial cells supporting the brain vasculature (Cambier et al., 2005; McCarty et al., 2005; McCarty et al., 2002; Proctor et al., 2005). Although in vitro blocking experiments have suggested that  $\alpha v\beta 3$  and  $\alpha v\beta 5$  integrins promote angiogenesis,  $\beta 3/\beta 5$ -null mice are viable and show increased pathological angiogenesis (Hodivala-Dilke et al., 1999; Huang et al., 2000a; Reynolds et al., 2002). Furthermore, Tie2-Cre-mediated deletion of  $\alpha v$  integrins in endothelial cells does not

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result in developmental vascular defects (McCarty et al., 2005), but instead leads to colitis through loss of  $\alpha_v$  integrins in hematopoietic cells causing defects in the activation of TGF $\beta$  (Lacy-Hulbert et al., 2007; Travis et al., 2007). These results suggest that  $\alpha_v$  integrins, although not essential, have some involvement in vascular development, but their exact roles remain uncertain.

To test the roles of endothelial  $\alpha_5\beta_1$  integrin in developmental angiogenesis we generated an integrin  $\alpha_5$ -floxed mouse line and ablated  $\alpha_5$  in endothelial cells using *Tie2*-driven expression of Cre recombinase. Surprisingly,  $\alpha_5$  conditional knockout mice were viable and lacked any immediately obvious phenotype. Characterization of  $\alpha_5$  knockout endothelial cells suggested compensation by  $\alpha_v$  integrins, which we tested by generating endothelium-specific  $\alpha_5$ ;  $\alpha_v$  double-knockout mice. Interestingly, these mice form normal vasculature initially but most die at mid-gestation due to vascular remodeling defects. These results shed further light on the complex interplay among integrins in controlling vascular development.

## MATERIALS AND METHODS

### Generation of $\alpha_5$ integrin-floxed mice

A conditional  $\alpha_5$  integrin targeting vector (see Fig. S1B in the supplementary material) contained a thymidine kinase (TK) negative-selection cassette, an Frt-flanked PGK-neo cassette and the 255 bp exon 1 of  $\alpha_5$  integrin flanked by loxP sites. R1 embryonic stem (ES) cells were electroporated, selected and screened for correct recombination and single integration. The PGK-neo cassette was removed by transient expression of Flip recombinase. Two karyotyped, correctly targeted ES cell clones (2H2 and 3G3) gave germline transmission and identical results. Cre-mediated excision of exon 1 was confirmed by PCR genotyping and Southern blotting (see Fig. S1B,C in the supplementary material).

### Mouse strains

All mouse lines used were on a 129S4:C57BL/6 mixed background. *Tie2-Cre<sup>Tg</sup>* (*Tek-Cre*) (Kisanuki et al., 2001) (Jackson Laboratories) mice were crossed with  $\alpha_5^{+/-}$  mice (Yang et al., 1993).  $\alpha_5$ -cKO crosses were set up as follows:  $\alpha_5^{\text{floxed/floxed}} \times \alpha_5^{+/-}$ ; *Tie2-Cre* or  $\alpha_5^{\text{floxed/floxed}} \times \alpha_5^{\text{floxed/+}}$ ; *Tie2-Cre*.  $\alpha_5/\alpha_v$  double-floxed mice, i.e.  $\alpha_5^{\text{floxed/floxed}}$ ,  $\alpha_v^{\text{floxed/floxed}}$ ; *R26R<sup>lacZ/lacZ</sup>* and  $\alpha_5^{\text{floxed/floxed}}$ ;  $\alpha_v^{\text{floxed/floxed}}$ ; Immorto<sup>Tg</sup> mice, were generated by intercrossing  $\alpha_5^{\text{floxed/floxed}}$  mice with  $\alpha_v^{\text{floxed}}$  mice (Lacy-Hulbert et al., 2007) and *R26-lox-STOP-lox-lacZ* reporter (*R26R-lacZ*) mice (Soriano, 1999) (Jackson Laboratories) or Immorto mice (Jat et al., 1991) (Charles River Laboratories).  $\alpha_5$ ;  $\alpha_v$  conditional double-knockout crosses ( $\alpha_5/\alpha_v$ -cdKO) were generally made by crossing  $\alpha_5^{\text{floxed/floxed}}$ ;  $\alpha_v^{\text{floxed/floxed}}$ ; *R26R-lacZ* mice to  $\alpha_5^{+/-}$ ;  $\alpha_v^{+/-}$ ; *Tie2-Cre<sup>Tg/Tg</sup>* mice.  $\alpha_v^{+/-}$  mice have been reported previously (Bader et al., 1998). Genotyping was performed in-house (see Fig. S1D in the supplementary material) or by Transnetyx.

### Histology, whole-mount staining and immunohistochemistry

E14.5 embryos were fixed in Bouin's (Sigma), paraffin embedded, serially sectioned and analyzed after Hematoxylin and Eosin (H&E) staining. Whole-mount ears or trachea were fixed overnight in 4% glutaraldehyde/PBS; embryos were fixed overnight in methanol:DMSO (4:1). Tissue was blocked with wash buffer (PBS/0.5% Tween 20) containing 5% normal goat serum. Antibody incubations were in 1:1 PBS-diluted blocking buffer overnight at 4°C, followed by six 1-hour washes. Tissues were embedded in Fluoromount-G (Southern-Biotech).

Tissues for sectioning were either cryofixed in Tissue-Tek OCT (Sakura Finetek) or tissues/embryos were fixed in IHC Zinc Fixative (BD-Pharmingen) and paraffin embedded. Cryosections were postfixed for 10 minutes in acetone at -20°C. Sections were blocked with PBS containing 5% normal goat serum (depleted of fibronectin by gelatin-Sepharose for fibronectin staining). Primary antibody incubations were overnight at 4°C. Secondary antibody incubations were 1 hour at room temperature. Antibodies were: from Millipore, integrin  $\alpha_4$  (9C10),  $\alpha_5$  (5H10-27),  $\alpha_v$  (AB1930),  $\beta_1$  (MB2.1) and PECAM1 (CD31) (390<sup>IHC</sup>); from Sigma, smooth-muscle actin-Cy3 (1A4), vinculin (hVin-1); from Abcam, LYVE1

(Ab14917) and pericentrin (Ab4448). Secondary goat anti-mouse, anti-rat and anti-rabbit antibodies conjugated with Alexa 488, Alexa 594 or Alexa 647 were from Invitrogen and goat anti-rat-HRP from Jackson ImmunoResearch. Staining of whole mouse embryos for  $\beta$ -galactosidase (*lacZ*) activity followed a standard protocol (Nagy, 2003).

### FACS analysis

E10.5 yolk sacs or embryos were minced and incubated for 10 (yolk sac) or 40 (embryos) minutes at 37°C in Dulbecco's Modified Eagle's Medium containing 0.1% collagenase type I (Worthington) and 12 U/ml DNase. Subsequently, cells were incubated for 10 minutes in 1 $\times$  Versene (Invitrogen) and strained through a 70- $\mu$ m nylon mesh (Falcon). Endothelial cell lines (see below) were detached using sequential collagenase and Versene treatments. Antibody incubations were for 30 minutes at 4°C in PBS containing 2 mM EDTA and 0.5% BSA, using antibodies conjugated to either FITC, PE, APC or biotin, followed by conjugated streptavidin (BD-Pharmingen). Antibodies were obtained from BD-Pharmingen: integrin  $\alpha_4$  (R1-2),  $\alpha_5$  (5H10-27),  $\alpha_v$  (RMV-7), CD34 (RAM34); ICAM2 (CD102) (2C4), PECAM1 (CD31) (390), VE-cadherin (CD144) (11D4.1). Analysis was on a FACSCalibur high-throughput sampler (BD Biosciences) and data were analyzed using FlowJo software (Treestar).

### Isolation of endothelial cell lines and cell culture

Endothelial cells were isolated from mice carrying the Immorto gene (Jat et al., 1991) with a 1-hour collagenase treatment.  $\alpha_5$ -KO and control lung (mLEC) or brain endothelial (mBEC) cells were isolated from adult  $\alpha_5^{\text{floxed/-}}$ ; *Tie2-Cre* or  $\alpha_5^{\text{floxed/+}}$ ; *Tie2-Cre* Immorto mice. Cells were grown to subconfluency on coated plates (see below) and immune cells were negatively selected with anti-CD18 (BD-Pharmingen, C71/16) followed by positive selection for endothelial cells with conjugated anti-ICAM2 antibodies using MACS beads (Miltenyi Biotec). After expansion, several mLEC preparations were selected as PECAM1<sup>+</sup>. Eventually, all endothelial cell lines were subcloned by FACS sorting for ICAM2<sup>+</sup> cells followed by limited dilution cloning.  $\alpha_5/\alpha_v$  double-floxed mLEC clones ( $\alpha_5^{\text{floxed/floxed}}$ ;  $\alpha_v^{\text{floxed/floxed}}$ ), derived from adult lungs, were incubated with AdCre (Gene Transfer Vector Core, University of Iowa, USA) to excise the  $\alpha_5$  and  $\alpha_v$  genes, and the  $\alpha_5/\alpha_v$ -dKO cells were isolated by FACS sorting for ICAM2<sup>+</sup> and  $\alpha_5^-$   $\alpha_v^-$  cells followed by limited dilution cloning. Embryonic endothelial cells (eECs) were isolated from the heads and tails of E13.5 embryos.  $\alpha_5$ -KO and control cell lines (mLEC and mBEC) were grown on 0.1% gelatin-coated plates. The eECs,  $\alpha_5^{\text{floxed/floxed}}$ ;  $\alpha_v^{\text{floxed/floxed}}$  control and their AdCre-derived  $\alpha_5/\alpha_v$ -dKO mLECs were grown on plates coated with 20  $\mu$ g/ml Matrigel basement membrane matrix (BD Biosciences).

Cells were maintained at 33°C in low-glucose DME/Ham's-F12 (1:1), 20% normal bovine serum, 50  $\mu$ g/ml endothelial mitogen (Biomedical Technologies, MA, USA) and 20 U/ml mouse interferon- $\gamma$  (Millipore). For experiments, cells were transferred to a 37°C incubator and depleted of interferon- $\gamma$ . Endothelial cells were reconstituted by retroviral expression of human  $\alpha_5$  integrin subcloned into LZRS-ms-IRES-zeo (Taverna et al., 1998; van der Flier et al., 2002).

### Immunofluorescent staining of cells

Cells were grown overnight on coated glass coverslips: mLECs and mBECs were plated on 10  $\mu$ g/ml fibronectin (BD Biosciences), whereas eECs were plated on a mix of 20  $\mu$ g/ml Matrigel and 10  $\mu$ g/ml human fibronectin. Cells were fixed for 10 minutes in 4% paraformaldehyde/PBS (or for 10 minutes in methanol at -20°C for  $\alpha_v$  integrin), washed and permeabilized for 10 minutes at room temperature with PBS containing 0.2% Triton X-100. Cells were blocked and incubated overnight at 4°C with primary antibody in PBS/2% BSA. Sections were incubated for 1 hour at room temperature with secondary antibodies and embedded in Vectashield mounting medium with DAPI (Vector Laboratories).

### Fibronectin binding and assembly assays

Ninety-six-well tissue culture plates were coated with the indicated concentrations of fibronectin, washed and blocked with 5% BSA, and 20,000 endothelial cells/well were allowed to adhere for 2 hours in DMEM/0.2% BSA at 37°C. Plates were washed three times; adherent cells were fixed with 4% formaldehyde and stained with 0.1% Crystal Violet.

After washes and permeabilization in 50  $\mu$ l PBS/0.2% Triton X-100, the OD<sub>540</sub> was measured in a plate reader. Two to three independent experiments were performed in triplicate.

Endothelial cells were seeded on Matrigel-coated or gelatin-coated 6-well plates (400,000 cells/well) in fibronectin-depleted medium. For incorporation of exogenous fibronectin, after culture overnight the medium was changed to fibronectin-depleted medium containing 10  $\mu$ g/ml exogenous biotinylated human fibronectin. At the times indicated, medium was collected, cells were washed with PBS containing 1 mM Ca<sup>2+</sup> and Mg<sup>2+</sup> and solubilized in 0.5 ml DOC buffer [2 mM EDTA, 1% sodium deoxycholate, 20 mM Tris pH 8.5, Complete Mini-protease Inhibitors (Roche)]. After passing eight times through a 22G needle, DOC-insoluble material was spun down for 20 minutes at 20,000 *g* at 4°C and solubilized in 120  $\mu$ l 2 $\times$  reducing SDS-PAGE loading buffer. Reduced (100 mM DDT) samples were loaded on 4–12% gels. For fibronectin immunostaining, 15,000 cells/well were plated on coated 8-well Lab Tek Permanox coverslips (Nunc). Treatments and immunostainings were as described above.

### Immunoblotting

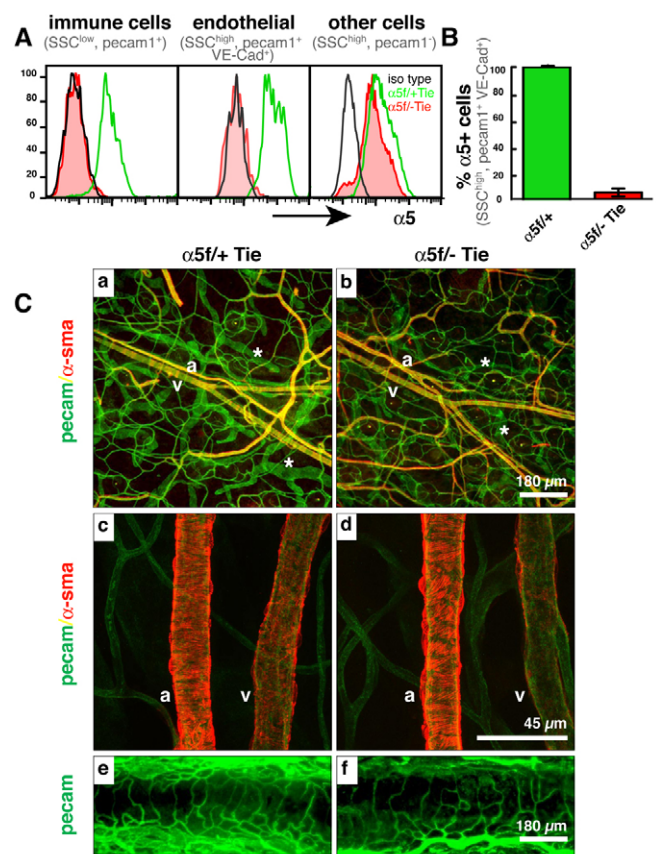
Novex Tris-glycine precast gels (Invitrogen) were used and wet-transferred to nitrocellulose. Where indicated, samples were treated with PNGaseF (500 U/sample, New England Biolabs). Blots were blocked and incubated with antibodies in 5% non-fat dried milk, 0.2% NP40, Tris-buffered saline (pH 8). Primary antibodies were integrin  $\alpha$ 5 (AB1928),  $\alpha$ v (AB1930) and GAPDH (MAB374) (all from Millipore), vimentin (Sigma), and rabbit anti-fibronectin (297.1; generated in our laboratory). HRP-conjugated secondary antibodies were from Jackson ImmunoResearch: goat anti-rabbit and sheep anti-mouse IgM and HRP-streptavidin. Blots were developed using Western-Lighting ECL (PerkinElmer)

## RESULTS

### Mice lacking endothelial $\alpha$ 5 integrin exhibit no obvious vascular phenotypes

Since complete integrin  $\alpha$ 5 knockout ( $\alpha$ 5-KO) mice die at ~E11, preventing functional studies at later stages, we developed mouse lines with a floxed  $\alpha$ 5 integrin gene (see Fig. S1B–D in the supplementary material). To study the function of  $\alpha$ 5 integrin in angiogenesis we crossed  $\alpha$ 5<sup>fllox/fllox</sup> mice to those bearing a *Tie2-Cre* transgene, which is expressed in both endothelial and hematopoietic cells (Koni et al., 2001; Srinivasan et al., 2007). PCR genotyping and Southern blotting confirmed Cre-mediated excision of the floxed  $\alpha$ 5 allele (see Fig. S1C,D in the supplementary material). We obtained several mice with a maternal germline-excised  $\alpha$ 5-null allele, as *Tie2-Cre* is frequently expressed in the female germline and occasionally the male germline (de Lange et al., 2008; Koni et al., 2001). Intercrosses of those  $\alpha$ 5<sup>+/-</sup> mice confirmed the previously reported  $\alpha$ 5-KO phenotype (Yang et al., 1993).  $\alpha$ 5-null embryos at E10.5 (*n*=8) were severely growth retarded, posterior somites were incompletely formed and embryos showed reduced vascularization (data not shown). To exclude potential complications arising from germline-mediated excision of the  $\alpha$ 5<sup>fllox</sup> allele in subsequent experiments, we intercrossed mice carrying the *Tie2-Cre* transgene and an  $\alpha$ 5-null allele with  $\alpha$ 5<sup>fllox/fllox</sup> mice.

Unexpectedly, we obtained normal Mendelian ratios of offspring from such  $\alpha$ 5<sup>fllox/fllox</sup>  $\times$   $\alpha$ 5<sup>+/-</sup>; *Tie2-Cre*<sup>Tg/+</sup> crosses ( $\chi^2$  test, *P*>0.3; see Table S1 in the supplementary material). FACS analysis for  $\alpha$ 5 integrin expression in cell populations of freshly isolated samples from E10.5 embryos (Fig. 1A,B) showed complete loss of  $\alpha$ 5 integrin protein in both hematopoietic and endothelial cells derived from the yolk sacs or from the integrin  $\alpha$ 5 conditional knockout ( $\alpha$ 5-cKO) embryos themselves. This confirmed that  $\alpha$ 5 integrin was indeed efficiently depleted early during embryonic development.



**Fig. 1. Tie2-Cre-mediated depletion of  $\alpha$ 5 integrin in endothelial cells results in no obvious vascular phenotypes.** (A) FACS analysis of cells dissociated from E10.5 mouse yolk sacs, gated on immune cells (SSC<sup>low</sup>, PECAM1<sup>+</sup>) and endothelial cells (SSC<sup>high</sup>, PECAM1<sup>+</sup>, VE-Cad<sup>+</sup>). Black line, isotype control antibody; green,  $\alpha$ 5 integrin on cells from  $\alpha$ 5<sup>fllox/+</sup>; *Tie2-Cre* embryos; red,  $\alpha$ 5 integrin on cells from  $\alpha$ 5<sup>fllox/-</sup>; *Tie2-Cre* embryos. Note the complete loss of  $\alpha$ 5 integrin from endothelial and hematopoietic cells but not from control 'other' cells (PECAM1<sup>-</sup>). For details of FACS analysis, see Figs S2 and S4 in the supplementary material. (B) Bar chart indicating loss of  $\alpha$ 5 integrin expression on greater than 95% of endothelial cells (SSC<sup>high</sup>, PECAM1<sup>+</sup> VE-Cad<sup>+</sup>) isolated from E10.5  $\alpha$ 5<sup>fllox/-</sup>; *Tie2-Cre* embryos. (C) Apparently normal vasculature in integrin  $\alpha$ 5 conditional knockout ( $\alpha$ 5-cKO) mice ( $\alpha$ 5f<sup>-</sup>Tie versus  $\alpha$ 5f<sup>+</sup>Tie control) as seen by whole-mount staining for endothelial (PECAM1) and smooth muscle ( $\alpha$ -SMA) cells in (a–d) ear skin and (e,f) trachea. Arteries (a), veins (v) and lymphatics (asterisk) are indicated. Scale bars: 180  $\mu$ m in a,b,e,f; 45  $\mu$ m in c,d.

No obvious vascular defects were visualized by whole-mount staining for platelet endothelial adhesion molecule [PECAM1 (CD31)] and smooth muscle  $\alpha$ -actin ( $\alpha$ -SMA; ACTA2) of vasculature of the inner-ear skin or trachea of adult  $\alpha$ 5-cKO mice (Fig. 1C). Similarly, immunohistochemical staining of sections from various other vascular beds showed no clear differences from controls (data not shown).

The  $\alpha$ 5<sup>fllox/-</sup>; *Tie2-Cre* ( $\alpha$ 5-cKO) mice did not develop any obvious spontaneous vascular or hematopoietic phenotypes for a period of 18 months. In addition, FACS analysis of the immune system showed no disparities in subsets of hematopoietic cells in adult  $\alpha$ 5-cKO mice (data not shown). These unexpected results suggested that deletion of  $\alpha$ 5 integrin from endothelial cells does not replicate the global  $\alpha$ 5-null phenotype. Together, these data

show that early developmental deletion of  $\alpha 5$  integrin in endothelial cells has no obvious effects on developmental vasculogenesis or angiogenesis.

### Integrin $\alpha 5$ -deficient endothelial cells adhere to fibronectin in vitro but redistribute integrin $\alpha v$ to focal adhesions

We isolated immortalized endothelial cell lines from adult lungs (mLEC) and brains (mBEC) using  $\alpha 5$ -cKO crosses into which a conditional SV40 large T antigen was introduced (Jat et al., 1991). FACS analysis and immunofluorescence staining showed that the clones were positive for ICAM2 and often PECAM1 and VE-cadherin (cadherin 5) (data not shown). FACS analysis and immunoblots confirmed complete loss of  $\alpha 5$  integrin in these endothelial cell lines.

$\alpha 5$ -KO endothelial cells showed reduced adhesion to fibronectin, which was rescued by ectopic (over)expression of human  $\alpha 5$  integrin (see Fig. S2 in the supplementary material). Extended adhesion times or increased fibronectin coating abolished this difference in adhesion between  $\alpha 5$ -KO and control cells (see Fig. S2C in the supplementary material). Immunofluorescence staining for vinculin of  $\alpha 5$ -KO endothelial cell cultures (mBECs, mLECs) plated on fibronectin consistently showed fewer, but larger and more peripherally localized, focal adhesions (see Fig. S3 in the supplementary material). We also observed relocalization of  $\alpha v$  integrins from a diffuse surface expression in control cells to a focal adhesion localization in  $\alpha 5$ -KO cells (see Fig. S3 in the supplementary material). FACS analysis revealed no, or minor, upregulation of  $\alpha v$  (see Fig. S2B in the supplementary material) or  $\beta 3$  (not shown) integrin in  $\alpha 5$ -KO endothelial cells. The relocalization and modest surface upregulation of  $\alpha v$  integrins in  $\alpha 5$ -KO endothelial cells suggested that  $\alpha v$  integrin heterodimers (i.e.  $\alpha v\beta 1$ ,  $\beta 3$ ,  $\beta 5$ ,  $\beta 6$  or  $\beta 8$ ) might be functionally compensating for loss of  $\alpha 5$  integrins in endothelial cells. To explore this hypothesis, we knocked out both  $\alpha 5$  and  $\alpha v$  integrins in endothelial cells using *Tie2-Cre* ( $\alpha 5/\alpha v$ -dcKO).

### Mice lacking both $\alpha 5$ and $\alpha v$ integrins in endothelium die at ~E14.5 and have organizational defects in their great vessels

We crossed doubly homozygous integrin  $\alpha 5$ ;  $\alpha v$  floxed mice carrying the *R26R-lacZ* reporter gene ( $\alpha 5^{flox/flox}$ ;  $\alpha v^{flox/flox}$ ; *R26R<sup>lacZ/lacZ</sup>*) to doubly heterozygous  $\alpha 5$  and  $\alpha v$  integrin-null mice

carrying the *Tie2-Cre* allele ( $\alpha 5^{+/-}$ ;  $\alpha v^{+/-}$ ; *Tie2-Cre<sup>Tg/Tg</sup>*). Whenever possible, we used mice homozygous for the *Tie2-Cre* transgene to increase the numbers of offspring carrying a *Tie2-Cre* allele. These crosses generate four potentially informative genotypes:  $\alpha 5/\alpha v$  conditional doubly hemizygous control mice ( $\alpha 5^{flox/+}$ ;  $\alpha v^{flox/+}$ ; *Tie2-Cre*, hereafter designated  $\alpha 5/\alpha v$ -cdHemi),  $\alpha 5$ -cKO and  $\alpha v$  conditional hemizygous ( $\alpha 5^{flox/-}$ ;  $\alpha v^{flox/+}$ ; *Tie2-Cre*, hereafter  $\alpha 5$ -cKO/ $\alpha v$ -cHemi),  $\alpha 5$  conditional hemizygous and  $\alpha v$ -cKO ( $\alpha 5^{flox/+}$ ;  $\alpha v^{flox/-}$ ; *Tie2-Cre*, hereafter  $\alpha 5$ -cHemi/ $\alpha v$ -cKO) and the  $\alpha 5/\alpha v$  conditional double knockout ( $\alpha 5^{flox/-}$ ;  $\alpha v^{flox/-}$ ; *Tie2-Cre*, hereafter  $\alpha 5/\alpha v$ -cdKO mice).

Table 1 shows that embryos lacking both  $\alpha 5$  and  $\alpha v$  integrins in endothelial cells exhibit embryonic lethality and also that fewer than half of the expected  $\alpha 5$ -cKO/ $\alpha v$ -cHemi mice survive (Table 1). Strikingly, there were no obvious vascular defects in the  $\alpha 5$ -cHemi/ $\alpha v$ -cKO mice, suggesting that hemizygosity for  $\alpha 5$  integrin is sufficient for survival in the absence of  $\alpha v$  integrins. As expected,  $\alpha 5$ -cHemi/ $\alpha v$ -cKO mice phenocopied the *Tie2-Cre*  $\alpha v$ -cKO mice and developed colitis at several months of age (Lacy-Hulbert et al., 2007). These  $\alpha 5/\alpha v$  *Tie2-Cre* crosses thus showed a genetic interaction between  $\alpha 5$  and  $\alpha v$  mutations, which could indicate overlap, compensation or convergence of the functions of these integrins. FACS analyses of E10.5 embryos confirmed efficient loss of both integrins (see Fig. S4 in the supplementary material).

Analysis of timed matings confirmed that endothelial deletion of both the  $\alpha 5$  and  $\alpha v$  integrin genes causes embryonic lethality. Most embryos died at ~E14.5 and showed severe dorsal edema and sometimes hemorrhage (Fig. 2A). Similarly, a subgroup of  $\alpha 5$ -cKO/ $\alpha v$ -cHemi embryos was embryonic lethal (Table 1). Strikingly, all the embryos, as well as their vasculature, seemed normal until at least E11.5, as determined by whole-mount staining for PECAM1 (Fig. 2Ba-d; see Fig. S5 in the supplementary material) and for  $\beta$ -galactosidase expressed from the *R26R-lacZ* reporter in  $\alpha 5/\alpha v$ -cdKO embryos (Fig. 2Be-f).

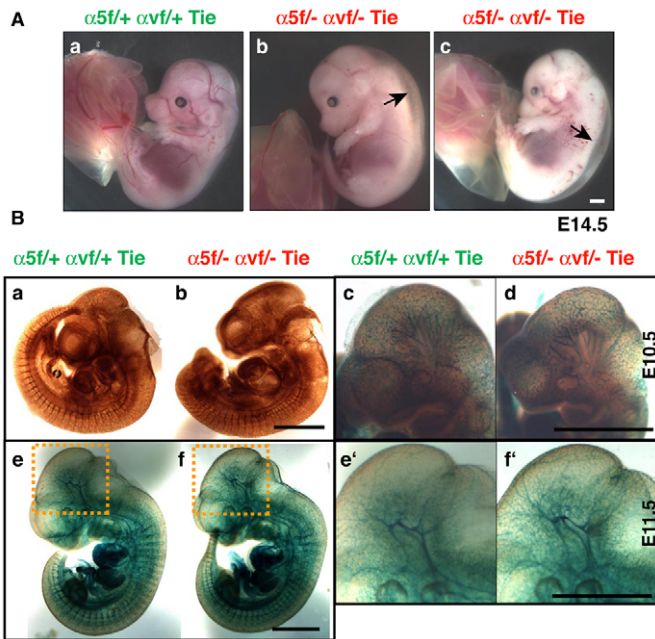
A small number of  $\alpha 5/\alpha v$ -cdKO mice survived (<4% of expected) and were found to lack both  $\alpha 5$  and  $\alpha v$  integrins on their hematopoietic cells as determined by FACS analysis (data not shown). These adult  $\alpha 5/\alpha v$ -cdKO mice appeared to have normal vasculature and lymphatics, compared with control littermates, when examined by whole-mount vascular staining of ear skin and trachea (see Fig. S6 in the supplementary material) or by sectioning (data not shown). The results on early embryos and on the rare

**Table 1. Genotypes of progeny from  $\alpha 5^{flox/flox}$ ;  $\alpha v^{flox/flox} \times \alpha 5^{+/-}$ ;  $\alpha v^{+/-}$ ; *Tie2-Cre* crosses**

Stage	Genotype			
	$\alpha 5^{flox/+}$ ; $\alpha v^{flox/+}$ ; <i>Tie2-Cre</i>	$\alpha 5^{flox/-}$ ; $\alpha v^{flox/+}$ ; <i>Tie2-Cre</i>	$\alpha 5^{flox/+}$ ; $\alpha v^{flox/-}$ ; <i>Tie2-Cre</i>	$\alpha 5^{flox/-}$ ; $\alpha v^{flox/-}$ ; <i>Tie2-Cre</i>
P21	144 (46%)	48* (15%)	115 (37%)	5* (2%)
E9.5	4	5	6	2
E10.5	39	31	31	48
E11.5	10	16	20 (0, 1)	16
E12.5	25	17	23 (0, 2)	31 (0, 1)
E13.5	9	5	6	12 (1, 0)
E14.5	21	25 (0, 5)	22 (0, 3)	31 (22, 4)
E15.5	8	10 (0, 4)	10	11 (3, 4)
E16.5	7	7 (0, 3)	2	5 (0, 5)
E17.5	2	0	2	1 (0, 1)
E18.5	8 (0, 1)	12 (0, 1)	6	8 (1, 6)

For postnatal progeny (P21), both numbers (total, 312) and percentages are given. For embryos, total numbers are listed and in parentheses are indicated first the number of embryos with edema/death and second the number of absorbed embryos. Note the onset of edema and death in integrin-deficient embryos starting at E14.5.

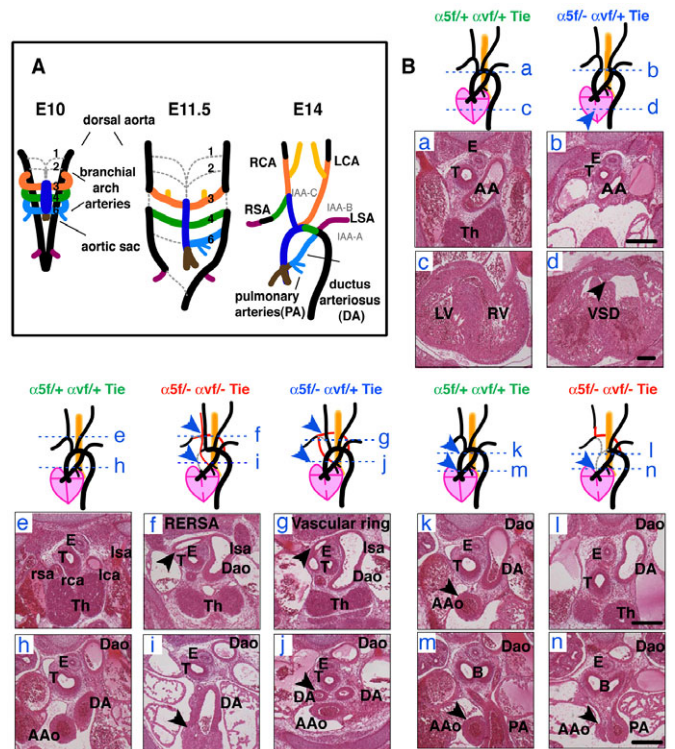
\*Significantly different from expected; Poisson regression model,  $P < 0.001$ .



**Fig. 2. Integrin  $\alpha 5/\alpha V$  endothelial conditional double-knockout ( $\alpha 5/\alpha V$ -cdKO) embryos develop severe edema at  $\sim E14.5$  but have normal vasculature at earlier stages. (Aa-c)** Most mice doubly deficient for  $\alpha 5$  and  $\alpha V$  integrins in endothelial and immune cells show edema (arrows) and hemorrhage at  $\sim E14.5$  ( $\alpha 5f^{-/-} \alpha Vf^{-/-} Tie2$  versus  $\alpha 5f^{+/+} \alpha Vf^{+/+} Tie2$  control). **(B)** Whole-mount vascular staining for **(a, b)** PECAM1 (HRP) or for **(c-f')**  $\beta$ -galactosidase (X-gal) using the *R26R-lacZ* reporter strain crossed into the experimental crosses in control conditional doubly hemizygous and conditional double-knockout embryos (as labeled) at E10.5 and E11.5. Neither method reveals any obvious vascular defects in early embryos deficient for integrins. The boxed regions in e and f are shown at higher magnification in e' and f'. Scale bars: 1 mm.

surviving  $\alpha 5/\alpha V$ -cKO mice show that it is possible to construct apparently normal vasculature in the absence of any  $\alpha 5$  or  $\alpha V$  integrins in the endothelium.

Despite normal vasculogenesis and angiogenesis, the majority of  $\alpha 5/\alpha V$ -cdKO embryos die by E14.5. This prompted us to analyze the formation of the heart and great vessels. Serial sectioning of three E14.5 litters revealed severe cardiovascular defects in  $\alpha 5/\alpha V$ -cdKO and  $\alpha 5$ -cKO/ $\alpha V$ -cHemi embryos (Fig. 3B and see Table S2 in the supplementary material). In normal embryos at this stage ( $\sim E14.5$ ), extensive branchial arch artery remodeling has occurred and the embryonic circulation is established, with the symmetrical branchial arch arteries reorganized into the aortic arch and the major arteries supplying the head and neck (carotid) and upper limbs (subclavian) (Fig. 3A). The left sixth branchial artery forms the temporary ductus arteriosus (DA) that shunts 80% of the blood from the pulmonary arteries directly into the descending aorta, bypassing the non-functional lungs in the embryo. All the  $\alpha 5/\alpha V$ -cdKO and  $\alpha 5$ -cKO/ $\alpha V$ -cHemi embryos displayed ventricular septation defects (VSDs), whereas the ventricles were separated in the control ( $\alpha 5/\alpha V$ -cdHemi) and  $\alpha 5$ -cHemi/ $\alpha V$ -cKO embryos at this stage.  $\alpha 5/\alpha V$ -cdKO embryos also frequently had aortic arch abnormalities, including a retro-oesophageal subclavian artery, vascular ring and absent ascending aorta (Fig. 3B and see Table S2 in the supplementary material). These results indicate that the endothelial  $\alpha 5$  and  $\alpha V$  integrins together play roles in the development or remodeling of the branchial arch arteries.



**Fig. 3. Cardiovascular defects in  $\alpha 5/\alpha V$ -cdKO embryos.**

**(A)** Schematic of the embryonic remodeling process of the symmetric branchial arch arteries into the aortic arch and great vessels (E14.5). Some vessels regress (gray dashed lines), while others are stabilized. **(Ba-n)** Sections and schematics illustrating the heart and great-vessel defects found in integrin conditional double-knockout (cdKO) and hemizygous (cdHemi) mice. Genotypes (in endothelial cells) are indicated and color-coded for this and subsequent figures as follows: green,  $\alpha 5^{flox+/+}; \alpha V^{flox+/+}; Tie2-Cre$  ( $\alpha 5/\alpha V$ -cdHemi); blue,  $\alpha 5^{flox-/-}; \alpha V^{flox+/+}; Tie2-Cre$  ( $\alpha 5$ -cKO;  $\alpha V$ -cHemi); red,  $\alpha 5^{flox-/-}; \alpha V^{flox-/-}; Tie2-Cre$  ( $\alpha 5/\alpha V$ -cdKO). Schematics illustrate the observed phenotypes and the blue dashed lines refer to the locations of the corresponding sections. Control hearts and great arteries (a, c, e, h, k, m); atrophic (or interrupted, not shown) aortic arch and ventricular septation defect (VSD) (b, d); interrupted aortic arch and retro-oesophageal right subclavian artery (RERSA) (f, i); vascular ring (g, j); interrupted aortic arch, loss of ascending aorta and RERSA (l, n). (a) Control aortic arch; (b) atrophic aortic arch; (c) separated left and right ventricles; (d) VSD; (e) normal position of great arteries ventral to the trachea; (f) retro-oesophageal right subclavian artery (arrowhead); (g) vascular ring around trachea (arrowhead), which results from maintenance of left sixth branchial arch artery and left dorsal aorta in A; (h) aorta in plane of ductus arteriosus (DA); (i) tiny remnant of ascending aorta (arrowhead) in plane of DA; (j) maintenance of both left and right sixth branchial arch arteries resulting in vascular ring; (k) control ascending aorta in plane of DA; (l) absence of ascending aorta in plane of DA; (m) control ascending aorta in plane of pulmonary artery; (n) remnant of ascending aorta in plane of pulmonary artery (arrowhead). AA, aortic arch; AAo, ascending aorta; B, bronchi; Dao, descending aorta; E, oesophagus; lca, left carotid artery; lsa, left subclavian artery; LV, left ventricle; rca, right carotid artery; rsa, right subclavian artery; RV, right ventricle; T, trachea; Th, thymus. Scale bars: 200  $\mu m$ .

In two  $\alpha 5/\alpha V$ -cdKO mice that survived, we checked castings of the major arteries for aortic arch abnormalities. We found a patent ductus arteriosus (PDA) in one of the two adult  $\alpha 5/\alpha V$ -cdKO mice (see Fig. S7E in the supplementary material) and PDA in several of the  $\alpha 5$ -cKO/ $\alpha V$ -cHemi mice (data not shown), all of mixed

background. This led us to check 10- to 20-week-old  $\alpha 5$ -cKO mice ( $\alpha 5^{\text{flox/flox}}$ ; *Tie2-Cre*) of which 9/10 had PDA (see Fig. S7A-D in the supplementary material). These mice were from  $\alpha 5^{\text{flox/flox}} \times \alpha 5^{\text{flox/+}}$ ; *Tie2-Cre/+* crosses on a C57BL/6 N7 background and about half of the  $\alpha 5^{\text{flox/flox}}$ ; *Tie2-Cre* ( $\alpha 5$ -cKO) from this cross seemed to be lost before weaning (see Table S3 in the supplementary material). However, in other crosses using a mixed 129S4:C57BL/6 background and  $\alpha 5^{\text{flox/+}}$ ; *Tie2-Cre* mice the DA was closed from postnatal day (P) 1 onwards, indicating a role of potential genetic background modifiers. These results indicate that endothelial  $\alpha 5$  or  $\alpha v$  integrins play roles in the development or remodeling of the vasculature.

### Integrin $\alpha 5/\alpha v$ -cdKO mice have normal branchial arch and cardiac cushion development

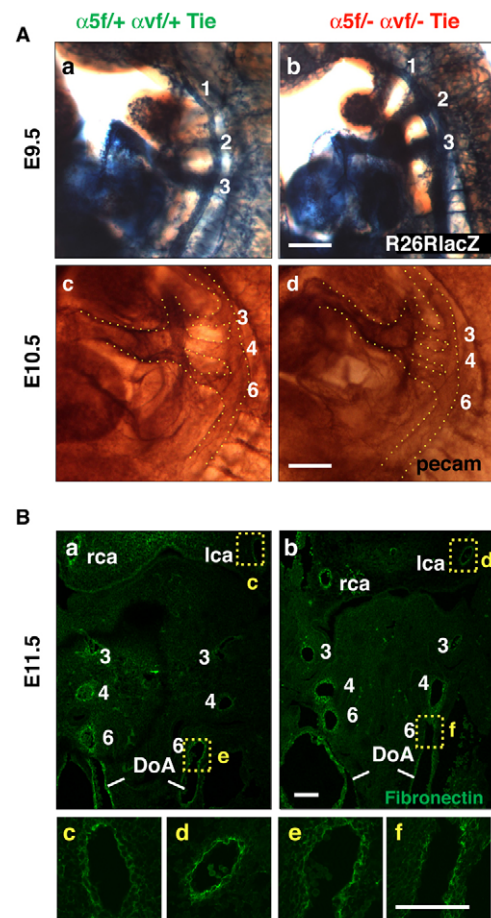
We next sought to define when and why defects arise in the great arteries of  $\alpha 5/\alpha v$ -cdKO embryos. We first investigated whether the development of branchial arch arteries was normal (Fig. 4). The branchial arch arteries developed symmetrically and normally between E9.5 and E11.5 in  $\alpha 5/\alpha v$ -cdKO embryos, as shown by whole-mount staining for PECAM1 and expression of the *R26R-lacZ* reporter (Fig. 4). Intracardial India ink injections also indicated normal symmetrical branchial arch formation (data not shown), as did histological analysis of branchial arch artery development in cdKO mice up to E11.5 (Fig. 4B and see Fig. S8G,H in the supplementary material). Furthermore, we detected comparable levels of staining for fibronectin around the vasculature of control and cdKO embryos (Fig. 4B).

We also checked whether the cardiac cushions formed normally in  $\alpha 5/\alpha v$ -cdKO mice. Endothelial cells migrate out of the endothelium into the cardiac jelly to form cardiac cushions, which contribute to the formation of the cardiac valves and closure of the septum (Kisanuki et al., 2001; Savolainen et al., 2009; Webb et al., 1998) and affect the hemodynamics in the embryo, which could play a crucial role in the vascular remodeling process (Yashiro et al., 2007). However, whole-mount *R26R-lacZ* reporter staining of E10.5 and E11.5  $\alpha 5/\alpha v$ -cdKO embryos did not show any differences in cardiac cushion formation (see Fig. S8 in the supplementary material). In the outflow tract cushions, most cells are derived from the neural crest and thus are not labeled by *Tie2-Cre*; *R26R-lacZ* (Kisanuki et al., 2001). However, the outflow tract cushions also appeared to have normal numbers of cells and exhibited comparable immunohistochemical staining for fibronectin (data not shown).

Great-artery remodeling phenotypes are found in many mutants in which neural crest cell migration or differentiation of neural crest cells into smooth muscle cells covering the branchial arch arteries is affected. However, we did not detect consistent differences in smooth muscle cell coverage of the branchial arch arteries between E10.5 and E11.5, indicating that the neural crest cells reach the branchial arches and, at least at these stages, differentiate into smooth muscle cells in a fashion comparable to that of their control littermates (data not shown). Therefore, no obvious defects were found in the formation of the branchial arches or of the cardiac cushions that could readily explain the subsequent arterial remodeling defects.

### Integrin $\alpha 5/\alpha v$ -dKO endothelial cells do not assemble normal fibronectin fibrils

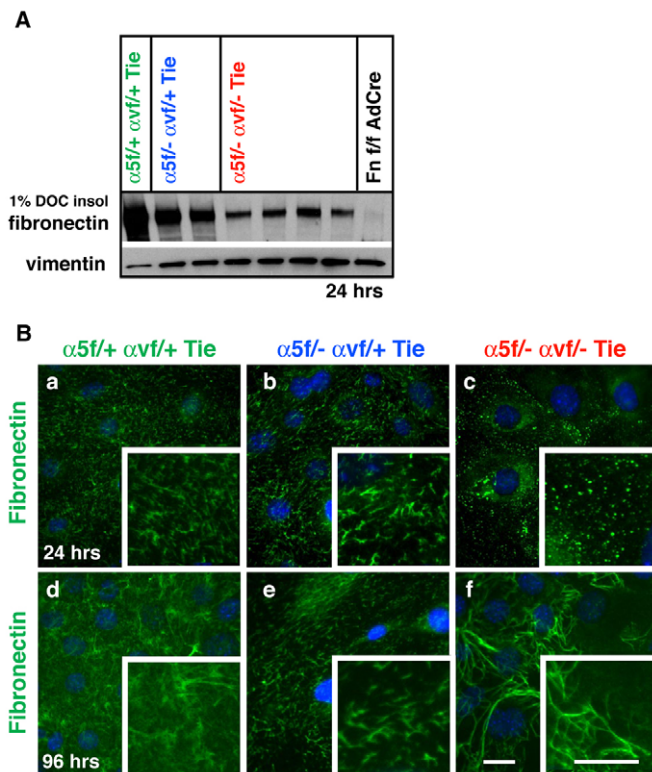
To analyze further the endothelial phenotype of  $\alpha 5/\alpha v$ -cdKO mice, we isolated dKO cell lines using two approaches. Adult lung-derived dKO endothelial (dKO-mLEC) cells were generated in



**Fig. 4. Normal development of branchial arch arteries and fibronectin deposition until E11.5.** (Aa-d) Whole-mount  $\beta$ -galactosidase reporter expression at E9.5 (a,b) and whole-mount PECAM1 staining at E10.5 (c,d) showing normal symmetric formation of branchial arch arteries in  $\alpha 5/\alpha v$ -cdKO (b,d) as compared with control cHemi (a,c) mouse embryos. (Ba-f) Frontal sections of E11.5 embryos showing symmetric branchial arch formation and similar fibronectin staining in (a) cHemi and (b)  $\alpha 5/\alpha v$ -cdKO embryos. Higher magnification images are shown of (c,d) carotid fibronectin staining and (e,f) the left sixth arch artery. Note the similar fibronectin staining pattern of several cell layers around the arteries. lca, left carotid artery; rca, right carotid artery; DoA, dorsal aorta. Scale bars: 100  $\mu\text{m}$ .

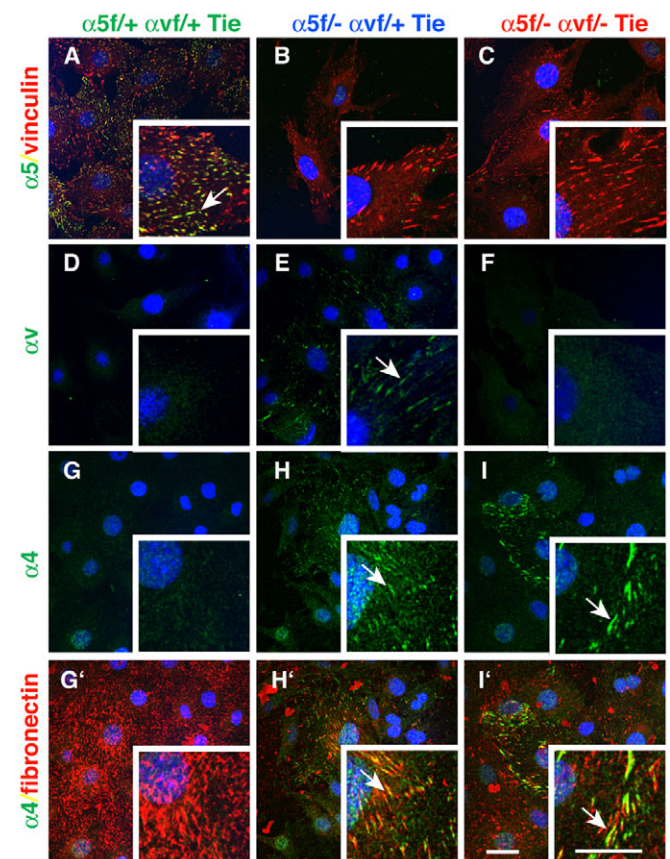
vitro from  $\alpha 5^{\text{flox/flox}}$ ;  $\alpha v^{\text{flox/flox}}$  Immorto endothelial cells by AdCre-mediated excision of both  $\alpha 5$  and  $\alpha v$  integrins. We also isolated ECs from E13.5 experimental cdKO crosses carrying the Immorto gene. All  $\alpha 5/\alpha v$ -dKO cell lines failed to adhere effectively to fibronectin (see Fig. S9B in the supplementary material; data not shown) and were therefore maintained on Matrigel-coated tissue culture plates.

As mentioned above, integrin  $\alpha 5$  plays a major role in fibronectin matrix assembly (Wierzbicka-Patynowski and Schwarzbauer, 2003), so we analyzed fibronectin fibrillogenesis by determining the amount of fibronectin incorporated into 1% DOC-insoluble matrix fractions.  $\alpha 5$ -KO cells assembled less fibronectin than did control cells,  $\alpha 5/\alpha v$ -dKO ECs assembled even less DOC-insoluble fibronectin, and dKO-mLEC cells assembled almost none (Fig. 5A; see Fig. S10 in the supplementary material; data not shown). These differences were also seen in immunofluorescence



**Fig. 5. Integrin  $\alpha5$ -KO and  $\alpha5/\alpha V$ -dKO cells assemble less fibrillar fibronectin.** (A) Immunoblot of 1% DOC-insoluble fibronectin matrix assembled by embryonic endothelial cell (eEC) lines plated for 24 hours on Matrigel. Note the slightly reduced insoluble fibronectin in  $\alpha5$ -KO endothelial cells and even further reduced insoluble fibronectin in  $\alpha5/\alpha V$ -dKO cells. The right-hand lane shows that fibronectin-null cells do not produce endogenous (insoluble) fibronectin (negative control). (Ba-f) Fibronectin fibrillogenesis as detected by immunofluorescence analyses of control  $\alpha5$ -KO and  $\alpha5/\alpha V$ -dKO endothelial cells after 24 and 96 hours plating on Matrigel. Similar results were obtained after plating on Matrigel containing 10  $\mu$ g/ml fibronectin. Note the small aggregates of fibronectin instead of fibrils in the dKO cells at 24 hours (c) and the delayed formation of long, wavy fibronectin fibrils in the dKO cell lines at 96 hours (f). Scale bars: 30  $\mu$ m.

analyses of endogenous fibronectin assembly.  $\alpha5$ -KO endothelial cells synthesized an extensive fibrillar network from 24 hours onwards (Fig. 5Bb,e), but the fibrils appeared slightly thicker and shorter than those of the control cells (Fig. 5Ba,d). However, both dKO-eEC (Fig. 5Bc,f) and dKO-mLEC (not shown) cells deposited only small fibronectin aggregates at 24 hours and only some subsets of dKO-eEC assembled long wavy fibronectin fibrils after prolonged cell culture (Fig. 5Bc,f). Both the DOC-insolubility and immunofluorescence results were obtained for several independent cell lines, as well as for the incorporation of exogenously added soluble fibronectin (10  $\mu$ g/ml) (data not shown). Interestingly, the different appearance of fibronectin fibers in long-term eEC cultures correlated with  $\alpha4$  integrin expression (see Fig. S9A in the supplementary material; data not shown). dKO cell lines completely negative for  $\alpha4$  integrin expression did not assemble fibrillar fibronectin (data not shown). Indeed, it has been shown that  $\alpha4$  integrin can assemble fibronectin in an RGD-independent



**Fig. 6. Embryonic endothelial  $\alpha5$ -KO and  $\alpha5/\alpha V$ -dKO cells show relocalization of  $\alpha V$  and  $\alpha4$  integrins when plated on fibronectin.** Cells were cultured overnight on a mixture of fibronectin (10  $\mu$ g/ml) and Matrigel (20  $\mu$ g/ml) and stained for integrins, vinculin or fibronectin. (A-C)  $\alpha5$  integrin staining showing loss of  $\alpha5$  in both  $\alpha5$ -KO and  $\alpha5/\alpha V$ -dKO eEC lines. (D-F) Relocalization of  $\alpha V$  integrin to focal adhesions in cells plated on fibronectin in both  $\alpha5$ -KO (H) and  $\alpha5/\alpha V$ -dKO cells (I) but not in control cells (G). (G'-I') Merged images of  $\alpha4$  and fibronectin (same frames as G-I), showing colocalization of  $\alpha4$  in focal adhesions with fibrillar fibronectin. Arrows point to focal adhesions containing co-stained integrin and fibronectin. Scale bars: 30  $\mu$ m.

manner (Sechler et al., 2000). Furthermore, we found that  $\alpha4$  integrin relocalized just as  $\alpha V$  did (Fig. 6; see also Fig. S3 in the supplementary material) into focal adhesions in  $\alpha5$ -KO-eEC and dKO-eEC. These findings suggest that, when both  $\alpha5$  and  $\alpha V$  are depleted,  $\alpha4$  integrin, if expressed, can assemble fibronectin into a fibrillar network, although the fibronectin fibers seem qualitatively different from those formed by  $\alpha5$  and/or  $\alpha V$  integrins.

## DISCUSSION

The results presented here document several novel findings. First, there is the surprising result that mice lacking endothelial  $\alpha5$  integrin are viable and fertile and have no obvious defects in developmental angiogenesis. Second, we provide evidence for genetic interaction between mutations in the  $\alpha5$  and  $\alpha V$  integrin genes and for overlapping functions and/or compensation between these integrins and perhaps others. Third, even in the absence of both  $\alpha5$  and  $\alpha V$  integrins, initial vasculogenesis and

angiogenesis proceed normally, at least up to E11.5, including the formation of intact and ostensibly normal yolk sac and embryonic vasculature and development of the branchial arches. Finally, in the absence of endothelial  $\alpha 5$  and  $\alpha v$  integrins, but not of either alone, there are extensive defects in remodeling of the great vessels and heart. These results have to be considered in light of cell biological data on the expression and distribution of these and other integrins and the assembly of their common ligand, fibronectin.

### The dispensability of endothelial $\alpha 5$ integrin

Complete deletion of  $\alpha 5$  in knockout mice (Francis et al., 2002; Yang et al., 1993),  $\alpha 5$ -KO teratomas (Taverna and Hynes, 2001) and ES cells (Francis et al., 2002) and several  $\alpha 5\beta 1$  integrin blocking studies (Bhaskar et al., 2007; Kim et al., 2000) have implicated  $\alpha 5\beta 1$  integrin in both developmental and pathological angiogenesis. To understand more about the role of endothelial  $\alpha 5\beta 1$  in angiogenesis, we depleted  $\alpha 5$  integrin specifically in endothelial cells. In contrast to  $\alpha 5$ -KO mice, which die at E11 because of vascular defects, we found that mice lacking endothelial  $\alpha 5$  integrin are viable and that  $\alpha 5$ , like  $\alpha v$  (this study) (McCarty et al., 2005), is not required for developmental angiogenesis. This suggests that in  $\alpha 5$ -KO embryos, the reported somite and neural crest defects might also contribute to the lethal phenotype (Goh et al., 1997; Yang et al., 1993).

The lack of a vascular phenotype raises several questions and possibilities as to how endothelial  $\alpha 5$  integrin could be dispensable for angiogenesis, whereas its major ligand, fibronectin, is essential. The data suggest that the timing, efficiency or delay in  $\alpha 5$  protein depletion in endothelial cells are not responsible for the lack of developmental vascular defects. Therefore, endothelial  $\alpha 5\beta 1$  integrin is dispensable on endothelial cells, certainly after E10.5. Its role as a receptor and assembler of fibronectin must be substituted by another (integrin) receptor or by  $\alpha 5\beta 1$  expression on another cell type.

### Genetic interactions and overlapping functions of endothelial $\alpha 5$ and $\alpha v$ integrins

We have reported previously that endothelial  $\alpha v$  integrins are completely dispensable for angiogenesis (McCarty et al., 2005), even though they may play some role in its regulation. Here we show that mice lacking endothelial  $\alpha 5$  and a single  $\alpha v$  allele show increased lethality, whereas the homozygous deletion of both genes leads to almost complete embryonic lethality. This suggests that  $\alpha 5$  and  $\alpha v$  integrins either overlap or converge in function, or that they can compensate for one another in some way. These developmental genetic data are complemented by cell biological data suggesting redeployment of  $\alpha v$  integrins in the absence of endothelial  $\alpha 5$  integrin. When  $\alpha 5$ -null endothelial cells spread on fibronectin,  $\alpha v$  integrins relocate to focal contacts, a position usually occupied by  $\alpha 5$  integrins in wild-type cells. This happens in the absence of major upregulation of  $\alpha v$  protein levels. The fact that  $\alpha v$  integrins can also mediate adhesion to fibronectin and assemble fibronectin-rich matrices, albeit less well than does  $\alpha 5\beta 1$ , is consistent with earlier work (Takahashi et al., 2007; Wennerberg et al., 1996; Yang and Hynes, 1996). Therefore,  $\alpha 5$ -null endothelial cells can interact with fibronectin through  $\alpha v$  integrins and this presumably underlies the lack of obvious embryonic phenotypes in the absence of  $\alpha 5\beta 1$  integrin on endothelial cells. However, this is unlikely to be the full story, as discussed below.

### Angiogenesis in the absence of endothelial $\alpha 5$ and $\alpha v$ integrins

The first 10 days of development proceed apparently normally in the absence of all the major fibronectin receptors on endothelial cells. Embryonic lethality does not commence until E14.5, after vasculogenesis and angiogenesis and the formation of the branchial arch network. This is in contrast to Tie2-Cre-mediated endothelial-specific ablation of all  $\beta 1$  integrins (Carlson et al., 2008; Lei et al., 2008; Tanjore et al., 2008) and to endothelial cKOs of several downstream signaling molecules, such as ILK (Friedrich et al., 2004) and FAK (PTK2) (Braren et al., 2006), all of which result in early embryonic death at ~E11 due to a variety of placental, yolk sac or vascular defects. These findings suggest that some endothelial  $\beta 1$  integrin(s) and integrin-mediated signaling are essential for early developmental angiogenesis. A few animals lacking  $\alpha 5$  and  $\alpha v$  integrins on endothelial cells complete development and survive. These mice appear to be healthy, although we have not obtained sufficient numbers to investigate their fertility and fecundity or their capabilities in pathological angiogenesis. Nonetheless, it is surprising, given the existing data implicating these integrins in angiogenesis, that they appear to be dispensable on endothelial cells for normal development, routinely to mid-gestation and occasionally into postnatal life.

### Vascular remodeling in the absence of endothelial $\alpha 5$ and $\alpha v$ integrins

The embryonic lethality in the great majority of mice lacking  $\alpha 5$  and  $\alpha v$  integrins in their endothelial cells appears to arise from defects in remodeling of the great vessels and heart from E11.5 onwards. The embryos exhibit a variety of defects including VSDs, misrouting and interruptions of the aorta and other vessels. We found a second defect related to branchial arch remodeling in  $\alpha 5$ -cKO mice, i.e. PDA in adult mice. PDA accounts for 14% of congenital heart defects in humans and the phenotype correlates with flow volume and symptoms may range from none to morbidity at birth (Schneider and Moore, 2006). Interestingly, one report has suggested that reduced fibronectin assembly in the vessel wall leads to PDA (Mason et al., 1999). This might correlate with the modest reduction in fibronectin assembly by  $\alpha 5$ -KO endothelial cells in vitro. We detected the PDA phenotype in mice backcrossed to C57BL/6J (N7), and about half of the expected  $\alpha 5^{\text{flox/flox}}$ ; *Tie2-Cre* mice are lost before weaning. Thus, the  $\alpha 5$ -cKO PDA phenotype seems to be background dependent, in line with other studies showing that 129S4 and C57BL/6 genetic modifiers play a significant role in  $\alpha 5\beta 1$  and fibronectin function (Astrof et al., 2007; George et al., 1997; Yang et al., 1999).

Two distinct classes of model could explain these vascular remodeling defects: local and systemic. Since it has been established previously that fibronectin is strongly expressed in the cardiac cushions that form the valves (French-Constant and Hynes, 1988; Mjaatvedt et al., 1987; Peters and Hynes, 1996; Roman and McDonald, 1992) and apparently plays a role in the migration of cushion cells into the cardiac jelly (Icardo et al., 1992; Loeber and Runyan, 1990), we investigated the possibility that defects in cushion formation might lead to defects in vascular flow and thus to the remodeling defects seen in the  $\alpha 5/\alpha v$ -cdKO embryos. However, we did not detect defects in cushion formation at E10.5/11.5; cells migrate into the cushions in apparently normal numbers and deposit fibronectin. These results do not rule out subsequent failures in valve development and/or systemic effects that could disrupt normal blood flow with consequential effects on vessel remodeling. A second group



of hypotheses – that local defects in integrin-mediated adhesion and signaling, assembly of extracellular matrix or migration of vascular cells could underlie the remodeling defects – are also likely possibilities and receive some support from the cell biological analyses presented.

### Fibronectin assembly in the absence of endothelial $\alpha 5$ and $\alpha v$ integrins

Our *in vitro* data show decreased fibronectin fibril formation by  $\alpha 5/\alpha v$ -dKO endothelial cell lines, suggesting that a defect in matrix assembly might contribute to the *in vivo* phenotypes. However, we have been unable to detect any consistent reduction in assembled fibronectin in the basement membrane underlying the endothelial cell layer in any of our cKO mice. This could reflect technical limitations in immunohistochemical detection of assembled fibronectin. Furthermore, the *in vitro* experiments show that fibronectin aggregates still form in  $\alpha 5/\alpha v$ -dKO ECs, indicating that one might not expect absolute loss of fibronectin but rather a quantitative or qualitative difference in fibril formation in the proximity of the  $\alpha 5$ -deficient endothelial cells. These might well be difficult or impossible to detect by immunohistochemical staining for fibronectin. Higher resolution methods, such as electron microscopy (EM) or immuno-EM, might be required.

### Potential involvement of other receptors

The continued deposition of fibronectin fibrils by at least some  $\alpha 5/\alpha v$ -dKO endothelial cell lines suggests that some other integrin or even a different class of receptor might be able to replace this function of  $\alpha 5$  and  $\alpha v$  integrins *in vitro*, and conceivably also *in vivo*. As mentioned above, deletion of  $\beta 1$  integrin in endothelial cells has significantly more severe effects on vascular development than the deletion of  $\alpha 5$  and  $\alpha v$  integrins that we report here. There are twelve possible  $\beta 1$  integrins, of which only two would be deleted in our experiments ( $\alpha 5\beta 1$  and  $\alpha v\beta 1$ , along with  $\alpha v\beta 3$ ,  $\alpha v\beta 5$ ,  $\alpha v\beta 6$  and  $\alpha v\beta 8$ , if present). It is not certain which of these integrins are present on endothelial cells at the relevant periods of development. Although integrin receptors for laminins and collagens could well play a role, we are particularly interested in receptors for fibronectin, given that it is known to be essential for angiogenesis and heart and vascular development (Astrof and Hynes, 2009). Among the  $\beta 1$  integrins, several have been reported as fibronectin receptors, most notably  $\alpha 4\beta 1$ ,  $\alpha 8\beta 1$  and  $\alpha 9\beta 1$ . We have observed a correlation between the expression of  $\alpha 4\beta 1$  integrin by  $\alpha 5/\alpha v$ -dKO endothelial cell lines and their ability to assemble abnormal fibronectin fibrils. The complete knockout of  $\alpha 4$  integrin does not block angiogenesis but does lead to defects in coronary vessels, probably as a secondary consequence of defects in the formation of the epicardium (Sengbusch et al., 2002; Yang et al., 1995) and defective mural cell coverage of dilated cranial vessels at E10 (Grazioli et al., 2006). Embryos deficient in both  $\alpha 4$  and  $\alpha 5$  integrins show the  $\alpha 5$  phenotype, without enhancement (Yang et al., 1999). Mice lacking  $\alpha 4$  integrin in endothelial cells are viable and lack a vascular phenotype (Priestley et al., 2007). These data argue against a major role for endothelial  $\alpha 4$  integrin in normal vascular development but do not rule out its potential to compensate for the absence of  $\alpha 5$  and  $\alpha v$  integrins in our experiments. More elaborate compound mouse models that lack several integrins will be needed to test these hypotheses further.

### Potential roles of $\alpha 5$ and $\alpha v$ integrins in other vessel-associated cell types

Quite apart from any function of  $\alpha 5\beta 1$  in vascular endothelial cells, it is likely that  $\alpha 5$  integrin also plays roles in pericytes and/or smooth muscle cells that support the vasculature. Indeed, staining of  $\alpha 5$ -cKO tissue cryosections of intestine and skeletal muscle for  $\alpha 5$  integrin showed the persistence of vessel-associated  $\alpha 5$  immunoreactivity (data not shown), making it difficult to confirm endothelial knockout of  $\alpha 5$  integrin by histology, even though it can be demonstrated by isolation of endothelial cells from embryos or adult tissues. This probably represents  $\alpha 5$  integrin expression in pericytes and smooth muscle cells. Therefore, we hypothesize that the  $\alpha 5\beta 1$  integrin of such vessel-associated cell types might contribute to fibronectin assembly. Recently, mural cell-specific  $\beta 1$  cKO using platelet-derived growth factor receptor  $\beta$  (PDGFR $\beta$ )-Cre resulted in a severe vascular phenotype: the mice showed hemorrhage and dilated vessels and were perinatal lethal (E18-P10) (Abraham et al., 2008). Another example of integrin function in perivascular cells comes from the depletion of  $\alpha v$  integrin in glial cells, which normally stabilize the vasculature of the brain, resulting in cerebral hemorrhage (McCarty et al., 2005), whereas there is no cerebral vascular phenotype in endothelial  $\alpha v$ -cKO mice. We did not observe a similar trans function for  $\alpha 5\beta 1$  integrin in the brain as Nestin-Cre-mediated knockout of  $\alpha 5$  integrin in the nervous system showed no embryonic cerebral hemorrhage, nor any other obvious vascular phenotype (our unpublished results).

### Implications for the inhibition of angiogenesis

It is intriguing that  $\alpha 5\beta 1$  antagonists, currently in clinical trials, have been shown to inhibit angiogenesis *in vitro*, whereas we report that developmental angiogenesis seems unaffected by loss of endothelial  $\alpha 5$ . Strikingly, a similar contradiction has been found for  $\alpha v\beta 3$  and  $\alpha v\beta 5$  antagonists that appear to be anti-angiogenic *in vitro*, whereas the respective integrin KO mice show an increased pathological angiogenesis response (Reynolds et al., 2002). One hypothesis in the latter case is that  $\alpha v\beta 3$  and  $\alpha v\beta 5$  act as negative regulators of angiogenesis (Hynes, 2002a; Hynes, 2007). The experimental timing of integrin depletion might also play a role. Acute inhibition of  $\alpha 5$  integrin in adult mice might offer fewer opportunities for compensation or plasticity in the adult organism than does depletion during embryogenesis. Our observation that several embryo-derived EC lines expressed some  $\alpha 4$  integrin and formed delayed fibronectin fibrils, whereas adult-derived and *in vitro*-depleted  $\alpha 5/\alpha v$ -dKO ECs did not express  $\alpha 4$  integrin nor assemble fibrils, might also hint at such a mechanism. These hypotheses deserve further testing; for instance, inducible endothelial  $\alpha 5$ -cKO could address the difference between depletion of endothelial  $\alpha 5$  in the adult versus the embryo. Another possible explanation is that there might be a fundamentally different function for  $\alpha 5\beta 1$  in developmental as distinct from pathological angiogenesis. For example,  $\alpha 5\beta 1$  integrin has been implicated in tumor angiogenesis (Taverna and Hynes, 2001) and has been found to be upregulated *in vivo* in a cerebral hypoxia model (Milner et al., 2008). In addition, developmental lymphangiogenesis seems to be unaffected, in contrast to a recent study showing that  $\alpha 5\beta 1$  blockade affects pathological lymphangiogenesis (Okazaki et al., 2009). Therefore, it would be interesting to test the role of endothelial  $\alpha 5$  integrin in pathological models of angiogenesis, in order to validate endothelial cells as targets for the blocking reagents in those models.

## Conclusions

The results reported here reveal further complexity in the roles of integrins in vascular development. Although various lines of evidence implicate  $\alpha 5$  and  $\alpha v$  integrins in vascular development and angiogenesis, it is clear that these processes can proceed almost, or completely, normally in the absence of either one of these integrin subunits and, to a significant extent, in the absence of both. These results raise intriguing questions about overlapping functions or compensation among integrins. This possibility pertains most strongly to the fibronectin receptor integrins because that ligand is the most essential extracellular matrix protein for vascular development. Since none of the clinical trials using antagonists of these integrins has yet proven their efficacy as anti-angiogenic agents, it might be worth considering the possibility of targeting both subunits at the same time. Basic developmental questions about which integrins play important roles in vascular development, and in which cells and cellular functions, require further investigation. This will be challenging given the number of potential players, their embryonic lethality and possible overlap in function.

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## Competing interests statement

The authors declare no competing financial interests.

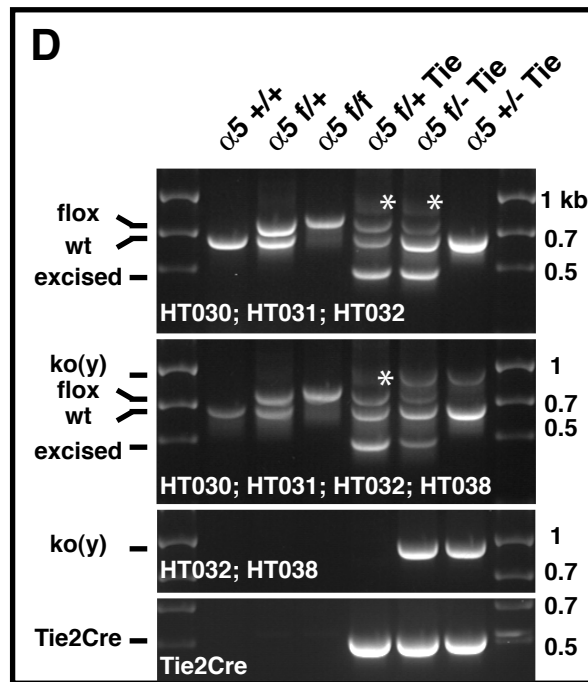
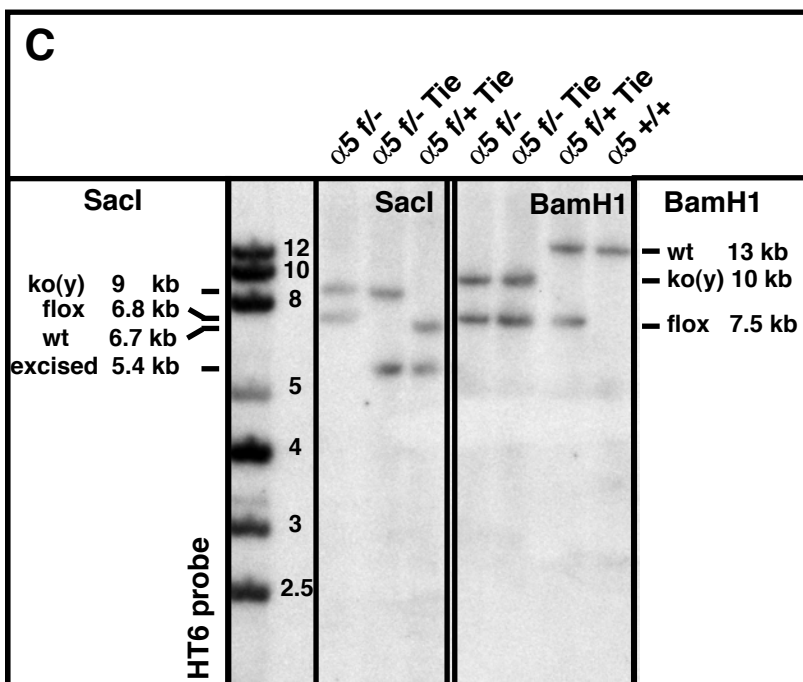
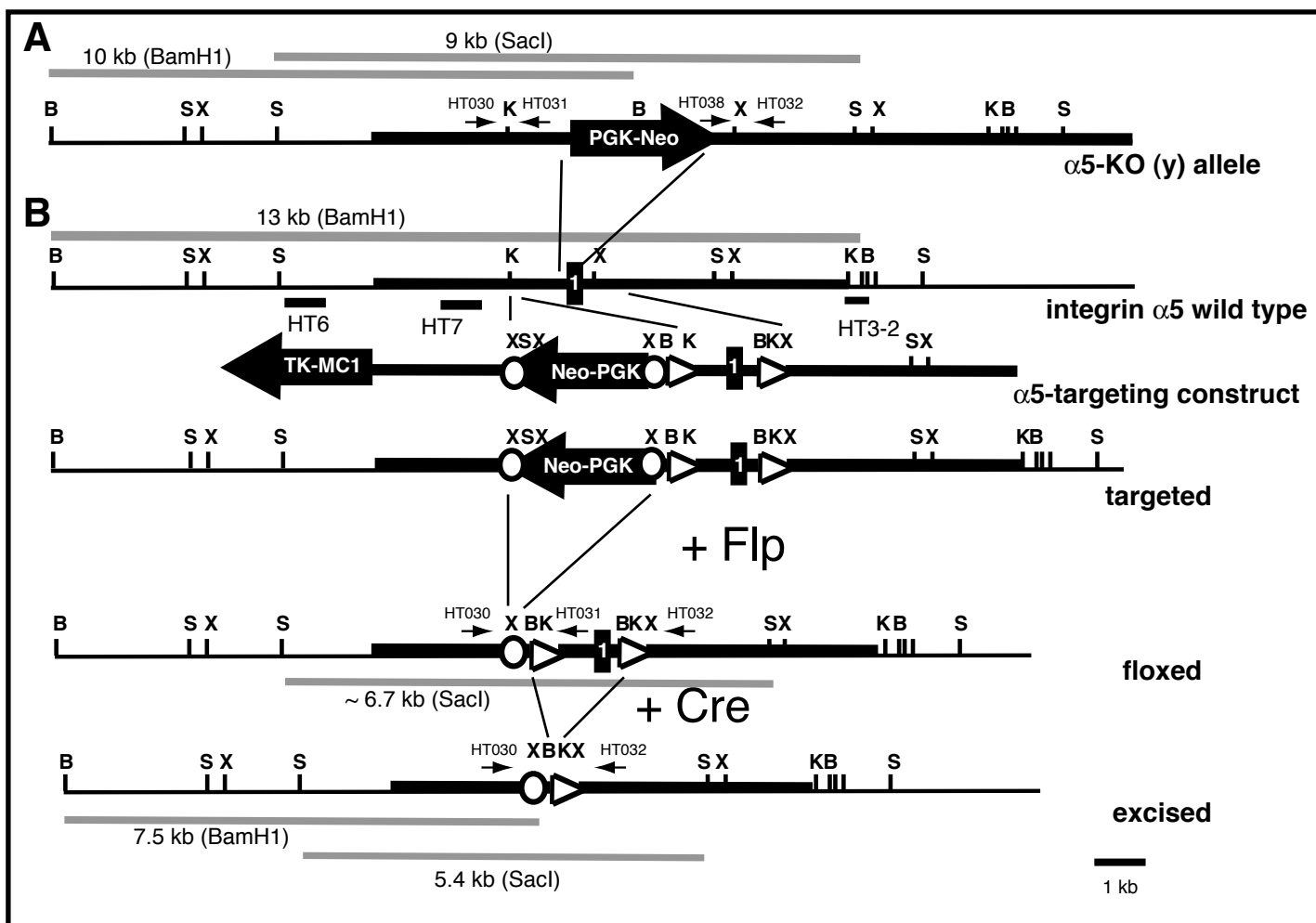
## Supplementary material

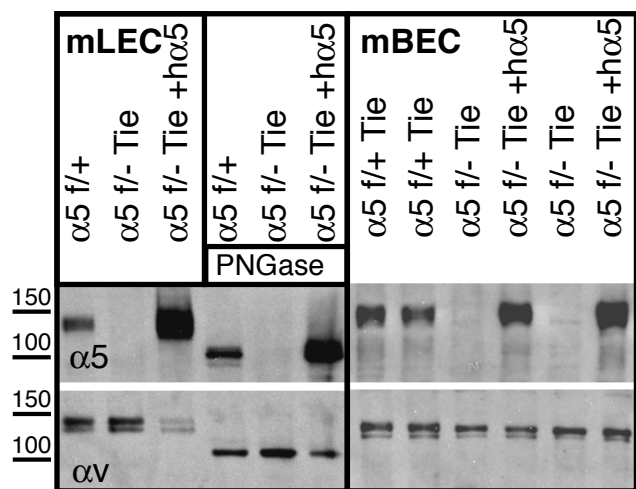
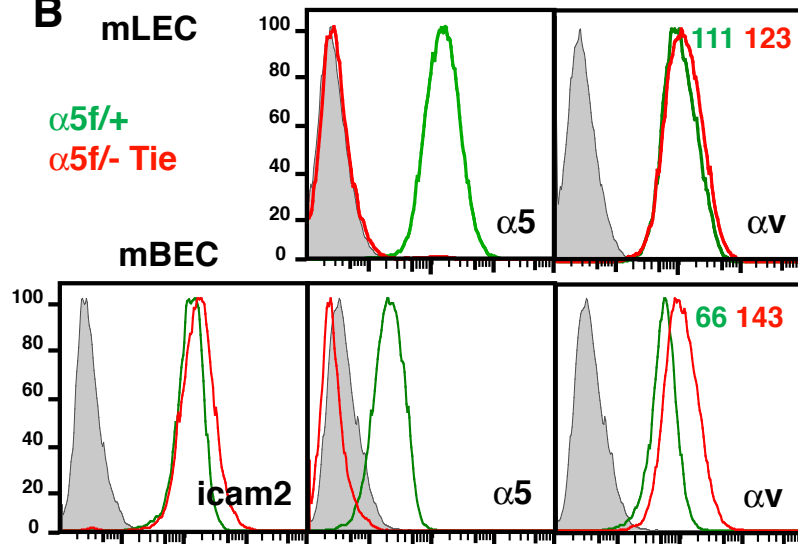
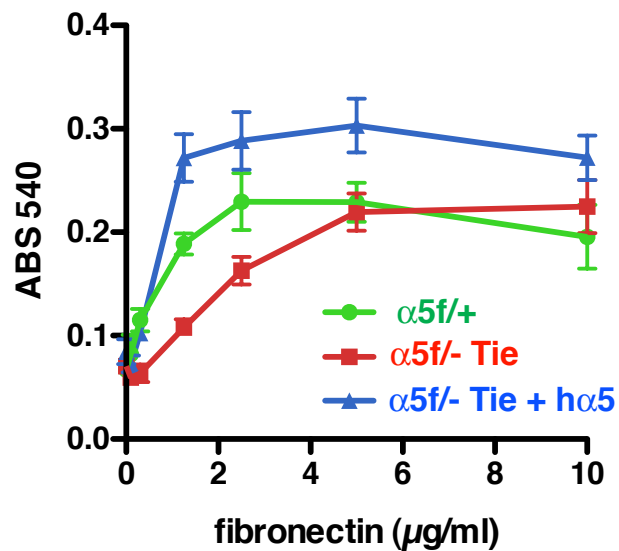
Supplementary material for this article is available at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.049551/-/DC1>

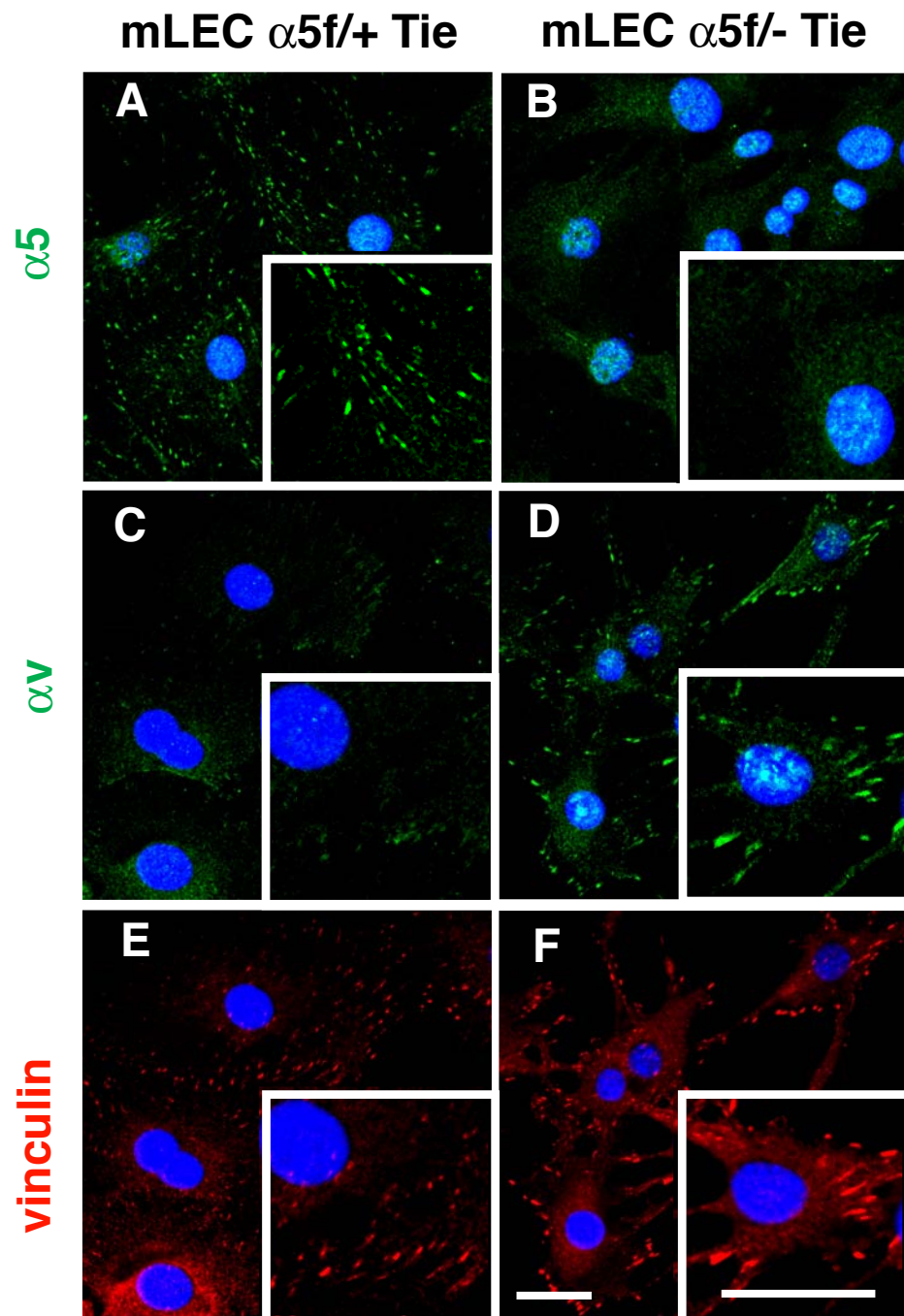
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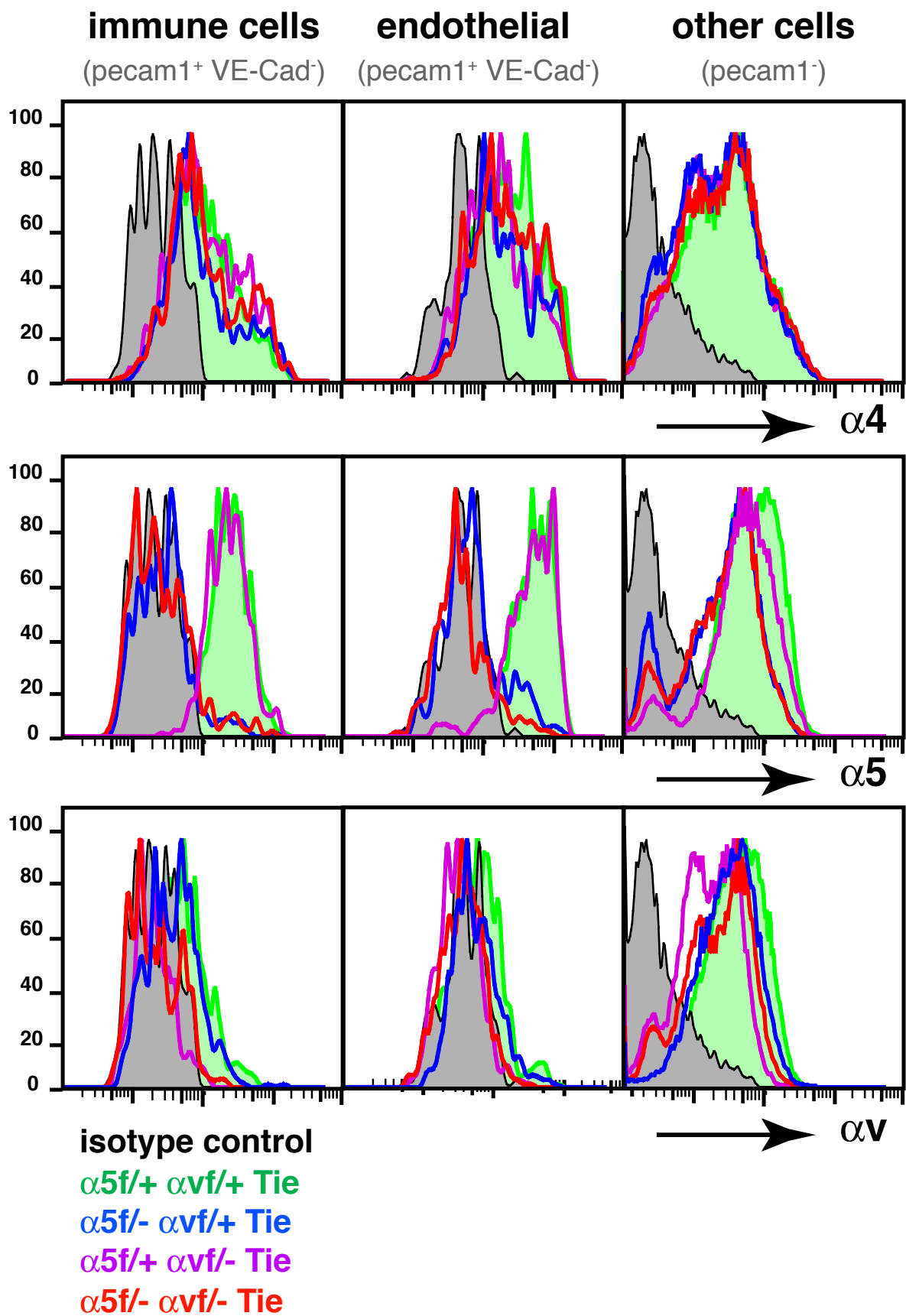
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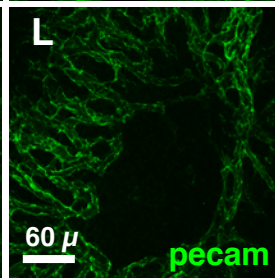
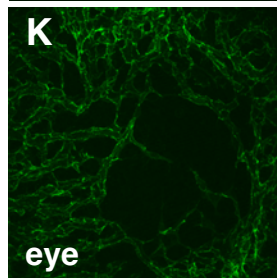
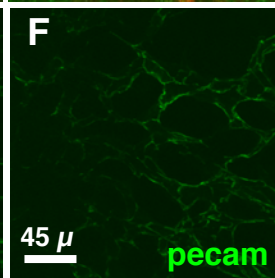
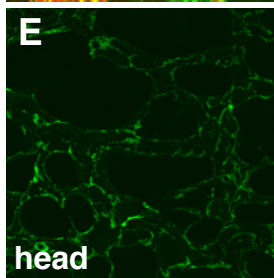
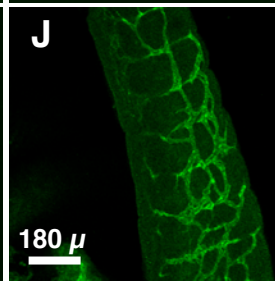
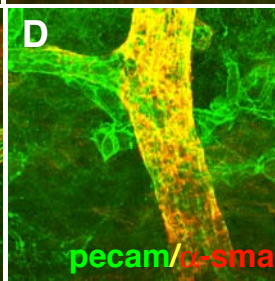
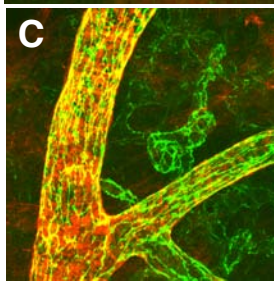
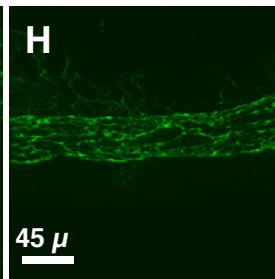
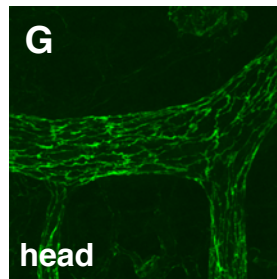
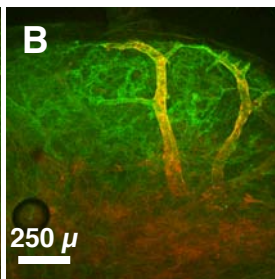
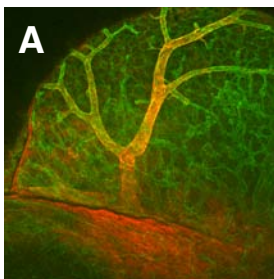


$\alpha 5f/+ \alpha vf/+$  Tie

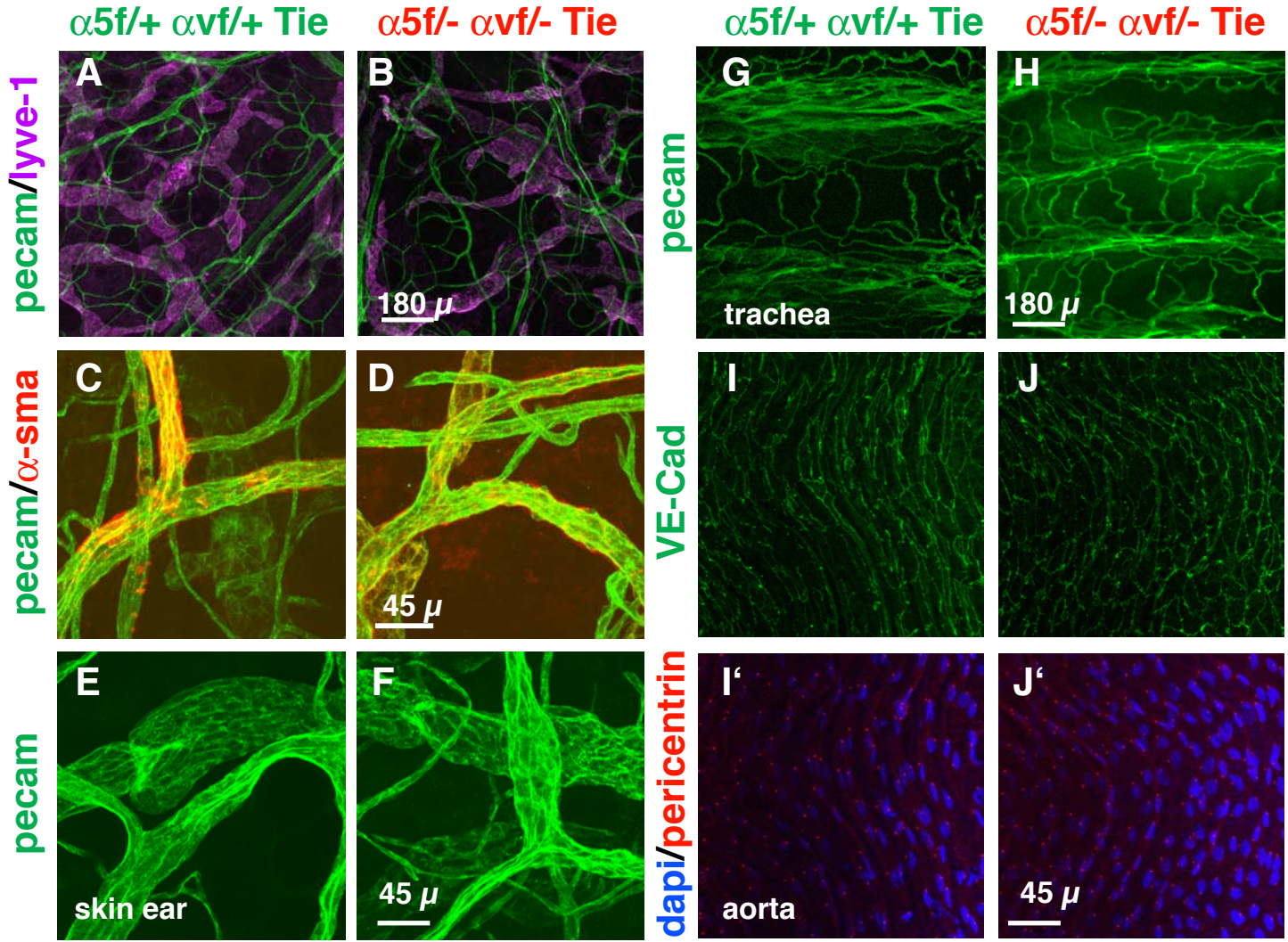
$\alpha 5f/- \alpha vf/-$  Tie

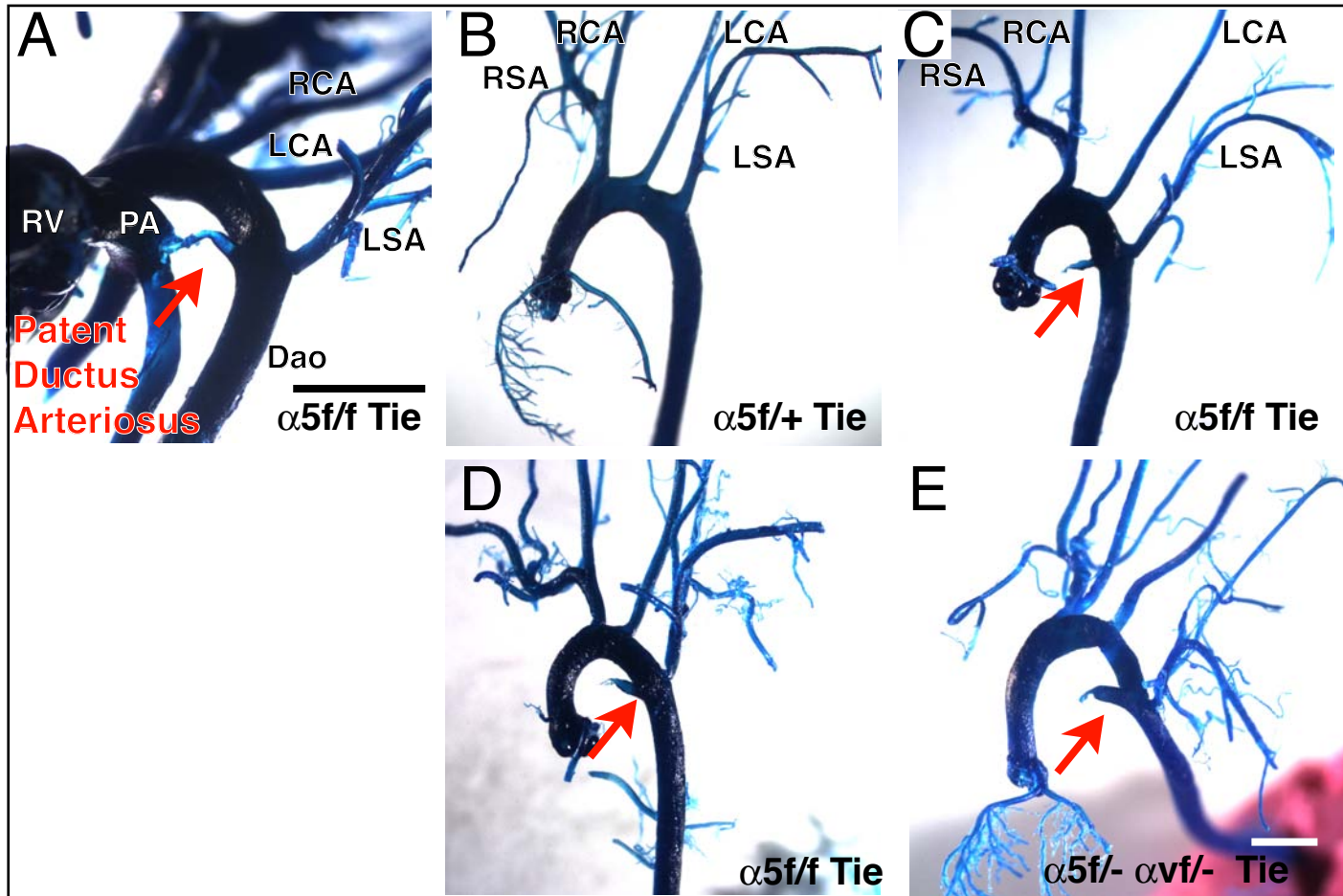
$\alpha 5f/+ \alpha vf/+$  Tie

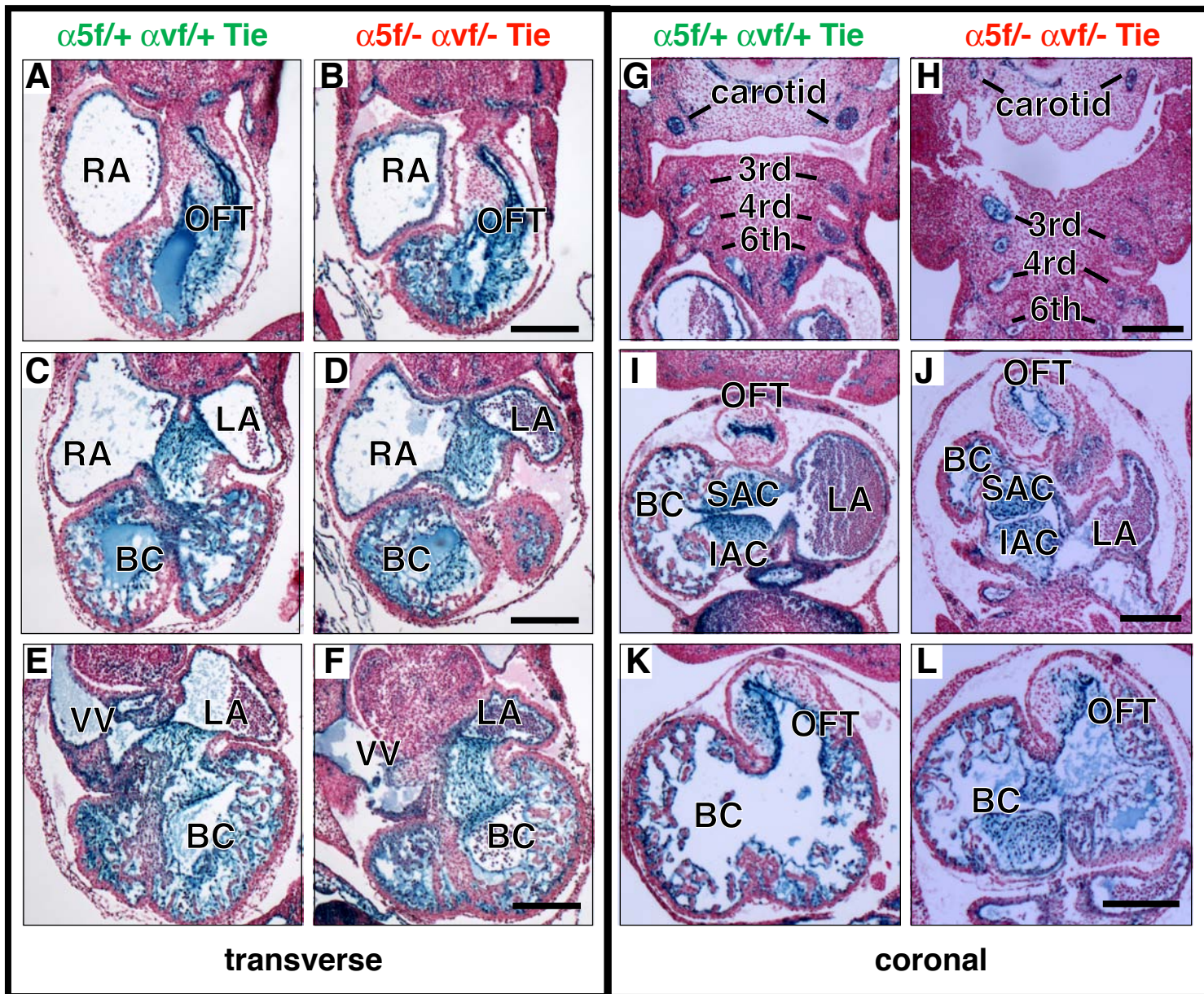
$\alpha 5f/- \alpha vf/-$  Tie

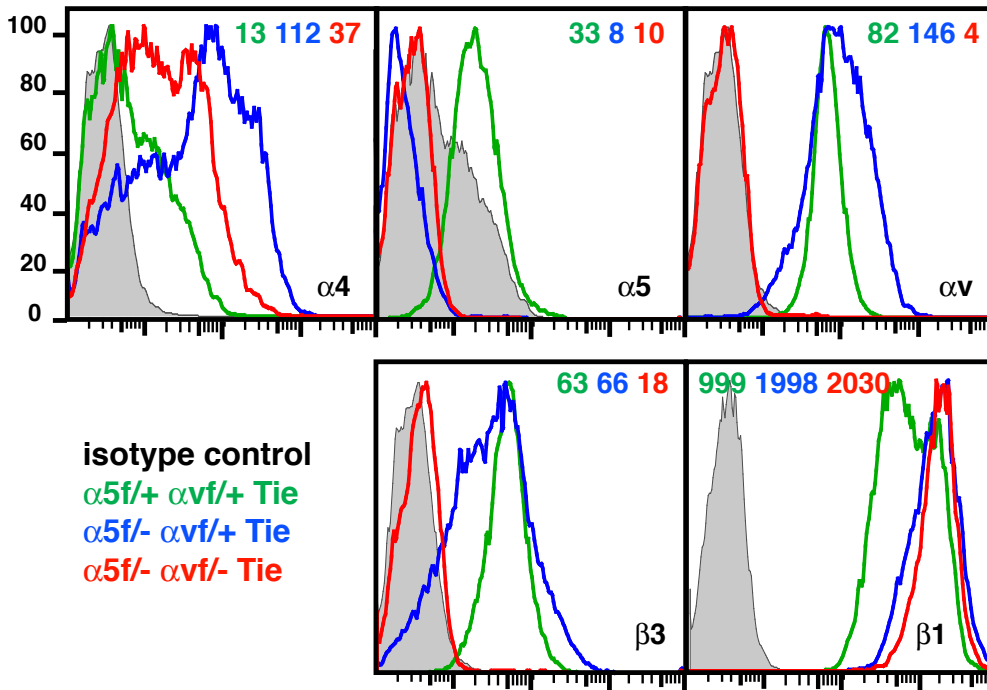
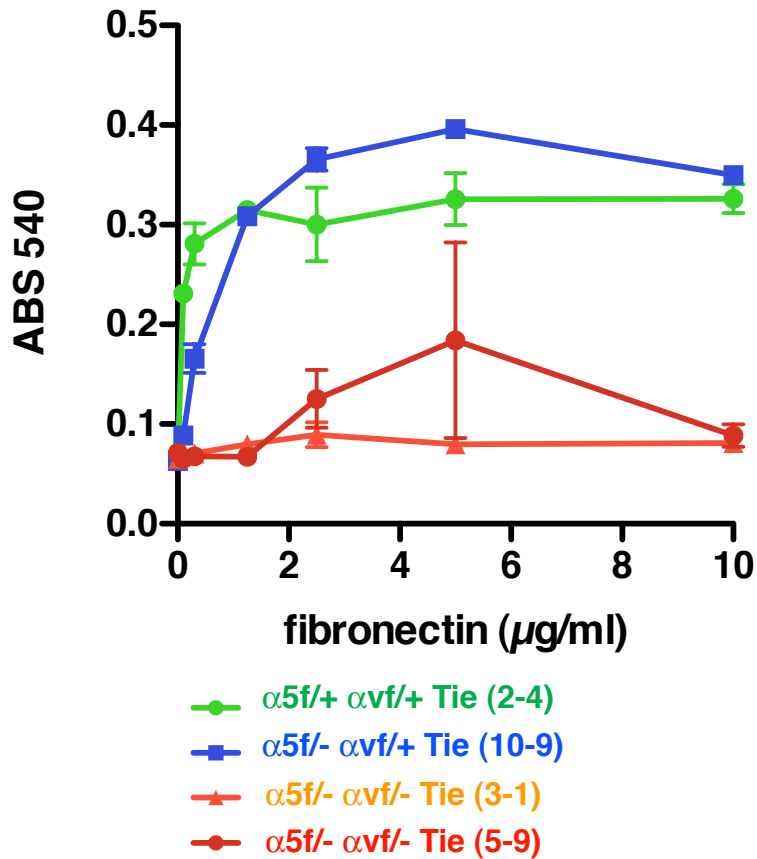


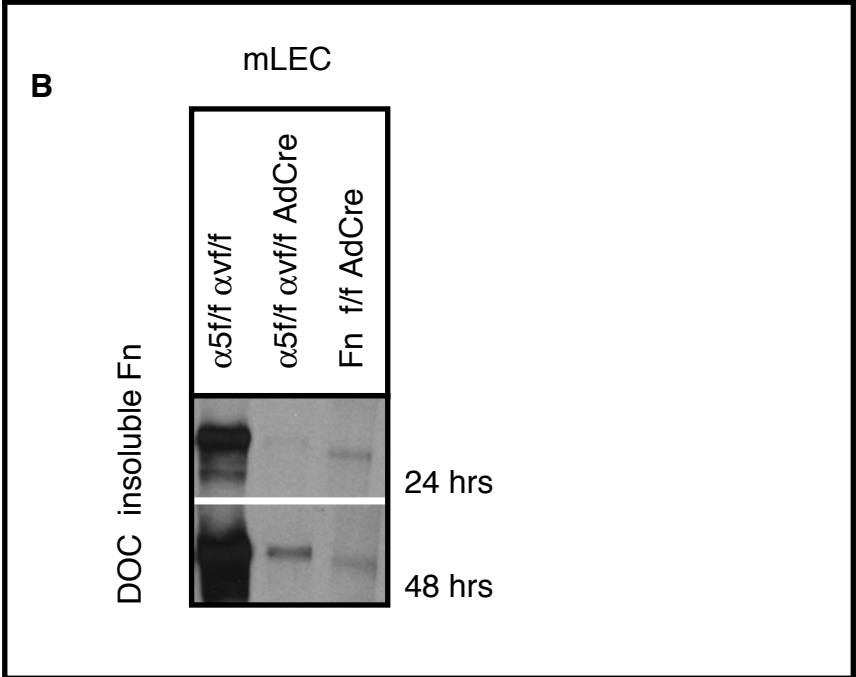
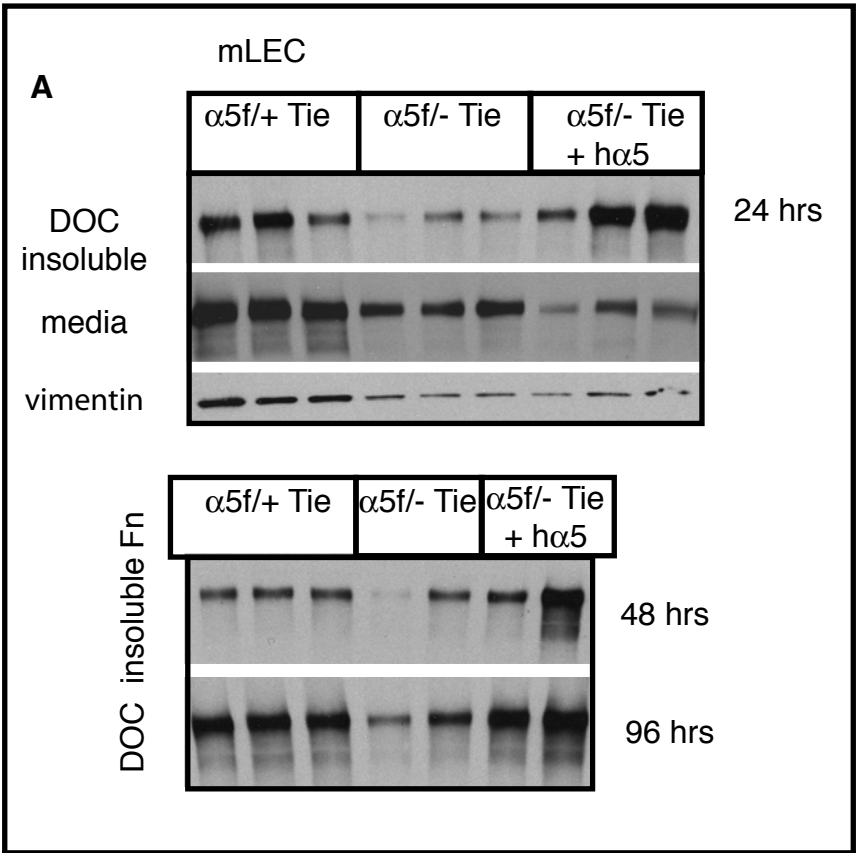








**A****B**



**Table S1. Genotypes of progeny from  $\alpha 5^{\text{flox/flox}}$   $\times$   $\alpha 5^{+/-}$ ; *Tie2-Cre/+* crosses at weaning**

Stage	$\alpha 5^{\text{flox/+}}$	$\alpha 5^{\text{flox/+}}$ ; <i>Tie2-Cre</i>	$\alpha 5^{\text{flox/-}}$	$\alpha 5^{\text{flox/-}}$ ; <i>Tie2-Cre</i>
~ P21	133 (28%)	125 (26%)	113 (24%)	102 (22%)

$\chi^2$  test;  $P=0.3$ ; no significant divergence from Mendelian ratios.

**Table S2. Overview of cardiovascular defects observed in serially sectioned E14.5 embryos**

Genotype	Great artery connections	VSD	RERSA	Aortic arch defects
<i>α5<sup>flox/+</sup>; αv<sup>flox/+</sup>; Tie2-Cre</i>				
1198-10	Normal	Closed	Absent	None
<b>α5/αv-cdHemi</b>	<b>1/1 normal</b>	<b>None</b>	<b>None</b>	<b>None</b>
<i>α5<sup>flox/-</sup>; αv<sup>flox/-</sup>; Tie2-Cre</i>				
1198-3	Defective	VSD	RERSA	Tiny AAo
1198-4	Defective	VSD	Absent	IAA-C
1198-9	Defective	VSD	RERSA	Very thin AA at AA-C
1199-2	Fragile	VSD	Absent	Thin AA
1261-1	Defective	VSD	RERSA	AAo missing
1261-4	Defective	VSD	RERSA	IAA-C (or B)
<b>α5/αv-cdKO</b>	<b>5/6 defective</b> <b>1/6 fragile</b>	<b>6/6</b>	<b>4/6</b>	<b>Frequent defects</b>
<i>α5<sup>flox/-</sup>; αv<sup>flox/+</sup>; Tie2-Cre</i>				
1198-1	Normal	VSD	Absent	Thin at AA-C region
1198-6	Normal	Small VSD	Absent	Thin AA
1198-8	Defective	VSD	RERSA	Vascular ring (double DA)
1198-11	Normal	VSD	Absent	Long thin AA
1199-1	Normal	VSD	Absent	AA dilated shape
1261-7	Fragile	VSD	Absent	Stenosis of DAo
1261-3	Fragile	VSD	Absent	Thin AA
<b>α5-cKO; αv-cHemi</b>	<b>1/7 defective</b> <b>2/7 fragile</b> <b>4/7 normal</b>	<b>7/7</b>	<b>1/7</b>	<b>Occasional defects</b>
<i>α5<sup>flox/+</sup>; αv<sup>flox/-</sup>; Tie2-Cre</i>				
1198-2	Normal	Closed	Absent	Absent
1261-5	Normal	Closed	Absent	Absent
<b>α5-cHemi; αv-cKO</b>	<b>2/2 normal</b>	<b>0/2</b>	<b>0/2</b>	<b>None</b>

Embryos are organized by genotype and litter embryo number. Great-artery connections: normal, appears as control; fragile, some parts of vessels look distorted or thin but all the connections are normal; defective, defects in vessel organization. VSD column: VSD, ventricular septation defect; closed, ventricular septation; RERSA, retro-oesophageal right subclavian artery. Aortic arch (AA) column, description of defects: A Ao, ascending aorta; (I)AA-B/C, interrupted aortic arch type (see Fig. 3A).

### Table S3. Genotypes of progeny from $\alpha 5^{\text{flox/flox}}$ $\times$ $\alpha 5^{\text{flox/+}}$ *Tie2-Cre* crosses at weaning

$\alpha 5^{\text{flox/+}}$	$\alpha 5^{\text{flox/+}}; \textit{Tie2-Cre}$	$\alpha 5^{\text{flox/flox}}$	$\alpha 5^{\text{flox/flox}}; \textit{Tie2-Cre}$
32 (29%)	33 (30%)	29 (27%)	15* (14%)

\* $\chi^2$  test;  $P=0.05$ ; marginally significant divergence from Mendelian ratios.