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LETTER

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A temporally-restricted pattern of endothelial cell collagen 4 alpha 1 expression during embryonic development determined with a novel knockin Col4a1-P2A-eGFP mouse line

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Summary

Classical collagen type IV comprising of a heterotrimer of two collagen IV alpha 1 chains and one collagen IV alpha 2 chain is the principal type of collagen synthesized by endothelial cells (EC) and is a major constituent of vascular basement membranes. In mouse and man, mutations in genes that encode collagen IV alpha 1 and alpha 2 result in vascular dysfunction. In addition, mutations in genes that encode the Ephrin receptor B4 (EPHB4) and the p120 Ras GTPase-activating protein (RASA1) that cause increased activation of the Ras mitogen-activated protein kinase (MAPK) signaling pathway in EC result in vascular dysfunction as a consequence of impaired export of collagen IV. To understand the pathogenesis of collagen IV-related vascular diseases and phenotypes it is necessary to identify at which times collagen IV is actively synthesized by EC. For this purpose, we used CRISPR/Cas9 targeting in mice to include immediately after the terminal *Col4a1* codon a sequence that specifies a P2A peptide followed by enhanced green fluorescent protein (eGFP). Analysis of eGFP expression in *Col4a1-P2A-eGFP* mice revealed active embryonic EC synthesis of collagen IV alpha 1 through mid to late gestation followed by a sharp decline before birth. These results provide a contextual framework for understanding the basis for the varied vascular abnormalities resulting from perturbation of EC expression and export of functional collagen IV.

KEYWORDS
basement membrane, blood vessels, capillary malformation–arteriovenous malformation, developmental angiogenesis, endothelial cell, Ras signal transduction, type IV collagen, vascular disease

1 | RESULTS AND DISCUSSION

Collagen IV is a non-fibrillar collagen that is essential for basement membrane stability, function and integrity (Pöschl et al., 2004; Wu & Ge, 2019). In vertebrates, there are six collagen IV isoforms, namely collagen IV alpha 1 to alpha 6, encoded by *COL4A1* to *COL4A6* genes respectively (Pöschl et al., 1988; Wu & Ge, 2019). These isoforms assemble into three distinct heterotrimeric collagen IV protomers

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in vivo. Classical collagen IV comprises two alpha 1 chains and a single alpha 2 chain (Wu & Ge, 2019). The two other collagen IV forms comprise of alpha 3 through alpha 6 monomers. Collagen IV protomer assembly takes place in the endoplasmic reticulum prior to cell export via the secretory pathway. Outside of the cell, protomers assemble into networks with other components of the extracellular matrix. Collagen IV protomers contain three domains: a short 7S amino terminus domain, a long collagenous triple helical domain comprising of Gly-Xaa-Yaa repeats, and a C terminus non-collagenous domain. Within the blood vasculature, classical collagen IV is the principal type of collagen present in basement membranes and is synthesized primarily by endothelial cells (EC) (Kramer et al., 1985; Wang & Su, 2011).

Mutations in *COL4A1* and *COL4A2* genes in humans are associated with genetically dominant vascular abnormalities that include intracerebral hemorrhage, leukoencephalopathy, intracranial aneurysm, and microbleeds (Gould et al., 2005; Jeanne et al., 2012; Jeanne & Gould, 2017; Kuo et al., 2012). Most mutations are missense mutations within the triple helical domain or are splice site substitutions that may result in the deletion of an entire exon (Jeanne & Gould, 2017; Kuo et al., 2012). Mice that express the same mutated forms of collagen IV alpha 1 or alpha 2 exhibit similar vascular abnormalities (Alavi et al., 2016; Jeanne et al., 2015; Jeanne & Gould, 2017; Kuo et al., 2012; van Agtmael et al., 2010). Exactly how *COL4A1* and *COL4A2* mutations cause vascular dysfunction is uncertain, although misfolding of collagen IV monomers in EC resulting in induction of an unfolded protein response in this cell type and/or an inability of EC to export sufficient collagen IV for deposition into basement membranes have both been proposed (Firtina et al., 2009; Gould et al., 2007; Jeanne et al., 2012). Notably, in most patients with *COL4A1* and *COL4A2* mutations, vascular defects are present at birth or appear in childhood (Jeanne & Gould, 2017; Kuo et al., 2012). Likewise, in *Col4a1* and *Col4a2* mutant mice, vascular lesions manifest early and are often evident during embryonic development or perinatally (Alavi et al., 2016; Jeanne et al., 2015; Jeanne & Gould, 2017; Kuo et al., 2012; van Agtmael et al., 2010). This is consistent with a pathogenic role for mutations during development of the blood vasculature rather than vascular maintenance. Indeed, an exon 41-skipped form of *COL4A1* causes blood vascular lesions in mice only when its expression is induced during development or perinatally, but not once mice have reached 3 weeks of age (Jeanne et al., 2015).

Another vascular disease in which abnormal folding and export of collagen IV may contribute to pathogenesis is capillary malformation-arteriovenous malformation (CM-AVM) (Eerola et al., 2003). CM-AVM is an autosomal dominant disorder caused by inactivating mutations of *RASA1* that encodes the RASA1 Ras GTPase-activating protein (CM-AVM1) or the Ephrin receptor B4 (EPHB4) growth factor receptor tyrosine kinase (CM-AVM2) (Amyere et al., 2017; Eerola et al., 2003; Revencu et al., 2013). Studies of conditional RASA1- and EPHB4-deficient mice have revealed that in EC, RASA1 and EPHB4 act together to limit activation of the Ras-mitogen active protein kinase (MAPK) signaling pathway (Chen et al., 2019, 2022, 2023). Consequently, induced EC-specific disruption of *Ephb4* or *Rasa1* genes during embryonic developmental angiogenesis results in dysregulated EC Ras-MAPK signal transduction that causes EC apoptosis. Mechanistically, dysregulated Ras-MAPK signaling results in an increased abundance of collagen proline and lysine hydroxylases in the ER that are thought to over-hydroxylate collagen IV monomers. In turn, this impairs collagen IV folding leading to EC apoptosis, again as a result of an unresolved unfolded protein response and/or EC detachment from an incompletely formed basement membrane (Chen et al., 2019, 2022, 2023). Like vascular defects in patients with *COL4A1* and *COL4A2* mutations, vascular lesions in CM-AVM are present at birth in the vast majority of cases (Amyere et al., 2017; Revencu et al., 2013). Furthermore, from late gestation through adult life, disruption of *Ephb4* or *Rasa1* genes in mice does not result in spontaneous blood vascular defects (Chen et al., 2019, 2022, 2023; Lapinski et al., 2012, 2017). Thus, the absence of functional EPHB4 or RASA1 in EC during development is necessary for the appearance of vascular lesions.

One possible explanation of a requirement for *COL4A1*, *COL4A2*, *RASA1* and *EPHB4* mutations to be present during early life for vascular lesions to manifest is that past the point of developmental angiogenesis in mid-late gestation, EC cease to engage in high-rate collagen IV synthesis. Collagens are recognized to be among the most stable proteins in the animal kingdom (Shoulders & Raines, 2009). Thus, once vascular basement membranes are formed, EC may not need to continue to engage in high-rate collagen IV synthesis for normal vascular functioning. However, at which times EC are actively engaged in collagen IV synthesis is uncertain, in part because of the long-term stability of collagen IV protein. Hence, the presence of collagen IV protein in vascular basement membranes does not necessarily indicate that the adjacent EC are actively involved in collagen IV synthesis. To address this, in the current study, we generated a novel knockin Col4a1-P2A-eGFP reporter mouse that identifies cells actively synthesizing collagen IV alpha 1. Analysis of these mice reveals a temporally-restricted pattern of high-rate EC collagen IV synthesis to the embryo that is consistent with the observed blood vascular phenotypes in humans and mice with mutations in genes that encode COL4A1, COL4A2, RASA1 and EPHB4.

To analyze the temporal expression of collagen IV alpha 1 throughout development, we generated a knockin mouse model in which the *Col4a1* promoter drives the expression of a *Col4a1-P2A-eGFP* allele (Figure 1a). The P2A peptide causes the ribosome to fail to make a peptide bond between a proline and a glycine residue in the P2A sequence during translation, resulting in the separate synthesis of collagen IV alpha 1 and GFP from the same mRNA (Kim et al., 2011; Sharma et al., 2012). Thus, with this approach, the eGFP protein, which would remain within the cell cytoplasm and nucleus, can be used as a readout of active collagen IV alpha 1 synthesis. Furthermore, any influence of an eGFP fusion upon folding of collagen IV alpha 1 monomers is circumvented. To generate a *Col4a1-P2A-eGFP* allele, we used CRISPR/Cas9 targeting to introduce a double-stranded DNA break in the terminal exon 52 of the *Col4a1* gene that contains the *Col4a1* stop codon (Figure 1a). Targeting was performed in fertilized eggs in the presence of a long single-stranded DNA donor to introduce the *P2A-eGFP* coding sequence after the last *Col4a1* codon

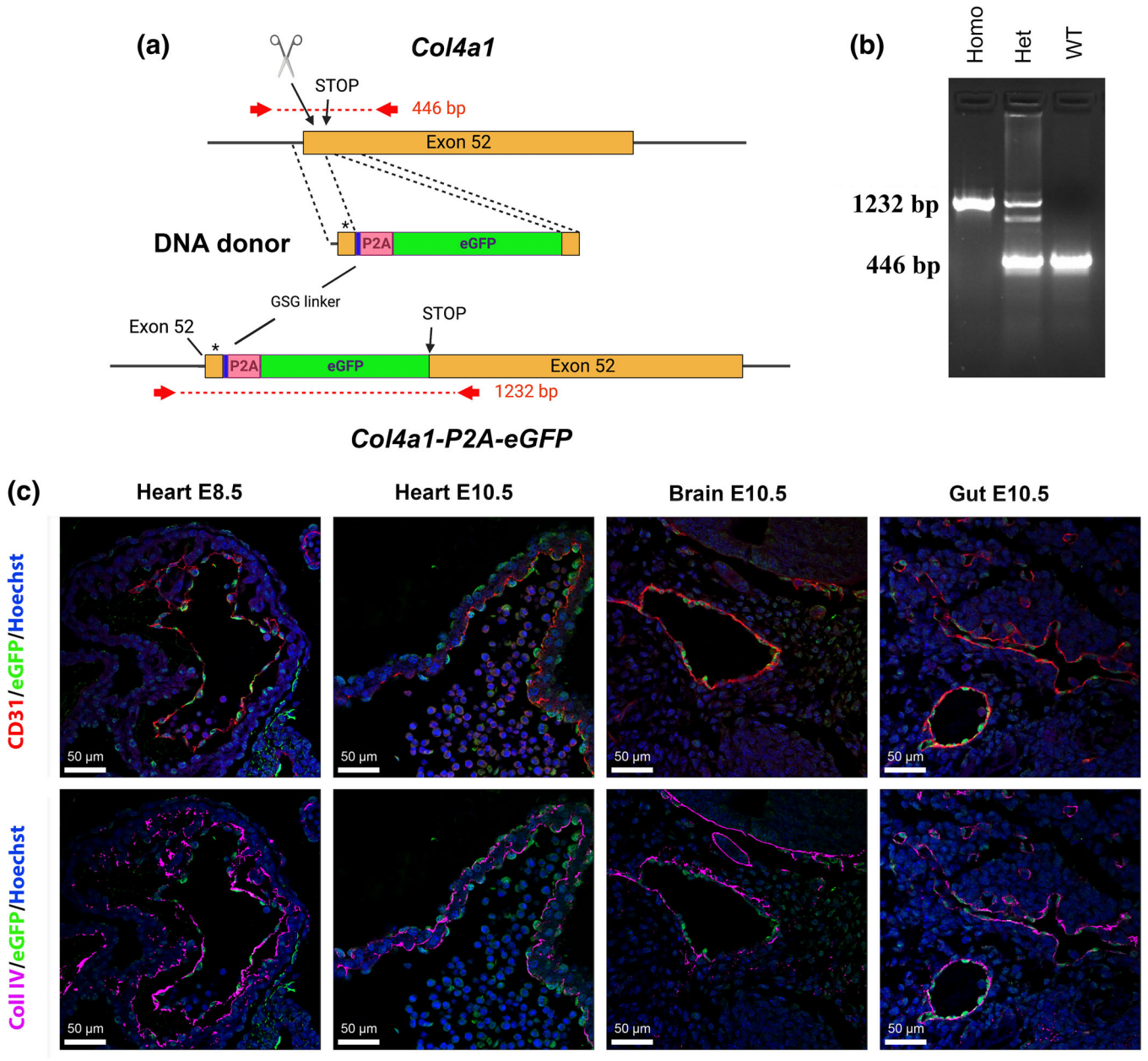


FIGURE 1 Generation and genotyping of Col4a1-P2A-eGFP mice. (a) Top, CRISPR/Cas9 was used to induce a double-stranded DNA break into exon 52 of the *Col4a1* locus, 5′ of the *Col4a1* stop codon. Middle, A long single-stranded DNA donor with indicated *Col4a1* 5′ and 3′ homology arms (solid line and orange) was used to introduce DNA encoding a P2A peptide (pink, preceded by a GSG linker in blue) and eGFP (green) immediately 3′ to and in frame with the terminal *Col4a1* codon. An asterisk denotes conservative mutations in the DNA donor to block Cas9 cutting. Bottom, organization of the targeted *Col4a1-P2A-eGFP* allele showing the position of the stop codon 3′ of the *eGFP* coding sequence. Location of primers used to distinguish *Col4a1* and *Col4a1-P2A-eGFP* alleles are indicated in red. (b) PCR genotyping of tail DNA using primers indicated in (a). Shown are examples of wild-type mice and mice heterozygous and homozygous for the *Col4a1-P2A-eGFP* allele. (c) Shown are tissue sections of E8.5 and E10.5 Col4a1-P2A-eGFP embryos stained with anti-GFP (green), anti-CD31 (red), and anti-collagen IV (purple) antibodies and Hoechst (blue). Note active synthesis of collagen IV alpha 1 in EC of endocardium, and in blood vessels of the developing brain and gut.

by homology-directed repair (Figure 1a). Of 27 live born mice, PCR screening identified three possible heterozygous Col4a1-P2A-eGFP founders that were confirmed by Sanger sequencing. However, only one of these founders survived to adulthood and was able to transmit the targeted allele. Sanger sequencing identified indels in the non-targeted homologous *Col4a1* allele in each founder. Since indels occurred in the terminal *Col4a1* exon it is likely that they resulted in the expression of dominant negative forms of collagen IV alpha 1 that impacted collagen IV alpha 1 protomer assembly, hence accounting for the lethality. Mice that inherited the *Col4a1-P2A-eGFP* allele from the surviving founder showed normal development and no increased lethality in either heterozygous or homozygous form.

For experiments that evaluated eGFP expression in EC during development, we set up timed matings between heterozygous Col4a1-P2A-eGFP mice and wild-type mice. Embryos were harvested at 2 day intervals from E8.5 to E18.5 of development and genotype was determined by PCR (Figure 1b). As negative controls, we used littermate wild-type embryos. In initial experiments, we examined frozen sections prepared from paraformaldehyde-fixed embryos for native GFP fluorescence. However, fluorescence could not be detected, most likely because the expression level of GFP was below a detection threshold. Therefore, in subsequent experiments, we stained sections from formalin-fixed paraffin-embedded embryos with anti-GFP antibodies to increase detection sensitivity. Using this approach, eGFP was readily detected within EC that were identified by co-staining for the CD31 marker (Figures 1–4).

Tissues examined included heart, liver, lung, intestine, kidney, brain and skin. In general, expression of eGFP within EC of organs was first detected from the time of onset of organogenesis. Thus, in E8.5–E10.5 embryos, eGFP-positive EC could be identified within EC of the endocardium of the developing heart and within blood vessels of the developing gut and brain (Figure 1c and Table 1). At E12.5 and E14.5, eGFP-positive EC were identified in the same structures as well as in EC of blood vessels of the heart myocardium, liver, lung, kidney and skin (Figure 2, Table 1 and data not shown). In all examined organs, expression of eGFP in EC was maintained though E16.5 (Figure 3), although in some organs such as the heart, lung and kidney, the percentage of eGFP-positive EC within blood vessels was substantially reduced compared to earlier time points (Table 1). In addition to EC, vascular smooth muscle cells (SMC) have been reported to synthesize collagen IV (Hospelhorn et al., 1992). However, using smooth muscle actin (SMA) as a marker of SMC, we were unable to identify any eGFP-positive SMC in the vasculature of any organ during embryonic development, for example, in vessels of the E10.5 gut

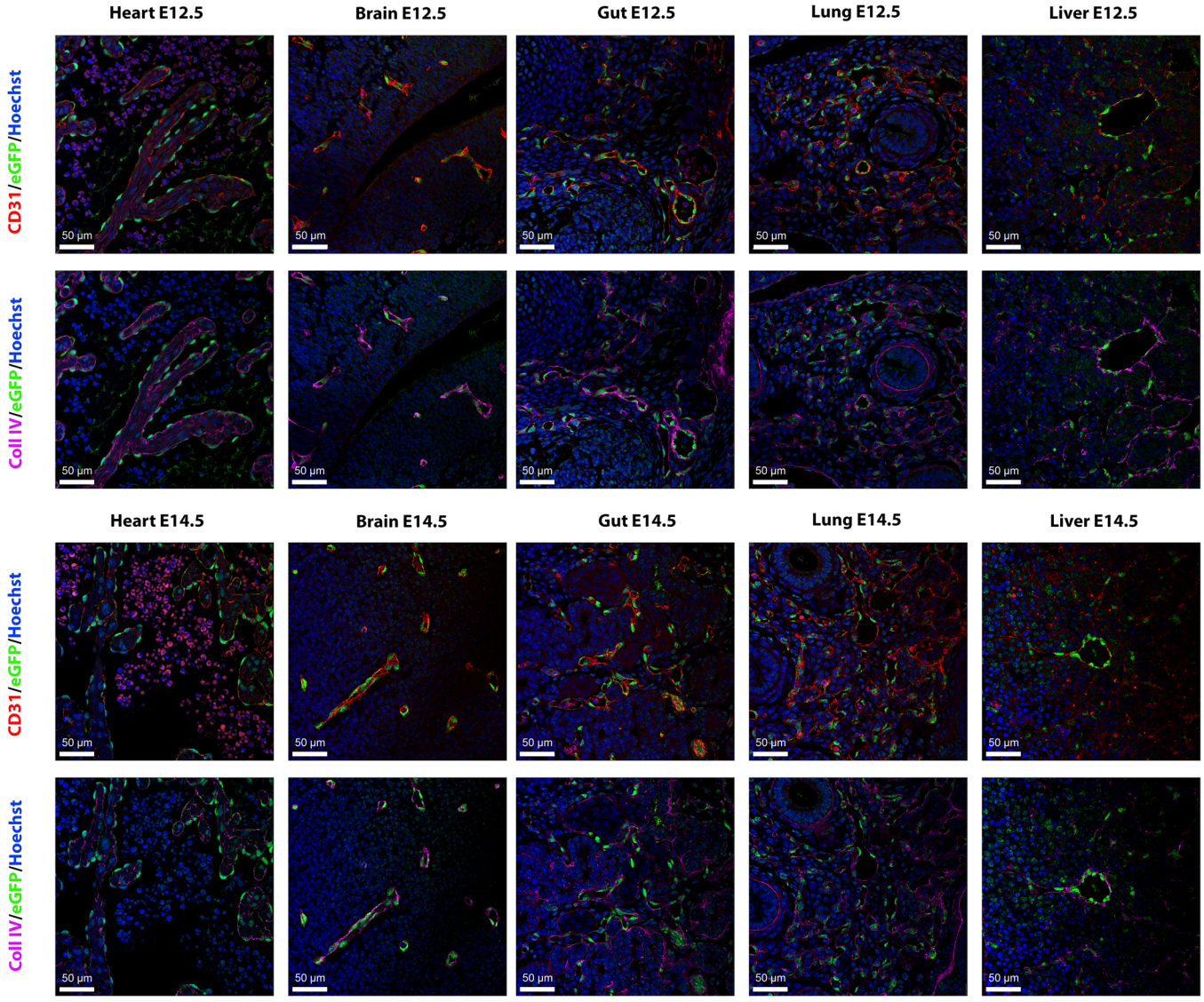


FIGURE 2 EC expression of collagen IV alpha 1 in mid-late gestation embryos. Shown are tissue sections of E12.5 and E14.5 Col4a1-P2A-eGFP embryos stained with anti-GFP (green), anti-CD31 (red), and anti-collagen IV (purple) antibodies and Hoechst (blue). Note active synthesis of collagen IV alpha 1 in EC of endocardium, and in blood vessels of the developing brain, gut, lung and liver.

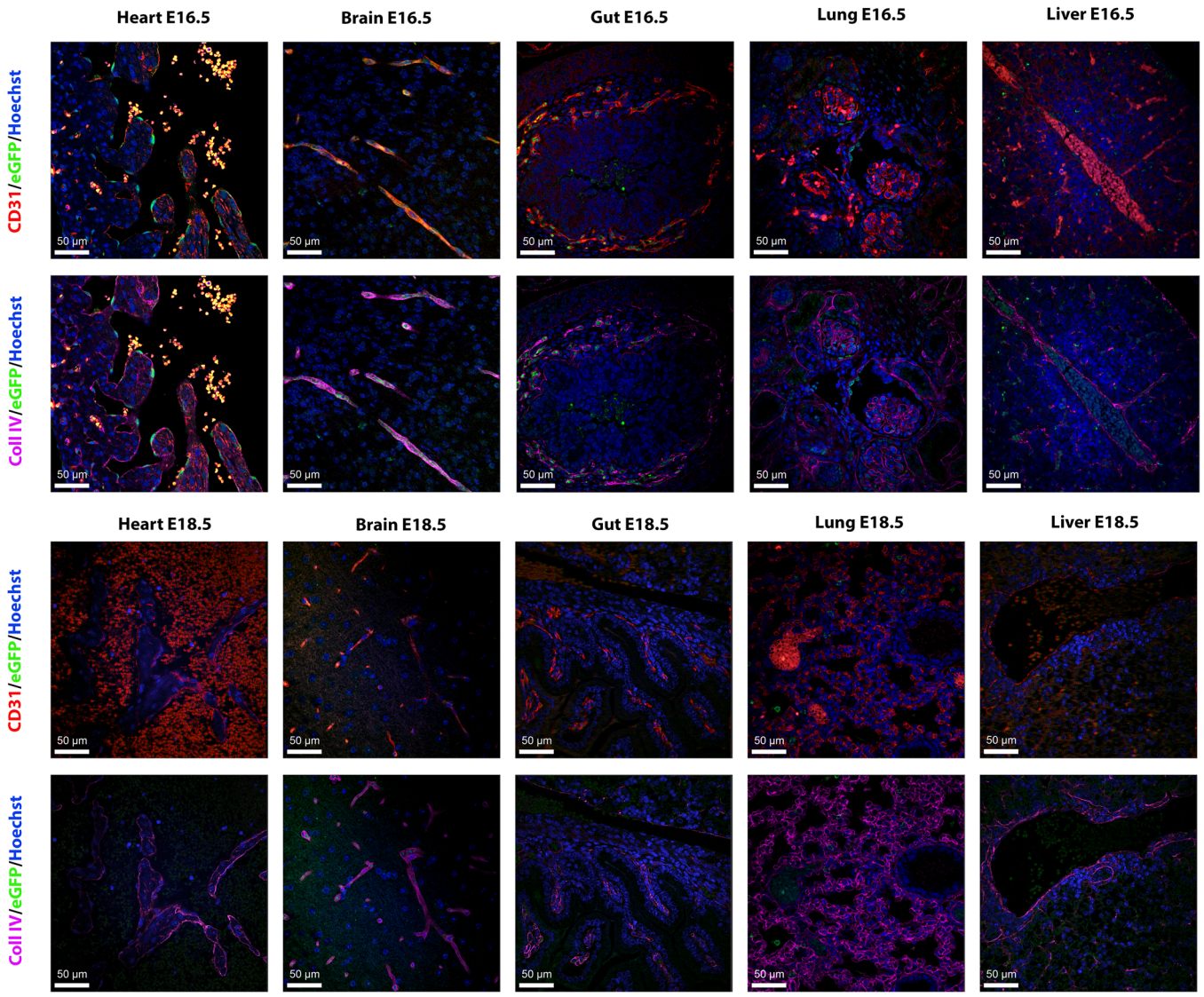


FIGURE 3 EC expression of collagen IV alpha 1 in late gestation embryos. Shown are tissue sections of E16.5 and E18.5 Col4a1-P2A-eGFP embryos stained with anti-GFP (green), anti-CD31 (red), and anti-collagen IV (purple) antibodies and Hoechst (blue). Note active synthesis of collagen IV alpha 1 in EC of all examined organs at E16.5 but absence of detectable collagen IV alpha 1 synthesis at E18.5.

(Figure 4). Strikingly, by E18.5 of development, eGFP-positive EC could also not be detected in any of the examined organs (Figure 3 and Table 1). Furthermore, eGFP-positive EC were not detected in any organs of adult mice (Figure 5, Table 1).

The finding that EC synthesis of collagen IV alpha 1 is mostly confined to embryonic development is consistent with and provides at least part of an explanation for the temporally-restricted blood vascular phenotypes of mice induced to express mutant collagen IV alpha 1 or to lose expression of RASA1 or EPHB4 (Alavi et al., 2016; Chen et al., 2019, 2022, 2023; Jeanne et al., 2015; Jeanne & Gould, 2017; Kuo et al., 2012; Lapinski et al., 2012, 2017; van Agtmael et al., 2010). In addition, findings provide a basis for the occurrence of vascular lesions at birth or in early childhood in most patients with inherited mutations in *COL4A1*, *COL4A2*, *RASA1* and *EPHB4* genes (Amyere et al., 2017; Jeanne & Gould, 2017; Kuo et al., 2012; Revencu et al., 2013). In mice, although disruption of *Rasa1* and *Ephb4* genes after birth does not result in spontaneous blood vascular abnormalities, both retinal and pathological angiogenesis are impaired (Chen et al., 2019, 2022, 2023). However, in these situations, it is expected that EC would re-engage in synthesis of collagen IV as part of the angiogenic process that would necessitate de novo production of basement membrane. Whether EC can be observed actively engaging in collagen IV synthesis during angiogenesis in our model has yet to be determined. It is also important to point out that our findings do not exclude the possibility that, after E18.5, some EC that are not engaged in angiogenesis may participate in low-level active collagen IV synthesis that is below a detection threshold in our model. Similarly, SMC may engage in low-level collagen IV synthesis that is below a detection threshold in our model. Nonetheless, findings with the current model are consistent with the

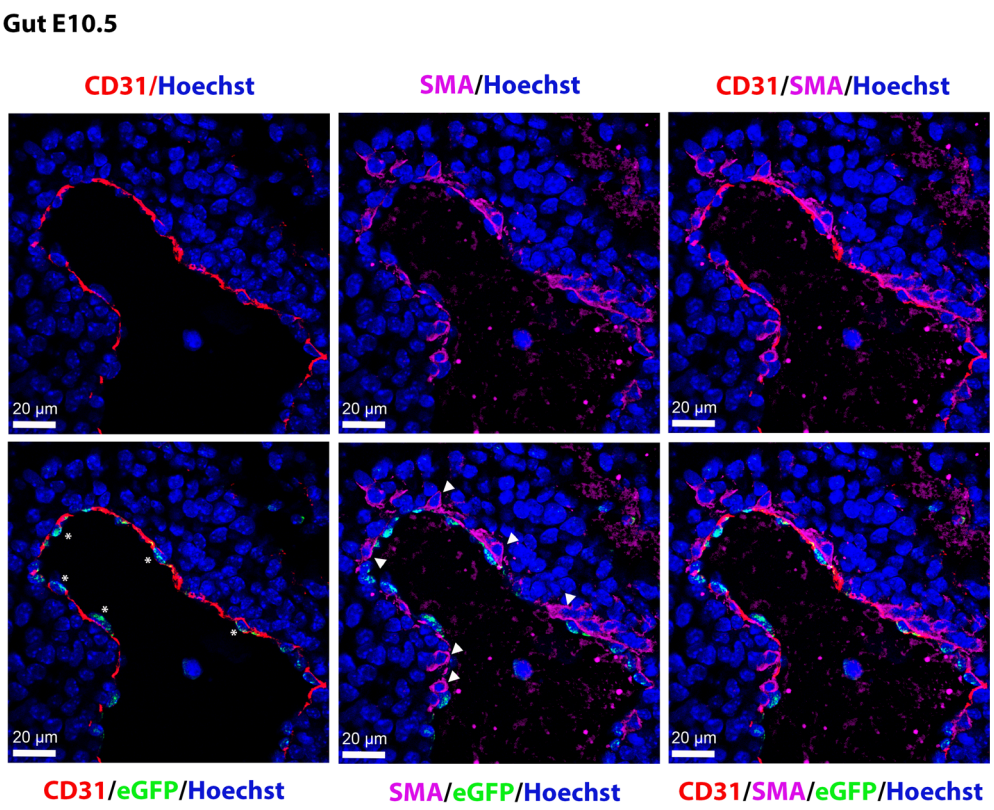


FIGURE 4 Predominant expression of collagen IV alpha 1 in EC of mid gestation embryos. Shown are sections of the primitive gut of E10.5 Col4a1-P2A-eGFP embryos stained with anti-GFP (green), anti-CD31 (red), and anti-SMA (purple) antibodies and Hoechst (blue). Note detectable active synthesis of collagen IV alpha 1 in EC (asterisks) but not SMC (arrowheads).

	E8.5	E10.5	E12.5	E14.5	E16.5	E18.5	Adult
Myocardium	–	–	90 ± 4	94 ± 3	58 ± 14	0	0
Endocardium	100 ± 0	89 ± 6	99 ± 1	100 ± 0	42 ± 24	0	0
Liver	–	–	70 ± 12	100 ± 0	50 ± 22	0	0
Lung	–	–	84 ± 5	80 ± 6	15 ± 7	0	0
Gut	–	67 ± 20	78 ± 8	73 ± 16	21 ± 13	0	0
Kidney	–	–	55 ± 0	64 ± 13	13 ± 5	0	0
Brain	–	95 ± 3	90 ± 8	84 ± 7	94 ± 4	0	0
Skin	–	–	87 ± 9	90 ± 7	80 ± 3	0	0

TABLE 1 Endothelial cell expression of eGFP in embryonic and adult Col4a1-P2A-eGFP mice.

Note: Shown is the mean ± 1 SEM of the percentage of vessels in each organ with at least one eGFP +ve EC (see Methods). (–) indicates that an organ is absent at the indicated stage of development.

notion that the majority of vascular collagen IV is synthesized by EC in mid to late gestation.

2 | METHODS

2.1 | Generation and genotyping of *Col4a1-P2A-eGFP* mice

A *Col4a1-P2A-eGFP* allele was generated by CRISPR/Cas9 targeting and homology-directed repair in fertilized eggs of C57BL/6 X SJL F2 hybrid mice using methods described previously (Quadros et al., 2017). The overall gene targeting strategy is shown in Figure 1. A sgRNA (5′ATGTTCTTCTCATGCACACT3′) was used to induce a Cas9 cut in the sixth codon upstream of the *Col4a1* termination codon in exon 52. The long single-stranded DNA donor comprised of a 5′ 101 bp homology arm (up to but not including the *Col4a1* termination codon) and a 3′ 101 bp homology arm (homologous to the sequence immediately downstream of the *Col4a1* termination codon) in between which was inserted DNA encoding a GSG linker, a P2A peptide from porcine teschovirus-1 and eGFP including a termination codon (Cormack et al., 1996; Kim et al., 2011; Quadros et al., 2017). The DNA sequence in the 5′ homology arm corresponding to the sgRNA recognition site was mutated to prevent recutting by Cas9. Eggs were micro-injected into C57BL76 mice to produce Col4a1-P2A-eGFP founder mice.

Founders were initially identified by PCR of tail DNA using forward (5′ AGAACCACAGACCCCAGTTG3′) and reverse (5′AGCGGG

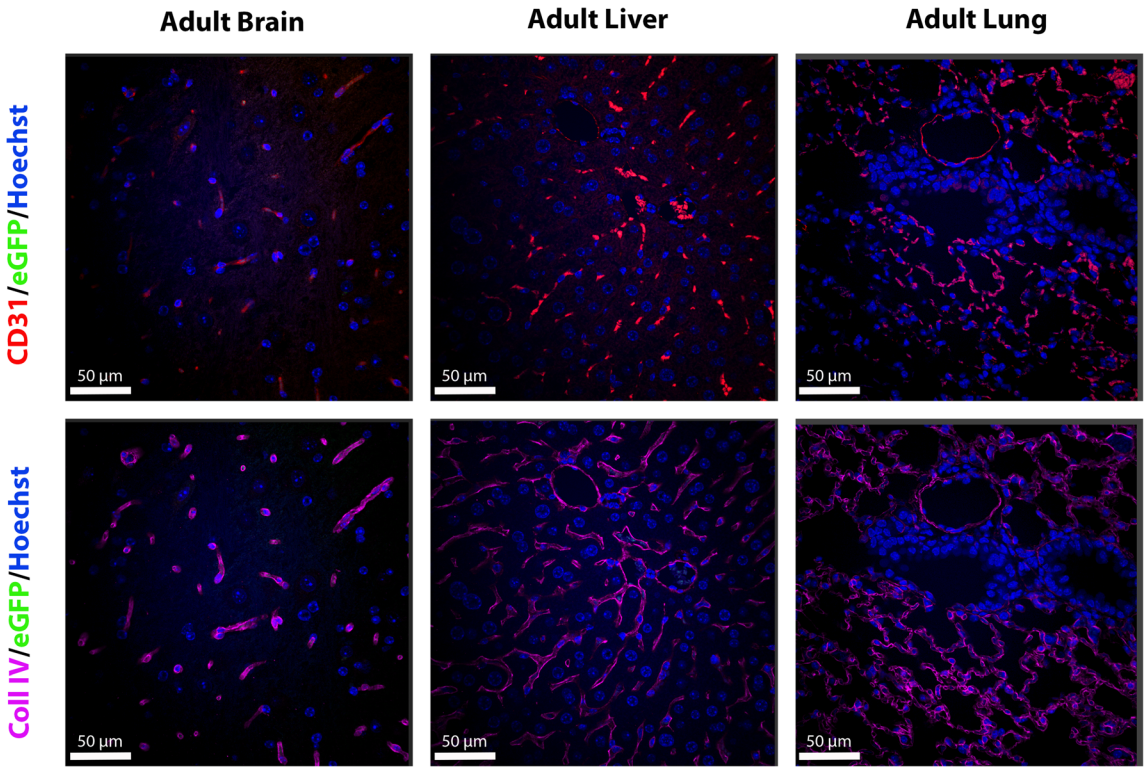


FIGURE 5 Absence of detectable expression of collagen IV alpha 1 in adult tissues. Shown are tissue sections of 3-month-old adult Col4a1-P2A-eGFP mice stained with anti-GFP (green), anti-CD31 (red), and anti-collagen IV (purple) antibodies and Hoechst (blue). Note absence of detectable active synthesis of collagen IV alpha 1 in adult tissues.

GTGTGTTAGTTACG3′) primers based upstream and downstream of the 5′ and 3′ DNA donor homology arms respectively (Figure 1a). Intended targeting was confirmed by Sanger sequencing of PCR products. Founder mice were subsequently bred with C57BL/6 mice to achieve germline transmission of the *Col4a1-P2A-eGFP* allele. Following germline transmission, mice were crossed with C57BL/6 mice to generate heterozygous Col4a1-P2A-eGFP mice and wild type controls for use in experiments. Genotyping used the same primers to identify founders. Bands generated from the *Col4a1-P2A-eGFP* allele and wild type alleles are readily distinguished (see Figure 1b). PCRs were performed using GoTag green master mix (Promega, Madison, WI, USA) with final primer concentrations of 0.25 µM each. Cycling conditions were an initial denaturation step at 95°C for 2 min, an amplification step consisting of 35 cycles of denaturation (30 s at 95°C), annealing (30 s at 56°C) and elongation (50 s at 72°C), followed by a final extension at 72°C for 10 min.

All experiments performed with mice followed the University of Michigan guidelines and were approved by the university committee on the use and care of animals.

2.2 | Tissue preparation

Timed matings were set up to generate embryos at different stages of development from E8.5 to E18.5. The morning the vaginal plug was seen was designated E0.5. Embryos were surgically removed from euthanized dams and were fixed in 10% formalin for 24 h and subsequently embedded in paraffin. In addition, organs (lungs, heart, brain, liver, kidneys, skin) were recovered from 3-month-old Col4a1-P2A-eGFP mice and wild-type controls and processed similarly. Sections (5 µm) of the formalin-fixed paraffin-embedded tissues (FFPE) were prepared and subsequently stained as indicated below.

2.3 | Immunofluorescence staining

FFPE sections were deparaffinized using xylene and rehydrated using ethanol and then treated with 3% hydrogen peroxide for 30 min. Slides were then boiled in Diva Decloaker solution (Biocare Medical, CA, USA) for 30 min for antigen retrieval. Blocking was performed by incubation in 0.1% Triton X-100 in PBS with 10% donkey serum followed by incubation with primary antibody against GFP (chicken anti-mouse GFP, Novus Biologicals, NB100-1614; 1:200 dilution) for 1 h. After washing, sections were incubated with secondary donkey anti-chicken biotin SP antibody (Jackson Immunoresearch, 703-065-155; 1:1000 dilution) for 1 h before additional washing and incubation with streptavidin-HRP (Akoya Biosciences, NEL741001KT) for 30 min. After additional washes, fluorescein (Akoya Biosciences, NEL741001KT; 1:500) was added to the slides and incubated for 1 min. Monoclonal antibodies against CD31 (rat anti-mouse CD31,

Dianova SZ31; 1:50 dilution), collagen IV (goat anti-mouse collagen IV, Southern Biotech, 1340-1; 1:100 dilution), and SMA (goat anti-alpha smooth muscle actin, Novus Biologicals, NB300-978; 1:100 dilution) were incubated with sections overnight before washing and incubation with secondary donkey anti-rat Alexa Fluor 594 antibody (Jackson Immunoresearch, 712-586-150; 1:200 dilution), donkey anti-goat Alexa Fluor 647 antibody (Jackson Immunoresearch, 705-606-147; 1:200 dilution) and Hoechst (1:1000) for 30 min and washed. After final washes, slides were mounted with ProLong Gold Antifade (ThermoFisher).

2.4 | Imaging

For quantitation, slides were examined on a BX60 fluorescent microscope. For each time point and organ, three randomly selected fields of 20 μm × 22 μm from at least two embryos were imaged and the percentage of vessels with at least one eGFP positive EC was determined for each field. To obtain representative high-quality images (Figures 1–5) slides were viewed and photographed on a Nikon A1R confocal microscope (Nikon).

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DATA AVAILABILITY STATEMENT

All data that support the findings of the study are available within the article.

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