

Review



Rapid Way to Generate Mouse Models for *In Vivo* Studies of the Endothelium

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ABSTRACT

A single layer of squamous endothelial cells (ECs), the endothelium, regulates the flow of substance and fluid into and out of a tissue. The endothelium is also involved in vasculogenesis, the formation of new blood vessels, which is a crucial process for organ development in the embryo and fetus. Because most murine mutations of genes involved in EC development cause early embryo lethality, EC-specific conditional knockout (cKO) mouse models are indispensable for *in vivo* studies. cKO mice including the floxed allele can be generated through advanced approaches including embryonic stem cell-mediated gene targeting or the CRISPR/Cas system. EC-specific mouse models can be generated through further breeding of floxed mice with a Cre driver line, the latest information of which is available in the Jackson Cre Repository or the EUCOMMTOOLS project. Because it takes a long time (generally 1–2 years) to generate EC-specific mouse models, researchers must thoroughly design and plan a breeding strategy before full-scale mouse experiments, which saves time and money for *in vivo* study. In summary, revolutionary technical advances in embryo manipulation and assisted reproduction technologies have made it easier to generate EC-specific mouse models, which have been used as essential resources for *in vivo* studies of the endothelium.

Keywords: Endothelium; Mice, knockout; Cre recombinase

INTRODUCTION

The endothelium forms the interface between circulating blood or lymph in the lumen and the rest of the vessel wall. The endothelial cells (ECs) that line the interior surface of blood and lymphatic vessels are the barrier between tissue and vessels and regulate the flow of fluid and substances into and out of a tissue.^{1,2} Vascular ECs are part of the circulatory system including the heart, arteries, veins, and capillaries.³ The endocardium is the endothelium of the interior surface of the heart chambers, the impairment of which can critically damage health. The loss of homeostasis of endothelial function, namely endothelial dysfunction, is a hallmark of vascular disease and is frequently a crucial early step in atherosclerosis.⁴ Dysfunctional endothelium frequently appears in patients with diabetes mellitus, hypertension, coronary artery disease, and hypercholesterolemia, as well as smokers,

finally leading to atherosclerosis and thrombosis. Dysfunctions of the endothelium are also predictive markers of future adverse cardiovascular events⁵ and present in inflammatory diseases, including rheumatoid arthritis and systemic lupus erythematosus.^{6,7}

Hemangioblasts differentiate into several distinct cell lineages which finally make up of the endothelium.⁸ Several cell lineages form the network of the heart, blood vessels, circulating blood cells, and supporting tissues. For normal cardiovascular development, a range of several signal transduction pathways are quantitatively and/or temporally regulated, including basic fibroblast growth factor, vascular endothelial growth factor (VEGF), angiopoietin/Tie, ephrin/Eph, and the DSL/Notch pathways. All of these pathways have been studied *in vivo* using various genetically engineered mouse (GEM) models, including conventional/conditional knockout (cKO), knock-in, and transgenic mice.

Most deletion mutations of genes involved in EC development have been shown to cause embryo lethality between embryonic day (E) 9.5 and E11.5.⁹ This makes it difficult for researchers to investigate the function of these genes in later development and in neonates, including their roles in determining vessel identity, hematopoiesis, and endothelial-mesenchymal transition. To overcome this problem, cKO strategies were developed about 20 years ago, with which numerous cKO models have been generated, analyzed, and then reported.¹⁰ In general, to generate cKO mouse models, locus of X-over P1 (*loxP*) sites are inserted into introns flanking critical exon(s) or in intergenic regions or flanking regulatory regions such as promoters and enhancers. To conditionally inactivate the target gene, Cre recombinase-expressing driver mice must be crossed with a cKO mouse model. In Cre recombinase-expressing cells, the protein recognizes *loxP* sites and removes the intervening exon(s). In this way, the targeted gene can be conditionally and functionally inactivated in the specific cell or tissue where the Cre recombinase is expressed.

Cre-*loxP* recombination was developed by Dr. Brian Sauer in the late 1980s.^{11,12} Dr. Jamey Marth and colleagues applied this Cre-*loxP* recombination system to mouse models and demonstrated that *loxP*-flanked genomic DNA sequences could be deleted in a highly efficient manner.¹³ *loxP* originates from bacteriophage P1 and consists of 34 bp, composed of two 13-bp recognition sequences and an 8-bp spacer/core sequence, which is palindromic (inverted repeated) and has an orientation. In addition to the wild-type *loxP* sequence, there are several *loxP* mutants, including *lox511*, *lox5171*, and *lox2272*.¹⁴ These mutant *loxP* sequences allow us to generate more complicated mouse models in which the targeted genes are controlled in a more sophisticated and delicate manner.

In addition to wild-type Cre recombinase, an inducible version of Cre recombinase, Cre/ERT2, has been developed.¹⁵ Cre/ERT2 was created by fusing Cre recombinase cDNA with a mutated version of the estrogen receptor ligand binding domain (ERT2). This fusion creates a specific receptor for the tamoxifen metabolite 4-hydroxytamoxifen (4-OHT). When Cre/ERT2 protein is expressed, the protein remains sequestered in the cytoplasm in complex with a heat shock protein (Hsp90). When tamoxifen is added, 4-OHT binds to ERT2, which interrupts the interaction with Hsp90 and allows Cre/ERT2 to translocate to the nucleus. Translocated Cre/ERT2 can recognize *loxP* sequences in the nucleus. As such, tamoxifen administration can induce Cre recombination temporally or spatially with minimal toxicity. These Cre/ERT2 mouse models are especially useful in studies on the endothelium and vasculogenesis, where conventional gene inactivation frequently induces lethality during early embryo development.

Among *Cre* transgenic mice, even if the same DNA construct is used in the generation of transgenic mice, it has been shown to create different *Cre* expression patterns.^{16,17} The difference in *Cre* expression in a mouse model could have critical effects regarding the interpretation of *in vivo* phenotypes. Therefore, when we design *in vivo* experiments using *Cre* transgenic mouse models, the origin of the *Cre* alleles and their expression patterns must be considered. In addition, it is recommended that Cre protein expression or recombination activity is assessed before starting a full-scale animal experiment by using Cre reporter mice or direct analysis of Cre expression/activity.

Although Cre/ERT2 fusion protein is a powerful tool in studies on the endothelium and vasculogenesis, the protein also has drawbacks.^{18,19} In addition to the differences in expression and limitations regarding characterization for wild-type *Cre* transgenic mouse models, there is a difference in the susceptibility of floxed alleles to Cre/ERT2, which is dependent on the dosage of tamoxifen and the administration method. Cre/ERT2 recombinase is also known to have the potential to produce unexpected Cre activity in the absence of tamoxifen which is called “leakiness” and should be considered during the design of experiments. Taking these findings together, it is recommended to determine the recombination efficiency and timing in target tissues for each targeted allele and the dosage and methods of tamoxifen administration before starting a full-scale animal experiment.

Although the *Cre/loxP* system is the most commonly used system for controlling gene expression in studies of the endothelium, the tetracycline-inducible system (tet-On and tet-Off) is also used for cell type-/tissue-specific regulation of a target gene or transgene in mice.^{20,21} While tetracycline or doxycycline, a non-toxic, synthetic tetracycline derivative, can inhibit a fusion protein composed of the VP16 transactivator and the tetracycline repressor (tTA) in the tet-Off system, they activate a fusion protein of the VP16 and the reverse tetracycline repressor (rtTA) in the tet-On system. Adding tetracycline causes a conformational change of tTA/rtTA, which increases the affinity for a unique 19 bp DNA binding site (tetO sequence) and enhances the recruitment of the transcriptional machinery. Adding or withdrawing tetracycline or doxycycline regulates the activity of the transactivator, which controls transgene expression driven by a promoter containing the tet-operator (tetO) sequence.

While the key advantages of the tetracycline-inducible system is graded and reversible transcriptional regulation by tetracycline or doxycycline, it has the disadvantage of leakiness of transgene transcription, in that the tTA or rtTA protein has some affinity for the tetO binding sequence in the absence of an inducer.²² Although extensive efforts have been made to optimize the sensitivity to an inducer, including increasing such sensitivity, decreasing basal activity, optimizing the tTA/rtTA target promoter, and introducing such positive feedback regulation of tTA/rtTA gene transcription, there is still a high risk of leakiness which continues to limit the usefulness of the tetracycline-inducible system.^{23,24}

Together, *Cre/loxP* and/or the tet-On/Off system have commonly used to achieve cell type-/tissue-specific transgene expression or target gene regulation. A strategy combining the *Cre/loxP* and the tet-On/Off system has been frequently used in the same cell or tissues in studies on the endothelium and vasculogenesis, which synergizes and enhances the specificity and tight regulation of target gene expression.

CURRENT TRENDS FOR GENERATING cKO MICE

Just a few years ago, mouse embryonic stem (mES) cells were used for the generation of conventional KO and cKO mice through homologous recombination with embryo manipulation, microinjection, and assisted reproductive technologies (ARTs), which were developed in the 1980s and are still considered as standard methods.¹⁰

Since 2005, large-scale and high-throughput projects for the generation of gene-targeting constructs and mutant ES cell clones for over 20,000 protein-coding murine genes have been launched, including the Knockout Mouse Project (KOMP), the European Conditional Mouse Mutagenesis (EUCOMM) Program, and the Internal Knockout Mouse Consortium (IKMC).²⁵ These projects utilized C57BL/6N mouse ES cells, so all mice generated were in the C57BL/6N background. To date, over 5,000 knockout mice have been generated using these ES cell clones, which are readily available in the repositories of the International Mouse Phenotyping Consortium (IMPC).²⁶

Recently genome editing technologies including clustered regularly interspaced short palindromic repeats/CRISPR-associated protein (CRISPR/Cas) have revolutionized the field of embryo manipulation and the generation of genetically modified organisms. Transgenesis, and gene knockout and knock-in can be readily achieved by directly injecting or electroporating Cas9 protein/mRNA, single guide RNA (sgRNA), and/or donor DNA into mouse zygotes.^{27,28} These genome editing technologies have resulted in dramatic improvements in the time and costs associated with generating GEM including cKO.

GENERATING FLOXED (*tm1c*) MICE FROM KNOCKOUT-FIRST (*tm1a*) MICE

The original KOMP/EUCOMM strategy involved “knockout-first” conditional allele targeting which allows both conditional targeting of a gene of interest and report-tagging (**Fig. 1A**).²⁶ An *IRES-lacZ* cassette is located 5' of a *loxP*-flanked, promoter-driven, neomycin resistance cassette, which lies upstream of the critical/targeted exon. The *IRES-lacZ* cassette includes the mouse En2 splice acceptor and the SV40 polyadenylation sequence, which can effectively create null alleles in mice. A third *loxP* is located after the critical exon to facilitate its removal and finally inactivate the target gene. This allele is referred to as “targeted mutation 1a” (*tm1a*) and is designated as “knockout-first”, as the insertion of the *IRES-lacZ* transposon could itself disrupt splicing of the target gene (**Fig. 1A**). Although the *tm1a* allele can have its own phenotype and not be used for conditional mouse experiments, the allele is very versatile because it can be converted into the *tm1b* allele for conventional KO and the *tm1c* allele for cKO.

To produce the floxed (*tm1c*) allele, the *lacZ* and *neomycin* cassette must be removed using a site-specific Flp recombinase that recognizes *FRT* sequences. When heterozygous *tm1a* mice are crossed with transgenic C57BL/6N mice expressing an enhanced variant of *Saccharomyces cerevisiae* FLP1 recombinase (FlpE) in all tissues, the offspring have both the *tm1a* allele and the *FlpE* allele and the *FRT*-flanked region of *tm1a* is excised and recombined. Upon crossing with wild-type mice, the *lacZ* and *neomycin*-resistance cassette is removed and the target gene expression is restored. Although the critical exon(s) is flanked by 2 *loxP* sequences, the *tm1c* mouse is expected to be phenotypically the same as wild-type mice and to have normal

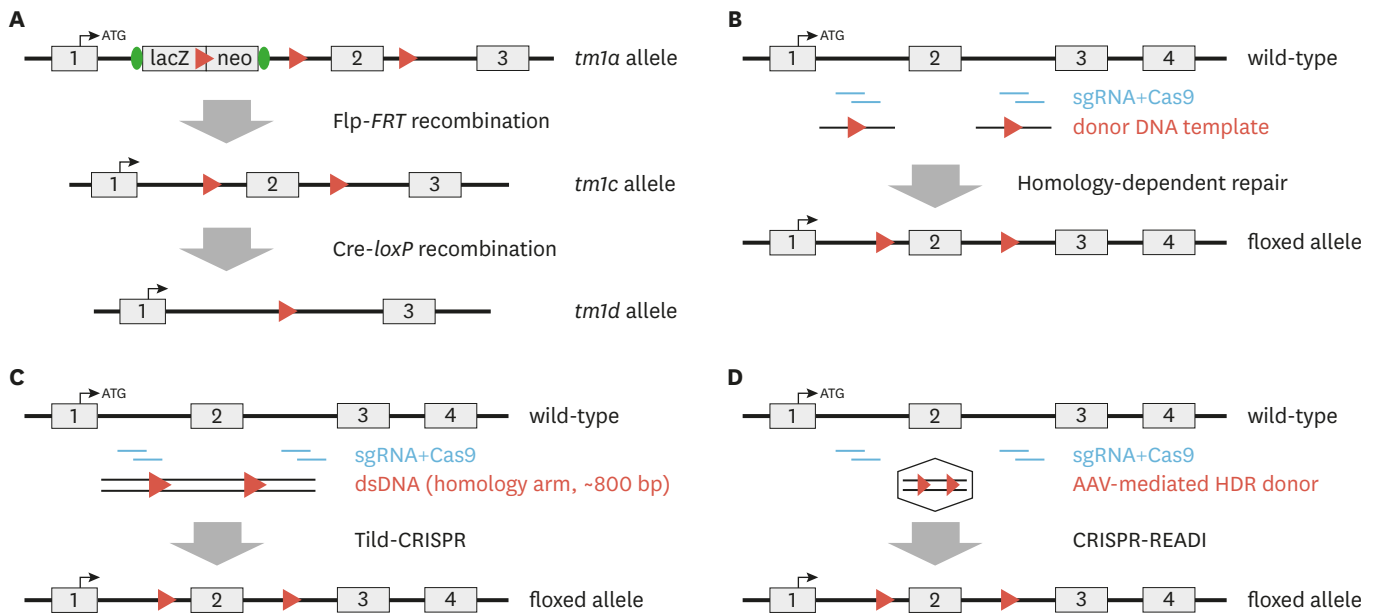


Fig. 1. Several strategies for generating cKO mice. Schematic diagrams for floxed alleles generated from the *tm1a* allele and through the CRISPR/Cas9 system. (A) Construct of *tm1a*, *tm1c*, and *tm1d* alleles. *tm1a* allele has the *lacZ-neomycin* cassette and 2 *loxP* sequences flanking critical exon(s) of the target gene. Flp-*FRT* recombination in the *tm1a* allele results in creation of the *tm1c* allele, with which the *tm1d* allele could be generated following Cre-*loxP* recombination. Rectangle, exon; triangle, *loxP* sequence; ellipse, *FRT* sequence. (B) The 2-donor methods. Through simultaneous or serial insertion of 2 *loxP* sequences using 2 sgRNAs and 2 oligodeoxynucleotides as donors, floxed mice could be generated. (C) Tild-CRISPR. Injection of transgene donor with 800 bp homology arms into a mouse zygote could generate cKO mice with 2 *loxP* sequences. (D) CRISPR-READI. This approach involves combining AAV-mediated donor delivery with Cas9/sgRNA RNP electroporation. cKO, conditional knockout; *tm1*, targeted mutation 1; CRISPR/Cas, clustered regularly interspaced short palindromic repeats/CRISPR-associated protein; Tild-CRISPR, targeted integration with linearized dsDNA-CRISPR; *loxP*, locus of X-over P1; CRISPR-READI, CRISPR RNA electroporation and AAV donor injection; sgRNA, single guide RNA; HDR, homology-directed repair.

splicing during mRNA processing. To create an EC-specific knockout of the target gene, cell type- or tissue-specific Cre driver mice of one's choosing can be used.

GENERATING cKO MICE WITH CRISPR/Cas9 TECHNOLOGY

The CRISPR/Cas9 system has recently been developed and revolutionized the way of generating genetically modified animal models including mouse models.²⁷⁻²⁹ Through the direct injection of Cas9 protein/mRNA, sgRNA, and/or donor DNA into embryonic cells, various different GEM models can be generated. When sgRNA and Cas9 endonuclease are injected into cells, the guide RNA comprises a region complementary to the target gene and Cas9 protein having 2 nuclease domains can generate a double-stranded DNA break in the genome. This double-stranded break would be preferentially repaired by non-homologous end-joining (NHEJ), potentially resulting in insertions or deletions (indels). Most of these indel mutations generate a translation stop codon behind the target sequence, causing silencing of the target gene. The simplicity and efficiency of this technology led it to surpass the conventional gene targeting methods and dramatically saved time and money in creating GEM models, including conventional/conditional KO, as well as transgenic and reporter mice.

Since publication of the first report describing GEM can be generated with CRISPR/Cas9 technology, various attempts have been made to generate cKO mice. Among them, 3 strategies for generating cKO mice are introduced in this review, including the 2-donor methods,^{30,31} Tild-CRISPR,³² and CRISPR-READI.³³

THE 2-DONOR METHODS

In the early stages of the technological development of genome editing, cKO mice were generated by the simultaneous insertion of 2 *loxP* sequences using 2 sgRNAs and 2 oligodeoxynucleotides (ODNs) as donors (**Fig. 1B**).^{30,31} However, when these methods were used, the success rate of obtaining cKO with 2 *loxP* sequences on the same chromosome was very low. The reason for the low efficiency of generating cKO mice may have been that the efficiency of ODN-mediated homologous recombination is much lower than that of NHEJ and double-stranded DNA breaks in one allele at the same time can easily lead to deletion of the target region. Most of the mice with one correct *loxP* sequence exhibit indel mutations at the other CRISPR/Cas9 cutting site. These mice cannot be used for insertion of the second *loxP* sequence because of disruption of the sequence complementary to the second sgRNA. Even when 2 *loxP* sites are successfully inserted, the 2 *loxP* sequences are located in different alleles in 50% of the founders. Other mutant mice have been found in which the entire sequence between the 2 cutting sites is deleted at a high frequency.

Alternatively, sequential methods are also commonly used, in which 2 *loxP* sequences are inserted in 2 steps.^{34,35} In the first step, mice with one *loxP* site are generated and then, using these carrier mice as embryo donors, a second *loxP* sequence is inserted through microinjection or electroporation. Although such sequential methods require one or 2 additional generations of breeding (if breeding is required to expand the colony with one *loxP*) and thus take a long time (up to 1 year), they are still superior to the conventional embryonic stem cell-mediated method because there is no need for targeting vector construction, mES cell culture, chimera breeding, germline transmission, or the removal of a drug-resistance cassette.

TILD-CRISPR AND CRISPR-READI

Given the challenges of simultaneously inserting both *loxP* sites using 2 sgRNAs and 2 oligos, various CRISPR-mediated strategies have been developed for generating cKO mice. These include tissue-specific expression of Cas9 through Cre-activated gene expression³⁶ or tissue-specific promoters,³⁷ inserting a Cre-regulated artificial small intron into the gene,³⁸ electroporating the 2 *loxP* sites into zygotes and 2-cell embryos on 2 consecutive days,³³ and using a long single-stranded DNA that contains both *loxP* sites as a donor.^{39,40}

Among these alternatives, targeted integration with linearized dsDNA-CRISPR (Tild-CRISPR) is a method using transgene donor with 800-bp homology arm (**Fig. 1C**).³² Additional methods include efficient additions with ssDNA inserts-CRISPR (Easi-CRISPR), a method using a long single-stranded DNA as a DNA donor,^{39,40} and CRISPR RNA electroporation and AAV donor injection (CRISPR-READI), a method combining AAV-mediated donor delivery and Cas9/sgRNA electroporation (**Fig. 1D**).³³

Yao *et al.*³² reported the new methods of Tild-CRISPR for generating cKO mice (**Fig. 1C**). In this method, a PCR-amplified or precisely enzyme-cut transgene donor with 800 bp homology arms is injected with Cas9 mRNA and sgRNA into mouse zygotes. Using donor DNA having a critical exon flanked by 2 *loxP* sequences, cKO mice can be generated through Tild-CRISPR. The authors reported that this method was highly generalizable because it was proven to work well for 9 loci, including 4 loci (*Actb*, *Nanog*, *Sox2*, *Cdx2*) in targeting mouse embryos and 6 loci (*Cdx2*, *Dbh*, *Sp8*, *Nr3c2*, *Lhx6*, *Rosa26*) in generating knock-in mice. They suggested that,

compared with the previously reported Easi-CRISPR using ssDNA as a donor, the Tild-CRISPR method has 2 clear advantages: higher DNA knock-in efficiency than Easi-CRISPR and the lack of any size limitation for insertions. They proved that various insertions, from 0.8 to 6.0 kb, could be precisely integrated into different loci by Tild-CRISPR. Finally they suggested that, compared with previous targeting strategies, Tild-CRISPR showed much higher efficiency for generating conditional alleles in mouse embryos, as well as in brain tissues.

Chen *et al.*³³ developed the CRISPR-READI approach which combines AAV-mediated donor delivery with Cas9/sgRNA RNP electroporation (**Fig. 1D**). CRISPR-READI was shown to be applicable to most widely used knock-in strategies requiring donor lengths within the 4.9 kb AAV packing capacity. Using an AAV donor including a critical exon flanked by 2 *loxP* sequences, cKO mice can be generated through CRISPR-READI. With this method, the authors showed that large site-specific modifications could be made in the mouse genome with high efficiency and throughput. Overall, the authors suggested that CRISPR-READI was an efficient, high-throughput, microinjection-free approach for generating cKO mice.

CHOOSING *Cre* TRANSGENIC MICE FOR ENDOTHELIUM-SPECIFIC STUDIES

Over several decades, a number of *Cre* mouse lines expressing *Cre* recombinase in the whole body or restricted cells have been developed. Among these, cell type-specific *Cre* driver lines have fully potentiated cKO models. To generate EC-specific models, the first step is to determine which *Cre* line is suitable for your study.

Here, 2 representative *Cre* repositories are introduced: The JAX *Cre* Repository and The EUCOMMTOOLS project. The JAX *Cre* Repository aims to provide the scientific community with a centralized, comprehensive set of well-characterized *Cre* driver lines and related information resources (jax.org). The Jackson Laboratory offers over 300 *Cre* tool strains, including *Cre*-expressing strains, inducible *Cre* strains, and *Cre* reporter strains. The EUCOMMTOOLS project has generated a new inducible *Cre* driver line resource that is available to researchers via the INFRAFRONTIER repository (mousephenotype.org/data/order/creline). This resource includes over 220 *Cre* driver lines, in which tamoxifen-inducible *Cre* expression has replaced the coding elements of genes of the *tm1a* allele, ensuring restricted tissue expression of *Cre*. Restricted *Cre* expression has been characterized and confirmed in individual *Cre* driver mice. The background of all EUCOMMTOOL *Cre* driver mouse lines is C57BL/6N, which is recognized as a unique resource of IMPC.

EC-SPECIFIC TRANSGENIC/KNOCK-IN MICE

This review summarizes the most commonly used EC-specific mouse models. In constitutively active pan-endothelial *Cre* transgenic mice, *Cre* recombinase activity is driven by enhancer and promoter regions that are generally considered to be expressed in all EC population from early in development into adulthood.⁴¹

There are important difference between constitutively active pan-endothelial alleles, including wide variations in the expression in different vascular beds and in the onset of enhancer/promoter activity. Therefore, a detailed understanding of the precise expression

Table 1. Endothelial cell-specific transgenic or knock-in mouse models

Gene	Mouse line	Repository	States
<i>Tek-cre</i>	<i>Tg(Tek-cre)1Ywa</i>	RBRC, Jackson, EMMA	Embryo, sperm, live
<i>Tek-cre</i>	<i>Tg(Tek-cre)2352Rwng</i>	Jackson	Sperm
<i>Tek-cre</i>	<i>Tg(Tek-cre)12Flv</i>	Jackson	Live
<i>Tek-cre/ERT2</i>	<i>Tg(Tek-cre/ERT2)1Arnd</i>	EMMA	Embryo, live
<i>Tek-rtTA</i>	<i>Tg(Tek-rtTA, TRE-lacZ)1425Tpr</i>	Jackson	Sperm
<i>Tek-RFP</i>	<i>Tg(Tek-RFP, -cre)3Narl</i>	RMRC-NLAC	Sperm
<i>Cdh5-cre</i>	<i>Tg(Cdh5-cre)1Spe</i>	Jackson	Live
<i>Cdh5-cre</i>	<i>Tg(Cdh5-cre)7Mlia</i>	Jackson	Live
<i>Cdh5-cre</i>	<i>Tg(Cdh5-cre, GFP)21Msri</i>	EMMA	Embryo
<i>Cdh5-cre/ERT2</i>	<i>Tg(Cdh5-cre/ERT2)1Rha</i>	TAC	Live
<i>Cdh5-tTA</i>	<i>Tg(Cdh5-tTA)D5Lbjn</i>	Jackson	Sperm
<i>Kdr^{tm1(cre)}</i>	<i>Kdr^{tm1(cre)Sato}</i>	Jackson	Sperm
<i>Kdr-mCherry</i>	<i>Tg(Kdr-mCherry)1Medi</i>	Jackson	Sperm
<i>Pdgfb-cre/ERT2</i>	<i>Tg(Pdgfb-cre)1Frut</i>	Jackson	Live
<i>Bmx-cre.ERT2</i>	<i>Tg(Bmx-cre/ERT2)1Rha</i>	TAC	Embryo, sperm
<i>Apln-cre/ERT2</i>	<i>Apln^{tm1.1(cre/ERT2)Bzsh}</i>	Zhou's lab	
<i>Fabp4-cre</i>	<i>Tg(Fabp4-cre)1Rev</i>	Jackson	Embryo, sperm

RBRC, Riken BioResource Research Center; Jackson, the Jackson Laboratory; EMMA, the European Mouse Mutant Archive; Embryo, cryopreserved embryos; Sperm, cryopreserved sperm; Live, live mouse; RMRC-NLAC, the Rodent Model Resource Center-the NARL's National Laboratories Animal Center; TAC, Taconic Biosciences Inc.; Zhou's lab, Bin Zhou in University of Chinese Academy of Sciences, Shanghai, China (reference 70).

pattern of Cre recombinase is important when interpreting any phenotypes generated with these models. Here, the most frequently cited EC-specific Cre mouse lines and their availability are summarized in **Table 1**; additional mouse models with the EC-specific tetracycline inducible system or fluorescence expression are also introduced.

MOUSE LINES WITH *Tek/Tie2* PROMOTER

The *Tek* (TEK receptor tyrosine kinase, also referred to *Tie2*) promoter is the most commonly used in studies of the endothelium. *Tek/Tie2* encodes an angiopoietin receptor, a member of the receptor tyrosine kinase family, which is expressed in all ECs during early embryo development, as well as in adulthood.⁴²⁻⁴⁴ *Tek/Tie2* protein has a unique extracellular region that is composed of 2 immunoglobulin-like domains, 3 fibronectin type II repeats and 3 epidermal growth factor-like domains. The ligand (angiopoietin-1) binding to *Tek/Tie2* receptor mediates a signaling pathway of embryonic vascular development.

Studies have shown that *Tek^{-/-}* mice exhibit embryo lethality during early embryogenesis, impaired vascular branching in the embryo and yolk sac, abnormal cardiac development, and occasional hemorrhages.^{42,43,45-47}

About 20 years ago, Schlaeger *et al.*⁴⁸ reported that a combination of the *Tek/Tie2* promoter region and an intron enhancer sequence can drive strong EC-specific gene expression. Finally, 2 groups generated *Tek/Tie2-cre* transgenic mouse lines using *Cre* cDNA placed between a 2.1 kb promoter region and a 10 kb region from intron 1: *Tg(Tek-cre)1Ywa*⁴⁹ and *Tg(Tek-cre)12Flv*.⁵⁰ In both transgenic mice, *Cre* expression was first observed from E7.5 in a subset of cells in the extra embryonic yolk sac, became pan-endothelial at E9.5, and remained so throughout development. During embryo development, both transgenic mice showed Cre recombinase activities in the endocardium, endocardial cushions, and all valves of the heart by E13.5. In adults, reporter expression mediated by *Cre* expression was shown in all vascular beds and in ECs of the lymphatic vasculature.

Despite these 2 transgenic mice having been generated using the same expression vector and strategy, there was a subtle difference in *Cre* expression between them, probably because of position effects of transgenic integration sites or copy number variation. While *Tg(Tek-cre)IYwa* transgenic mice showed *Cre* expression within ECs of the embryo by E8.5 with patchy activity observed in the dorsal aorta and in a small subset of ECs, *Tg(Tek-cre)12Flv* transgenic mice showed strong endothelial *Cre* expression in all small blood vessels as well as the dorsal and umbilical vessels at E8.5.

In *Tg(Tek-cre)IYwa* transgenic mice, insertion within intron 1 of *Sorcs2* resulted in a 204 bp deletion (36,382,105 to 36,382,309 on mouse chromosome 5; GRCm37/mm9 genome build), leading to reduced and variable expression. In *Tg(Tek-cre)12Flv*, the transgene integrated into chromosome 13, causing a 241.3 kb deletion and disrupting the adjacent genes: 5-methyltetrahydrofolate-homocysteine methyltransferase reductase (*Mtrr*), FAST kinase domains 3 (*Fastkd3*), *1700001L19Rik*, and adenylate cyclase 2 (*Adcy2*). The deletion resulted in functional knock-out of *Mtrr*, *Adcy2*, and *Fastkd3* in homozygous mice.

Braren *et al.*⁵¹ generated another *Tek-cre* transgenic mouse. This mouse was generated by the pronuclear injection of DNA including *Cre* cDNA and the *Tek* promoter into FVB/N donor eggs. The founder line 2352 was subsequently established and referred to as *Tg(Tek-cre)2352Rwng*. In the transgenic mice, Cre recombinase is expressed in most ECs and blood islands of the extraembryonic mesoderm by E7.5, in dorsal aorta by E8.5, and is very strongly expressed in the vasculature of the head by E9.

Teng *et al.*⁵² used the murine *Tek* promoter and enhancer coupled with the tet-On regulatory elements to generate a transgenic mouse, *Tg(Tek-rtTA, TRE-lacZ)1425Tpr*, in which a β -galactosidase reporter can be regulated and selectively expressed in the adult murine vascular endothelium. The transgenic mice were generated by 2 transgenic constructs being co-injected into B6CBA donor eggs. The transgenic mice were viable, fertile, normal in size, and showed no physical or behavioral abnormalities. The administration of tetracycline or doxycycline selectively induced the expression of β -galactosidase in the nuclei of vascular endothelium in various tissues: aorta, heart, brain, lung, kidney, liver, spleen, uterus, prostate, stomach, skeletal muscle, and intestine. The effective induction of β -galactosidase was achieved after 3 days of oral doxycycline administration and persisted with continuous treatment. Taking these findings together, this transgenic model can be widely applied to studies on EC-specific function.

The *Tg(Tek-cre/ERT2)1Arnd* transgenic mouse has a similar construct to the constitutively active *Tek-cre* transgenic model, in which *Cre/ERT2* cDNA is placed between the *Tek* promoter and intron 1 enhancer sequence.⁵³ Forde *et al.*⁵³ confirmed transgene expression in ECs of the liver and kidney of the mice by immunohistochemical analysis. The authors reported that these transgenic mice facilitated gene targeting exclusively in ECs. Tamoxifen administration induced highly efficient Cre recombination in the vast majority of ECs, as monitored using an EGFP reporter mouse line. Taking these finding together, these inducible *Cre* transgenic mice will be a very useful resource to investigate the role of EC-specific function in adulthood and to temporarily induce the expression of transgenes in the endothelium.

The *Tg(Tek-RFP, cre)3Narl* mouse was also generated and maintained at the NARL's National Laboratories Animal Center-the Rodent Model Resource Center, Taiwan. This mouse was generated by the insertion of a construct involving an *RFP-IRES-cre* cassette in the *Tek* genomic

sequence after exon 3. The red fluorescent protein and Cre recombinase were expressed under the control of the *Tek* promoter.

MOUSE LINES WITH *Cdh5* PROMOTER

Cadherin 5 or vascular endothelial cadherin (VE-cadherin) is a type of cadherin also known as CD144 (cluster of differentiation 144). This protein is localized at EC junctions, where it mediates cell adhesion and plays a crucial role in vessel assembly. *Cdh5*^{-/-} mice die during early embryogenesis because of abnormal somite and heart development and severe vascular defects.

Alva *et al.*⁵⁴ generated a transgenic mouse line, *Tg(Cdh5-cre)7Mlia*, in which *Cre* expression is under the control of the *VE-cadherin* promoter. A region 2.2 kb from the *Cdh5* transcription start site has been known to include both an enhancer to independently drive EC expression and a negative regulator element to inhibit *Cdh5* expression in other cell types. *Tg(Cdh5-cre)7Mlia* mice generated with a construct including the 2.2 kb regulatory region of the *Cdh5* promoter showed *Cre* activity as early as E7.5 in the yolk sac and this activity progressively increased from E8.5 to E13.5. While *Cre* was expressed only sporadically within the ECs of the dorsal aorta at E10.5 and variably between littermates at early stages, its activity increased during development in all of the observed blood and lymphatic vasculature by E14.5 and into adulthood. On the basis of *Cre* expression observed in the adult quiescent vasculature and the constitutive nature of the *VE-cadherin* promoter in adults, this model should be more advantageous for analyzing the effects of gene deletion than other EC-specific *Cre* transgenic mice.

Another constitutive *Cdh5-cre* transgenic mouse is *Tg(Cdh5-cre)1Spe*.⁵⁵ This mouse has not only the same 2.2 kb upstream fragment of the *Cdh5* gene as in *Tg(Cdh5-cre)7Mlia*, but also has a flanking 2 kb insulator sequence from the chicken *γ-globin* gene. In this model, *Cre* expression was detected in the yolk sac and vasculature of E9.5 to E10.5 conceptuses, but was curiously absent from the vasculature in the head at this point. *Cre* expression was also observed in the liver of E15.5 fetuses and nearly all adult blood cells from the mice were derived from *Cre*-expressing progenitors.

The *Cdh5-cre/ERT2* transgenic mouse is one of the inducible transgenic lines mostly widely used in studies of the endothelium. Of 3 *Cdh5-cre/ERT2* transgenic mice lines previously reported, *Tg(Cdh5-cre/ERT2)1Rha* mouse is the most popular, available from Taconic BioSciences Inc. In this mouse, the *Cre/ERT2* cDNA sequence is inserted at the start codon of the *Cdh5* gene in a P1 artificial chromosome (PAC) to generate the transgene construct, with which transgenic mice are generated through pronuclear injection. Tamoxifen administration to the mice between P1 and P6 induced pan-EC *Cre*-mediated recombination in both retina and brain endothelium.^{56,57} Tamoxifen injection at 2 weeks after birth have also been reported to generate efficient *Cre* recombination in the endocardium.

Sun *et al.*⁵⁸ have generated EC-specific *Cre* driver mice, *Tg(Cdh5-rTA)D5Lbjn*, by cloning tTA behind the EC-specific *Cdh5* promoter. Of 4 founders, the D5 line was analyzed further and is currently used. tTA expression was monitored by breeding the mouse with a reporter strain exhibiting tetracycline-responsive promoter-driving β-galactosidase expression. With double transgenic mice, these researchers showed that the β-galactosidase was expressed in the blood vessels at E13.5 and the retinal vasculature, which exclusively occurred in ECs by costaining for anti-vascular endothelial growth factor receptor 2 (VEGFR-2) and CD31.

MOUSE LINES WITH *KDR* PROMOTER

Kinase insert domain receptor (*KDR*) encodes a VEGF receptor, a receptor tyrosine kinase that is absolutely required for VEGF-induced ECs proliferation, migration, and survival.⁵⁹ *KDR* is also known as fetal liver kinase 1 (Flk1) or VEGFR-2. *KDR* is widely expressed in early ECs and also robustly in the precursor cells that give rise to endothelial, hematopoietic, and muscle cell lineages.⁶⁰

Kdr^{tm1(cre)Sato} mice are knock-in mice whose allele was generated by the replacement of exon 1 with an *NSL-cre-PGK-neomycin* cassette via homologous recombination.⁶¹ These transgenic mice begins to express Cre protein slightly earlier than E8.5. However, the usefulness of these mice as an EC-specific Cre line is limited because of the active *Cre* expression in hematopoietic cells, the skeletal and cardiac muscle.

To characterize cell and tissue dynamics during the formation of the cardiovascular system in mice, Larina *et al.*⁶² generated a novel transgenic mouse line, *Tg(Kdr-mCherry)244Mema*, in which an mCherry reporter with an attached myristoylation motif is placed under the control of the *Kdr* regulator region. The *Kdr* promoter is located 5' and the enhancer element 3' of the mCherry reporter. These transgenic mice were shown to be viable, fertile, and did no developmental abnormalities. High expression of mCherry, a red fluorescent protein, was observed in the embryonic endothelium and endocardium, and capillaries in adult mice. In the transgenic mice, EC membranes were brightly labeled with mCherry. It was suggested that this mouse model is suitable for the characterization of cardio dynamics.

MOUSE LINES WITH PLATELET-DERIVED GROWTH FACTOR B (*PDGFB*) PROMOTER

PDGFB is a member of the platelet-derived growth factor family and a high affinity ligand for PDGFB receptors A and B. This protein exists either as a homodimer or as a heterodimer with PDGFA, with the dimer components being connected by disulfide bonds. PDGFB expression is observed in ECs and plays a crucial role in the correct arrangement of pericytes and smooth muscle cells in the vessel wall.⁶³ Expression of this protein is observed in capillary and arterial ECs in E11.5 embryos, but is downregulated in the mature vasculature, although its expression remains in capillaries into adulthood.⁶⁴ Notably, PDGFB is not exclusively expressed in the vasculature, as its expression is also observed in pericytes, neurons, megakaryocytes, and smooth muscle.

Claxton *et al.*⁶⁴ reported that *Tg(Pdgfb-icre/ERT2)1Fruit* was generated by insertion of *Cre/ERT2* cDNA at the start codon of the *Pdgfb* gene in a PAC. The PAC construct was randomly integrated into the mouse genome, generating *Tg(Pdgfb-icre/ERT2)1Fruit* mice through pronuclear injection. Tamoxifen injection into the mice at P1 to P4 induced efficient pan-endothelial Cre recombination at P5.⁶⁵ Tamoxifen administration in adult mice also induced pan-endothelial Cre recombination in coronary vessels (recombination efficiency, up to 99% of all coronary vascular ECs and 0% in the endocardium).⁶⁶ When tamoxifen was administered by gavage in adult mice, Cre recombination was observed in most capillary beds except the liver or the central nervous system after 4 days, even though no *Cre* expression was observed in larger arteries and veins at this stage.⁶⁷

MOUSE LINES WITH *Bmx* PROMOTER

BMX is a member of the Tec tyrosine kinase family that is expressed in arterial ECs of fetal and adult tissues.⁶⁸ It is a member of the Tec family of nonreceptor tyrosine kinase and expressed in arterial ECs of fetal and adult tissues. The Tec tyrosine kinase family has homology with the *Drosophila* Src28 tyrosine kinase and contains SH3 and SH2 domains upstream of the TK domain. Integrins activate BMX, which is mediated by PTK2/FAK1, a key mediator of integrin signaling events, leading to regulation of the actin cytoskeleton and cell mobility.

In 2013, Ehling *et al.*⁶⁸ generated and reported *Tg(Bmx-cre/ERT2)Rha*, which has been used as an inducible transgenic arterial EC-specific *Cre* mouse. To create this mouse, *Cre/ERT2* cDNA was inserted downstream of the *Bmx* promoter in a PAC, which was randomly integrated into the mouse genome.⁶⁹ Tamoxifen administration through intraperitoneal injection from P10 to P13 induced *Cre* recombination specifically within arterial ECs in the P28 retina and adult intestine, ovary, and uterus. Kidoya *et al.*⁶⁹ reported *Cre* activity in these transgenic mice from E10.5 onward, with reporter expression in smooth muscle α -actin-positive arteries at E14.5.

MOUSE LINES WITH *Apln* PROMOTER

Tian *et al.*⁷⁰ generated *Apln^{tm1.1(cre/ERT2)Bzsh}* mice by the insertion of *Cre/ERT2* cDNA at the *Apln* locus, deleting exon 1; these mice express an inducible *Cre* recombinase specifically in developing coronary vessels. The adult knock-in mice showed significantly lower EC-specific *Cre* expression 7 days after tamoxifen injection than in the embryo, indicating that, with these knock-in mice, sprouting vessels can be distinguished from mature, quiescent vessels. In adult mice, tamoxifen injection did not induce *Cre* recombination in the retina, indicating that *Cre* activity was lacking in mature, quiescent ECs. Furthermore, the induction of *Cre* activity by tamoxifen was observed in various tissue injury models with the stimulation of sprouting angiogenesis. In these models, including numerous cancer, hindlimb ischemia–reperfusion, and myocardial infarction models, the induction of *Cre* recombination was seen in newly formed vessels, a subset of which were actively proliferating.⁷¹ In the knock-in mice bred with reporter mice, the reporter activity was more localized in the peripheral angiogenic front of the P6 retina than that in *Tg(Chd5-cre/ERT2)Rha* mice, indicating that the *Apln^{tm1.1(cre/ERT2)Bzsh}* allele targets angiogenic ECs in the postnatal retina.

MOUSE LINES WITH FATTY ACID BINDING PROTEIN 4 (FABP4) PROMOTER

FABP4, also known as adipocyte FABP or adipocyte protein 2, act as a carrier protein for fatty acids that is primarily expressed in adipocytes and macrophages. It plays an important role in the development of insulin resistance and atherosclerosis in relation to metaflammation. FABP4 is also expressed in most ECs, including coronary vessel ECs but not endocardial ones.

Barak *et al.*⁷² generated *Tg(Fabp4-cre)IRev* mice in which *Cre* expression is driven by a 5.4 kb *Fabp4* promoter/enhancer randomly integrated into the mouse genome through pronuclear injection.⁷³ In these transgenic mice, abundant *Cre* activity was detected in coronary ECs and brown and white adipose tissue through northern blotting and a functional assay. No significant expression of *Cre* was observed in other tissues including liver, heart, and skeletal muscle.

BREEDING FOR GENERATION OF EC-SPECIFIC MOUSE MODELS

To generate EC-specific mouse models, several generation of breeding of cKO mice with *Cre* or other transgenic mice is required, which takes a long time (from several months to several years). Therefore, researchers must thoroughly design and plan the breeding strategy before starting a full-scale mouse experiment.

For the generation of floxed (*tm1c*) mice from *tm1a* mice, a 2-step breeding strategy is required (Fig. 2A). First, heterozygous *tm1a* mice must be crossed with *FlpE* recombinase transgenic mice on a congenic C57BL/6N background. The offspring including both the *tm1a* allele and the *FlpE* allele are crossed with wild-type C57BL/6N, resulting in removal of the *lacZ* and neomycin-resistance cassette and the generation of a floxed allele. To obtain the heterozygous

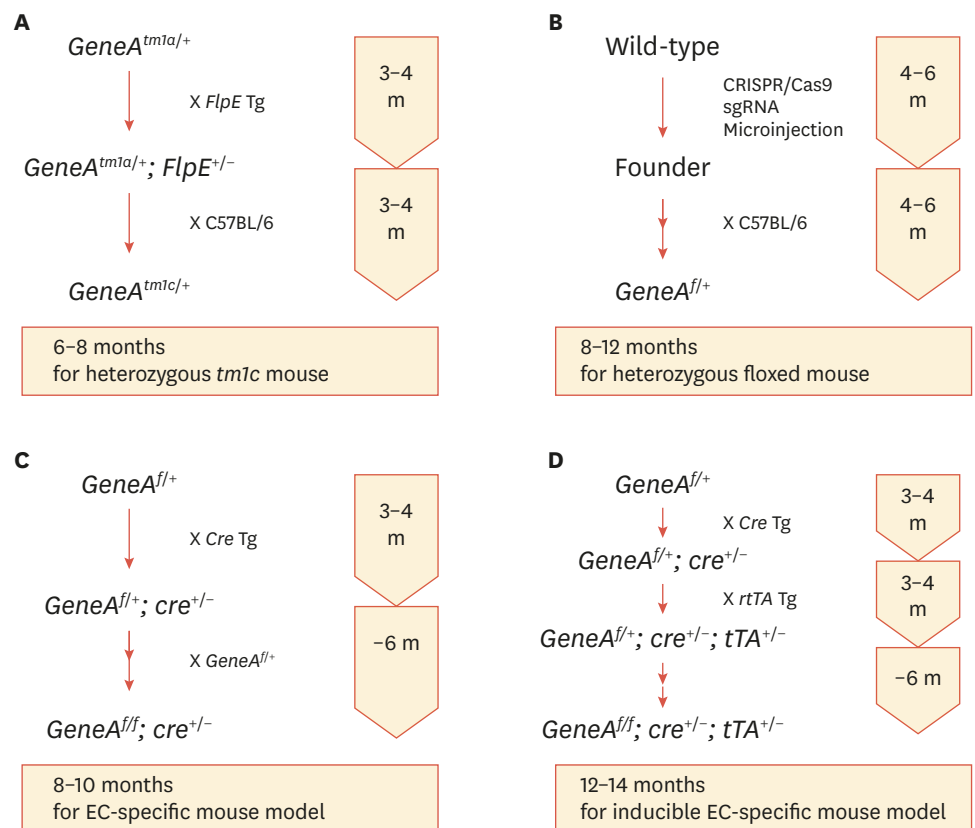


Fig. 2. Breeding strategies for generating endothelial cell-specific mouse models. (A) Generation of cKO mice with the floxed (*tm1c*) allele using *tm1a* mice. When heterozygous *tm1a* mice are crossed with *FlpE* transgenic mice, the offspring have both the *tm1a* and the *FlpE* alleles. Upon further crossing wild-type mice, both *lacZ* and neomycin cassettes are removed and the floxed (*tm1c*) allele is generated. (B) Generation of cKO mice with 2 *loxP* sequences through the CRISPR/Cas9 system. Through direct injection or electroporation of Cas9 protein/mRNA, sgRNAs, and donor DNAs into mouse embryonic cells, cKO mice with 2 *loxPs* could be generated. (C, D) To obtain EC-specific mouse model, the first step is breeding with *Cre* driver mice. Among offspring, mice with both the floxed and the *Cre* alleles could be identified. Further breeding with heterozygotes would generate homozygous cKO mice with the *Cre* allele. To create EC-specific inducible mice, breeding once more with *tTA* or *rtTA* transgenic mice is required, in contrast to the case for mice with *Cre*-expression specifically in EC. cKO, conditional knockout; *tm1*, targeted mutation 1; *loxP*, locus of X-over P1; CRISPR/Cas, clustered regularly interspaced short palindromic repeats/CRISPR-associated protein; sgRNA, single guide RNA; EC, endothelial cell; *tTA*, tetracycline repressor; *rtTA*, reverse tetracycline repressor; Tg, transgenic.

mice including only the *tm1c* allele, breeding once more with the wild-type is required. To obtain heterozygous *tm1c* floxed mice from *tm1a* mice, 6 to 8 months is typically required.

For the generating of cKO mice using CRISPR/Cas9 technology, microinjection or electroporation methods with mouse embryos are generally used (**Fig. 2B**). sgRNA and Cas9 protein/mRNA are injected or electroporated into mouse embryonic cells and then the embryos are transferred to pseudopregnant mice. Among the offspring, founder mice can be identified by PCR, cloning and Sanger sequencing. After the founder mice including 2 *loxP* sequences are bred with the wild-type, heterozygous floxed mice will be produced. It generally takes 8 to 12 months to obtain cKO mice using CRISPR/Cas9 technology.

To obtain an EC-specific mouse model, floxed mice need to be bred with a Cre driver mouse line. Among the offspring, mice with both the floxed and the *Cre* alleles could be identified. Crossbreeding with heterozygous mice would generate homozygous floxed mice with the *Cre* allele (**Fig. 2C**). This generally takes 8 to 10 months. To obtain an EC-specific inducible mouse model, breeding once more with tTA or rtTA transgenic mice is required, in contrast to the case for the *Cre*-expressing EC-specific model (**Fig. 2D**). Therefore, it takes 12 to 14 months to finally obtain EC-specific inducible mouse models.

CONCLUSION

For studies of the endothelium, cKO mouse models are indispensable because deletion mutations of genes involved in endothelium development and vasculogenesis frequently cause early embryo lethality. Owing to the recent revolutionary technical development of generating cKO mouse models with 2 *loxPs*, it is relatively easy for researchers to obtain them. EC-specific mouse models could be generated through breeding floxed mice with endothelial-specific transgenic mice including Cre driver, rtTA transgenic, and fluorescent protein expression lines, allowing researchers to generate more compound and versatile mouse models for studies of the endothelium. Considering the long time required to generate EC-specific mouse models, it is essential to make a plan for efficient breeding to save time and money. Taking the findings presented here together, recent technical progress for creating genetically modified mouse models has expanded the opportunities for more scientists to use such models and obtain more *in vivo* information on the endothelium and vasculogenesis.

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