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Blood flow-mediated gene transfer and siRNA-knockdown in the developing vasculature in a spatio-temporally controlled manner in chicken embryos

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ABSTRACT

We describe a method by which early developing vasculature can be gene-manipulated independently of the heart in a spatio-temporally controlled manner. Lipofectamine 2000 or 3000, an easy-to-use lipid reagent, has been found to yield a high efficiency of transfection when co-injected with *GFP* DNA within a critical range of lipid concentration. By exploiting developmentally changing patterns of vasculature and blood flow, we have succeed in controlling the site of transfection: injection with a lipid-DNA cocktail into the heart before or after the blood circulation starts results in a limited and widely spread patterns of transfection, respectively. Furthermore, a cocktail injection into the right dorsal aorta leads to transgenesis of the right half of embryonic vasculature. In addition, this method combined with the siRNA technique has allowed, for the first time, to knockdown the endogenous expression of *VE-cadherin* (also called *Cdh5*), which has been implicated in assembly of nasant blood vessels: when *Cah5* siRNA is injected into the right dorsal aorta, pronounced defects in the right half of vasculature are observed without heart defects. Whereas infusion-mediated gene transfection method has previously been reported using lipid reagents that were elaborately prepared on their own, Lipofectamine is an easy-use reagent with no requirement of special expertise. The methods reported here would overcome shortcomings of conventional vascular-transgenic animals, such as mice and zebrafish, in which pan-endothelial enhancer-driven transgenesis often leads to the heart malformation, which, in turn, indirectly affects peripheral vasculature due to flow defects. Since a variety of subtypes in vasculature have increasingly been appreciated, the spatio-temporally controllable gene manipulation described in this study offers a powerful tool to understand how the vasculature is established at the molecular level.

1. Introduction

Blood vessel networks are distributed widely in the vertebrate body, and they deliver essential substances including oxygen, nutrients, and hormones to tissues and organs (Adams and Alitalo, 2007; Potente et al., 2011). The vascular system also plays important roles in a variety of functional events such as homeostasis, tissue morphogenesis, regeneration, and cancer metastasis (Carmeliet, 2005; Makita et al., 2008; Saito et al., 2012). During blood vessel formation in embryogenesis, endothelial precursor cells called angioblasts emerge in mesenchymal populations, and subsequently differentiate into endothelial cells to form nascent blood vessels by the process of vasculogenesis (Herbert and Stainier, 2011). Following vasculogenesis, blood vessels undergo angiogenesis, during which time they change their structures by extending, sprouting, pruning, and remodeling (Adams and Alitalo, 2007; Herbert and Stainier, 2011). Finally, mature vasculatures become associated with type-specific perivascular cells (Jain, 2003) to complete arterial-venous differentiation.

An increasing body of experimental evidence has advanced our understanding of the molecular and cellular mechanisms by which blood vessel formation is regulated. The major approaches used for the analyses have been the endothelial-specific loss- or gain-of-function of genes, for which transgenic animals in mice and zebrafish serve as powerful experimental models (De Val and Black, 2009; Hogan and Schulte-Merker, 2017; Stahl et al., 2010). In these experiments, pan-endothelial drivers, such as Tie 1 or Tie2, are often used, and this

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leads to a transgenesis of the entire cardiovascular system, including both the heart and vessels (Korhonen et al., 1995; Schlaeger et al., 1997). If heart development is affected by the transgene (for example by interfering with ALK5 or Smad4 expression), blood flow is frequently undermined, and this loss of mechanical flow would indirectly cause the malformation of peripheral vasculature (De Val and Black, 2009; Lan et al., 2007; Sridurongrit et al., 2008). This makes it difficult to distinguish between the direct and indirect effects of transgenesis in peripheral vasculatures.

Recent reports have shown that the vasculature contains numerous subtypes of blood vessels. There is not only the arterio-venus specification, but also the region-specific diversity of shapes and functions of vessel branches, which are being increasingly appreciated (Augustin and Koh, 2017; Pardanaud et al., 1996; Sato et al., 2008). However, how these diverse types of peripheral vasculature are established remains poorly explored, mainly because methods that allow region-specific gene manipulations of vasculature have been limited.

Early chicken embryos are flat in structure, in which a variety of vascular subtypes can be seen, including dorsal aortae, intersegmental vessels, and yolk sac vasculature as early as at embryonic day 2 (E2). Rosselo and Torres (2010) reported that the *in ovo* electroporation into developing chick dorsal aortae resulted in local transgenesis (Rossello and Torres, 2010). However, as we confirmed (unpublished data), the efficiency of such gene transfer is low. Other groups (Bollerot et al., 2006; Decastro et al., 2006) have reported techniques of liposome-mediated transfection into chicken embryonic vasculature, but these methods require high expertise of liposome construction and preparation. So far, no method has been reported to knockdown endogenous gene(s) in chicken vasculature.

Here, we demonstrate methods that enable efficient and local transgenesis of vasculature in chicken embryos using the commercially available reagent Lipofectamine, which has been widely and commonly used for gene transfection into cultured cells. Following infusion with a cocktail containing Lipofectamine and *GFP* cDNA, GFP-positive endothelial cells were found in a variety of vasculature subtypes. By exploiting blood flow and vascular patterns that change in a stage-dependent manner, we were able to control transfected areas by changing the site and time point of injection along the developmental stages. In addition, our transfection technique allowed local knockdown of the endogenously expressed *VE-cadherin* (also called *Cdh5*) gene by small interfering RNA (siRNA). The methods described here allow us to spatially and temporally regulate gain-of-function and loss-of-function gene expression in the developing vasculature, without requiring complicated liposome preparation protocols or transgenic animals.

2. Materials and Methods

2.1. Embryological manipulations

Fertilized chicken eggs were commercially obtained from the poultry farm Shiroyama Farm (Kanagawa, Japan). Embryos were staged according to Hamburger and Hamilton (1951) or somite stage (ss) (Hamburger and Hamilton, 1951). All animal experiments were conducted with the ethical approval of Kyoto University.

2.2. Expression vectors

pCAGGS-GFP and pCAGGS-mCherry plasmids were described previously (Momose et al., 1999; Yokota et al., 2011). The open reading frame (ORF) of chicken *Cdh5* was PCR-amplified using following primers:

Cdh5-Fw, 5′-CCACCGGTCGCCACCATGAAGAAGCTTATTCTGCT-3’; Cdh5-Rv, 5′-GCCCTTGCTCACCATCTCGAGGGAATACACAAAATCTTCAT-3’.

pCMV-Cdh5GFP plasmid was made by fusing the Cdh5 ORF to pCMV-GFP (Clontech) using In-Fusion HD Cloning Kits (Clontech). pCMV-GFP linearized vector was amplified from pEGFP-N1 (Clontech) using following primers:

GFP-N1-Fw, 5′-ATGGTGAGCAAGGGCGAGGA-3’; GFP-N1-Rv, 5′-GGTGGCGACCGGTGGATCCC-3’.

Cdh5mutGFP, which was resistant to Cdh5-siRNA#1, was made using PrimeSTAR Mutagenesis Basal Kit (Takara) with following primers (capital letters indicate replaced nucleotides):

Cdh5mut-Fw, 5′-atcTTTAgaGccTccTAGCaaGtttattatcaaggtttctgat-3’.

Cdh5mut-Rv, 5′-aaaCttGCTAggAggCtcTAAAgatcggttgtttcttctgtca-3’.

2.3. siRNAs

We designed siRNAs to interfere with *Cdh5* expression, referring to technical information of BLOCK-iT RNAi Designer (Invitrogen) and siRNA Target Finder (Genscript). The following 19-mer sequences were selected:

Cdh5-siRNA#1 (at positions 369–387 of ORF), 5′-GCTGGAACCACCATCTAAA-3’; Cdh5-siRNA#2 (at positions 1052–1070 of ORF), 5′-CAACAATTACCATTGAAGT-3’; Cdh5-siRNA#3 (at positions 1470–1488 of ORF), 5′-GGTAATCATCAGGATTTCA-3’; Luc-siRNA (negative control interfering with Luciferase expression), 5′-GGATCCTATCCGAAGGCAA-3’.

All sense and anti-sense RNA oligos were synthesized by Eurofins Genomics. Annealing of sense and anti-sense RNA oligos was prepared according to the manufacturer’s instruction. Rhodamine labeling of siRNA (Cdh5-siRNA#1) were made using *Label* IT siRNA Tracker Kit (TAKARA).

2.4. mRNAs

Luciferase and *Cdh5mut* capped mRNAs were prepared from pBS-Luciferase and pBS-Cdh5mut, respectively, using T7 mScript Standard mRNA Production System (CellScript).

2.5. Cell culture

Chicken fibroblast-derived DF1 cells were maintained with Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) at 38.5 °C, 5% CO_2. For evaluation of Cdh5-siRNA efficacy, pCMV-Cdh5GFP (1,800 ng) and pCAGGS-mCherry (1,000 ng) were co-transfected into DF1 cells along with 30 pmol of each siRNA using Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, DF1 cells were fixed overnight in phosphate buffered saline (PBS) containing 4% paraformaldehyde (PFA) at 4 °C. Fluorescent images were obtained using AZ-C1 macro-confocal microscope system (Nikon). For cell counting analyses, images were processed using ImageJ (NIH).

2.6. Visualization of blood vessel

In vivo visualization of blood vessels was performed by infusion with fluorescent ink (Takase et al., 2013) or *Lens culinaris* lectin (LCA-lectin) (Jilani et al., 2003). For the ink injection, chicken embryos were infused with 0.5–1 μl of highlighter ink (PILOT spotliter; 1:10 dilution in PBS; Takase et al., 2013) through the heart using a micropipette pulled from a glass capillary (Narishige, GD-1) with a vertical micropipette puller (Narishige, PC-10). For lectin infusion, 0.5–1 μl of 0.5 mg/ml rhodamine labeled LCA-lectin (Vector laboratories) was used. Manipulated embryos were incubated at 38.5 °C for an additional period of 5 min before harvest. Fluorescent images were obtained using the Leica MZ10 F microscope (Leica) with the DS-Ri1 camera (Nikon).

2.7. In vivo transfection into embryonic vasculature

For plasmid DNA transfection, Lipofectamine 2000 (Invitrogen), Lipofectamine 3000 (Invitrogen), ViaFect (Promega), FuGENE HD (Promega) and SuperFect (QIAGEN) were used. Each transfection

reagent was diluted with Opti-MEM at 0, 25, 50, 75 or 100% (v/v), and plasmid DNA solution in Opti-MEM in was separately prepared at 600 ng/μl. Subsequently, the diluted transfection reagent and DNA solution were mixed at a final concentration of 0, 12.5, 25, 37.5 or 50% (v/v) and 300 ng/μl, respectively, and incubated at room temperature for 5 min to allow the lipid–DNA complex to form.

Using a glass micropipette, 0.8 μl of the lipid–DNA complex solution was injected into the heart or the right dorsal aorta (R-DA) at the appropriate embryonic stages. For siRNA transfection, Lipofectamine 2000, siRNA and BLOCK-iT Alexa Fluor Red Fluorescent Control (Invitrogen) were diluted with Opti-MEM at a final concentration of 12.5% (v/v), 5 μM and 2.5 μM, respectively. For siRNA and mRNA transfection, Lipofectamine 2000, rhodamine-labeled Cdh5-siRNA#1 and *Luciferase* or *Cdh5mut* mRNA were diluted with Opti-MEM at a final concentration of 12.5% (v/v), 4 μM and 360 ng/μl (*Luciferase* mRNA) or 600 ng/μl (*Cdh5mut* mRNA), respectively.

2.8. Frozen sections

Chicken embryos were fixed overnight in PBS containing 4% PFA at 4 °C. Frozen sections (10 μm thick) of fixed embryos were prepared with a cryostat (MICROM, HM500 OM). The sections were washed in PBS three times (each 5 min), and were sealed by FluorSave reagent (Calbiochem, 345789) containing 4′6′-diamidino-2-phenylindole dihydrochloride (DAPI). Fluorescent images were obtained using the Axioplan 2 microscope with Apotome system (Carl Zeiss).

2.9. RNA probes and in situ hybridization

cDNA fragment of chicken *Cdh5* was as described (Sato et al., 2008). cDNA of chicken *Tie2* was isolated by Dr. Ohata (Nara Institute of Science and Technology, NAIST). Digoxigenin-labeled RNA probe was prepared according to the manufacturer's instruction (Roche). Whole-mount and section i*n situ* hybridizations were performed as previously described (Tonegawa et al, 1997, 2003). Whole-mount images were obtained using the Leica MZ10 F microscope with the DS-Ri1 camera, and section images were obtained using the Axioskop 2 microscope (Carl Zeiss) with the DS-L4 camera (Nikon).

2.10. Immunohistochemistry

Frozen sections were washed in PBS three times (each 5 min). After blocking with 1% blocking reagent (Roche, 1096176)/PBS for 1 h at room temperature (RT), the sections were incubated overnight at 4 °C with a 1:300 dilution of anti-ACTIVE caspase-3 rabbit polyclonal antibody (Promega, G7481), or a 1:1000 dilution of anti-phospho-Histone-H3 (pH3) rabbit polyclonal antibody (Millipore, 06–570) in 1% blocking reagent/PBS. The sections were washed three times in PBS (each 5 min), and were reacted with a 1:500 dilution of Alexa 488 goat anti-rabbit IgG (Invitrogen, A-11008) in 1% blocking reagent/PBS for 1 h at RT. After washing three times in PBS (each 5 min), the sections were sealed by FluorSave reagent containing DAPI. Fluorescent images were obtained using an Axioplan 2 microscope with Apotome system, and obtained images were processed using ImageJ.

For immunostaining of chick hind limb, a hind limb was dissected from fixed E6 embryo and washed in 1% Tween 20/PBS (PBST) three times (each 10 min). After blocking with 1% blocking reagent/PBST for 1 h at RT, the specimen was incubated overnight at 4 °C with a 1:300 dilution of anti-ACTIVE caspase-3 rabbit polyclonal antibody in 1% blocking reagent/PBST. The specimen was washed three times in PBST (each 1 h), and was reacted with a 1:500 dilution of Alexa 488 goat anti-rabbit IgG in 1% blocking reagent/PBST for overnight at 4 °C. After washing three times in PBST (each 1 h), fluorescent images were obtained using the Leica MZ10 F microscope with the DS-Ri1 camera.

To obtain the rate of proliferation or apoptosis in DAs, the number of immune-stained endothelial cells was divided by the circumferential length of DA, which was subsequently compared between control and siRNA-treated DAs.

2.11. Whole embryo culture and time-lapse live imaging

The whole-mount embryo culture was performed as described previously (Chapman et al., 2001) with a minor modification: a siRNA-transfected embryo was taken out from an egg shell and mounted on a filter paper ring. The embryo was cultured ventral-side up in an incubation chamber AZPU (TOKAI HIT) connected to the AZ-C1 macro-confocal microscope system. Blood flow speed was measured by filming movements of infused fluorescent beads (7.0–7.9 μm in diameter) (Spherotech, FP-7052-2) using the Leica MZ10 F microscope with the DS-Ri1 camera.

3. Results

3.1. Reagents for in vitro transfection can be used for vascular transgenesis in live and intact chicken embryos

Lipofectamine 2000 (Invitrogen) is a widely used reagent for the *in vitro* transfection into cultured cells. We asked if this reagent could also be used for *in vivo* transfection to target developing vasculatures in chicken embryos. We injected 0.8 μl of transfection cocktail (a solution containing Lipofectamine 2000 (lipid) and DNA) into the heart at embryonic day 2 (E2; HH14) at 5 different concentrations of lipid, 0%, 12.5%, 25%, 37.5%, and 50%, along with 300 ng/μl of pCAGGS-GFP (Fig. 1A). After 17 h, relatively high levels of GFP signals were observed in the heart and nearby vessels in embryos that received the lipid concentration of 12.5% or 25% (Fig. 1C, n = 25; Fig. 1D, n = 40, Table 1), whereas a cocktail at concentrations of 37.5% or 50% resulted in barely detectable/no GFP expression or embryonic death (Fig. 1E, F, n = 10 each, Table 1). Expression levels of GFP were categorized into three classes: no expression (−), moderate (+), and high (++). The highest GFP level with the lowest frequency of embryonic death was achieved with the concentrations of 12.5% or 25% of Lipofectamine (2000) (Table 1).

We also tested other commercially available reagents for transfection, Lipofectamine 3000 (Invitrogen), ViaFect (Promega), FuGENE HD (Promega), and SuperFect (Qiagen) at the concentration of 25%. Among these, only Lipofectamine 3000 yielded GFP signals comparable to those by Lipofectamine (2000) (Table 1). For further studies, we used Lipofectamine 2000at 12.5%, since it is less expensive than Lipofectamine 3000.

To further scrutinize transfected sites in embryos, we visualized blood vessels of cocktail-injected embryos by ink perfusion prior to harvest 17 h after transfection (Fig. 1G) (Takase et al., 2013, see also Materials and Methods). In addition to the heart, GFP signals were widely distributed in blood vessels including intersegmental vessels (ISVs) and yolk sac vasculature (YS) (Fig. 1H–K). Whereas some circulating blood cells were also transfected (data not shown), most non-circulating GFP-positive cells were found to overlap with perfused ink. We also co-labeled GFP-positive cells with perfused LCA-lectin, a conventionally used endothelial marker. In transverse sections of the dorsal aorta and yolk sac vascular plexus, GFP-positive cells were thin and flat in shape, and co-labeled with LCA-lectin (Fig. 1L–N). Together, endothelial cells were successfully transfected with infused *GFP* DNA using Lipofectamine 2000 in chicken embryos.

3.2. Site-specific transgenesis of developing vasculature

We examined whether GFP-transfected sites in the vasculature would differ if the developmental stage and site of cocktail injection were changed. When the lipid-DNA cocktail is injected into a beating heart, the cocktail solution must be delivered by blood flow. In contrast, if injected into an early-formed heart before the circulation starts, the cocktail

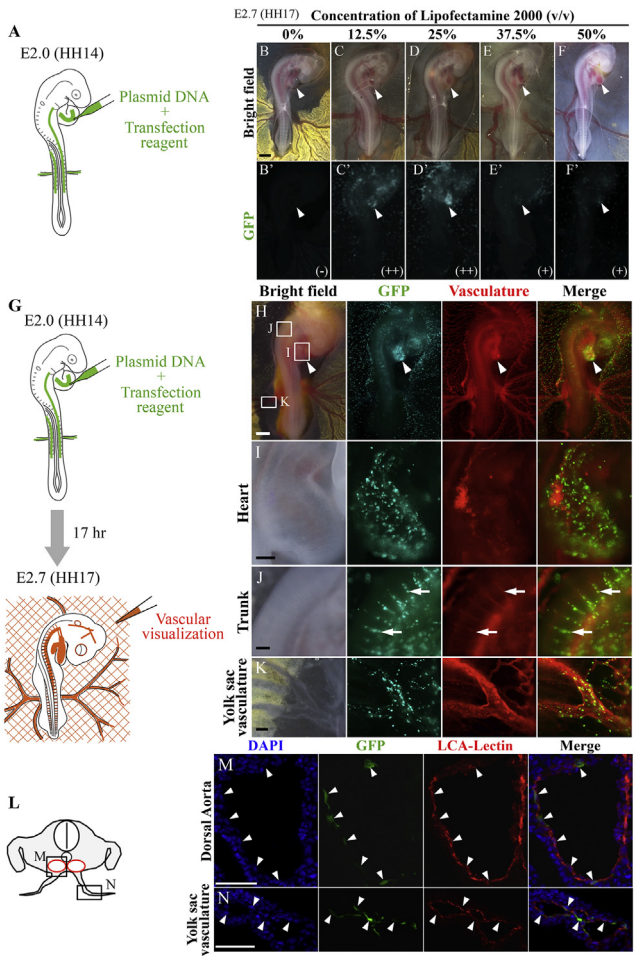


Fig. 1. Commercially available reagents can be used for vascular transgenesis in chicken embryos. (A) A diagram showing injection with a liposome-DNA cocktail. A cocktail containing pCAGGS-GFP plasmid (300 ng/μl) and transfection reagent, shown in green, was injected into the heart of E2 (HH14) embryos, and GFP signals were assessed 17 h after transfection (E2.7; HH17). Transfection efficiency was classified into three levels (-, +, ++) based on GFP expression in the heart. (B–F) GFP expression in transfected embryos at E2.7. Embryos receiving the concentration of Lipofectamine (2000) at 12.5% and 25% exhibited high level of GFP expression (++), whereas embryos receiving 37.5% and 50% of Lipofectamine displayed a moderate level (+). (G) A diagram showing injection with a liposome-DNA cocktail and vascular visualization. A cocktail containing pCAGGS-GFP plasmid (300 ng/μl) and Lipofectamine 2000 (12.5%), shown in green, was injected into the heart of E2 (HH14) embryos. Vascular networks of transfected embryos were visualized by perfusion with fluorescent ink at E2.7 (HH17) shown in red. (H–K) GFP expression and vascular networks of transfected E2.7 embryo. (H) Widespread GFP expression was observed in the vasculature, and a high level of GFP signals was detected in the heart (arrowheads), into which the lipid–DNA cocktail was injected. (I–K) GFP signals in the heart (I), intersegmental vessels (arrows) (J), and yolk sac vasculature (K). (L) Transverse sections of the trunk region of transfected embryo at E2.7. (M–N) GFP signals were detected in endothelial cells co-labeled with infused LCA-Lectin. Arrowheads indicate endothelial cells of the DA (L) and YS (N), respectively. DA, dorsal aorta; CV, cardinal vein. Scale bars: 1 mm for (B–F, H), 250 μm for (I–K), and 50 μm for (M–N).

would spread only locally. Likewise, the delivery pattern of the cocktail must be different if injected into one of paired dorsal aortae. In this way, we compared GFP-transfected areas in vasculature by testing three different developmental stages, 13 ss, 16 ss and 19 ss, and two different sites of injection, the heart and right dorsal aorta (R-DA) (Fig. 2). Ink was perfused prior to harvest to confirm that the transfected cells were confined to endothelial cells.

When injected into the R-DA at the pre-circulation stage (13 ss; no blood flow), an GFP-positive area was within a limited range in the anterior YS of the right side of embryo (R-YS) (Fig. 2A, H). In contrast, when injected into the R-DA after the circulation started (16 ss), the GFP-positive area was expanded, and the right vitelline artery (R-VA) was highly labeled (Fig. 2B, I). When injected into the heart at the same stage (16 ss), the transfected area was seen in both sides of embryo including YSs and VAs (Fig. 2C, F).

Fig. 2D–J summarize relative levels of GFP expression assessed in 7 different areas of embryos, the heart, right and left DAs, right and left YSs, and right and left VAs. The cocktail was injected into different stages and different sites; three different embryonic stages of 13 ss (pre-circulation), 16 ss (onset of circulation), and 19 ss (circulation established), and two different injection sites, the heart and R-DA, were tested.

When injected into the heart of pre-circulation (13 ss), the cocktail remained in limited areas of YSs of both sides of embryos (Fig. 2E). In contrast, when injected into the heart after the circulation started (16 ss), the cocktail spread widely, and GFP expression was preferentially observed in VAs, in which blood flow was predominant compared to their neighboring vascular plexus (capillaries) (Fig. 2F). After the establishment of blood circulation, the cocktail injected into the heart spread over the entire vasculature (Fig. 2G, GFP-positive cells in DA, YS, VA are shown). When injected into the right DA, the right half of vasculature exhibited sequential changes in GFP distribution which was similar to those in the case of the heart injection (Fig. 2H–J). Together, site-specific transfection in early chicken vasculature is enabled by adjusting both developmental stages (pre-circulation vs post-circulation) and injection sites of a lipid–DNA cocktail.

siRNA-mediated *Cdh5* knockdown: *In vitro* assay for evaluation of efficiency.

To test if the aforementioned method could readily accomplish siRNA-mediated gene knockdown in chicken embryonic vasculature, we used the *VE-Cadherin* gene (also called *cadherin 5* (*Cdh5*), which is implicated to be important for the assembly of nascent blood vessels in mice (Crosby et al., 2005). We prepared three siRNAs against the *Cdh5* ORF (Cdh5-siRNA#1, #2, #3) (Fig. 3A).

To evaluate the knockdown efficiency by these Cdh5-siRNAs, we performed *in vitro* assay using chicken fibroblast-derived DF1 cells. The cells were co-transfected with expression vectors of GFP-tagged Cdh5 (Cdh5GFP) and mCherry, the latter being used as an internal control of transfection efficiency, along with each of three Cdh5-siRNAs (Fig. 3B). Luciferase-siRNA (Luc-siRNA) was used as a negative control. DF1 cells co-transfected with Cdh5GFP, mCherry, and Luc-siRNA displayed both GFP and mCherry signals (Fig. 3C). In contrast, intensity of GFP signals was reduced when co-transfected with Cdh5-siRNA: a relative ratio of the number of $Cdh5GFP^{+}$ cells to that of $mCherry^{+}$ cells (normalized by the index of Luc-siRNA control) was 0.03 ± 0.02 for Cdh5-siRNA#1, 0.55 ± 0.11 for Cdh5-siRNA#2, and 0.19 ± 0.09 for Cdh5-siRNA#3 (Fig. 3C, D, $n = 4$ for each). These results indicate that Cdh5-siRNAs prepared in this study could inhibit expression of exogenously introduced Cdh5GFP in culture cells, with Cdh5-siRNA#1 exerting the highest efficiency.

3.3. Site-specific gene knockdown in developing vasculature

We next asked whether our flow-mediated gene transfer method was applicable to local knock-down of endogenous *Cdh5*. The three Cdh5-siRNAs were separately injected into the right dorsal aorta (R-DA) of 16 ss (HH12) embryos as a 0.8 μl lipid cocktail (12.5% of Lipofectamine, 2000, 5 μM of each siRNA, and 2.5 μM of Alexa Fluor Red Fluorescent Control, a fluorescent tracer of siRNA transfection) (Fig. 4A). When the embryos developed to HH16 (20 h post injection), vascular formation was assessed by yellow ink infusion, and siRNA-transfected sites were visualized by co-injected Alexa Fluor Red Fluorescent signals.

We found that the formation of R-YS and R-VA was markedly affected,

Table 1
Comparison of transfection efficiencies among commercial transfection reagents. A GFP-cocktail with each of transfection reagents (Lipofectamine, 2000, Lipofectamine 3000, ViaFect, FuGENE HD, and SuperFect) was injected into the heart at HH14, and GFP signals were assessed after 17 h in the heart. See text for details. -, no GFP expression; +, low or moderate GFP expression; ++, high GFP expression.

Transfection reagents (Company)	Type of reagents	Concentration of reagents (%)	No. injected embryos	Viability†	Efficiency†		
					++	+	–
Lipofectamine 2000 (Invitrogen)	Liposomal	0	10	10 (100%)	0	0	10
		12.5	25	25 (100%)	19 (76%)	5	1
		25	40	40 (100%)	31 (77.5%)	9	0
		37.5	10	10 (100%)	1 (10%)	8	1
		50	10	5 (50%)	0	4	1
Lipofectamine 3000 (Invitrogen)	Liposomal	25	10	10 (100%)	8 (80%)	2	0
ViaFect (Promega)	Non-liposomal	25	10	10 (100%)	0	6	4
FuGENE HD (Promega)	Non-liposomal	25	10	10 (100%)	0	10	0
SuperFect (Qiagen)	Non-liposomal	25	10	10 (100%)	0	3	7

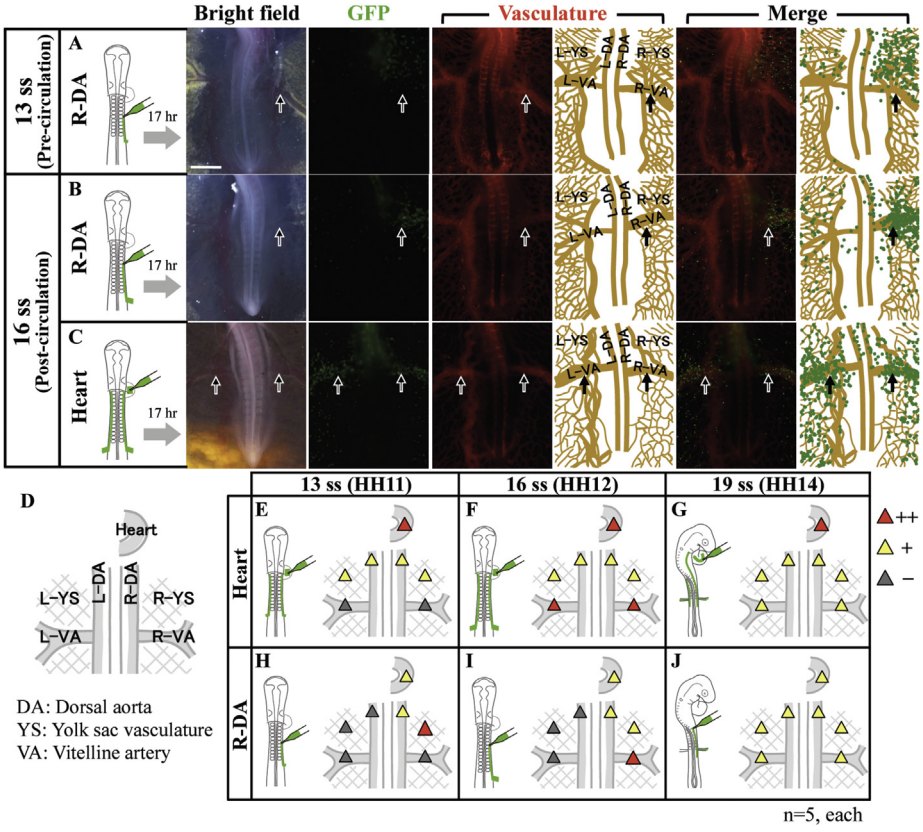


Fig. 2. Site-specific transgenesis of developing vasculature by adjusting developmental stages and injection sites. (A–C) A cocktail of pCAGGS-GFP plasmid and Lipofectamine 2000 was injected into the right dorsal aorta (R-DA) or heart at two different stages, 13 ss and 16 ss. GFP-signals were assessed 17 h after transfection. Black arrows indicate vitelline arteries. (A) When a cocktail was injected into R-DA at 13 ss of pre-circulation stage, GFP-transfected area was in a limited range within the anterior YS of the right side of embryo (R-YS) and R-DA. (B) When injected into R-DA at a more advanced stage of 16 ss, the GFP-transfected area was expanded with R-DA, R-YS and R-VA being labeled. (C) When injected into the heart of 16 ss embryo, the GFP-positive area was seen in both sides of embryo including DAs, YSs, and VAs. Scale bar: 1 mm. (D–J) Summary of GFP-transfection in 7 different areas (D) after cocktail injections in two different positions (heart and R-DA) and three stages (13 ss, 16 ss, 19 ss). GFP signal intensities were classified into three levels: high expression (red, ++), moderate expression (yellow, +), and low or no expression (grey, -).

as revealed by both ink perfusion and *in situ* hybridization with *Cdh5* mRNA (Fig. 4C, n = 29/32), whereas effects on the vasculature in the left side (L-YS and L-VA) were unrecognizable. Consistent with differential knockdown efficiencies among the three Cdh5-siRNAs (Fig. 3), embryos injected with Cdh5-siRNA#1 displayed most pronounced defects in vasculature, with siRNA#2 and siRNA#3 showing little and moderate activities, respectively (Fig. 4C–E; #1: n = 32, #2 and #3: n = 17). Vasculatures injected with Luc-siRNA-containing cocktail were indistinguishable from those of untreated embryos (Fig. 4B, n = 15/16).

To further scrutinize the effects on vascular formation by Cdh5-siRNA#1, DA and YS in the infused side (R-DA and R-YS) were examined in transverse sections, and compared with those in the non-infused side (L-DA and L-YS) (Fig. 4F–H). R-DA, positive for *Cdh5* and *Tie2*, was smaller than L-DA: circumferential length was 266.9 ± 47.1 μm (R-DA) and 580.9 ± 35.1 μm (L-DA) (Fig. 4G; n = 2/2). In the R-YS, few vascular structures were found in the transfected area whereas such effects were not observed in Luc-siRNA-infused embryos (Fig. 4H; n = 2/2).

The defects of R-YS vasculature could be either by a direct effect of the trasnfected Cdh5-siRNA#1 in this region, or by a secondary effect due to the reduced size (flow) of/in R-DA (Fig. 4G). To distinguish between these possibilities, we carried out time-lapse live imaging with Cdh5-siRNA#1-infused embryo to examine the blood flow in DAs. At 12 h after injection with Cdh5-siRNA#1 into the R-DA of 16 ss embryos, the Cdh5-siRNA#1-infused$^{R\text{-}DA}$ embryo was filmed for 30 s with 1 frame/second (Movie 1). Whereas the blood flow was seen in the R-DA, no/little circulation was observed in the Cdh5-siRNA#1-transfected area of R-YS (Movie 1, Suppl. Fig. 1; brackets and arrowheads). Importantly, the non-transfected area of YS (negative for Alexa Fluor Red) even in the right side of embryo exhibited normal vasculature. Thus, it is likely that the vascular disruption in the R-YS was elicited by knockdown of endogenous Cdh5 in this region, and not by a secondary effect of blood flow cessation.

Supplementary video related to this article can be found at https://doi.org/10.1016/j.ydbio.2019.08.001.

To further corroborate this notion, we compared sheer stress (τ) between DAs of control- and knocked-down embryos, since τ is a landmark

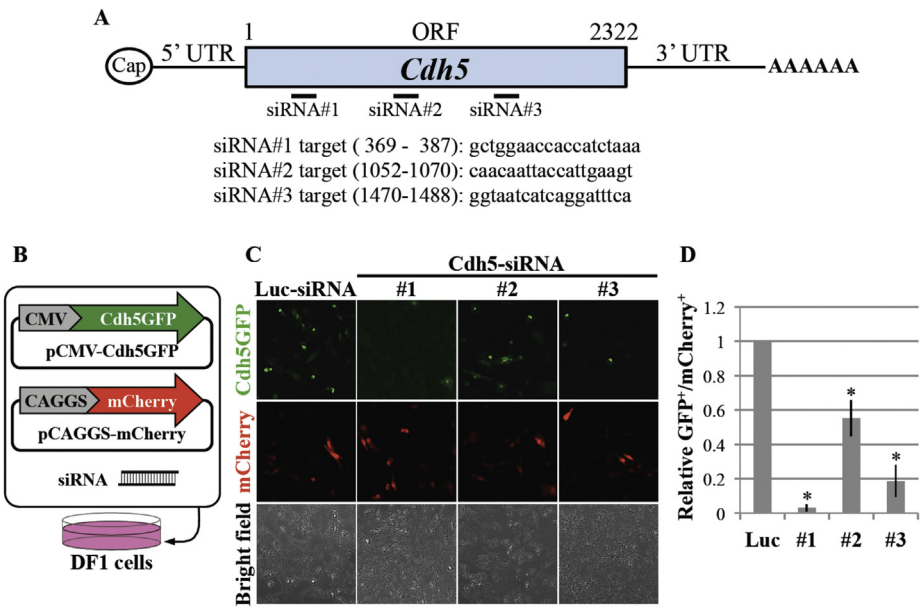


Fig. 3. *In vitro* evaluation of siRNAs against *Cdh5*. (A) Schematic structure of chicken *Cdh5* mRNA and siRNA-targeted regions. Three siRNAs against *Cdh5* (Cdh5-siRNA#1, #2 and #3) were designed to target the open reading frame (ORF). (B) pCMV-Cdh5GFP and pCAGGS-mCherry plasmids were co-transfected into DF1 cells with each of Luc-siRNA or Cdh5-siRNAs#1–3, and GFP intensity was assessed 24 h after transfection. (C) In Cdh5-siRNA#1–3 co-transfected DF1 cells, whereas the number of mCherry^{+} cells was comparable to that in Luc-siRNA co-transfected cells, the number of Cdh5GFP^{+} cells markedly decreased receiving Cdh5-siRNA#1 or -#3. (D) Quantitative representation of a relative ratio of the number of Cdh5GFP^{+} cells to the number of mCherry^{+} cells (n = 4 each). Error bars represent SD (standard deviation). *$P < 0.001$.

of vascular function. It is known that τ is directly and inversely proportional to the speed of blood flow and vessel diameter (or radius), respectively (Papaioannou and Stefanadis, 2005). We therefore measured the speed of blood flow (maximum) by filming infused fluorescent beads (7.0–7.9 μm in diameter), and combined it with the average radius obtained from the circumferential length of DAs to calculate τ. The maximum speed (μm/s) in R-DA was smaller than that of L-DA in Cdh5-siRNA#1-infused$^{R\text{-}DA}$ embryo (Fig. 4I; 2,102 ± 353 μm/s for R-DA and 3,828 ± 229 μm/s for L-DA). The relative τs in four DAs, L-DAs and R-DAs in control- and knocked-down embryos, were 1.00, 0.94, 0.88, and 1.04 (Fig. 4J; relative $\tau_{L\text{-}DA\ (Luc)} = 1.00$), indicating that the R-DA of Cdh5-siRNA#1-infused$^{R\text{-}DA}$ embryo retains normal vascular function.

Although minor expression of GFP was observed when R-DA was infused at 16 ss (Fig. 2I), the heart function was apparently normal because: 1) the heart beat rate was almost identical between control- and knocked-down embryos (Fig. 4I; 90.3 ± 2.1 for Luc-siRNA, 90.3 ± 7.9 for Cdh5-siRNA#1), and 2) the formation and remodeling of YS in the non-transfected left side of embryo took place normally, the process known to be susceptible to changes in blood flow.

To know what caused the size reduction of Cdh5-siRNA#1-infused R-DA, we examined cell proliferation with pH3 and apoptosis with ACTIVE-caspase3. The number of pH3-positive cells in R-DAs was 1.28 ± 0.26 for Luc-siRNA, and 1.41 ± 0.37 for Cdh5-siRNA#1 (n = 6 for each, $P = 0.79$; Suppl. Fig. 2A and B), and the number of pH3-positive cells in YS was 1.55 ± 0.24 for Luc-siRNA, and 1.67 ± 0.35 for Cdh5-siRNA#1 (n = 5 each, $P = 0.79$; Suppl. Fig. 2A and B). Thus, no significant differences in these cellular events were detected in DAs and YSs between control- and knocked-down embryos. We detected few apoptotic cells in DAs and YSs of control- and knocked-down embryos (Suppl. Fig. 2C), whereas the anti-ACTIVE-caspase3 antibody we used in this study successfully detected apoptotic cells in E6.5 chick hind limb as expected (Fernandez-Teran et al., 2006; Suppl. Fig. 2D). Thus, although it remains unknown how the Cdh5-knockdown caused the vascular defects, one possibility is that the knock-downed cells might have detached from blood vessels.

Together, these studies provide proof-of-concept that the blood flow-mediated transfection *in vivo* with Lipofectamine 2000 can be used for siRNA-knockdown. Importantly, this method allows a site-specific knockdown in vasculature, which has been difficult by conventional genetics such as using Tie2-Cre/flox mice.

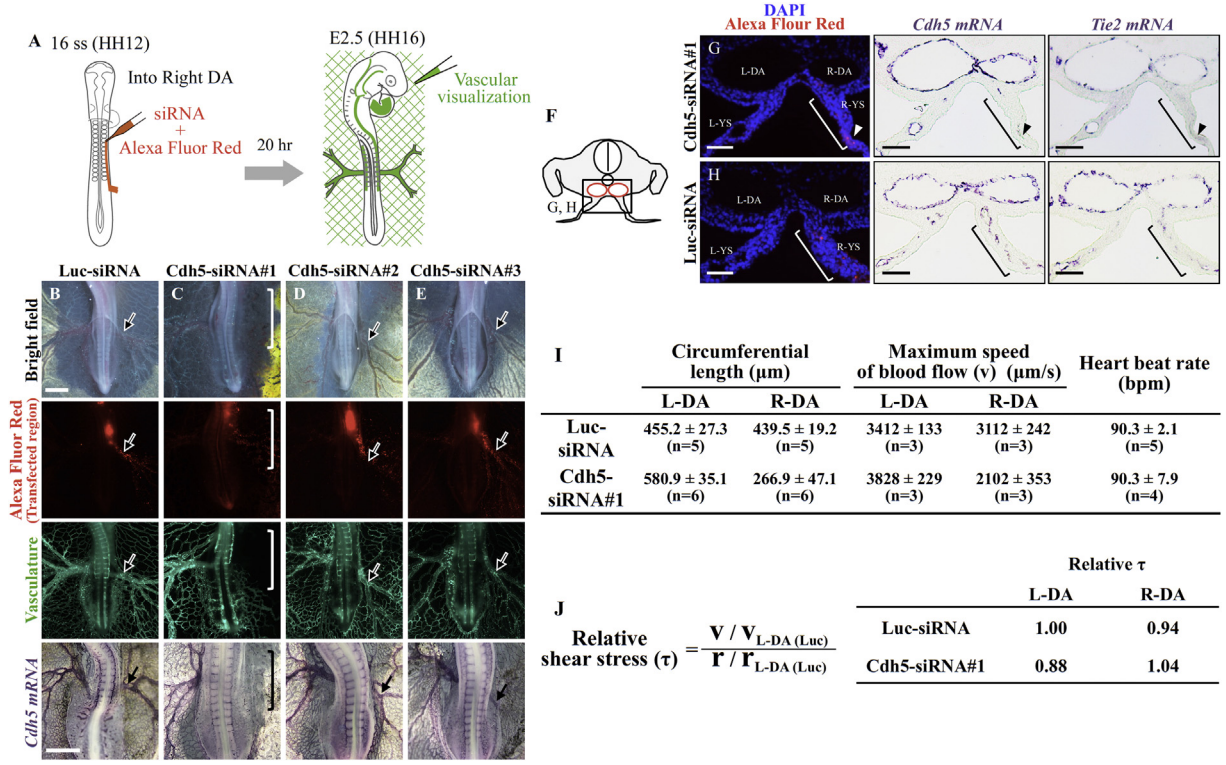
3.4. Rescue of Cdh5 siRNA-knockdown by co-transfection with Cdh5 mRNA

To determine if the vascular defects observed in the siRNA#1-injected embryos were elicited specifically by Cdh5-specific knockdown, we performed rescue experiments using siRNA-resistant *Cdh5* mRNA (Fig. 5). mRNA of the *Cdh5 mutant (Cdh5mut)*, in which the Cdh5-siRNA#1-recognizing region of the gene was mutated (Fig. 5A), was expected to be resistant to Cdh5-siRNA#1-mediated degradation, but sensitive to Cdh5-siRNA#3. To validate this resistance, DF1 cells were co-transfected with Cdh5mutGFP-encoding cDNA along with either Cdh5-siRNA#1 or Cdh5-siRNA#3 (Fig. 5A). Whereas knockdown efficiency (GFP^{+} cells/mCherry^{+} cells) by Cdh5-siRNA#3 was similar to that in Fig. 3 (0.18 ± 0.11; n = 4), Cdh5-siRNA#1 failed to inhibit Cdh5mutGFP (1.11 ± 0.10; n = 4) (Fig. 5B and C). Thus, Cdh5mutGFP was resistant against Cdh5-siRNA #1-mediated degradation.

Finally, we asked if *Cdh5mut* mRNA would rescue the *in vivo* vascular phenotype elicited by Cdh5-siRNA#1. Right dorsal aorta (R-DA) of 16 ss embryo was injected with a 0.8 μl cocktail containing Cdh5-siRNA#1 and *Cdh5mut* mRNA (12.5% of Lipofectamine, 2000, 4 μM of rhodamine-labeled Cdh5-siRNA#1, and 360 ng/μl of *Luciferase* mRNA or 600 ng/μl of *Cdh5mut* mRNA), and vascular formation was assessed after 20 h at HH16 (Fig. 5D). When *Luciferase* mRNA and Cdh5-siRNA#1 were co-injected as a control, the vasculature was disrupted in the rhodamine-labeled area as seen in Fig. 4C (Fig. 5E, n = 7/7). In contrast, when *Cdh5mut* mRNA was co-transfected with Cdh5-siRNA#1, overt structures of vitelline artery and yolk sac vasculature with blood circulation were recognized in the rhodamine-labeled area (Fig. 5F, n = 7/10). Thus, we conclude that the vascular defects seen in Cdh5-siRNA#1-transfected embryos are caused by Cdh5-specific knockdown and not by off-target effects. The results also show that the transfection method described in this study is applicable not only for DNA/plasmid, but also for mRNA products.

4. Discussion

We have demonstrated that blood flow-mediated gene transfer into developing vasculature of chicken embryos enables efficient transgenesis in a spatio-temporally controlled manner. The gene transfer is achieved by infusion with a cocktail containing DNA/RNA and Lipofectamine, the latter being a commercially available easy-to-use reagent. By exploiting stage-dependent patterns of blood flow combined with the siRNA



I

	Circumferential length (μm)		Maximum speed of blood flow (v) (μm/s)		Heart beat rate (bpm)
	L-DA	R-DA	L-DA	R-DA	
Luc-siRNA	455.2 ± 27.3 (n=5)	439.5 ± 19.2 (n=5)	3412 ± 133 (n=3)	3112 ± 242 (n=3)	90.3 ± 2.1 (n=5)
Cdh5-siRNA#1	580.9 ± 35.1 (n=6)	266.9 ± 47.1 (n=6)	3828 ± 229 (n=3)	2102 ± 353 (n=3)	90.3 ± 7.9 (n=4)

J

$$\text{Relative shear stress } (\tau) = \frac{V / V_{\text{L-DA (Luc)}}}{r / r_{\text{L-DA (Luc)}}}$$

	Relative τ	
	L-DA	R-DA
Luc-siRNA	1.00	0.94
Cdh5-siRNA#1	0.88	1.04

Fig. 4. Site-specific *Cdh5* knockdown in developing vasculature caused local disruption of vascular network. (A) A cocktail containing siRNA, Alexa Fluor Red, and Lipofectamine 2000 was injected into the R-DA of 16 ss embryos, followed by examination 20 h after transfection (E2.5). Vascular networks of E2.5 (HH16) embryos were visualized by perfusion with fluorescent ink, shown in green. (B–E) Phenotypes of embryos transfected with siRNAs (shown on the top). Bright field, Alexa Fluor Red, and vasculature photos were taken of the same embryos. Specimens for *in situ* hybridization with *Cdh5* mRNA were different from those shown above. Arrows indicate R-VA. (B) Luc-siRNA-transfected embryos showed normal vascular networks (n = 15/16). (C) Cdh5-siRNA#1-transfected embryos exhibited a local disruption of vasculature corresponding to the transfected area in the right side including R-YS and R-VA (brackets) (n = 29/32). (D, E) Embryos transfected with Cdh5-siRNA#2- or -#3 displayed mild (#3) or almost undetectable (#2) phenotypes compared to those with Cdh5-siRNA#1 (n = 17 each). (F) Transverse sections of the trunk region of siRNA-transfected embryo at E2.5. (G) R-DA in Cdh5-siRNA#1-transfected embryos was smaller in diameter, and R-YS was locally disrupted (Cdh5/Tie2 double negative) (n = 2/2). (H) Luc-siRNA-transfected control embryos (n = 2/2). siRNA-transfected areas are revealed by Alexa Flour Red (brackets). (I) Measurements of vascular parameters of DAs (circumferential length and maximum speed of blood flow) and heart beat rates of Luc-siRNA and Cdh5-siRNA#1 transfected embryos. A cocktail containing siRNA, Alexa Fluor Red, and Lipofectamine 2000 was injected into the R-DA of 16 ss embryos, and measurements were performed after 12 h (E2.2). The circumferential length of the DA was measured in transverse sections of trunk region. For the measurement of maximum blood flow speed (v), embryos infused with fluorescent beads were filmed *in ovo* for 10 s with 10 frames/second. The heart beat rate was directly counted *in ovo*. (J) Relative shear stress (τ) in L-DAs and R-DAs of Luc-siRNA and Cdh5-siRNA#1 transfected embryos, respectively. The average radius (r) was obtained from the circumferential length of DA. The relative τ in L-DA of Luc-siRNA ($\tau_{\text{L-DA (Luc)}}$) was set as a reference. There were no significant differences in relative τ between these four DAs. Bpm, beats per minute. Scale bars: 1 mm for (B–E) and 50 μm for (G, H).

technique, we have shown, for the first time, that endogenous gene expression of *Cdh5* can be knocked down in a site-specific manner in developing vasculature.

4.1. Lipofectamine is useful for infusion-mediated gene transfer

We have found that among commercially available reagents commonly used for gene transfection into cultured cells, Lipofectamine 2000 and -3000, the liposomal reagents, give higher efficiency than the non-liposomal reagents, ViaFect, FuGENE HD, and SuperFect (Fig. 1, Table 1). This suggests that liposomal components are critical for the infusion-mediated transfection at least into early vasculatures *in ovo*, and this is consistent with the previous reports in which elaborately prepared liposomal constructs were used for DNA transfection into vasculatures in chicken embryos (Bollerot et al., 2006; Decastro et al., 2006). Bollerot et al. developed their original reagent by mixing lipid RPR209120 (synthesized in their laboratory) and the neutral helper lipid DOPE. Decastro et al. prepared their original reagent by mixing three lipids, cationic lipid DOSPA, DOPE, and mPEG–SS–DOPE (synthesized on their own). Both groups succeeded in transfection with infused genes into developing vasculature, and Bollerot et al. further conducted siRNA-knockdown of the co-infused exogenous *GFP* gene. These pioneering techniques, however, required high expertise to prepare reagents. In the meantime, commercially available reagents, such as Lipofectamine 2000 or -3000, have been extended to *in ovo* transgenesis of primordial germ cells (PGCs) in chickens, for which Lipofectamine-DNA cocktail is infused into blood stream (PGCs in avians are translocated by blood circulation) (Tyack et al., 2013). Indeed, we have found that Lipofectamine reagents exhibit high transfection efficiency in developing vasculature. Although we have not directly compared, the transfection efficiency by Lipofectamine used in the current study appears to be at least comparable with, if not superior to, the efficiency described in the aforementioned previous studies using liposomes prepared by their own (Bollerot et al., 2006; Decastro et al., 2006).

In our hands, whereas the transfection efficiency is comparable between Lipofectamine 2000 and Lipofectamine 3000, the former costs less than the latter. For both maximizing the efficiency of gene transfer and minimizing embryonic lethality, the concentration of Lipofectamine in the DNA-lipid cocktail is critical, which should be in the range of 12.5%–25%. This concentration-sensitivity of Lipofectamine might be the reason why this popular reagent has been overlooked for *in vivo/in ovo* transfection.

Further optimization might enable other commercially available liposome-based reagents to be used as a convenient vehicle for endothelial transfection.

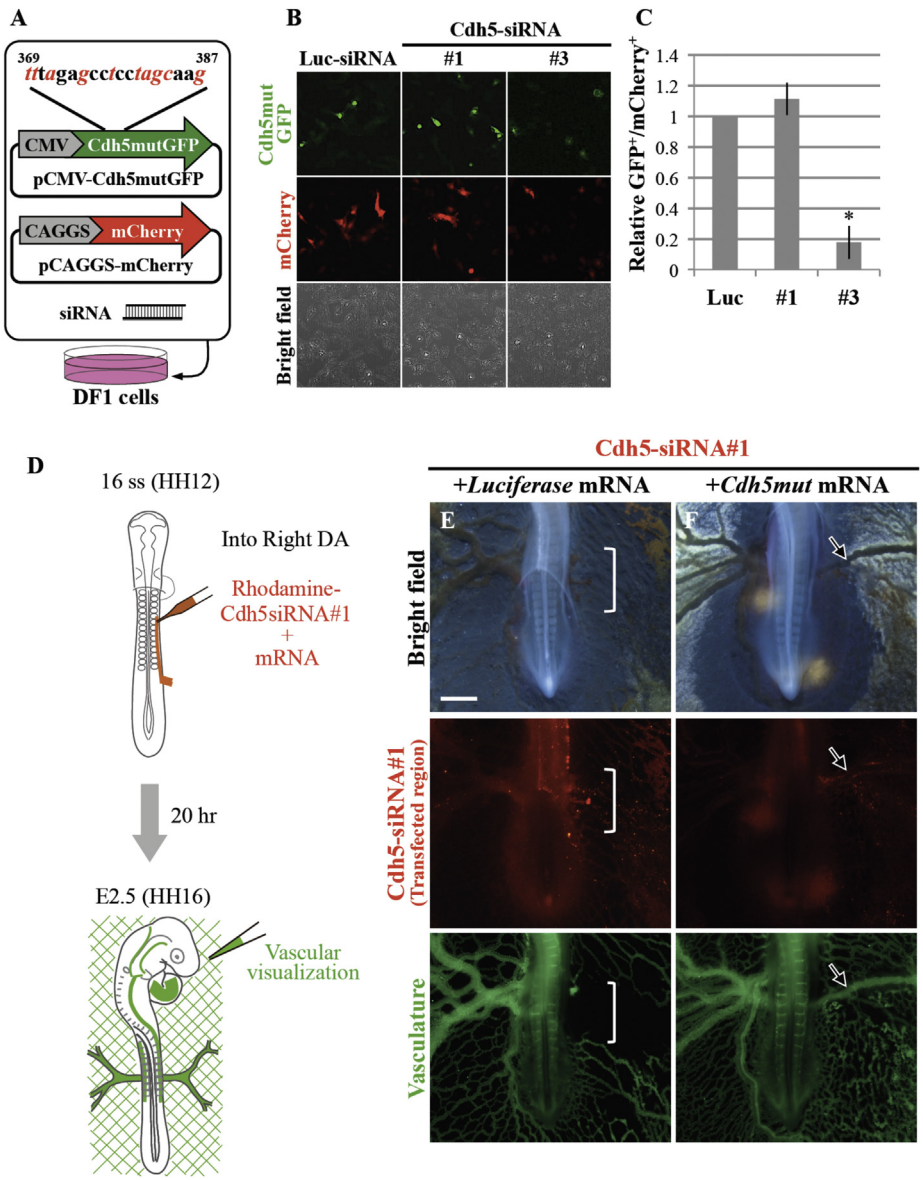


Fig. 5. Rescue of Cdh5 siRNA-knockdown phenotypes by co-transfection with Cdh5 mRNA which was resistant to Cdh5-siRNA#1. (A) pCMV-Cdh5mutGFP (red letters are replaced nucleotides) and pCAGGS-mCherry plasmids were co-transfected into DF1 cells with each siRNA (Luc-siRNA, Cdh5-siRNA#1, and -#3), and assessed 24 h after transfection. (B) Cdh5mutGFP and mCherry expression in DF1 cells co-transfected with siRNAs shown on the top. Compared to the number of GFP-positive cells co-transfected with Luc-siRNA, that of Cdh5-siRNA#3 co-transfected cells was smaller. However, the number of GFP-positive cells co-transfected with Cdh5-siRNA#1 was comparable to that of Luc-siRNA-cells. (C) Quantitative representation of a relative ratio of the number of GFP^+ cells to that of $mCherry^+$ cells (n = 4 each). Thus, Cdh5-siRNA#1 failed to knockdown Cdh5mutGFP, whereas Cdh5mutGFP was susceptible to Cdh5-siRNA#3. Error bars represent SD (standard deviation). $^*P < 0.001$. (D) A cocktail containing rhodamine-labeled Cdh5-siRNA#1, mRNAs of *Luciferase* or *Cdh5mut*, and Lipofectamine 2000 was injected into the R-DA of 16 ss embryos, which were assessed 20 h after transfection (E2.5) with vascular visualization by ink perfusion (green). (E, F) Phenotypes of embryos co-transfected with Cdh5-siRNA#1 and mRNA shown on the top. White brackets indicate transfected area (rhodamine-positive). (E) Embryos co-transfected with Cdh5-siRNA#1 and *Luciferase* mRNA exhibited the local disruption of R-VA in the rhodamine-labeled region (n = 7/7) as seen in Fig. 4C. (F) However, such defects of R-VA by Cdh5-siRNA#1 was rescued when co-transfected with *Cdh5mut* mRNA (n = 7/10).

4.2. Blood flow-mediated site-specific gene manipulation in early vasculature

We were able to control targeted sites of vascular transgenesis in two different ways. One is by controlling infusion sites, which results in different areas of transfection. The other one is by controlling time points of infusion: since the early patterns of blood flow and vasculature changes drastically as development proceeds, an infused cocktail also spreads differently in a stage-dependent manner even when the cocktail is infused into the same site.

Differential sites of infusion: When we inject a *GFP*-Lipofectamine cocktail into the heart after the circulation is established, vasculatures in both right and left halves are equally transfected in addition to the heart (Fig. 2F). In contrast, when the cocktail is infused into the right dorsal aorta, the vasculature in only the right half of embryo/extraembryo is positive for GFP (Fig. 2I).

Differential time points of infusion: Our infusion-mediated gene transfer in vasculature takes advantage of the blood flow. When a cocktail is infused into the heart at 13 ss when the blood flow is about to start, the transfected area is limited (Fig. 2E). In contrast, when the cocktail is infused into the heart at 19 ss when the blood flow is more prominent than 13 ss, the infused reagents are more widely spread in the vasculature, resulting in a wide area of transgenesis (Fig. 2G).

4.3. Vascular subtype-specific knockdown of endogenous genes using siRNAs

We have described that the endogenous *VE-cadherin/Cdh5* gene in vasculature can be locally knocked down by combining Lipofectamine-mediated transfection with siRNAs. The importance of Cdh5 for the vascular assembly has been appreciated since an infusion of function-blocking Cdh5 antibody causes disassembly of vessels in mice (Crosby et al., 2005). However, investigations at high resolution of the role of Cdh5 in the peripheral vasculature have largely been hampered, since transgenic animals such as mice or zebrafish, in which the Cdh5 gene is knocked out/down, exhibit early lethality mainly because of a heart failure (Carmeliet et al., 1999; Gory-Faure et al., 1999; Montero-Balaguer et al., 2009). In the current study, we have succeeded, for the first time, in knocking down of the endogenous *Cdh5* in a restricted site of the right half vasculature, which results in severe defects of blood vessels. In embryos infused with Cdh5-siRNA#1 into R-DA (Cdh5-siRNA#1-infused$^{R\text{-}DA}$ embryo), not only heart beat/function but also sheer stress in

both transfected and non-transfected DAs remain unaffected (Fig. 4I and J). These findings further highlight a usefulness of our method for a local transgenesis in peripheral vasculature. Indeed, when the Cdh5-siRNA#1 cocktail was injected into a beating heart, the entire vasculature, including the heart, was severely affected, resulting in embryo-wide malformation (n = 18 for Luc-siRNA; n = 16 for Cdh5-siRNA#1; Suppl. Fig. 3). Such phenotype is reminiscent of malformed *Cdh5*-knockout mice, which die at mid-gestation as a result of severe cardiovascular anomalies (Carmeliet et al., 1999; Gory-Faure et al., 1999).

In summary, the method described in this study enables efficient gene transfer into early chicken vasculature without affecting heartbeat. Infusion of a cocktail with Lipofectamine, an easy-use reagent, delivers plasmid DNAs, mRNAs, and siRNAs to developing vasculatures. Vascular type-specific transfection can be achieved by adjusting infusion sites and developmental stages of embryos. This method could also be applicable to other experimental animals, facilitating investigations of the formation of peripheral vascular systems, and their roles in tissue and organ formation.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ydbio.2019.08.001.

References

Adams, R.H., Alitalo, K., 2007. Molecular regulation of angiogenesis and lymphangiogenesis. Nat. Rev. Mol. Cell Biol. 8, 464–478.
Augustin, H.G., Koh, G.Y., 2017. Organotypic vasculature: from descriptive heterogeneity to functional pathophysiology. Science 357.
Bollerot, K., Sugiyama, D., Escriou, V., Gautier, R., Tozer, S., Scherman, D., Jaffredo, T., 2006. Widespread lipoplex-mediated gene transfer to vascular endothelial cells and hemangioblasts in the vertebrate embryo. Dev. Dyn. 235, 105–114.
Carmeliet, P., 2005. Angiogenesis in life, disease and medicine. Nature 438, 932–936.
Carmeliet, P., Lampugnani, M.G., Moons, L., Breviario, F., Compernolle, V., Bono, F., Balconi, G., Spagnuolo, R., Oosthuyse, B., Dewerchin, M., et al., 1999. Targeted deficiency or cytosolic truncation of the VE-cadherin gene in mice impairs VEGF-mediated endothelial survival and angiogenesis. Cell 98, 147–157.
Chapman, S.C., Collignon, J., Schoenwolf, G.C., Lumsden, A., 2001. Improved method for chick whole-embryo culture using a filter paper carrier. Dev. Dyn. 220, 284–289.
Crosby, C.V., Fleming, P.A., Argraves, W.S., Corada, M., Zanetta, L., Dejana, E., Drake, C.J., 2005. VE-cadherin is not required for the formation of nascent blood vessels but acts to prevent their disassembly. Blood 105, 2771–2776.
De Val, S., Black, B.L., 2009. Transcriptional control of endothelial cell development. Dev. Cell 16, 180–195.
Decastro, M., Saijoh, Y., Schoenwolf, G.C., 2006. Optimized cationic lipid-based gene delivery reagents for use in developing vertebrate embryos. Dev. Dyn. 235, 2210–2219.
Fernandez-Teran, M.A., Hinchliffe, J.R., Ros, M.A., 2006. Birth and death of cells in limb development: a mapping study. Dev. Dyn. 235, 2521–2537.
Gory-Faure, S., Prandini, M.H., Pointu, H., Roullot, V., Pignot-Paintrand, I., Vernet, M., Huber, P., 1999. Role of vascular endothelial-cadherin in vascular morphogenesis. Development 126, 2093–2102.
Hamburger, V., Hamilton, H.L., 1951. A series of normal stages in the developing chick embryo. J. Morphol. 88, 49–92.
Herbert, S.P., Stainier, D.Y., 2011. Molecular control of endothelial cell behaviour during blood vessel morphogenesis. Nat. Rev. Mol. Cell Biol. 12, 551–564.
Hogan, B.M., Schulte-Merker, S., 2017. How to plumb a pisces: understanding vascular development and disease using zebrafish embryos. Dev. Cell 42, 567–583.
Jain, R.K., 2003. Molecular regulation of vessel maturation. Nat. Med. 9, 685–693.
Jilani, S.M., Murphy, T.J., Thai, S.N., Eichmann, A., Alva, J.A., Iruela-Arispe, M.L., 2003. Selective binding of lectins to embryonic chicken vasculature. J. Histochem. Cytochem. 51, 597–604.
Korhonen, J., Lahtinen, I., Halmekyto, M., Alhonen, L., Janne, J., Dumont, D., Alitalo, K., 1995. Endothelial-specific gene expression directed by the tie gene promoter in vivo. Blood 86, 1828–1835.
Lan, Y., Liu, B., Yao, H., Li, F., Weng, T., Yang, G., Li, W., Cheng, X., Mao, N., Yang, X., 2007. Essential role of endothelial Smad4 in vascular remodeling and integrity. Mol. Cell. Biol. 27, 7683–7692.
Makita, T., Sucov, H.M., Gariepy, C.E., Yanagisawa, M., Ginty, D.D., 2008. Endothelins are vascular-derived axonal guidance cues for developing sympathetic neurons. Nature 452, 759–763.
Momose, T., Tonegawa, A., Takeuchi, J., Ogawa, H., Umesono, K., Yasuda, K., 1999. Efficient targeting of gene expression in chick embryos by microelectroporation. Dev. Growth Differ. 41, 335–344.
Montero-Balaguer, M., Swirsding, K., Orsenigo, F., Cotelli, F., Mione, M., Dejana, E., 2009. Stable vascular connections and remodeling require full expression of VE-cadherin in zebrafish embryos. PLoS One 4, e5772.
Papaioannou, T.G., Stefanadis, C., 2005. Vascular wall shear stress: basic principles and methods. Hellenic J. Cardiol. 46, 9–15.
Pardanaud, L., Luton, D., Prigent, M., Bourcheix, L.M., Catala, M., Dieterlen-Lievre, F., 1996. Two distinct endothelial lineages in ontogeny, one of them related to hemopoiesis. Development 122, 1363–1371.
Potente, M., Gerhardt, H., Carmeliet, P., 2011. Basic and therapeutic aspects of angiogenesis. Cell 146, 873–887.
Rossello, C.A., Torres, M., 2010. Gene transfer by electroporation into hemogenic endothelium in the avian embryo. Dev. Dyn. 239, 1748–1754.
Saito, D., Takase, Y., Murai, H., Takahashi, Y., 2012. The dorsal aorta initiates a molecular cascade that instructs sympatho-adrenal specification. Science 336, 1578–1581.
Sato, Y., Watanabe, T., Saito, D., Takahashi, T., Yoshida, S., Kohyama, J., Ohata, E., Okano, H., Takahashi, Y., 2008. Notch mediates the segmental specification of angioblasts in somites and their directed migration toward the dorsal aorta in avian embryos. Dev. Cell 14, 890–901.
Schlaeger, T.M., Bartunkova, S., Lawitts, J.A., Teichmann, G., Risau, W., Deutsch, U., Sato, T.N., 1997. Uniform vascular-endothelial-cell-specific gene expression in both embryonic and adult transgenic mice. Proc. Natl. Acad. Sci. U. S. A 94, 3058–3063.
Sridurongrit, S., Larsson, J., Schwartz, R., Ruiz-Lozano, P., Kaartinen, V., 2008. Signaling via the Tgf-beta type I receptor Alk5 in heart development. Dev. Biol. 322, 208–218.
Stahl, A., Connor, K.M., Sapieha, P., Chen, J., Dennison, R.J., Krah, N.M., Seaward, M.R., Willett, K.L., Aderman, C.M., Guerin, K.I., et al., 2010. The mouse retina as an angiogenesis model. Investig. Ophthalmol. Vis. Sci. 51, 2813–2826.
Takase, Y., Tadokoro, R., Takahashi, Y., 2013. Low cost labeling with highlighter ink efficiently visualizes developing blood vessels in avian and mouse embryos. Dev. Growth Differ. 55, 792–801.
Tonegawa, A., Funayama, N., Ueno, N., Takahashi, Y., 1997. Mesodermal subdivision along the mediolateral axis in chicken controlled by different concentrations of BMP-4. Development 124, 1975–1984.
Tonegawa, A., Kasai, T., Takahashi, Y., 2003. Systematic screening for signaling molecules expressed during somitogenesis by the signal sequence trap method. Dev. Biol. 262, 32–50.
Tyack, S.G., Jenkins, K.A., O'Neil, T.E., Wise, T.G., Morris, K.R., Bruce, M.P., McLeod, S., Wade, A.J., McKay, J., Moore, R.J., et al., 2013. A new method for producing transgenic birds via direct in vivo transfection of primordial germ cells. Transgenic Res. 22, 1257–1264.
Yokota, Y., Saito, D., Tadokoro, R., Takahashi, Y., 2011. Genomically integrated transgenes are stably and conditionally expressed in neural crest cell-specific lineages. Dev. Biol. 353, 382–395.