Short Communication

DOCK180 Is a Rac Activator That Regulates Cardiovascular Development by Acting Downstream of CXCR4

Fumiyuki Sanematsu, Masanori Hirashima, Mélanie Laurin, Ryosuke Takii, Akihiko Nishikimi, Keiko Kitajima, Guo Ding, Mamiko Noda, Yuzo Murata, Yoshihiko Tanaka, Sadahiko Masuko, Toshio Suda, Chikara Meno, Jean-François Côté, Takashi Nagasawa, Yoshinori Fukui

Rationale: During embryogenesis, the CXC chemokine ligand (CXCL)12 acts on endothelial cells to control cardiac development and angiogenesis. Although biological functions of CXCL12 are exerted in part through activation of the small GTPase Rac, the pathway leading from its receptor CXC chemokine receptor (CXCR)4 to Rac activation remains to be determined.

Objective: DOCK180 (dedicator of cytokinesis), an atypical Rac activator, has been implicated in various cellular functions. Here, we examined the role of DOCK180 in cardiovascular development.

Methods and Results: DOCK180 associates with ELMO (engulfment and cell motility) through the N-terminal region containing a Src homology 3 domain. We found that targeted deletion of the Src homology 3 domain of DOCK180 in mice leads to embryonic lethality with marked reduction of DOCK180 expression at the protein level. These mutant mice, as well as DOCK180-deficient mice, exhibited multiple cardiovascular abnormalities resembling those seen in CXCR4-deficient mice. In DOCK180 knocked down endothelial cells, CXCL12-induced Rac activation was impaired, resulting in a marked reduction of cell motility.

Conclusions: These results suggest that DOCK180 links CXCR4 signaling to Rac activation to control endothelial cell migration during cardiovascular development. (Circ Res. 2010;107:1102-1105.)

Key Words: cardiovascular development ▪ endothelial cells ▪ CXCL12 ▪ Rac ▪ DOCK180

During embryogenesis, progenitor cells migrate to specific locations, where they differentiate into specialized cells that make up different tissues and organs. This process is coordinately regulated by extrinsic guidance cues. The CXC chemokine ligand (CXCL)12 is one of such cues critical for cardiovascular development. Mice lacking CXCL12 or its receptor CXC chemokine receptor (CXCR)4 exhibit ventricular septal defect (VSD) and poor vascularization of the gastrointestinal tract.1,2 In developing heart, CXCR4 is predominantly expressed on endocardial cells in the atrioventricular (AV) canal,3 which are destined to participate in formation of the membranous septa and valves. Similarly, the expression of CXCR4 in developing vessels is limited to arterial endothelial cells.4 Thus, CXCL12 acts on endothelial cells to control cardiac development and angiogenesis during embryogenesis.

On binding to CXCR4, CXCL12 induces dissociation of heterodimeric G proteins into α and βγ subunits, which results in the activation of a variety of signaling pathways including Rac. Like other small GTPases, Rac cycles between GDP-bound inactive and GTP-bound active states, and stimulus-induced formation of the active Rac is mediated by guanine nucleotide exchange factors (GEFs). However, the mechanism controlling Rac activation downstream of CXCR4 is unknown during cardiovascular development.

DOCK180 (dedicator of cytokinesis), a mammalian ortholog of Caenorhabditis elegans CED-5 and Drosophila melanogaster Myoblast City,5,6 controls cell migration and

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DOCK180 area of the back at E14.5 (Figure 1A; Online Figure III). In this study, we mediate the GTP-GDP exchange for Rac by means of its DHR-2 (also known as Docker) domain.10,12 In this study, we examined the functions of DOCK180 during cardiovascular development.

**Methods**

Biochemical, histological, and functional analyses were performed by standard methods. An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

**Results and Discussion**

DOCK180 associates with ELMO (engulfment and cell motility) through the N-terminal region containing a Src homology (SH)3 domain and a putative α-helical region.7,13 To understand the mechanism of protein decrease in the mutant animals, we examined protein degradation using CHO cells stably expressing green fluorescent protein (GFP)-tagged WT DOCK180 or its mutant bearing the same deletion as in DOCK180d/d mice (designated SH3del). Pulse-Chase experiments revealed that the SH3del mutant was more rapidly degraded than WT DOCK180 (Figure 1C). The proteasome inhibitor MG132 blocked this degradation, but bafilomycin had no effect, indicating that deletion of the SH3 domain leads to proteasome-dependent degradation of DOCK180 (Figure 1C). When the SH3del mutant was transiently expressed with ELMO1 in HEK293T cells, its binding to ELMO1 was modestly diminished (Figure 1D), in agreement with a nonessential role of this domain for ELMO-binding.13 However, whereas ubiquitination of WT DOCK180 was suppressed by coexpression of ELMO1 in the presence of MG132, such inhibitory effect was less profound for the SH3del mutant (Figure 1E), suggesting that the interaction of DOCK180 SH3 domain with ELMO is required to inhibit ubiquitination and degradation of DOCK180.

DOCK180d/d mice exhibited severe edema, we hypothesized that they might have defects in cardiovascular development. Indeed, cardiac abnormalities such as submembranous VSD and double outlet right ventricle (DORV) were detected in all DOCK180d/d embryos examined at E14.5 (Figure 2A). Similar results were obtained when DOCK180−/− embryos were analyzed (Online Figure III). In both DOCK180d/d and DOCK180−/− embryos, mitral valve leaflets were also thickened and in some of them fused, which accounted for the blood retention in left atrium (Figure 2A; Online Figure III). Side-by-side analyses revealed that...
cardiac abnormalities of DOCK180<sup>d/d</sup> embryos resembled those seen in CXCR4-deficient (CXCR4<sup>−/−</sup>) mice (Figure 2A). When DOCK180 SH3 domain was specifically deleted in endothelial cells using Tie2-Cre transgenic mice, these mice also exhibited VSD and DORV (Online Figure IV), thus confirming the endothelial origin of the cardiac defects in DOCK180<sup>d/d</sup> embryos.

Cardiac endothelial cells in the AV canal undergo transforming growth factor-β–dependent epithelial–mesenchymal transformation (EMT) and migrate into an extracellular matrix referred to as the cardiac jelly. This process is important to form endocardial cushion from which AV valves and membranous septa originate. At E8.5, before EMT initiates, DOCK180<sup>d/d</sup> and WT littermates were indistinguishable concerning the heart development. However, the endocardial cushion of DOCK180<sup>d/d</sup> embryos showed a significantly decreased cellularity at E12.5 (Figure 2B). In heart explant assays, cardiac endothelial cells from both WT and DOCK180<sup>d/d</sup> embryos were widely spread on the gel, showing spindle-like mesenchymal morphology (Figure 2C). However, although cells from the WT explants effectively invaded the gel, the number of invading cells significantly decreased in the case of DOCK180<sup>d/d</sup> explants (Figure 2D). It is clear that DOCK180 is not required for EMT itself, because endothelial cells from WT and DOCK180<sup>d/d</sup> embryos comparably transformed into mesenchymal cells expressing α-smooth muscle actin (α-SMA) (Figure 2E).

Because CXCR4 is required for vascularization of the gastrointestinal tract, we examined whether DOCK180 also functions in vascular endothelial cells. During embryogenesis, the small intestine begins to develop as a simple tube called a midgut loop. When the midgut loop of WT embryos was analyzed at E12.5, many branches were found to arise from the α-SMA–positive superior mesenteric artery (Figure 3A). However, as in CXCR4<sup>−/−</sup> embryos, the interconnecting vessels between superior mesenteric artery and the capillary plexus were severely reduced in DOCK180<sup>d/d</sup> midgut loop (Figure 3A). Similarly, at E15.5, large vessels poorly developed in the stomach of DOCK180<sup>d/d</sup> embryos (Figure 3A).

To examine whether DOCK180 functions downstream of CXCR4, we purified arterial endothelial cells from WT and DOCK180<sup>d/d</sup> embryos. In chemotaxis assays, WT endothelial cells efficiently migrated to the lower chamber in response to either CXCL12 or vascular endothelial growth factor (VEGF). However, migratory response to CXCL12, but not to VEGF, was almost completely lost for DOCK180<sup>d/d</sup> endothelial cells (Figure 3B). Consistent with this finding,
CXCL12-induced peripheral membrane ruffle formation was scarcely detected in DOCK180<sup>−/−</sup> endothelial cells (Figure 3C). To obtain biochemical evidence that DOCK180 controls Rac activation downstream of CXCR4, we used human aorta endothelial cells. In these cells, knockdown of DOCK180 expression by RNA interference abrogated CXCL12-induced Rac activation (Figure 3D).

Here we have shown that DOCK180 is required for cardiovascular development. Importantly, DOCK180 knockdown does not affect the expression or localization of CXCL12 and CXCR4 in vivo (Online Figure V). In addition, DOCK180 and CXCR4 are coexpressed in cardiovascular and vascular endothelial cell lineage (Online Figure VI). Our results thus indicate that DOCK180 links CXCR4 signaling to Rac activation to control endothelial cell migration during cardiovascular development.

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**Disclosures**

None.

**References**


**Novelty and Significance**

**What Is Known?**

- The chemokine CXCL12 and its receptor CXCR4 are required for cardiac development and angiogenesis.
- On activation, Rac provides the force necessary to extend membrane protrusion in the direction of migration.
- DOCK180, an atypical Rac activator, controls cell migration and phagocytosis in vitro and myoblast fusion in vivo.

**What New Information Does This Article Contribute?**

- Mice with defective DOCK180 expression exhibit multiple cardiovascular abnormalities similar to those seen in CXCR4-deficient mice.
- DOCK180 regulates CXCL12-induced endothelial cell migration.

- DOCK180 is required for CXCR4-mediated Rac activation in endothelial cells.

Cardiovascular malformations are the most common congenital anomalies. Therefore, elucidation of the signaling pathways involved in heart and vessel patterning is important in clinical medicine. Although previous studies have shown that the chemokine CXCL12 and its receptor CXCR4 play important roles in cardiovascular development, the downstream signaling events are poorly understood. In this study, we show that DOCK180, an atypical Rac activator, controls cardiac development and angiogenesis in vivo by acting downstream of CXCR4 in endothelial cells. Our results provide the first evidence showing that DOCK180 is a key signaling molecule that regulates cardiovascular development.
DOCK180 Is a Rac Activator That Regulates Cardiovascular Development by Acting Downstream of CXCR4

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SUPPLEMENTAL MATERIAL

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Detailed Methods

Mice
For generation of DOCK180<sup>dd</sup> mice, we inserted two loxP sites flanking the second exon of DOCK180 gene via homologous recombination in embryonic stem cells (see Online Figure I). Mice harboring a loxP-flanked allele (DOCK180<sup>lox/+</sup>) were bread with EIIa-Cre transgenic mice<sup>1</sup> to delete the second exon encoding the SH3 domain in frame. The resultant progeny (DOCK180<sup>dd/+</sup>) were backcrossed with C57BL/6 mice for at least eight generations before use. CXCR4<sup>−/−</sup> mice have been described elsewhere.<sup>2</sup> Mice were kept under specific pathogen–free conditions. All experiments were performed in accordance with the guidelines of the committee of Ethics of Animal Experiments at Kyushu University.

Cells and Culture
HAECs were obtained from Cell applications and cultured with Endothelial Cell Basal Medium (Cell applications). Primary arterial endothelial cells were isolated from E13.5 embryos as previously described,<sup>3</sup> with some modifications. Briefly, E13.5 embryos were dissected and incubated for 30 minutes in DMEM medium containing 0.1% collagenase. Cells were incubated with biotinylated anti–EphB4 antibody (R&D systems) for eliminating venous endothelial cells with MACS separation system (Miltenyi Biotec). Arterial endothelial cells were then enriched by using fluorescein isothiocyanate (FITC)-conjugated anti–PECAM-1 antibody (MEC13.3, BD Biosciences) and anti–FITC antibody–coated microbeads (Miltenyi Biotec). Cells were cultured in a 96-well plate (IWAKI), which was coated with 10 µg/ml fibronectin, using EGM-2 medium and the EGM-2 bullet kit (Lonza).

Plasmids and siRNA
The gene encoding WT DOCK180 or SH3del mutant was created by polymerase chain reaction (PCR) using the cDNA prepared from WT or DOCK180<sup>dd</sup> embryo as a template. The amplified gene was subcloned into pCI or PBJ1 vector to express the C-terminally GFP-tagged fusion protein
in HEK293T cells or CHO cells, respectively. The pCDNA4 vector encoding ELMO1 with V5-tag has been described elsewhere, and pCGN-HA-Ubiquitin vector was obtained from Dr. K.-I. Nakayama (Kyushu University, Japan). The siRNA for human DOCK180 (ID#145822) was purchased from Ambion. For knock-down experiments, HAECs were transfected with the siRNA using Lipofectamine 2000 (Invitrogen) 2 days before assay.

Immunoblot Analyses

Transient transfection into HEK293T cells was performed with polyethylenimine as described elsewhere. HEK293T cell extracts were immunoblotted with anti–GFP antibody (Invitrogen), anti–V5 antibody (Invitrogen) or anti–HA antibody (Roche Diagnostics) to detect DOCK180, ELMO1 or ubiquitin, respectively. The expression of DOCK180 in embryos was examined with anti–DOCK180 antibody (C19, Santa Cruz Biotechnology). Rac activation assay was performed as described elsewhere. Briefly, aliquots of the cell extracts were kept for total lysate controls, and the remaining extracts were incubated with glutathione-S-transferase (GST)–fusion Rac–binding domain of PAK1 at 4 °C for 60 minutes. The bound proteins and the same amounts of total lysates were analyzed by SDS-PAGE, and blots were probed with anti–Rac antibody (23A8, Millipore).

Pulse Chase Experiments

After preincubation in methionine- and cystein-free medium for 1 hour, CHO transfectants stably expressing WT DOCK180 or SH3del mutant were pulsed with [35S]-methionine and cystein for 30 minutes at 37 °C. The medium was then changed to DMEM with or without MG132 (20 µM) or bafilomycin (100 nM), and cell lysates were prepared at the specified time points for immunoprecipitation with anti–GFP antibody (Invitrogen). Quantification of the intensity was carried out with BAS-2500 bio-imaging analyzer and the Image Gauge 4.0 software (Fujifilm).

Histological and Immunohistochemical Analyses

Tissues were fixed in 4% formaldehyde and stained with hematoxylin and eosin, or alcin blue and nuclear fast red. Vasculature of the midgut and stomach was analyzed by staining tissues with antibodies for PECAM-1 (MEC13.3) and α-SMA (1A4, DAKO) followed by Alexa Fluor 488- or Alexa Fluor 546-conjugated secondary antibodies (Invitrogen). In some experiments, embryos were stained with anti–PECAM-1 antibody followed by horseradish peroxidase (HRP)-conjugated secondary antibody (Biosource). The MF20 staining was performed as described previously. To assess peripheral ruffle formation, primary mouse endothelial cells were starved for 5 hours before
assay. After stimulation with CXCL12 (30 ng/ml) for 1 minute at 37 °C, cells were fixed with 4% paraformaldehyde for 5 minutes, soaked with 0.5% Triton X-100 for 5 minutes, and stained with Alexa 546-conjugated phalloidin (Invitrogen). Co-localization of CXCR4 and DOCK180 in the midgut and heart was visualized using ‘zinc fixed frozen methods’. Briefly, tissue samples were immersed in ice-cold zinc fixative (BD Biosciences) overnight with gentle shaking at 4 °C. After samples were immersed with graded sucrose solution, tissue sections (10 µm) were prepared, fixed with zinc fixative for 15 minutes, soaked with 0.5% NP-40 for 3 minutes, and blocked with 1% BSA in TBS/0.1% Tween-20. Samples were then stained with biotinylated anti–CXCR4 (2B11, BD Biosciences) and anti–PECAM-1 (MEC13.3) or anti–DOCK180 (C-19) antibodies followed by appropriately labeled secondary reagents.

**RT-PCR**

Total RNA from the midgut tissues was isolated with ISOGEN (NIPPON GENE). After treatment with RNase-free DNase I (Invitrogen), RNA samples were reverse-transcribed with oligo(dT) primers (Invitrogen) and Superscript III reverse transcriptase (Invitrogen). RT-PCR analysis was performed using the following primers: CXCL12: 5’-ACGCCAAGGTCGTCGCCGTGCTGG-3’ and 5’-GTTAGGGTAATACAATTCCTTAGA-3’, CXCR4: 5’-ATGGAACCGATCAGTGTGAG and 5’-CTGTTGGCCCTTGGAGTGTGACAGC-3’, hypoxanthine phosphoribosyl transferase (HPRT): 5’-CACAGGACTAGAACACCTGC-3’ and 5’-GCTGGTGAAGGACCTCT-3’.

**Whole-Mount In Situ Hybridization**

Embryos were fixed overnight at 4 °C in 4% paraformaldehyde in PBS and dehydrated in methanol. Hybridization was performed according to standard procedures. Briefly, genotyped embryos were rehydrated in PBS containing 0.1% Tween-20 (PBS-T) and incubated in proteinase K (10 µg/ml in PBS-T) for 45 minutes at room temperature. Digestion was stopped with 2 mg/ml glycine in PBS-T, and the embryos were refixed in 4% paraformaldehyde with 0.2% glutaraldehyde in PBS-T. Embryos were washed and hybridized overnight at 70 °C with the digoxigenin-labeled riboprobe for CXCL12. Samples were then incubated with pre-absorbed alkaline phosphatase-conjugated anti–digoxigenin antibody, and the detection of alkaline phosphatase activity was carried out using the NBT/BCIP color reaction.

**Chemotaxis Assay**

Chemotaxis assay was performed using a 48-well chemotaxis chamber (Neuro Probe). After
polycarbonate membranes (pore size, 5 µm) were coated with 10 µg/ml fibronectin, endothelial cells (7.5 x 10^3) were loaded into upper compartment, which was placed onto a chamber containing CXCL12 (100 ng/ml) or VEGF (100 ng/ml). Cells were allowed to migrate for 4 hours at 37 °C and stained with Crystal Violet.

**Heart Explant Assay**
The AV canal region dissected from E9.5 embryos were placed on rat type I collagen gels and allowed to firmly attach for 6-14 hours before addition of 150 µl of M199 explant medium (1 x M199 medium, 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.1% insulin, transferrin and selenium). After being maintained in M199 explant medium for 2 days, AV explants were stained with phalloidin and Hoechst 33342 (both from Invitrogen). Images were acquired with LSM510 META (Zeiss).

**In Vitro EMT Assay**
Cells were cultured in DMEM medium containing 2% FCS in the presence or absence of 1 ng/ml TGF-β2 (R&D systems) for 5 days. Cells were then fixed with 4% paraformaldehyde and stained with anti–α-SMA antibody.

**Statistical Analysis**
Data are expressed as a mean ± SEM. Differences between groups were tested by a two-tailed Student’s t-test.
**Supplemental Table**

**Online Table I.  Genotyping of mice derived from DOCK180^{d/+} intercrosses**

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\(^a\)Genotypes of mice at indicated post-natal (P) or embryonic (E) days were determined using PCR analysis.

\(^b\)Numbers in parentheses indicate the number of dead mice.
Supplemental References


Online Figure I. Strategy for generation of DOCK180\textsuperscript{d/d} mice. A, Targeting strategy. Exon 2 of the murine DOCK180 gene is indicated by gray box, and loxP sites are indicated by black arrowheads. The targeting construct, partial Cre–mediated recombinated allele and EIIa-Cre–mediated recombinated allele (ΔSH3 allele) are shown. Scale bar, 1 kbp. The deleted amino acid sequence is as follows: AFYNYDARGADELSQIQGDTVHILECYE. B, Strategy of genomic PCR in WT and ΔSH3 allele. Primers used for genotyping are as follows (5’ to 3’): primer 1, CCCTACATGACATGCTTTTCT; primer 2, TGCCTGGAAGAGCCTATATCT; primer 3, GCAACACACTGAATTCACTAGTT. C, Genomic PCR in WT and DOCK180\textsuperscript{d/d} embryos.
**Online Figure II. Impaired myoblast fusion in DOCK180<sup>d/d</sup> embryos.** Sections of intercostal muscle of E14.5 embryos were stained with the antibody for muscle-specific myosin heavy chain (MF20; green) and Hoechst (blue). The diameter of myofibers were compared among WT, DOCK180<sup>d/d</sup>, and DOCK180<sup>−/−</sup> embryos. ***, P<0.01. DOCK180<sup>d/d</sup> mice exhibited similar, but less severe, phenotypes as compared with those of DOCK180<sup>−/−</sup> embryos.
Online Figure III. Cardiac abnormalities in DOCK180\(^{-/-}\) embryos. A, Gross morphology of a DOCK180\(^{-/-}\) embryo at E14.5. Arrowhead indicates edema. B, Histology of cardiac tissues of E14.5 embryos. RA, right atrium; LA, left atrium; RV, right ventricle; LV, left ventricle; TV, tricuspid valve; MV, mitral valve; AV, aortic valve; PV, pulmonary valve; *, endocardial cushion. Arrowhead or arrow indicates VSD or DORV, respectively.
Online Figure IV. Histology of cardiac tissues of DOCK180\textsuperscript{lox/lox} (180\textsuperscript{lox/lox}) and Tie2-Cre transgenic DOCK180\textsuperscript{lox/d} (Tie2-Cre180\textsuperscript{lox/d}) embryos at E14.5. RA, right atrium; LA, left atrium; RV, right ventricle; LV, left ventricle; TV, tricuspid valve; MV, mitral valve; AV, aortic valve; PV, pulmonary valve; *, endocardial cushion. Arrowhead or arrow indicates VSD or DORV, respectively.
Online Figure V. Normal expression of CXCL12 and CXCR4 in the midgut of DOCK180<sup>dd</sup> embryos. A, RT-PCR analysis for the expression of CXCR4 and CXCL12 in the E12.5 midgut. d/d, DOCK180<sup>dd</sup>; +/+, WT. The gene encoding hypoxanthine phosphoribosyl transferase (HPRT) was used as a control. The numerals indicate amplification cycles. B, Whole-mount in situ hybridization analysis for the expression of CXCL12 in the E12.5 midgut. C, Immunohistochemical analysis for the expression of CXCR4 in the E12.5 midgut. Samples were stained with anti–CXCR4 and anti–PECAM-1 antibodies. Ma, superior mesenteric artery; Mv, superior mesenteric vein. Scale bar, 50 μm.
Online Figure VI. Expression of CXCR4 and DOCK180 in the midgut and endocardial cushion. Sections from the midgut (A) or heart (B) of E12.5 WT embryos were stained with anti–CXCR4 (green) and anti–DOCK180 (red) antibodies. Ma, superior mesenteric artery; ECC, endocardial cushion. Scale bar, 20 μm.