Generation and evaluation of a transgenic zebrafish for tissue-specific expression of a dominant-negative Rho-associated protein kinase-2

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Abstract

The Ras homologous (Rho) proteins are a family of small GTPases, which regulate the cytoskeleton and are related to stress fibers and focal adhesion. The Rho-associated protein kinases (ROCK) constitute part of the Rho effectors that regulate cell shape and movement via phosphorylation of the myosin light chain and actin depolymerizing factor/cofilin. ROCK members are widely expressed and play roles in various cell types during vertebrate development and morphogenesis; therefore, ROCK-knockout animals exhibit multiple defects mostly initiated at the embryonic stage. Analyzing the distinct roles of ROCK in cell shape and movement during the embryonic stages using live mammalian models is difficult. Here, we inhibited the Rho/ROCK pathway in zebrafish, which is a small fish that can be conveniently used as a developmental animal model in place of mammals. To inhibit the Rho/ROCK pathway, we designed a dominant-negative ROCK-2 (dnROCK-2) that lacked the kinase domain and was under the control of an upstream activation sequence (UAS). To evaluate the effects of expression of dnROCK-2, transgenic zebrafish lines were generated by mating strains expressing the construct with counterpart strains expressing the Gal4 activator in target tissues. In this study, we crossed the dnROCK-2-expressing line with two such Gal4-expressing lines; (1) SAGFF(LF)73A for expression in the whole body, and (2) Tg(*fli1a*: Gal4FF)^{ubs4} for endothelial cell-specific expression. The phenotypes of the fish obtained were observed by fluorescent stereomicroscopy or confocal microscopy. Overexpression of dnROCK-2 in the whole body resulted in an inhibition of development, notably in cephalic formation, at 1-day post-fertilization (dpf). Confocal microscopy revealed that Hensen's zone became unclear in the trunk muscle fibers expressing dnROCK-2. Endothelial cell-specific expression of dnROCK-2 caused abnormalities in cardiovascular formation at 2-dpf. These results suggest that dnROCK-2 can act as a dominant negative construct of the Rho/ROCK pathway to affect regulation of the cytoskeleton. This construct could be a convenient tool to investigate the function of ROCK members in other vertebrate cell types.

Keywords

Rho pathway, ROCK-2, dominant negative, zebrafish, cardiovascular development

Introduction

The Ras homologous (Rho) protein family belongs to the Ras superfamily of small GTPases [1]. In mammals, the Rho family of GTPases contains 20 members, such as cyclin-dependent kinase 1(Cdk1, previously known as Cdc2), Rac family small GTPase 1 (Rac1), and Ras homolog family member A (RhoA) [2]. The Rho family of proteins play various roles in cell shape, adhesion, or migration via regulation of filopodia, lamellipodia or stress fibers [3]. The Rho-associated protein kinases (ROCK) are Rho-associated serine/threonine kinases which play essential roles as Rho effectors. ROCK proteins directly regulate the phosphorylation of myosin - light chain and actin depolymerizing factor/cofilin after activation by binding to RhoA [4, 5]. In previous studies using mice, it has been shown that *Rock* genes are expressed in various cell types and tissues and animals with knockouts of these genes exhibit multiple defects most of which are initiated at the embryonic stage [6-9]. Analyzing the spatio-temporal effects in cell shape or migration using live animals is difficult. In particular, live observation without fixation is also required to visualize the fine details of the cellular machinery which depends on cytoskeleton dynamics.

Here, we use zebrafish as a live vertebrate model system to analyze the functions of the Rho-pathway, including ROCK proteins. Zebrafish are small freshwater fish and one of the most conventional vertebrate model alternatives to mammals. The fish embryo is fertilized, and development occurs outside the female body; thus, it is possible to observe developmental events in the live animal easier than in mammalian models [10]. The zebrafish genome contains four *rock* genes (*rock1*, *rock2a*, *rock2b*, *rock2bl*), however there are no reports so far of any generated mutants or knockout strains. Previous studies using antisense morpholino oligos have suggested that the *rock* genes are involved in the left-right asymmetry of internal organs, including development of the heart in the zebrafish embryo [11, 12]. Another study has demonstrated that a truncated ROCK2 protein (deleted N-terminal kinase domain) can act as a dominant-negative form in zebrafish development [13]. In this study, we set the objective of investigating the tissue-specific Rho inhibition in zebrafish embryos. To accomplish this, we used the Gal4/UAS tissue-specific gene expression system, a commonly used method in zebrafish (Asakawa et al., 2008). Here, we report on the generation of a Gal4-driven dominant-negative ROCK-2 (dnROCK-2) transgenic zebrafish line, and the observed phenotypes in ubiquitous or endothelial-cell specific dnROCK-2-expressing zebrafish embryos.

Materials & Methods

Animal experiments

All experimental protocols were carried out in accordance with and approved by the Ethics Review Board for Animal Experiments at Kyoto University (approval number: J-19-24). The minimum number of live animals was killed under anesthesia according to institutional guidelines.

Fish Husbandry

Adult fish were maintained at 27°C under a 14:10 h light/dark cycle. Embryos were maintained in 0.006 % sea salt water containing methylene blue. Hatched embryos were fed paramecia from 5 to 14 d post-fertilization (dpf), after which juvenile fish were fed live brine shrimp larvae and a powder diet.

Fish strains

The Riken-Mic strain was used for the generation of transgenic fish. Plasmids for the Gal4-UAS system (pT2MUASMCS) were provided by Dr. Koichi Kawakami. The DNA fragment for the dnROCK-2 peptide was synthesized by a commercial supplier (FASMAC Inc., Atsugi-city, Japan). The 821-1375 aa sequence of zebrafish ROCK-2a protein (NCBI Reference Sequence: NP_777288.1) was used as a dominant-negative molecule in this study. The *Tol2*-mediated transgenic method has been previously described [14]. The transgenic lines generated in this study were named Tg(UAS: mCherry-dnROCK-2)^{ko114Tg} and Tg(UAS: mCherry)^{ko115Tg} and have been registered in The Zebrafish Information Network (ZFIN, https://zfin.org). The Gal4 driver strain SAGFF(LF)73A was provided by the National BioResource Project Zebrafish (NBRP; https://shigen.nig.ac.jp/zebra/index_en.html). The transgenic strains Tg(*fli1a*:EGFP)^{y1}, and Tg(*fli1a*:GAL4FF)^{ubs4} also used in the assay have been previously published [15, 16]. To facilitate microscopic observation, the body-color background of the generated transgenic lines utilized the *casper* (*mpv17*$^{a9/a9}$; *mitfa*$^{w2/w2}$), a transparent zebrafish strain [17, 18].

Sample collection

To obtain dnROCK-2-expressing fish, SAGFF(LF)73A/Tg(UAS: GFP) double transgenic fish were crossed with either Tg(UAS: mCherry-dnROCK-2)^{ko114Tg} or Tg(UAS: mCherry)^{ko115Tg}

fish. Embryos exhibiting double fluorescent positive signals (GFP^+, RFP^+) were collected for microscopic observation. To generate endothelial cell-specific dnROCK-2-expressing fish, Tg(*fli1a*: Gal4FF)^{ubs4} fish were crossed with either Tg(*fli1a*: EGFP)^{y1}/Tg(UAS: mCherry-dnROCK-2)^{ko114Tg} or Tg(*fli1a*: EGFP)^{y1}/Tg(UAS: mCherry)^{ko115Tg} double transgenic fish. Embryos exhibiting double fluorescent positive signals (GFP^+, RFP^+) were collected for microscopic observation.

Microscopic observation

For stereomicroscopy, embryo is anesthetized with 0.01 % tricaine and placed on 1.0 % agar in petri dishes. Images were captured using a Leica M205C or MZ16FA microscope (Leica, Wetzlar, Germany). For confocal microscopy, embryo is placed in glass-bottomed dishes and images were acquired using a Leica TCS-SP8 confocal microscope (Leica).

Phalloidin staining

Embryos were fixed in 4 % paraformaldehyde in phosphate-buffered saline (PBS) overnight at 4°C. Fixed samples were then permeabilized with 0.5 % Triton X-100 in PBS at 25°C for 30 min and then washed with 0.1 % Tween-20 in PBS at 25°C for 1 h. Samples were labeled with 0.6 units/mL of Alexa Fluor 647 Phalloidin (Thermo Fisher Scientific, Waltham, MA, USA) overnight at 4°C. Labelled samples were washed with 0.1 % Tween-20 in PBS at 25°C for 1 h.

Results

Molecular design of dominant negative ROCK-2 for generating zebrafish transgenic lines

To generate our transgenic lines, we first synthesized a partial sequence of the zebrafish *rock2a* cDNA lacking the kinase domain (Fig. 1A, see also Fig. S1). The truncated Rock2a protein retains the Rho-binding motif, thus enabling it to act as a competitor against endogenous Rock2a (Fig. 1B). The mCherry red fluorescent protein, used as an expression marker, was then fused to the N-terminal of the truncated Rock2a, leading to the creation of the mCherry-fused truncated *rock2a* transgene (dnROCK-2; Fig. S2). We also prepared an intact mCherry transgene construct not containing any *rock2a*-derived sequences to be used as negative control in our experiments. Each component incorporated a quintuple repeat of upstream activation sequences (5 x UAS), and finally the obtained transgene cassettes (UAS: mCherry-dnROCK-2, UAS: mCherry) were introduced into zebrafish, resulting in the generation of the Tg(UAS: mCherry-dnROCK-2)^{ko114Tg} and Tg(UAS: mCherry)^{ko115Tg} zebrafish transgenic lines.

Whole body expression of the dnROCK-2 construct in zebrafish embryos

To validate the function of the dnROCK-2 construct in zebrafish, the Tg(UAS: mCherry-dnROCK-2)^{ko114Tg} was crossed with the SAGFF(LF)73A transgenic line, which ubiquitously expresses the Gal4 activator. The Gal4-driven fluorescent markers were visualized in whole embryos (Fig. 2A and B), suggesting that the mCherry-dnROCK-2 protein was distributed in the whole body of these embryos. Notably, we observed a number of developmental abnormalities in the dnROCK-2-expressing embryos. The observed effects showed individual variability, but two such typical embryos representing "mild" and "severe" effects are shown in Figure 2. Cephalic formation and trunk elongation were arrested and shrunken in the severely affected embryo at 28-hpf, whereas in the mild embryo, there were no apparent damage except in eye formation (Fig. 2A). However, both embryos exhibited serious defects in the morphogenesis of the whole body by 48-hpf. In the case of the severely affected embryo, overall development appeared to be arrested leading to a collapse of the body structure. Even in the mildly affected embryo, the body trunk was highly underdeveloped, and was accompanied by the presence of edema in the heart and yolk (Fig. 2B). The intensity of the mCherry fluorescence suggested that this phenotypic variation was dependent on the expression level of the transgene. More specifically, the severely

affected embryo exhibited a stronger red fluorescent signal when compared with the mildly affected embryo (Fig. 2A and B).

To validate the actin organization in the dnROCK-2-expressing zebrafish, filamentous actin (F-actin) in the trunk muscle was visualized using a fluorescent phalloidin reagent (Fig. 2C). There were no apparent differences in the number of muscular cells between the control and dnROCK-2-expressing embryos. The red fluorescence signal for dnROCK-2 was localized to the edge of the muscle fibers and the myotendinous junction. In contrast, the signal for mCherry alone was distributed in the nuclei and cytoplasm. However, there were no differences in phalloidin fluorescence intensities between the control and dnROCK-2-expressing embryos. The Hensen's zones in the myofibers were unclear in the muscle pioneer and fast muscle fibers of the dnROCK-2-expressing embryos (Fig. 2C and D).

Endothelial cell-specific expression of dnROCK-2 in zebrafish embryos

To assess the effect of tissue-specific inhibition of the Rho/ROCK pathway we generated transgenic zebrafish expressing dnROCK-2 specifically in endothelial cells. To accomplish this, the Tg(UAS: mCherry-dnROCK-2)^{ko114Tg} was crossed with the Tg(*fli1a*: Gal4FF)^{UBS4} line, which expresses the Gal4 activator in endothelial cells. Under bright field microscopy, some embryos were seen to exhibit heart edema, cephalic hemorrhage, or congestion in the trunk (Fig. 3A). The incidence rate of cardiovascular defects in dnROCK-2-expressing embryos was significantly higher than that in control embryos (Fig. 3B, see also Table. S1). Fluorescent microscopy showed a weaker signal in the intersegmental vessels (ISVs) of the dnROCK-2-expressing embryos relative to control embryos at 30-hpf (Fig. 3C). At 54-hpf, both the dorsal longitudinal anastomotic vessels (DLAVs) and parachordal lymphangioblasts (PLs) were partially lacking in the dnROCK-2-expressing embryos (Fig. 3D). However, serious morphological defects, such as highly underdeveloped body structure that was observed in the embryos with ubiquitous cell inhibition of ROCK, were not observed in embryos with endothelial cell-restricted inhibition.

Discussion

In this study, we generated two novel transgenic zebrafish lines to investigate the tissue specific-expression of the dominant-negative dnROCK-2 transgene and demonstrated that inhibition of ROCK in endothelial cells resulted in cardiovascular defects in zebrafish embryos. The molecular design of the dominant-negative construct was based on a previous study [13]. Our results for the Gal4/UAS-mediated ubiquitous expression of our dnROCK-2 transgene mostly appeared to mimic the phenotypes seen in a previous study, which was achieved through the overexpression of a dominant-negative ROCK-2 through mRNA injection [13]. The dnROCK-2 molecule used here would be expected to bind to all Rho GTPase proteins; thus, it would not just act as a competitor of Rock2 but also might function as a powerful and wide-ranging inhibitor of the Rho/ROCK pathway [19]. The expression of dnROCK-2 in the whole body led to severe developmental defects, including failure in the formation of the head and shrinking of the trunk. The defects observed regarding the development of eyes appeared to mimic the phenotype seen in the *Rock1*-knockout mice previously reported [8]. Furthermore, confocal microscopy indicated that inhibition of the Rho pathway resulted in a lack of the Hensen's zone in the muscle pioneers and fast muscle fibers. A previous report using mice indicated that ROCK proteins are expressed in skeletal muscle fibers [20]. Collectively, our results suggest a novel function of ROCK or other Rho-dependent pathway members in the development of skeletal muscle.

We also assesses the effect of endothelial cell-specific expression of the dnROCK-2 transgene in zebrafish embryos. Endothelial expression of dnROCK-2 resulted in cardiovascular defects, heart edema, cephalic hemorrhage, and congestion of the trunk. In mice, it has been suggested that *Rock* genes are expressed in the embryonic heart, and that the Rho-pathway is involved in cardiovascular development [6, 7]. In zebrafish, ROCK-dependent cytoskeletal remodeling has been shown to be related to morphogenesis of the heart [12]. Therefore, we propose that the edematous phenotype observed in our study might be associated with these heart-related functions. In contrast, there are no reports regarding defects, such as hemorrhage or congestion due to inhibition of the Rho pathway. We previously demonstrated that endothelial cell-specific expression of a dominant-negative Integrinβ1 (dnItgβ1) leads to cephalic hemorrhage and deformation of the DLAVs and PLs in zebrafish embryos at 2-dpf [21]. The dnItgβ1 has been reported to affect the formation of focal adhesions or the remodeling of actin filaments through binding to intracellular cytoskeleton-associated proteins, such as Talin or

Actinin [22]. This evidence suggests the possibility that the dnROCK-2 construct might work in close association with the integrin machinery in endothelial cells.

Finally, the Tg(UAS: mCherry-dnROCK-2)^{ko114Tg} zebrafish line generated in this study was useful in analyzing the tissue-specific effects of the expression of dnROCK-2 in zebrafish. This transgenic fish line could be used in further studies to investigate the function of the Rho-pathway not only in the vasculature but also in various other tissues.

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Competing Interest statement

The authors declare that they have no competing interests.

Author contributions

A.I. designed the study. A.I and Z.W. carried out the experiments and analysis. E.H. and A.S.F. contributed to the data analysis and discussion. A.I. wrote the manuscript.

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Figure and Legends

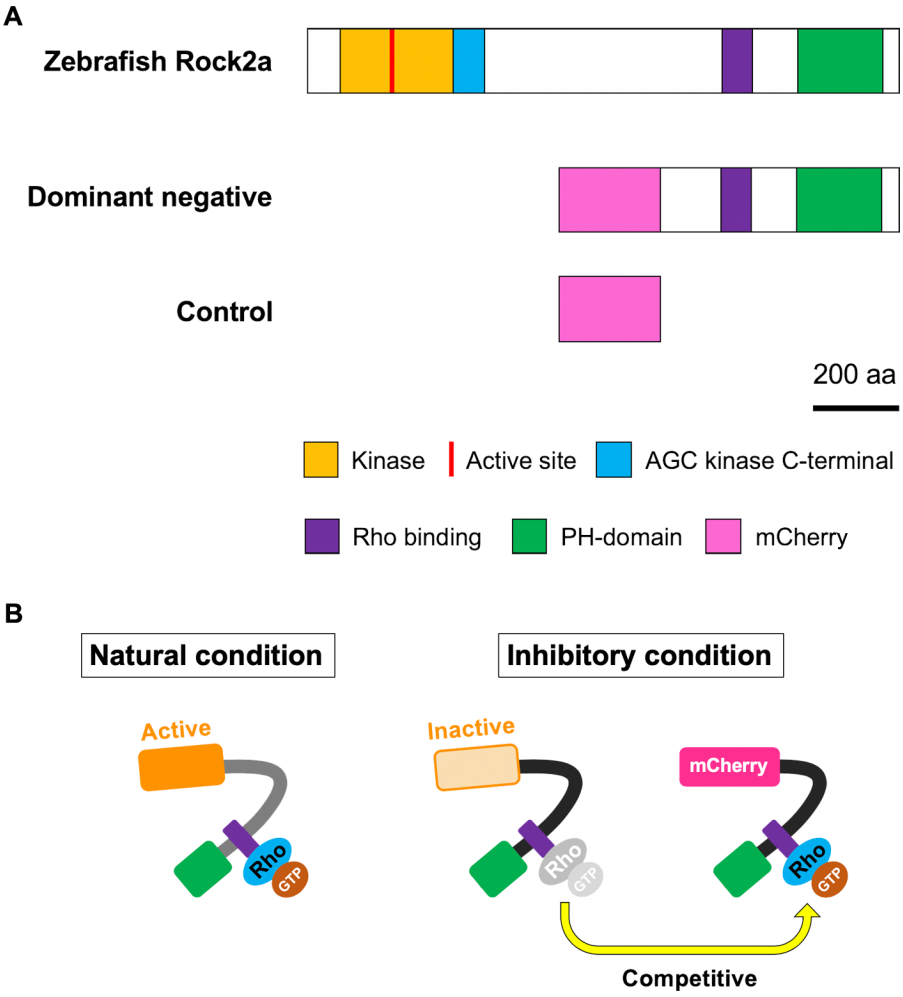

Figure 1. Schematic of the dominant negative ROCK-2 used for the generation of transgenic zebrafish and its proposed mechanism

(**A**) The colored boxes represent the different sequences of the domains in endogenous zebrafish Rock2a. The red line indicates the active site. In the dominant negative (dnROCK-2; DN) construct the kinase domain was replaced with mCherry, whereas the Rho-binding domain was conserved. The intact mCherry construct, used as a negative control, was obtained by deleting the functional domain from dnROCK-2. (**B**) Cartoon showing the predicted molecular mechanism of the dnROCK-2-mediated ROCK-2 inhibition. The native ROCK-2 protein is activated via binding to the GTP form of a Rho GTPase. The dnROCK-2 competes with the native ROCK-2 protein for the Rho-GTP but cannot be activated since it lacks the kinase domain. The removal of Rho-GTP from native ROCK-2 leads to its inactivation.

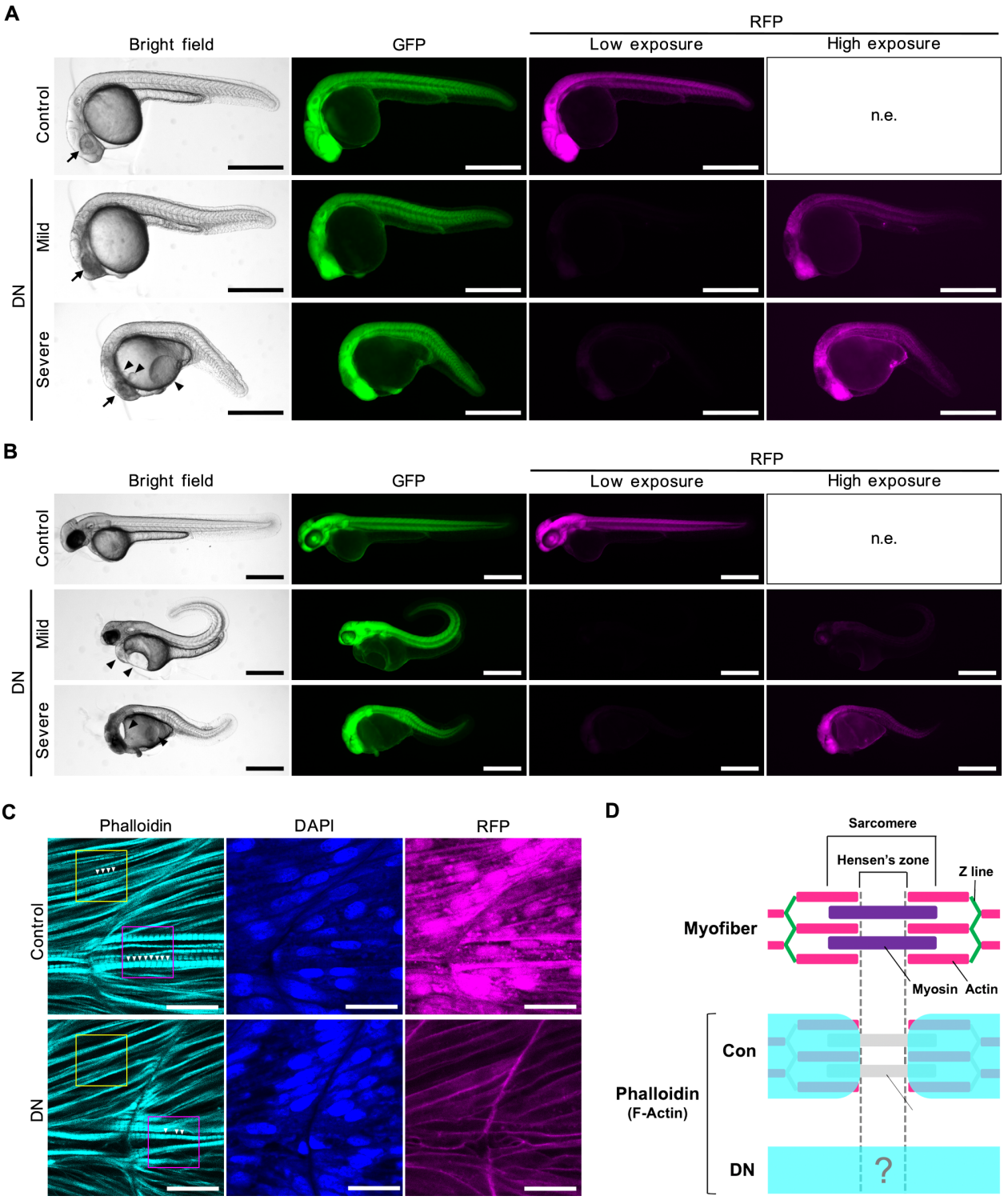

Figure 2. Ubiquitous expression of dnROCK-2 in zebrafish embryos

Typical images of transgenic zebrafish embryos ubiquitously expressing mCherry-dnROCK-2 (mild or severe phenotypes) or mCherry (control line) at 28 hpf (**A**) and 48 hpf (**B**). Both the Tg(UAS: mCherry-dnROCK-2)^{ko114Tg} and Tg(UAS: mCherry)^{ko115Tg} strains were crossed with the

SAGFF(LF)73A transgenic line. GFP is used as a Gal4-UAS expression marker. mCherry expression is indicated by RFP and shows the expression of mCherry-tagged dnROCK-2. The black arrows indicate eyes. The black arrowheads indicate edema in the heart and yolk. Scale bar, 500 μm. n.e., not examined. **(C)** Confocal image of muscle fibers in the trunk region of the transgenic lines. Phalloidin staining reveals the shapes of the muscle fibers through labelling of filamentous actin. The yellow squares indicate fast muscle fibers. The magenta squares indicate muscle pioneer cells. The white arrowheads indicate Hensen's zone observed on the myofibers. DAPI was used to stain cell nuclei. RFP indicates expression of the mCherry tagged dnROCK-2. Scale bar, 20 μm. (**D)** Cartoon of a hypothetical model to explain the confocal observation in **C**. The upper picture shows the molecular structure of the myofiber. The lower pictures provide a cartoon to explain the phalloidin fluorescent signals seen in the control or DN fish. Hensen's zone is an interspace between actin filaments, thus; it is visualized as a phalloidin-negative region on the myofibers. In the DN fish, Hensen's zone was unclear and the phalloidin-labeled fibers ended to be narrower.

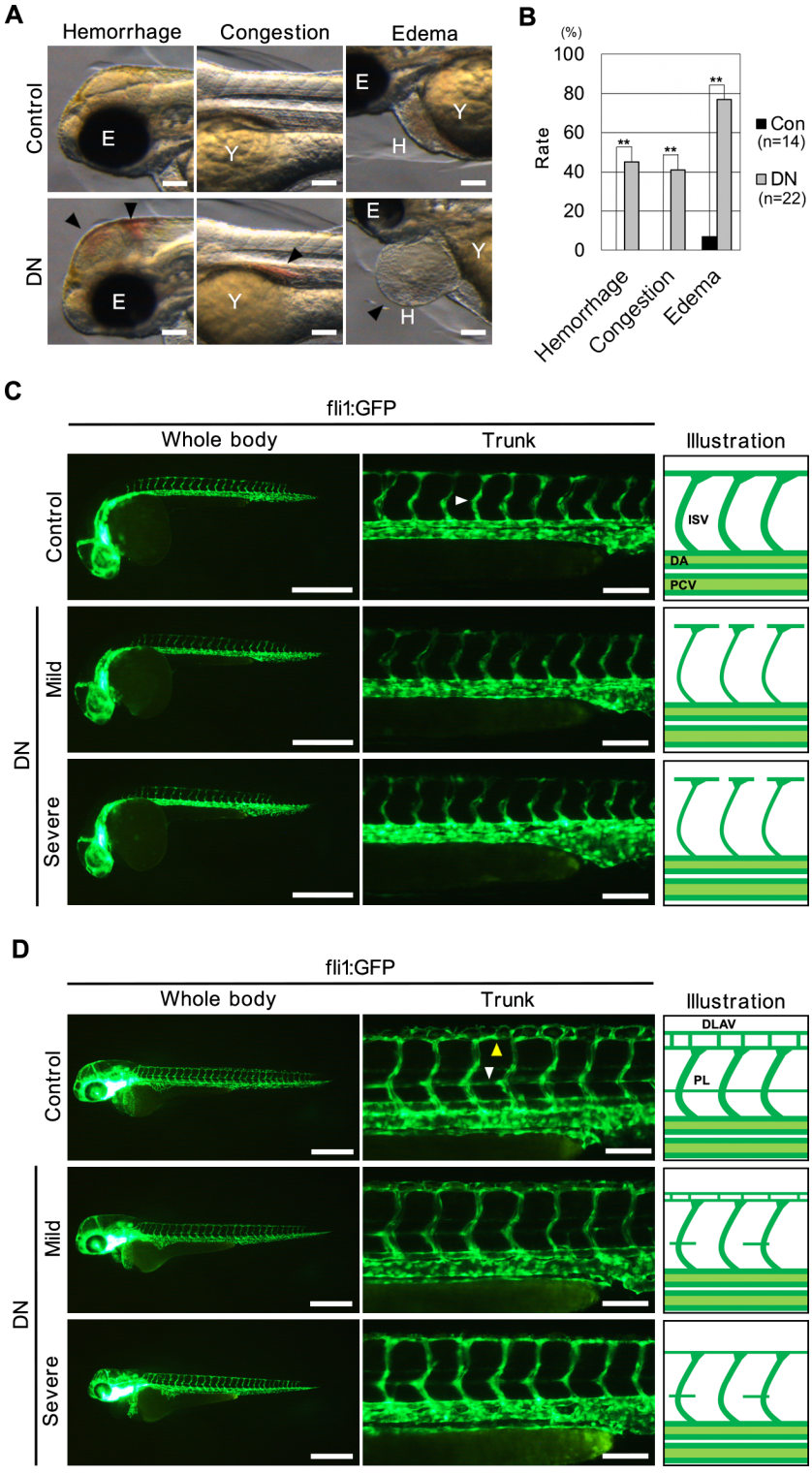

Figure 3. Endothelial cell-specific expression of dnROCK-2 in zebrafish embryos

(**A**) Typical phenotypes of hemorrhage, congestion, and edema seen in transgenic zebrafish embryos expressing an endothelial cell-specific mCherry-dnROCK-2 compared to mCherry

(control) at 30 hpf. The Tg(UAS: mCherry-dnROCK-2)^{ko114Tg} and Tg(UAS: mCherry)^{ko115Tg} strains were crossed with the Tg(*fli1a*: Gal4FF)^{ubs4} transgenic line. The black arrowheads indicate abnormalities in the DN embryo. E, eye. Y, yolk. H, heart. (**B**) Quantitation of the incidence rate of cardiovascular defects observed in transgenic zebrafish at 54-hpf. Student's *t* test was used for statistical analyses. The *p* values for Con vs. DN are 2.99×10^{-3} (hemorrhage), 5.72×10^{-3} (congestion) and 4.8×10^{-5} (edema). $^{**}p < 0.05$. Con, control. DN, dominant-negative. (**C**) GFP indicates the endothelial cells labelled by *fli1a*:EGFP. The enlarged images indicate the trunk region. The white arrowhead indicates an intersegmental vessel. The illustrations are cartoons to explain the observed phenotype. DA, dorsal aorta. PCV, posterior cardinal vein. ISV, intersegmental vessel. Scale bars, 500 μm (whole embryo) and 100 μm (enlarged trunk). (**D**) Images of the transgenic zebrafish embryos shown in **A** at 54 hpf. The white arrowhead indicates a dorsal longitudinal anastomotic vessel (DLAV). The yellow arrowhead indicates a parachordal lymphangioblast (PL). The illustrations are a cartoon to explain the observed phenotype. Scale bars, 500 μm (whole embryo) and 100 μm (enlarged trunk).