Supporting Information

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SI Materials and Methods

Zebrafish Husbandry, Heart Amputation, and Inhibitor Treatment. Zebrafish were raised and maintained as described (1) and used for heart amputation according to the described procedure (2). For testing the effects of PDGFR inhibitor (PDGFR inh. V; Calbiochem), wild-type fish were amputated and allowed to recover for 2 d. The fish were then kept in a 1-L tank (with 0.5 L of water) supplied with air on the bench. PDGFR inh. V (0.25 μM) was dissolved in DMSO and added to the fish water, which was changed every day. Control fish were treated with DMSO only. The fish were kept in the dark because PDGFR inh. V is light-sensitive. To identify the source of PDGF during zebrafish heart regeneration, we used a CD41-GFP transgenic zebrafish line in which thrombocytes (which are the nucleated equivalent of platelets in zebrafish) are marked (3).

RT-PCR. RT-PCR was performed using the following primer pairs: *snail1a* (NM_131066): F 5′-CAGATACCCGCTAGCAGAGC-3′, R 5′-ATGATGCGTCATCCTTCTCC-3′; *snail1b* (NM_130989): F 5′-AGCAGTGGAGAGGAGGATGA-3′, R 5′-GTGCTTGTGCAGGAGAGACA-3′; *snail2* (*NM_001008581*) (4): F 5′-CGGTTCGGAGAGCCCCAGGA-3′, R 5′-TGTGCAGCAGCGACATGCGA-3′; *twist1b* (NM_001017820): F 5′-AGGTTCTACAGAGTGACGAGC-3′, R 5′-GCACAGGATTCGAACTAGAGG-3′; and *twist1a* (NM_130984): F 5′-TGTGGCGCACGAGAGACT-3′, R 5′-GATCTATTCTGCATTGTGAC-3′ (5).

ISH. ISH was performed essentially as described (1). Briefly, digoxigenin-labeled RNA probes were transcribed using T7 or SP6 RNA polymerase (Invitrogen). After deparaffinization in toluene and digestion with proteinase K (7.5 μg/mL) in DEPC-PBS-T ween20 for 10 min, slides were postfixed in 4% (wt/vol) paraformaldehyde and 0.2% glutaraldehyde for 20 min and digested with proteinase K (10 mg/mL) for 10 min at 37 °C. Digoxigenin-labeled riboprobes were diluted at 1:1,000 in hybridization solution. The hybridization was performed for 24 h at 65 °C. After hybridization, slides were washed three times for 30 min each in 0.1× SSC at 65 °C. Detection was done using biotinylated antidigoxigenin antibody (Biogenex), followed by streptavidin-alkaline-phosphatase conjugate, and was visualized by NBT/BCIP substrate (Roche). Slides were counterstained with nuclear fast red (Vector Laboratories) and dehydrated and mounted for microscopic analysis.

In Vitro Epicardial Cell Culture Using Sham-Operated Hearts. Explant cultures of sham-operated hearts were used to determine the effect of PDGF-BB. A monolayer of epicardial cells formed after 3 d in culture. Human recombinant PDGF-BB (0.2 ng/μL; Sigma) plus DMSO or different inhibitors were added to the media for 1 d (from day 3 to day 4 in culture). PDGFR inhibitor (2 μM; PDGFR inh. V), ROCK inhibitor (2 μM, Y-27632; Tocris), and PI3K inhibitor (2 μM, LY-294002; Calbiochem) were used. DMSO was used as a control. For immunohistochemical analysis, the epicardial cells were fixed in 4% (wt/vol) paraformaldehyde and the hearts were subsequently removed. The epicardial cells were stained with phalloidin (1:100; Fluka) or antibodies for epicardin (1:100; Abcam) and ZO1 (1:100; Invitrogen) to be scored for formation of stress fiber and loss of epithelial phenotypes.

In Vitro BrdU Assay. Explant cultures of 4-dpa regenerating hearts were used for BrdU incorporation, with similarly aged sham-operated hearts as controls. A monolayer of epicardial cells formed after 3 d in culture. DMSO (control), PDGFR inhibitor (0.25, 0.5, 1, and 2 μM), ROCK inhibitor (2 μM), or PI3K inhibitor (2 μM) was added to the monolayer of epicardial cell culture for 1 d (from day 3 to day 4 in culture). BrdU (0.04 μg/μL) was added at the same time. The epicardial cells were first fixed in 4% (wt/vol) paraformaldehyde, and hearts were then removed. Epicardial cells were stained with anti-BrdU antibodies (1:100; Abcam), and the percentage of BrdU-positive proliferating cells was counted.

In Vivo BrdU Labeling. Amputated fish were treated with PDGFR inhibitor (0.25 μM) or DMSO as a control, as described above. The epicardium of zebrafish hearts was labeled by injecting ∼0.5 μL of 1 mg/mL Cell Tracker CM-DiI (Molecular Probes) into the pericardial cavity of adult zebrafish at 2 dpa. BrdU was given from 2–7 dpa. The hearts were collected at 7 dpa and processed for sectioning. BrdU labeling and immunostaining were then performed as described (1).

Quantification of Blood Vessel Formation. Hearts from 14-dpa *fli1a:EGFP* transgenic fish were collected and sectioned. Blood vessel formation, as visualized by *fli1a:EGFP* fluorescence, was quantified using ImageJ (National Institutes of Health) by measuring the total area of fluorescent signal within the scar area as a percentage of total scar area. Only obvious blood vessel-like structures with strong fluorescent signals were measured; fluorescent signals of fainter fluorescent puncta within the scar that were barely above background fluorescence were not included in the analysis. Measurements from multiple hearts were taken and pooled for statistical analysis.

EM. The regenerating zebrafish hearts were fixed with 2% (wt/vol) glutaraldehyde in phosphate buffer, postfixed with 1% osmium, and embedded in Epon. The areas of interest were dissected and confirmed by light microscopy in longitudinal sections of the embryo stained with toluidine blue solution. The ultrathin sections were cut onto one-hole grids, stained with uranyl and lead, and examined with Morgagni 268 (FEI).

Immunostaining of Thrombocytes. Zebrafish thrombocytes were fixed in 4% (wt/vol) formaldehyde and centrifuged onto poly-L-lysine (1 μg/mL)–coated coverslips (500 × *g*, 2.5 min). Cells were washed briefly in PBS, permeabilized with 0.1% Triton X-100 in PBS for 5 min, and blocked (1% BSA in PBS) overnight before antibody labeling. Cells were incubated in 1 μg/mL primary rabbit polyclonal anti-PDGF-B antibodies (H-55, sc-7878; Santa Cruz Biotechnology) diluted in blocking buffer for 2 h and then washed three times in PBS. Thrombocytes were incubated in secondary antibody conjugated to Alexa Fluor 568 (Molecular Probes) at a dilution of 1:500 (room temperature, 1.5 h), followed by three washes in PBS. Coverslips were mounted onto microscope slides using Aqua-Mount (Polysciences). Fluorescent images of thrombocytes were visualized by fluorescence microscopy using a Nikon Eclipse TE-2000E microscope equipped with a 60× (N.A. = 1.4) differential interference contrast objective with a 1.5× optivar (Nikon). Images were captured with an Orca-II ER cooled CCD camera (Hamamatsu) equipped with an electronic shutter, and the shutter and image acquisition were controlled by Metamorph software (Universal Imaging, Molecular Devices).

1. Lien CL, Schebesta M, Makino S, Weber GJ, Keating MT (2006) Gene expression analysis of zebrafish heart regeneration. *PLoS Biol* 4:e260.
2. Poss KD, Wilson LG, Keating MT (2002) Heart regeneration in zebrafish. *Science* 298: 2188–2190.
3. Lin HF, et al. (2005) Analysis of thrombocyte development in CD41-GFP transgenic zebrafish. *Blood* 106:3803–3810.
4. Locascio A, Manzanares M, Blanco MJ, Nieto MA (2002) Modularity and reshuffling of Snail and Slug expression during vertebrate evolution. *Proc Natl Acad Sci USA* 99: 16841–16846.
5. Yeo GH, et al. (2009) Phylogenetic and evolutionary relationships and developmental expression patterns of the zebrafish twist gene family. *Dev Genes Evol* 219: 289–300.

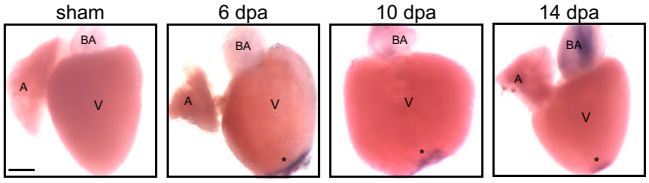

Fig. S1. Whole-mount ISH of *pdgfrβ*. ISH using digoxigenin-labeled antisense probes against *pdgfrβ* in sham and 6-, 10-, and 14-dpa regenerating hearts. A, atrium; BA, bulbus arteriosus; V, ventricle. *Fibrin clot in the wound. (Scale bar = 200 μm.)

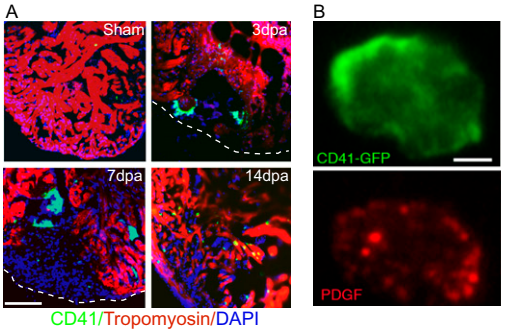

Fig. S2. Thrombocytes present at the wound site express *pdgfb*. (*A*) Thrombocytes marked by CD41-EGFP fluorescence are localized in the wound area from 3–14 dpa (CD41-GFP, green; DAPI, blue; tropomyosin, red). (Scale bar = 100 μm.) (*B*) Immunostaining of PDGF-B (red) in a CD41-EGFP-positive thrombocyte. The staining pattern indicated the localization of PDGF-B in α-granules of thrombocytes. (Scale bar = 2.5 μm.)

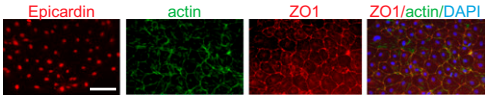

Fig. S3. Primary epicardial cell culture in vitro from sham-operated heart explants. Explant cultures of sham-operated hearts (*n* = 12 hearts) were cultured for 3 d on fibrin gels and then fixed and stained (actin, green; DAPI, blue; epicardin, red; ZO1, red). Actin is localized at the subcortical region. Each individual explant was imaged in at least three different fields of view; a representative image is shown. (Scale bar = 50 μm.)

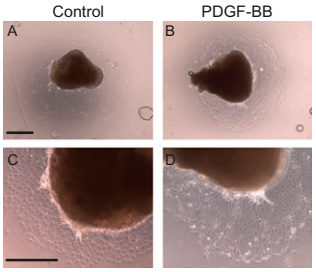

Fig. S4. PDGF-BB induces mobile cells to detach in vitro from sham-operated heart explants. Sham-operated heart explants were cultured for 3 d and then treated with recombinant PDGF-BB (*n* = 6) for 1 d (from day 3 to day 4 in culture). BSA was used as a control (*n* = 6). Each individual explant was imaged in at least three different fields of view; a representative image is shown. (Scale bars: *A* and *B*, 500 μm; *C* and *D*, 400 μm.)

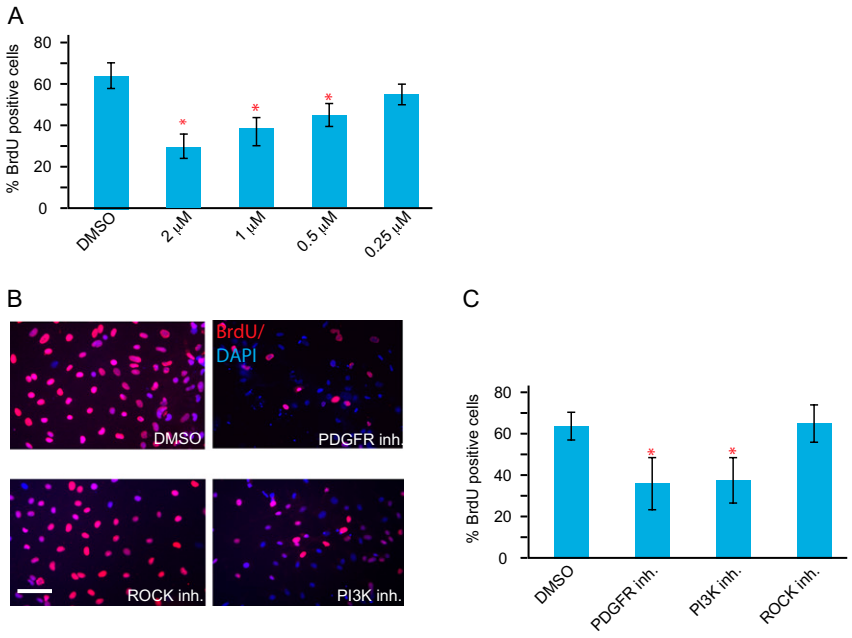

Fig. S5. PDGF signaling is required for DNA synthesis in 4-dpa epicardial cultures. (*A*) Dose-dependent effect of PDGFR inhibitor on DNA synthesis as percentage of BrdU-positive cells. Error bars indicate SEM. **P* < 0.05. (*B*) Monolayer of epicardial cells migrated out from the apex of 4-dpa regenerating hearts after 3 d in culture. BrdU was added to the culture at the same time as DMSO (*n* = 12 hearts), PDGFR inhibitor (2 μM, *n* = 11 hearts), ROCK inhibitor (2 μM, *n* = 9 hearts), or PI3K inhibitor (inh; 2 μM, *n* = 13 hearts) for 1 d (i.e., from day 3 to day 4 in culture). BrdU staining is shown in red, and DAPI staining is shown in blue. (Scale bar = 50 μm.) (*C*) DNA synthesis quantified as the percentage of BrdU-positive cells. Error bars indicate SEM. **P* < 0.01.

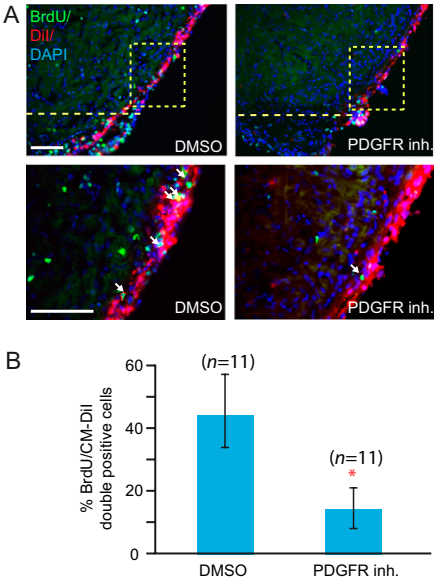

Fig. S6. PDGF signaling is essential for proliferation of epicardial cells during heart regeneration. Treatment of regenerating hearts with DMSO as a control or with PDGFR inhibitor (inh.). The hearts were treated from 2–7 dpa and collected at 7 dpa. (*A*) Epicardium of zebrafish hearts was labeled with Cell Tracker CM-DiI at 2 dpa (BrdU, green; CM-DiI, red). The dashed line marks the approximate position of the amputation plane. BrdU-positive epicardial cells near the wound (marked by arrows) were quantified. (Scale bars = 50 μm.) (*B*) Statistical analysis of in vivo BrdU incorporation in epicardial cells during heart regeneration. BrdU-positive cells in the boxed area in *A* were quantified. Eleven hearts (*n* = 11) were analyzed in each group. Error bars indicate SEM. **P* < 0.01.

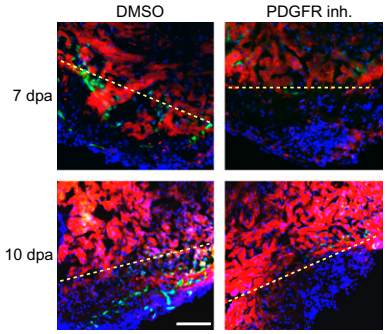

Fig. S7. PDGF signaling is required for endothelial cell formation. Treatment of *fli1a:EGFP* fish with PDGFR inhibitor (inh.) from 2 to 7 dpa or 10 dpa. The 7- or 10-dpa hearts were collected and processed (DAPI, blue; tropomyosin, red). Blood vessel formation is marked by *fli1a:EGFP* (green). The dashed lines mark the approximate position of the amputation plane. (Scale bar = 100 μm.)