Epicardial regeneration is guided by cardiac outflow tract and Hedgehog signalling

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In response to cardiac damage, a mesothelial tissue layer enveloping the heart called the epicardium is activated to proliferate and accumulate at the injury site. Recent studies have implicated the epicardium in multiple aspects of cardiac repair: as a source of paracrine signals for cardiomyocyte survival or proliferation; a supply of perivascular cells and possibly other cell types such as cardiomyocytes; and as a mediator of inflammation1–9. However, the biology and dynamism of the adult epicardium is poorly understood. To investigate this, we created a transgenic line to ablate the epicardial cell population in adult zebrafish. Here we find that genetic depletion of the epicardium after myocardial loss inhibits cardiomyocyte proliferation and delays muscle regeneration. The epicardium vigorously regenerates after its ablation, through proliferation and migration of spared epicardial cells as a sheet to cover the exposed ventricular surface in a wave from the chamber base towards its apex. By reconstituting epicardial regeneration ex vivo, we show that extirpation of the bulbous arteriosus—a distinct, smooth-muscle-rich tissue structure that distributes outflow from the ventricle—prevents epicardial regeneration. Conversely, experimental repositioning of the bulbous arteriosus by tissue recombination initiates epicardial regeneration and can govern its direction. Hedgehog (Hh) ligand is expressed in the bulbous arteriosus, and treatment with a Hh signalling antagonist arrests epicardial regeneration and blunts the epicardial response to muscle injury. Transplantation of Sonic hedgehog (Shh)-soaked beads at the ventricular base stimulates epicardial regeneration after bulbous arteriosus removal, indicating that Hh signalling can substitute for the influence of the outflow tract. Thus, the ventricular epicardium has pronounced regenerative capacity, regulated by the ventricular outflow tract and Hh signalling. These findings extend our understanding of tissue interactions during regeneration and have implications for mobilizing epicardial cells for therapeutic heart repair.

To assess the homeostatic properties of the epicardium, we used an inducible cell ablation system in adult zebrafish. Targeted expression of bacterial nitroreductase (NTR) depletes specific cell types via conversion of a non-toxic substrate, metronidazole (Mtz), to a cytotoxin10–12. We used tcf21 regulatory sequences, which in zebrafish drive the most widespread epicardial expression of known DNA elements13, to create an NTR transgenic line for lesioning this tissue without direct myocardial damage. After treatment of adult tcf21:NTR; tcf21:muceGFP animals with Mtz, ~90% of enhanced green fluorescent protein (eGFP)14 epicardial nuclei on average were ablated from the ventricular surface in large patches (Fig. 1a, b, f). To determine whether epicardial depletion affects the well-documented capacity of the zebrafish heart to regenerate15, we transiently incubated tcf21:NTR zebrafish with Mtz after resection of the ventricular apex. Mtz treatment reduced epicardial cell number in the 7 days post-amputation (dpa) injury site by ~45%, while reducing cardiomyocyte proliferation indices by ~33% (Fig. 1c, d and Extended Data Figs 1a, b, c). Myofibroblasts were represented similarly in vehicle- and Mtz-treated clutches by 14 dpa (Extended Data Fig. 1c). Injured ventricles of Mtz-treated animals displayed reduced vascularization and muscularization by 30 dpa (Fig. 1e and Extended Data Fig. 1d, e), associated with fibrin and collagen retention (Fig. 1e). By 60 dpa, ventricles from Mtz-treated zebrafish consistently showed normal muscularization and a large complement of tcf21:muceGFP+ cells, along with minor collagen deposits (Extended Data Fig. 1f). Thus, depletion of epicardial tissue inhibits cardiomyocyte proliferation and vascularization after resection injury, reducing the efficacy of heart regeneration.

These experiments suggested a high capacity of epicardial cells to regenerate after major depletion. To test this directly, we examined otherwise uninjured hearts at different times after epicardial ablation. Ventricular epicardial cells typically have a low proliferation index (Extended Data Fig. 2a). However, within 3 days of Mtz treatment (3 days post-inubcation (dpi)), many spared epicardial cells entered the cell cycle (Extended Data Fig. 2b, c). At 7 dpi, ventricles displayed quantifiable epicardial recovery that was more prominent at the chamber base (Fig. 1b). By 14 dpi, and as early as 7 dpi, ventricles were fully covered to their apices with tcf21:muceGFP+ epicardial cells (Fig. 1b, f). The temporal variation in recovery probably reflects variation in the location/pattern of epicardial cells spared by ablation among clutches, or in chamber size (Extended Data Fig. 3a). To examine the origins of the regenerated epicardium, we used inducible Cre-based genetic fate-mapping to label tcf21-expressing cells and their progeny permanently before injury16. Labelling and subsequent fate-mapping experiments indicated that pre-existing epicardial cells, and not a tcf21-negative precursor, are a primary source for regeneration (Fig. 1g, h). In sum, these experiments reveal that adult epicardium regenerates after substantial genetic ablation, through a mechanism of expansion by spared epicardial cells.

To expand our range of experimental manipulations, we refined protocols such that freshly dissected hearts contracted for several weeks ex vivo (Supplementary Video 1)14–15. When Mtz was added transiently to the culture medium for 1 day, ventricular epicardial cells were potently ablated. Epicardial layers of the atrium and the bulbous arteriosus (alternatively referred to as the outflow tract) were less effectively depleted (Fig. 2a), probably owing to differential expression of the NTR transgene among cardiac chambers (Extended Data Fig. 3b). Daily imaging of these hearts confirmed observations from in vivo experiments, demonstrating regeneration of the epicardium from base to apex that was typically completed in 2 weeks (Fig. 2a). Hearts from animals given partial ventricular resections in vivo showed a similar pattern of epicardial regeneration after ex vivo ablation (Extended Data Fig. 4a). Cardiac muscle regeneration was ineffective in explanted hearts in our experiments. Increases in cell number occurred concomitantly with movement across the myocardial surface during epicardial regeneration, with spared epicardial cell patches away from the leading edge eventually incorporated into the sheet (Fig. 2a). To identify possible intrinsic differences in epicardial cells from different ventricular regions, we examined behaviours of basal or

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Epicardial ablation and regeneration in vivo. a, Adult zebrafish heart. OFT, outflow tract. b, tcf21:NTR, tcf21:nuceGFP adults were incubated with Mzt or vehicle, and hearts were collected by random sampling at 3, 7 or 14 dpi. White dashed lines delineate ventricle. Numbers in bottom right corners are proportion of total animals with indicated phenotype. All 3 dpi ventricles showed major ablation, averaging ~90% loss. c, Left, schematic for tests of epicardial ablation on muscle regeneration. Right, ventricular cardiomyocyte proliferation at 7 dpi. Brackets indicate injury site. Arrowheads indicate proliferating cardiomyocytes. DAPI, 4',6-diamidino-2-phenylindole; WT, wild type. d, Quantified PCNA* cardiomyocyte indices in injury sites in experiments from c. ***P < 0.001, Mann–Whitney rank sum test; n = 18 (wild type) and 19 (tcf21:NTR) animals from two experiments. e, Section images of eGFP+ epicardial cells in ventricular surface (fibrin, collagen). One of eleven tcf21:nuceGFP and 8 of 12 tcf21:NTR; tcf21:nuceGFP ventricles showed myocardial gaps. Dashed line indicates approximate resection plane. ***P < 0.001, Fisher Irwin exact test. f, Quantified eGFP* nuclei from experiments in b. ***P < 0.001, Student’s two-tailed t-test. g, Left, CreER-based strategy for permanent labelling of tcf21* progeny. Right, section images of lineage-labelled eGFP* epicardial progeny up to 14 dpi, indicating derivation from pre-existing epicardium. Arrows indicate eGFP* cells spared by epicardial ablation. h, Quantified eGFP* cells from experiments in g. ***P < 0.001, Student’s two-tailed t-test; n = 10 (vehicle, 3 dpi), 13 (Mzt, 3 dpi) and 15 (Mzt, 14 dpi). c, g, Insets, high magnifications of boxed areas. Scale bars, 50 μm. Error bars indicate standard deviation (s.d.).

In these experiments, transplanted cells of either origin consistently repopulated the ventricular surface in a base-to-apex direction after transplantation (Extended Data Fig. 5a–d), revealing no proliferative bias in ventricular epicardial cells that could explain the directional flow of regeneration. To assess potential extrinsic influences on epicardial regeneration, we removed the atrium or bulbous arteriosus from its attachment at the ventricular base before epicardial ablation. Atrial extirpation did not noticeably affect the regeneration of ventricular epicardium (Fig. 2b and Supplementary Video 2). By contrast, removal of outflow tract tissue blocked epicardial cell recovery, an arrest that persisted for at least 2 weeks (Fig. 2c, d and data not shown). To test whether this arrest was solely a consequence of mechanical tissue disruption, we ablated the epicardium after host bulbous arteriosus removal, before grafting a non-transgenic bulbous arteriosus to the ventricular base 2 days later. In most of these tissue recombination procedures (13 of 21), host tcf21:nuceGFP* epicardium regenerated to cover the ventricle (Fig. 3a). This effect was not observed when a portion of donor ventricular apex was inverted and transplanted to the host ventricular base (Extended Data Fig. 5e). Complementary grafting experiments indicated that bulbous arteriosus could contribute epicardial cells to the ventricular surface, as a potential supplement to expansion of the ventricular epicardial cell pool (Extended Data Fig. 5f). Thus, our experiments indicate that outflow tract tissue provides an essential interaction for regeneration from existing ventricular epicardial cells.

To test whether outflow tract tissue is sufficient to stimulate epicardial regeneration, we ectopically positioned experimentally manipulated cardiac structures. Co-culture of several outflow tracts in a transwell assay with an epicardially ablated ventricle did not restore regeneration in the absence of host bulbous arteriosus (Extended Data Fig. 6a). Similarly, a bulbous arteriosus graft placed at the ventricular apex showed no evidence of directing regeneration of basally located host epicardial cells towards the apex (Extended Data Fig. 6b). Thus, we could not detect bulbous arteriosus effects requiring long-range diffusion through tissue or culture medium. Next, we transplanted a tcf21:nuceGFP* epicardial cell patch to the apex of an ablated host ventricle, after which we grafted a wild-type bulbous arteriosus to the
apex (Fig. 3b). Remarkably, the apical bulbous arteriosus was capable of stimulating apex-to-base regeneration from the nearby epicardial patch in a high proportion (21 of 32) of experiments, effectively reversing the stereotypical direction of recovery (Fig. 3c, d). Together, these experiments indicate that the cardiac outflow tract is necessary and sufficient for epicardial regeneration, and that this neighbouring tissue provides a short-range influence(s) that directs regeneration from base to apex.

To pursue the molecular nature of interactions between outflow tract and ventricular epicardium, we applied a small panel of signalling pathway effectors to epicardially ablated hearts cultured ex vivo. Among several compounds (Extended Data Fig. 7), the Smoothened (Smo) antagonist cyclopamine (CyA) blocked regeneration; nonetheless, regeneration initiated normally after drug washout (Fig. 4a). CyA treatment reduced spontaneous epicardial cell 5-ethynyl-2'-deoxyuridine (EdU) incorporation occurring in the first 2 days of explant culture, suggesting that intact Hh signalling promotes epicardial proliferation (Extended Data Fig. 8a, b). CyA also disrupted in vivo epicardial regeneration, not only in adults but in larvae, an additional developmental setting in which we identified base-to-apex regeneration (Fig. 4b, c and Extended Data Fig. 9a–c). CyA treatment from 2 to 4 days post-fertilization also reduced the initial epicardial occupancy of the larval ventricle (Extended Data Fig. 9d–f), indicating that epicardial regeneration recapitulates at least one pathway influential in morphogenesis. Finally, we observed inhibitory effects of CyA on the epicardial proliferative response to muscle resection in vivo, and in coverage of these injuries ex vivo (Extended Data Fig. 4b and Extended Data Fig. 8c, d).

Smo is an effector for several Hh family ligands, which have potent short-range effects in multiple contexts of embryonic development. Quantitative polymerase chain reaction (qPCR) revealed shha, ihhb and dhh ligand transcripts in adult atrium, ventricle and bulbous arteriosus, where in situ hybridization detected shha and dhh transcript signals in smooth muscle tissue (Extended Data Fig. 10a–d). Epicardial ablation injury boosted bulbous arteriosus and ventricular shha levels, as well as levels of ptch1 and gli2a in purified epicardial cells (Fig. 4d, e). Moreover, a shhaceGFP reporter strain visualized shha regulatory-sequence-driven fluorescence in smooth muscle and epicardial tissues of the bulbous arteriosus (Extended Data Fig. 10e). No additional in situ hybridization or shhaceGFP fluorescence patterns were detectable after epicardial ablation; however, apical resection injury induced fluorescence in ventricular epicardial tissue by 2 dpa (Extended Data Fig. 10f, f). To test whether local Hh ligand delivery is sufficient to substitute for the bulbous arteriosus, we removed atrium and bulbous arteriosus from cardiac explants, ablated epicardial cells, and applied
beads soaked with Shh protein to the exposed ventricular base. Shh-soaked beads stimulated epicardial regeneration (one-half or greater coverage) in 9 of 32 ventricles, whereas this level of recovery never occurred after transplantation of BSA-soaked beads (0 of 27 ventricles; Fig. 4f). We speculate that these effects of Hh on the epicardial sheet might involve cytoplasmic extensions or a factor transport system. Together, our findings support a model in which Hh ligand from the outflow tract, and possibly additional tissues, guides the base-to-apex regeneration of ventricular epicardium. In conclusion, we have identified a requirement for the mesothelial covering of the zebrafish heart for proficient muscle regeneration. Moreover, we show that the ventricular epicardium itself has high endogenous renewal capacity, vigorously regenerating as a sheet from the base of the chamber to its apex after genetic depletion. Our results point to the outflow tract as an unexpected signalling centre and source of Hh, and possibly additional influences, that can promote epicardial regeneration. It is likely that tissue regeneration is similarly regulated in trans in other contexts; for example, to maintain the mesothelium that lines abdominal organs. As a mediator of epicardial regeneration, Hh signalling can be integrated into new strategies to modulate repair of the damaged heart.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Author Contributions J.W., J.C. and K.D.P. designed the experimental strategy, analysed data, and prepared the manuscript. J.W. generated the transgenic system for epicardial cell ablation and performed in vivo regeneration experiments and analysis. J.C. developed the ex vivo culture assay and performed ex vivo regeneration experiments and analysis. A.L.D. performed histology and data analysis. All authors commented on the manuscript. 

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to K.D.P. (kenneth.poss@duke.edu).
METHODS

Zebrafish maintenance and procedures. Adult zebrafish of the Ekkwill and Ekkwill/AB strains were maintained as described and rescued through injuring experiments as described previously. Animals between 4 and 12 months of both sexes were used. Transgenic lines used in this study were Tg(tcf21:mCherry-NTR)79950 (tcf21:NTR, described later), Tg(tcf21:nuceGFP)141 (ref. 24), Tg(tcf21:CreER)142 (ref. 2), Tg(gata5a:lox-p-mCherry-lox-nuceGFP)143 (ref. 25), Tg(fli1a:eGFP)144 (ref. 26) and Tg(dbh:eGAPDH)145 (ref. 27). All transgenic strains were analysed as hemizygotes. For epicardial ablation experiments in adults, animals were bathed for 24 h in 10 mM Mtz (Sigma) as described and returned to water.22 If ablation was performed after ventricular resection, we used a protocol of daily changes of 1 mM Mtz solution for 3 days that had similar ablation effects (Extended Data Fig. 3c). This protocol corresponds with the early epicardial proliferative response plus to resection injury data not shown, and was intended to extend the ablation window and improve animal survival. For larval epicardial ablation, 6 hours post-fertilization (hpf) embryos were bathed for 48 h in 10 mM Mtz before washout. For ex vivo epicardial ablation, dissected hearts were bathed for 24 h in 1 mM Mtz before washout. Cyclopinone (Cya; Selleckchem) was dissolved in ethanol to a final concentration of 20 mM. CyA was used at 10 μM for in vivo treatment of adult animals and 5 μM for ex vivo culture and embryo treatments. For EdU incorporation experiments that followed epicardial ablation, animals were injected with 10 μM EdU 4 h before collection. Experiments with uninjured animals used three medial, longitudinal sections from each experimental arm. For lineage tracing, strains carrying tcf21:NTR, tcf21:CreER and gata5a:lox-p-mCherry-lox-nuceGFP transgenes were placed in a beaker of aqueous water containing 5 mM tamoxifen. Fish were maintained for 24 h, rinsed with fresh aquarium water, and returned to a recirculating aquatic system for 24 h, before repeating this incubation twice. After 3 days of rinsing, Mtz was added for an additional 24 h. As is common when using Cre-based tools, we could not genetically label all epicardial cells in these experiments or rule out minor contributions by tcf21-negative cells. Animal procedures were performed in accordance with Duke University guidelines.

Construction of tcf21:NTR zebrafish. The translational start codon of tcf21 in the bacterial artificial chromosome (BAC) clone DKEYP-7997F12 was replaced with the mCherry-NTR cassette by Red/ET recombineering technology (Gene Bridges)12. The 5’ and 3’ homologous arms for recombination were a 50-base pair (bp) fragment upstream and downstream of the start codon, and were included in PCR primers to flank the mCherry-NTR cassette. To avoid aberrant recombination between the mCherry-NTR cassette and the endogenous loxP site in the BAC vector, we replaced the vector-derived loxP site with an I-SceI site using the same technology. The final BAC was purified with Nucleobond BAC 100 kit (Clontech) and co-injected with 1-SceI into one-cell-stage zebrafish embryos.

Ex vivo cardiac explants. Adult hearts were rinsed several times in PBS after collection and cultured in dishes with DMEM medium (Life Technologies) supplemented with 2 mM L-glutamine (Life Technologies), 10% fetal bovine serum (HyClone, Thermo), 1% MEM non-essential amino acids (Life Technologies), 100 U ml⁻¹ penicillin, 100 μg ml⁻¹ streptomycin (Life Technologies) and 50 μM 2-mercaptoethanol (Life Technologies), while rotating at 150 r.p.m. Primocin (InvivoGen) was added to prohibit microbial contaminants during the first 3 days primary culture. For transplantation experiments, outgrowth tract or ventricular tissues were grafted by mounting in 1% low-melting-point agarose with ablated hearts in culture dishes, and covering with medium. After 2 days of culturing, attached tissues were released from the agarose; if transplanting an epicardial patch, the ventricular donor tissue was removed carefully using forceps. Fluorescent transgenes in these cardiac explants were monitored using a Leica MZ50FA stereo-fluorescence microscope.

Recombinant mouse Shh (C2511, N term protein (R & D Systems) was reconstituted at 100 μg ml⁻¹ in PBS containing 0.1% BSA. Affi-Gel Blue beads (Bio-Rad) were prepared by thoroughly washing the beads in PBS, then incubating them in the Shh solution for 2 h at room temperature. A solution with the same concentration of BSA protein was used as the control. The beads were then applied to the base of ventricular explants that were settled in low-melting-point agarose in serum-free DMEM supplemented as described earlier. After 24 h, the ventricles were released from the agarose with calcium- and magnesium-supplemented, serum-free DMEM. Under these ex vivo culture conditions we observed no increase in cardiomyocyte proliferation upon resection injury.

Preparation of outflow tract and ventricular epicardial cells and RT–qPCR. Adult hearts were dissected from tcf21:NTR or tcf21:NTR; tcf21:nuceGFP animals 3 and 7 days post-treatment with vehicle or Mtz. Outflow tracts were frozen in liquid nitrogen. Ventricular nuceGFP™ epicardial cells were isolated as described previously22 with modifications. Briefly, ventricles were collected on ice and washed several times to remove blood cells. Ventricles were digested in an Eppendorf tube with 0.5 ml HBSS plus 0.13 U ml⁻¹ Liberase DH (Roche) and 1% sheep serum at 37 °C, while stirring gently with a Spinbar magnetic stirring bar (Bel-Art Products). Supernatants were collected every 5 min and neutralized with sheep serum. Dissociated cells were spun down and sorted using a BD FACSVantage SE sorter for eGFP-positive cells. Total RNA was extracted using an RNeasy Micro isolation kit (Ambion) according to the manufacturer’s instructions. Reverse transcription was carried out using a SuperScript III First-Strand synthesis kit (Life Technologies). qPCR was carried out in triplicate using a Roche LightCycler 480 II system with the LightCycler 480 Probes Master Mix and a Universal Probe Library (UPL) (Roche). Rpl3a served as the control. Primers and the UPL numbers used in this study were: shha-f, AAGCCAGACATTCTGTCCT, and shha-r, CCTCTTGCCTGTCCGCTCGT, UPL #54; shhb-f, GCAAG TATGGATGTATCCGACAG, and shhb-r, TCTTGATTTAGCGACACTG, UPL #16; ihha-f, TGGGTCTACTATGCTAAGGCAAGC, and ihha-r, GTTGGTACTATGTCAAGGCAAAG, and ihhb-f, GACCCAGAGCTT, and ihhb-r, AAAACATGACATGGGTTTTGTT, UPL #156; ptc1-f1, TG GCTTAAAGGGCAGCATACT, and ptc1-r, GCCGGTGACACGTATCCCTC, UPL #87; ptch2-f, CCACTGAATCACTGAAGATTGA, and ptch2-r, GAATGC CCCTGAACACGCAC, UPL #68; gata5a-f, CTTCCCGGATGCTGT, UPL #68; gata5a-f2, CCTCAGAAATGGCAAGAG, and gata5a-r2, CGATCCGGATGTTGTTGT, UPL #39; gata5b-f, GCCGGCAGAATCTTCCAGCTCA, and gata5b-r2, CTAAACCTGGGCGGTCAA, UPL #48; rpl3a-f1, GGGGACGATTCAATAGGG, and rpl3a-r1, GAAAGAGCCAGGAGATG, UPL #147.

Histology. Analyses of cardiomyocyte proliferation were performed as described by counting MeD2™ and PCNA™ nuclei in wound sites24. To quantify vascular endothelial cells in the wound site by 30 dpa with fli1a:eGFP or tcf21:NTR; fli1a:eGFP animals, three medial, longitudinal sections were selected from each heart. Images of single optical slices of green fluorescence in the wound site were acquired using a ×20 objective (1,024 × 1,024 pixels) and pixel values were quantified in pixels by ImageJ software, and the ratio of eGFP™ area versus the length of the outlined apical wound was calculated for each heart. For quantification tcf21™ epicardial cells in the wound site at 7 and 30 dpa with tcf21:nuceGFP or tcf21:NTR; tcf21:nuceGFP animals, three medial, longitudinal sections were selected from each heart. eGFP™ cells were counted in the wound area, and the ratio of eGFP™ cells versus the length of the outlined apical wound was calculated for each heart. Acid Fuchs-Orange G and immunostaining were performed as described24. Primary antibodies used in this study were anti-myosin heavy chain (MHC; F59, mouse; Developmental Studies Hybridoma Bank), anti-GFP (rabbit; Invitrogen), anti-Mef2 (rabbit; Santa Cruz Biotechnology), anti-MLCK (mouse; K36; Sigma) and anti-PCNA (mouse; Sigma). Secondary antibodies (Invitrogen) used in this study were: Alexa Fluor 488 goat anti-rabbit; Alexa Fluor 594 goat anti-rabbit, goat anti-rat and goat anti-mouse; and Alexa 633 goat anti-mouse. EdU was detected through a click reaction as described previously25 with fluorescent azide (Alexa Fluor 594 or 647; Invitrogen). Whole-mounted and sectioned ventricular tissues were imaged using a Zeiss 700 confocal microscope.

Data collection and statistics. Clusters (or hearts collected from clusters) were randomized into different treatment groups for each experiment. No animal or sample was excluded from the analysis unless the animal died during the experiment. All experiments were performed with at least two biological replicates, using appropriate number of samples for each replicate. Sample sizes were chosen on the basis of previous publications and experiment types, and are indicated in each figure legend. For expression patterns, at least six fish were examined. For assessment of epicardial ablation and consequences on muscle regeneration, at least nine fish were examined. At least 12 hearts of each group were pooled for RNA purification and subsequent RT–qPCR. For ex vivo epicardial ablation experiments, at least six hearts were used for each treatment. An exception was the small compound screen, where at least four hearts were used for each drug. Quantification of cell proliferation and calculation of statistical outcomes were assessed by a person blinded to the treatments. All statistical values are displayed as mean ± s.e.m., with i.e., deviation. Sample size, statistical tests, and P values are indicated in the figures or the legends. Student’s t-tests (two-tailed) were applied when normality and equal variance tests were passed. The Mann–Whitney rank sum test was used when these failed. Fisher Irwin exact tests or chi-squared tests were used where appropriate.

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Extended Data Figure 1 | Epicardial ablation and responses to resection injury. **a**, tcf21:nuceGFP or tcf21:NTR; tcf21:nuceGFP clutchmates underwent resection injury and were treated for 3 days with 1 mM Mtz, before collection of ventricles at 7 (n = 8 animals per group) and 30 dpa (n = 11). tcf21:NTR; tcf21:nuceGFP wounds had fewer epicardial cells at 7 dpa and comparable occupancy by 30 dpa compared with tcf21:nuceGFP wounds. White dashed lines indicate wound edge. **b**, Quantification of eGFP⁺ epicardial cells at 7 and 30 dpa with respect to wound edge lengths from **a**. NS, not significant; Mann–Whitney rank sum test. **c**, tcf21:NTR clutchmates underwent resection injury and were treated for 3 days with 1 mM Mtz or vehicle with random separation into two groups for treatment. MLCK⁺ cells, indicating myofibroblasts, had comparable presence in both groups at 14 dpa (n = 7 for each group). **d**, tcf21:NTR; fli1a:eGFP clutchmates underwent resection injury and were treated for 3 days with 1 mM Mtz or vehicle with random separation into two groups for treatment. Epicardial ablation led to lower vascular density in 30 dpa wounds compared with controls. Red dashed lines indicate wound edge. **e**, Quantification of eGFP⁺ vascular endothelial pixel area in wounds of tcf21:NTR; fli1a:eGFP zebrafish treated with Mtz (n = 12) or vehicle (n = 6), or of fli1a:eGFP zebrafish treated with Mtz (n = 6), with respect to the wound edge lengths. Student’s two-tailed t-test. **f**, By 60 dpa, 55 days after epicardial ablation protocols, muscularization (top) and wound collagen deposition (bottom) were grossly normal (n = 23). Brackets indicate area of regeneration. **a, c, d**, Yellow dashed lines indicate the approximate amputation plane. Scale bars, 50 μm. Error bars indicate s.d.
Extended Data Figure 2 | Epicardial cell proliferation without injury and after epicardial ablation. a, Limited epicardial cell proliferation on the ventricular surface. tcf21:nuceGFP fish were injected with 10 mM EdU once daily for 3 days and collected 1 day after the last injection. 10^5 ventricular nuceGFP cells were assessed for EdU reactivity in 15 animals, from which 608 cells were positive (a 0.6% rate for 4 days EdU labelling). Whole-mount image is shown, and arrows in enlarged boxed area indicate eGFP^+ EdU^+ nuclei. b, tcf21:nuceGFP or tcf21:NTR; tcf21:nuceGFP fish were injected with 10 mM EdU at 3 days post-Mtz treatment, and hearts were collected 4 h later. Boxed areas in images of whole-mounted hearts show magnified views. a, b, Yellow arrows indicate representative eGFP^+ (green) EdU^+ (red) nuclei. c, fli1a:eGFP or tcf21:NTR; fli1a:eGFP fish were injected with 10 mM EdU at 3 days post-Mtz treatment, and hearts were collected 4 h later. Red arrows indicate representative eGFP^+ (green) EdU^+ (magenta) endocardial cell nuclei; yellow arrowheads, representative eGFP^+ (green) EdU^+ (magenta) vascular endothelial cell nuclei; white arrowheads, EdU^+ (magenta) nuclei within the ventricular lumen, ostensibly erythrocyte nuclei. Scale bars, 50 μm.
Extended Data Figure 3 | Mosaic NTR expression and patterns of spared epicardial cells after ablation. a, Whole-mounted examples of varied location/pattern of spared epicardial cells in ventricles from tcf21:NTR; tcf21:nuceGFP adult clutchmates 3 days after incubation with 10 mM Mtz. White dashed lines delineate ventricle. b, Differential expression of the NTR transgene among cardiac chambers. In adult tcf21:NTR; tcf21:nuceGFP hearts, eGFP expression is comparable in epicardial tissue covering the atrium, outflow tract (OFT) and ventricle. By contrast, NTR (red, indicated by mCherry) expression is patchy and/or weak in the atrium and outflow tract compared with ventricular expression. c, Section images of ventricles from tcf21:nuceGFP (left) or tcf21:NTR; tcf21:nuceGFP zebrafish (right) treated with 1 mM Mtz (right) for 3 days, and collected 2 days later. Ventricular epicardium was ablated effectively in these experiments. Scale bars, 50 μm.
Extended Data Figure 4 | Epicardial regeneration after ventricular resection. a, Hearts were removed from tcf21:NTR; tcf21:nuceGFP fish immediately after ventricular resection injuries, followed by 24 h of Mtz and a 24 h washout ex vivo. A base-to-apex pattern of epicardial regeneration was observed, in this example covering the apical wound by 11 dpa (n = 18; behaviour seen in all samples). Epicardial coverage of resection injuries in these ablation experiments is delayed compared to ventricles recovering with an intact epicardium (b, top). Yellow boxed area, magnified view of the apical wound. Red dashed lines delineate ventricle. b, Hearts were removed from tcf21:nuceGFP clutchmates immediately after apical resection injury and cultured ex vivo, before random separation into two treatment groups. Epicardial cells covered the wound area by 3 dpa (n = 11; behaviour seen in all samples), unless treated with CyA (n = 26; failed coverage in 20 of 26 ventricles). a, b, White dashed lines indicate apical wounds. Scale bars, 50 μm.
Extended Data Figure 5 | Ex vivo grafts and epicardial regeneration.

**a**, Schematic of the experimental design. **b**, **c**, Epicardial cells transplanted at the base of an epicardially ablated host regenerated towards the apex regardless of basal (b) (n = 25, behaviour seen in all samples) or apical (c) (n = 27, all samples) origin. **d**, Top, epicardial cells from the base of a transgenic donor ventricle were transplanted to the chamber midpoint of an epicardially ablated host ventricle and observed for regeneration. Bottom, transplanted cells eventually migrated towards the apex, not the base (n = 13; all samples). **e**, Top, after epicardial ablation, the host bulbous arteriosus was replaced with a non-transgenic donor ventricular apex and observed for regeneration. Bottom, ventricular epicardium showed little or no regeneration in these experiments (n = 7; behaviour seen in all samples). **f**, Left, after ex vivo epicardial ablation in a host tcf21:NTR ventricle, the host bulbous arteriosus was replaced with a donor tcf21:nuceGFP bulbous arteriosus. Right, the host ventricular surface contained different amounts of eGFP+ nuclei in these ventricles (n = 3; behaviour seen in all samples). **b–f**, Red dashed lines indicate epicardium or epicardial leading edge; white dashed lines delineate ventricle. **e, f**, Yellow dashed lines indicate donor apex (e) or bulbous arteriosus (f). Scale bars, 50 μm.
Extended Data Figure 6 | Context-specific effects of outflow tract on epicardial regeneration. a, Top, after ex vivo epicardial ablation and bulbous arteriosus removal, ventricles were co-cultured with ten outflow tracts in a transwell assay and observed for regeneration. Bottom, no evidence for epicardial regeneration was observed in these experiments (n = 9; behaviour seen in all samples). b, Left, after ex vivo epicardial ablation and bulbous arteriosus removal, a non-transgenic bulbous arteriosus (labelled as donor OFT) was transplanted to the apex and observed for regeneration. Right, no evidence for regeneration of eGFP⁺ epicardium from apex to base was observed in these experiments (n = 10; behaviour seen in all samples). Red dashed lines delineate epicardium; white dashed lines delineate ventricle; yellow dashed lines delineate donor outflow tract. Scale bars, 50 μm.
Extended Data Figure 7 | Small-scale screen for compounds that inhibit epicardial regeneration. Ex vivo ablation and regeneration of tcf21:NTR; tcf21:nuceGFP ventricles over 7 days. Mtz was added for 24 h to freshly isolated hearts, washed out, and compounds were added after 2 days (day 0). Hearts were treated with vehicle (n = 10), 10 μM DEAB (n = 5; Sigma-Aldrich), 100 nM LDN193189 (n = 4; Cayman Chemical), 10 μM SU5402 (n = 5; Santa Cruz Biotechnology), 1 μM cyclosporin A (n = 4; Sigma-Aldrich), or 0.1 μg ml\(^{-1}\) FK506 (n = 5; Sigma-Aldrich), in each case showing base-to-apex recovery (behaviour seen in all samples). The dissected hearts were randomly separated into groups for drug treatment. Red dashed lines indicate epicardial leading edge; white dashed lines delineate ventricle. Scale bars, 50 μm.
Extended Data Figure 8 | Epicardial proliferation is regulated by Hh signalling. a, Freshly dissected tcfl1:nuceGFP hearts were randomly separated into two groups and cultured for 47 h with vehicle (n = 11) or 5 μM CyA (n = 8). Then, 25 μM EdU was added to the medium for 1 h before collection at 48 h. CyA treatment decreases epicardial cell proliferation ex vivo. Arrows indicate representative eGFP− EdU+ (green) EdU+ (red) nuclei. b, Quantification of eGFP− EdU+ nuclei per mm2 on the ventricular surface, from hearts in a. **P < 0.01, Student’s two-tailed t-test. c, tcfl1:nuceGFP adult fish were subjected to partial ventricular resection surgery, and randomly separated into two groups for treatment with vehicle (n = 8) or 10 μM CyA (n = 10) from 2 to 3 dpa. Then, 10 mM EdU was injected intraperitoneally 1 h before collection. CyA treatment decreases epicardial cell proliferation in vivo. Arrowheads indicate representative eGFP− EdU+ (green) EdU+ (red) nuclei. d, Quantification of eGFP− EdU+ nuclei per mm2 on the ventricular surface, from hearts in c. ***P < 0.001; Mann–Whitney rank sum test. c, Yellow dashed lines indicate resection plane; white dashed lines delineate ventricle. a, c, Boxed areas, magnified views. Scale bars, 50 μm. Error bars indicate s.d.
Extended Data Figure 9 | Larval epicardial development and regeneration.
a, *tcf21:nuceGFP* or *tcf21:NTR; tcf21:nuceGFP* larval clutchmates were treated with 10 mM Mtz from 6 hpf to 54 hpf, and then imaged at different times from 3 to 5 dpf. *tcf21:nuceGFP* larvae show normal ventricular epicardial coverage at 3 dpf, while *tcf21:NTR; tcf21:nuceGFP* coverage is sparse. *tcf21:NTR; tcf21:nuceGFP* larvae with confirmed full ablation were imaged from 3 to 5 dpf, covering first the ventricular base and then the apex. Three different extents of regeneration at 5 dpf are shown: class I, greater than two-thirds coverage; class II, one-third to two-thirds coverage; and class III, some cells but less than one-third coverage. b, A subset of *tcf21:NTR; tcf21:nuceGFP* larvae with confirmed full ablations were randomly separated and treated with vehicle or CyA, which limited regeneration in most cases (class IV, no ventricular epicardial cells). c, Quantification of extents of regeneration from experiments in a and b. ***P < 0.001, chi-squared test; n = 54 embryos for vehicle, 51 for CyA. d, Epicardial morphogenesis visualized in *tcf21:nuceGFP* larvae. No epicardial cells are evident at or before 2 dpf. By 3 dpf, ventricles contained 17.6 ± 6 epicardial cells on average (n = 23), whereas 4 dpf larvae contained 45.2 ± 5.8 cells (n = 21). e, *tcf21:nuceGFP* larval clutchmates were randomly separated into two groups for treatment with vehicle or 5 μM CyA from 2 to 4 dpf. f, Quantification of ventricular eGFP+ epicardial cells from groups in e. ***P < 0.001, Student’s two-tailed t-test; n = 21 for each group. a, b, d, e, White dashed lines delineate ventricle. d, e, Boxed areas, magnified views. Scale bars, 50 μm. Error bars indicate s.d.
Extended Data Figure 10 | Hh ligand expression. a–c, Quantitative RT–PCR revealing *shha, ihhb* and *dhh* expression in atrium (a) or ventricle (b) in uninjured hearts and 3 days post-ablation, or in separated ventricular basal (the basal third of the chamber) and apical (the apical third) tissue after ablation (c). Three separate quantitative RT–PCR experiments on pooled tissues were performed, using a total of 90 zebrafish for experiments shown in a and b, and another 90 fish for c. *shhb* and *ihha* were not detected in these tissues. d, In situ hybridization (ISH) for *shha* or *dhh* in wild-type (WT) or tcf21:NTR clutchmate hearts at 3 days after Mtz treatment, indicating expression in outflow tract but not ventricle or atrium. Outflow tract of uninjured and epicardially ablated hearts showed comparable *shha* and *dhh* signals by ISH, a qualitative/semiquantitative assay. e, Section of adult *shha:eGFP* heart, indicating fluorescence in outflow tract tissues. Smooth muscle cells (MLCK, red) and epicardial cells (outer layer) in outflow tract showed clear eGFP signals, while there is no obvious eGFP fluorescence in ventricle and atrium. Valve mesenchyme also displays eGFP fluorescence. Arrowheads indicate eGFP signals in smooth muscle cells and epicardium. f, Ventricular resection induces *shha:eGFP* fluorescence in the basal ventricular epicardium at 2 dpa. Arrows indicate ventricular epicardial fluorescence. d, e, White dashed line indicate outflow tract (d) or atrioventricular junction (e). d–f, Boxed areas, magnified views. Scale bars, 50 μm. Error bars indicate s.d.