Notch signalling restricts inflammation and serpine1 expression in the dynamic endocardium of the regenerating zebrafish heart

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ABSTRACT
The zebrafish heart regenerates after ventricular damage through a process involving inflammation, fibrotic tissue deposition/removal and myocardial regeneration. Using 3D whole-mount imaging, we reveal a highly dynamic endocardium during cardiac regeneration, including changes in cell morphology, behaviour and gene expression. These events lay the foundation for an initial expansion of the endocardium that matures to form a coherent endocardial structure within the injury site. We studied two important endocardial molecules, Serpine1 and Notch, which are implicated in different aspects of endocardial regeneration. Notch signalling regulates developmental gene expression and features of endocardial maturation. Also, Notch manipulation interferes with attenuation of the inflammatory response and cardiomyocyte proliferation and dedifferentiation. serpine1 is strongly expressed very early in the wound endocardium, with decreasing expression at later time points. serpine1 expression persists in Notch-abrogated hearts, via what appears to be a conserved mechanism. Functional inhibition studies show that Serpine1 controls endocardial maturation and proliferation and cardiomyocyte proliferation. Thus, we describe a highly dynamic endocardium in the regenerating zebrafish heart, with two key endocardial players, Serpine1 and Notch signalling, regulating crucial regenerative processes.

KEY WORDS: Heart regeneration, Endocardium, Myocardium, Signalling, Serpine1, Notch

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The adult mammalian heart fails to regenerate after cardiac injury owing to a permanent deposition of massive fibrotic tissue, together with the inability to replenish lost cardiac muscle (Conrad et al., 1995). The zebrafish exhibits a remarkable capacity for organ regeneration (Gemberling et al., 2013). Localised damage to the zebrafish heart using the cryoinjury model induces processes similar to those in the infarcted mammalian heart: cell death, inflammatory response and cardiomyocyte proliferation and dedifferentiation. Thus, we describe a highly dynamic endocardium during cardiac regeneration, including changes in cell morphology, behaviour and gene expression. These events lay the foundation for an initial expansion of the endocardium that matures to form a coherent endocardial structure within the injury site. We studied two important endocardial molecules, Serpine1 and Notch, which are implicated in different aspects of endocardial regeneration. Notch signalling regulates developmental gene expression and features of endocardial maturation. Also, Notch manipulation interferes with attenuation of the inflammatory response and cardiomyocyte proliferation and dedifferentiation. serpine1 is strongly expressed very early in the wound endocardium, with decreasing expression at later time points. serpine1 expression persists in Notch-abrogated hearts, via what appears to be a conserved mechanism. Functional inhibition studies show that Serpine1 controls endocardial maturation and proliferation and cardiomyocyte proliferation. Thus, we describe a highly dynamic endocardium in the regenerating zebrafish heart, with two key endocardial players, Serpine1 and Notch signalling, regulating crucial regenerative processes.

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RESULTS
Endocardial cells reside and expand within the injured tissue after cryoinjury
To study the endocardium during heart regeneration we used the endocardial enhancer trap line ET33-mi60a, in which GFP is inserted close to the promoter of the Notch signalling modulator lunatic fringe (lfr) (Poon et al., 2010). We used the cryoinjury model of heart regeneration that faithfully recapitulates mammalian ischemic injury (Chablais et al., 2011; González-Rajal et al., 2011; Schnabel et al., 2011). We detected GFP expression throughout the ventricle but also within the cryoinjury site of ET33-mi60a hearts (Fig. 1A,B; sample sizes for all figures are provided in Tables S1, S2). GFP+ cells (GFP mRNA, Fig. 1C) expressed the endothelial erythroblast transformation-specific transcription factor Erg (Fig. 1D, Fig. S1A) and the endocardial development gene nfatc1a (Fig. 1E, Fig. S1B) (Larson et al., 2004; Wong et al., 2012). This suggests that GFP+ cells in the wound endocardium
Fig. 1. The endocardium expands at the injury site. (A) ET33-mi60a transgenic zebrafish heart, whole-mount views. Strong GFP expression is apparent in the cryoinjured region of the ventricle (arrowheads). (B) ET33-mi60a heart section. Immunohistochemistry (IHC) shows GFP+ endocardial cells in a myosin heavy chain (MF20−) area. (C) ET33-mi60a heart consecutive section to Fig. 1B showing GFPISH. (D-F) IHC, or IHC combined with FISH. GFP+ cells express Erg (D), nfatc1a (E) and Aldh1a2 (F). (B-F) Boxed areas are magnified on the right. (G-I) Volume rendering of ET33-mi60a;myl7:mRFP injured ventricles. Endocardium, green; myocardium, magenta. (J,J′) Volume rendering (J) and amplified optical section from the centre (J′) of an injured ventricle. (K) Imaris-based volume quantification. The volume of the mRFP− region was determined (blue, V_{injury site}) and used as a mask to label GFP+ cells in this region (yellow) and determine their volume (V_{GFP cells}). (L) Scatter plot showing the relative volume occupied by GFP+ cells in the injury site. Mean (red line)±s.d.; one-way ANOVA and Newman-Keuls test (see Table S3). (M) IHC showing Pcn1a+ GFP+ endocardial nuclei (arrowheads). Boxed areas magnified beneath. (N) Scatter plot showing percentage of Pcn1a+ cells among GFP+ cells within and adjacent (50 μm) to the injury site. Mean±s.d.; one-way ANOVA and Newman-Keuls test, *P<0.05, ***P<0.005 (see Table S4). (O) Schematic illustrating that endocardium proliferation precedes endocardial expansion at the injury site. is, injury site; a, atrium; ba, bulbus arteriosus. Dotted lines demarcate injured tissue. Scale bars: 200 μm in A-C,G-J; 20 μm in D-F; 50 μm in magnified views in B,C; 20 μm in magnified views in D,E,M; 100 μm in J.
region retain their endocardial/endothelial features after injury and are activated. These GFP+ wound endocardial cells expressed Aldh1a2 protein (Fig. 1F), consistent with previous results (Kikuchi et al., 2011). Likewise, the endocardial reporter Tg(βfli1a:GFP) (Lawson and Weinstein, 2002) presented GFP+ cells in the injury site (Fig. S1C).

We studied wound endocardium dynamics by high-resolution 3D confocal imaging. Optical clearing of ET33-mi60a:myl7:mRFP hearts by CUBIC (clear, unobstructed brain imaging cocktails and computational analysis; reagent 1; Susaki et al., 2014) allowed detection of endogenous fluorescence signals: GFP in the endocardium and membrane-bound RFP in cardiomyocytes (Rohr et al., 2008). At 24 hours post cryoinjury (hpci), strongly reduced mRFP fluorescence at the injury site relative to uninjured tissue indicated the absence of myocardial cells (Fig. 1G, Movie 1). Despite the overall reduced GFP fluorescence, we detected spared GFP+ cells within the injury site (Fig. 1G, Movie 1). To estimate the abundance of endocardial cells with respect to the injury site extension, we used Imaris software to 3D reconstruct confocal images and quantify the volume of the injury site, identified by the absence of mRFP+ cardiomyocytes (Fig. 1K; see Materials and Methods). We also quantified the volume of the injury site occupied by GFP+ cells (Fig. 1K). The relationship between these two values provided an estimate of the relative volume occupied by GFP+ endocardial cells over the course of regeneration (Fig. 1K,L). GFP+ cells in an uninjured region, surrounding cardiomyocytes, occupied a volume of 46.1±2.2% (Fig. S1D). At 24 hpci, GFP+ cells were rare at the injury site, occupying 3.5±2.1% of the injured volume (Fig. 1G,L, Fig. S1D, Movie 1). By 36 hpci, the GFP+ volume increased slightly but significantly (8.5±3.8%; Fig. 1L, Fig. S1E, Movie 2). At 3 dpci, most hearts exhibited a higher occupation by GFP+ cells (15.5±8.9%; Fig. 1H,L, Movie 3). The proportion of the injured volume occupied by GFP+ cells progressively increased until 7 dpci (Fig. 1L, Fig. S1E, Movies 4, 5) and was stable at 9 dpci (47.1±13.7%; Fig. 1L, Movie 6). The relative volume occupied by GFP+ cells continued to increase up to 3 months post cryoinjury (mpci), albeit more gradually (57.6±4.4% at 1 mpci and 74.6±12.6% at 3 mpci; Fig. 1L,L, Fig. S1E, Movies 7, 8). This might be due to the decrease in the total size of the injury site over this period of time (Fig. S1F), when cardiomyocytes had already begun to replace lost cardiac muscle (Fig. 1J, 1 mpci; Fig. S1E, 3 mpci), consistent with published data (Chalbais et al., 2011; Itou et al., 2012). Analysis of optical sections in 1 mpci hearts revealed that regions still lacking cardiomyocytes were filled with endocardial cells (Fig. 1J). Moreover, endocardial volume at 1 mpci and 3 mpci is beyond the value of an uninjured region of the heart (Fig. 1L, Fig. S1C), indicating that endocardial density is augmented at the injury site.

We next examined endocardial cell proliferation in ET33-mi60a hearts. GFP+ endocardial cells stained for proliferating cell nuclear antigen (Pena) were very rare in uninjured hearts (Fig. S1G; 0.45±0.15 cells/heart section). At 24 and 36 hpci, several GFP+ endocardial cells at the injury site were Pena+ (Fig. 1M,N). At 3 dpci, we observed a massive proliferative response of GFP+ cells adjacent to (Fig. 1M, N) and throughout (Fig. S1H) the injury site. Endocardial proliferation decreased at 5 dpci (Fig. 1N, Fig. S1I) and was almost abolished at 7 dpci (Fig. 1M,N). This shows that the highest proliferation coincides with the initiation of endocardial expansion and suggests that endocardial cell proliferation at 3 and 5 dpci contributes to the endocardial expansion from 3 to 7 dpci (Fig. 1O). The observation that maximal endocardial proliferation (3 dpci) occurs before the peak of myocardial proliferation (7 dpci) (Kikuchi et al., 2010; Sallin et al., 2014; Bednarek et al., 2015), and the presence of the endocardium at the injury site throughout regeneration (Kikuchi et al., 2010; Sallin et al., 2014; Bednarek et al., 2015), suggest that endocardial regeneration precedes myocardial regeneration.

Wound endocardium is highly dynamic during regeneration

We next analysed endocardial morphology at different time points after cryoinjury and conducted 3D imaging analysis at high magnification. In the undamaged region of the heart, a coherent network of endocardial cells with elongated morphology surrounded clusters of cardiomyocytes (Fig. 2A). Injury site endocardial cells at 24 hpci were similarly elongated (Fig. 2A). By contrast, we observed a dense and disorganised endocardial mesh at the injury site at 3 dpci (Fig. 2A). Endocardial cells appeared rounded, in clusters and displayed numerous filopodia-like protrusions (Fig. 2A, arrowheads). These co-stained with phalloidin (Fig. 2B, Fig. S2A), indicating an actin-rich cytoskeleton, one characteristic of filopodia (Mallavarapu and Mitchison, 1999; Mattila and Lappalainen, 2008). The high density of GFP+ cells was maintained at 9 dpci; however, cells seemed more orderly aligned than at 3 dpci, forming a coherent sheet (Fig. 2A). Moreover, filopodia-like protrusions were less abundant at 9 dpci than at 3 dpci (Fig. 2C,D).

We next examined expression of the endothelial cell-cell adhesion protein Cadherin 5 (Cdh5), which is involved in endocardial junction integrity (Mitchell et al., 2010). In situ hybridisation (ISH) showed strong cdh5 expression at the injury site, which was weaker throughout the ventricle and in the uninjured heart (Fig. S2B,C). We conducted fluorescent ISH (FISH) on ET33-mi60a heart sections and compared levels of cdh5 mRNA fluorescence of remote and wound endocardium (Fig. 2E,F). Relative cdh5 expression at the injury site increased up to 1.156 (±0.089) at 3 dpci (Fig. 2G), and even more at 7 dpci (1.820±0.142), indicating that injury endocardium maturation coincides with increasing levels of cdh5 expression (Fig. 2G). Also, more mature injury endocardium at 7 dpci was strongly associated with collagen fibres at 7 dpci, but not at 3 dpci (Fig. S2D,E).

The presence of filopodia-like protrusions, a characteristic of migrating cells (Ridley, 2011), together with the observed endocardial expansion suggested endocardial cellular migration within the injury site. To test this, we cultured cryoinjured hearts and performed live imaging of the regenerating endocardium in ET33-mi60a transgenic fish at ≈2-3 dpci. We observed that endocardial cells changed their location and that individual cells migrated short distances within the injury site (Fig. 2H, Movies 9, 10). We also detected endocardial cells sending out filopodia (Fig. 2I, Movie 10).

Overall, we show by morphological, gene expression and live imaging analyses a dynamic post-injury endocardium (Fig. 2), and distinguish an early activated, proliferative endocardium (3 dpci) from a more mature, organised, less-proliferative endocardial structure (7-9 dpci).

Notch pathway elements are expressed in the endocardium and are implicated in endocardial maturation and heart regeneration

Next, we examined the involvement of Notch, a crucial endocardial cell signalling pathway during development and regeneration (Raya et al., 2003; Zhang et al., 2013; Luxán et al., 2016), in the cryoinjured heart. The ligand Delta-like 4 (Dll4) is expressed in GFP+ wound endocardial cells in ET33-mi60a and Tg(βfli1a:GFP) hearts (Fig. 3A, Fig. S3A,E). notch1b expression was initially low
Fig. 2. Characteristics of injury endocardium at different stages of regeneration. (A) Volume rendering and corresponding optical sections of part of the remote and injured region from ET33-mi60a;myl7::mRFP hearts. Remote region contains elongated and coherent GFP* cells. At 1 dpci individual GFP* cells are seen at the injury site (is) (arrows). At 3 dpci dense, clustered endocardial cells with filopodia-like protrusions (arrowheads) are observed. At 9 dpci the endocardial cells are more organised, aligned and mostly lack filopodia-like protrusions. (B-B″) Vibratome section of ET33-mi60a heart stained for GFP and with phalloidin (for F-actin). Filopodia-like protrusions of wound endocardial cells show phalloidin staining (arrowheads). (C) Volume rendering of part of the injured region from ET33-mi60a;myl7::mRFP hearts. (D) Quantification of filopodia-like protrusions from comparable 3D images. Mean±s.d.; t-test, *P<0.05. (E) FISH combined with IF showing high cdh5 expression (red) in GFP* wound endocardium. Boxed areas magnified on the right. (F, G) cdh5 (red) fluorescence intensity measurements with ImageJ software: GFP* endocardium was selected (F, yellow) in the remote or wound region. Scatter plot (G) showing relative red fluorescence intensity comparing values of both regions. Mean±s.d.; t-test, **P<0.01. (H, I) Confocal still pictures from time-lapse movies (Movies 9, 10) of wound endocardial cells in cultured ET33-mi60a;myl7::mRFP hearts. The box in the schematic indicates the imaging region. Endocardial cells move (H, I, red arrowheads) and change their position (H, blue arrowhead). Dotted arrow (H) indicates the direction of cell migration. Endocardial cells present dynamic filopodia-like protrusions (I, asterisks). (J) Schematic showing wound endocardium characteristics. Filopodia-like protrusions are more abundant at early (3 dpci) than at later (9 dpci) phases. cdh5 expression increases when regeneration proceeds. Scale bars: 50 μm in A-C; 100 μm in E; 20 μm H, I; 10 μm in magnified views of B.
Fig. 3. Notch signalling elements are expressed in the endocardium and Notch signalling modulation affects injury endocardium maturation. (A) IHC showing Dll4 expression by GFP+ wound endocardial cells (arrowheads). (B) ISH showing notch1b expression in endocardial cells at the injury site (arrows) and lining injury-adjacent cardiomyocytes (arrowheads). (C-E) FISH for notch1b (C), notch2 (D) or lfng (E) combined with IHC showing transcripts in GFP+ wound endocardial cells (arrowheads). (F) Volume rendering of injured ET33-mi60a;myl7:mRFP ventricles after DMSO or RO treatment and of heat shocked (HS) Tg(UAS:NICD);ET33-mi60a;myl7:mRFP and control hearts. Treatment regimens are indicated at the top. (G) Scatter plot presenting relative wound GFP+ cell volume. Mean (dotted line)±s.d.; t-test. (H) Injury site optical sections showing the disorganisation and clustered appearance of endocardial cells and more filopodia-like protrusions (arrowheads) after RO treatment, and fewer filopodia-like protrusions after heat shock in Tg(UAS:NICD);ET33-mi60a;myl7:mRFP hearts. (I) Scatter plot showing the number of filopodia-like protrusions. For representative images used for quantification see Fig. S6A. Mean±s.d.; t-test **P<0.05. Dotted lines (A,C,D,E) delineate the injury site (is). Boxed areas are magnified on the right. Scale bars: 100 µm in A-E; 200 µm in F; 50 µm in H; 20 µm in magnified views in A,C,D,E; 25 µm in magnified views in H.
shown by unchanged expression of interferon expression and endocardial activation (Kikuchi et al., 2011), as endocardial cell expansion and maturation. In line with published overactivation impairs heart regeneration. control fish (Fig. S4G,H), suggesting that long-term Notch 90 dpci, regeneration between control and Tg(UAS:NICD) induced the expression of the Notch intracellular domain (NICD) [abbreviated to Tg(UAS:NICD)] transgenic fish. To study the requirement of Notch for cryoinjured heart regeneration, we used the γ-secretase inhibitor RO492909 (RO), which effectively reduces Notch activity in the zebrafish embryo and adult fin (Munch et al., 2013), and in the retina (Conner et al., 2014). In the injured heart, RO treatment diminished Notch target gene transcription (Fig. S4A) and impaired heart regeneration, as indicated by the increased amount of fibrotic tissue at 30 dpci (Fig. S4B,C), similar to observations made in the ventricular resection model (Zhao et al., 2014).

We examined the impact of increased Notch activity on regeneration using the inducible model Tg(hsp70l:Gal4);Tg(UAS:myc-notch1a-intra) [abbreviated to Tg(UAS:NICD)]. Heat shocks induced the expression of the Notch intracellular domain (NICD) (Scheer et al., 2001) and increased Notch target gene transcription in Tg(UAS:NICD) injured hearts (Fig. S4D). Differences in regeneration between control and Tg(UAS:NICD) hearts were not evident until 33 dpci (Fig. S4E,F), presumably owing to heat shock-induced slowing of regeneration (Gemberling et al., 2013). At 90 dpci, Tg(UAS:NICD) hearts retained more fibrotic tissue than control fish (Fig. S4G,H), suggesting that long-term Notch overactivation impairs heart regeneration.

Next, we examined the requirement of Notch for wound endocardial cell expansion and maturation. In line with published results (Zhao et al., 2014), manipulation of Notch signalling did not interfere with endocardial activation (Kikuchi et al., 2011), as shown by unchanged expression of aldh1a2 (Fig. S5A,B), and did not alter endocardial cell proliferation at 3 dpci (Fig. S5C-E). In ET33-mi60a transgenic fish, GFP expression was unaffected by RO treatment or pathway overactivation (Fig. S5G), allowing us to use this line for the following studies.

Notch signalling inhibition for 4 dpci did not significantly alter GFP+ wound endocardium expansion, which was similar in Tg(UAS:NICD);ET33-mi60a and control hearts (Fig. 3F,G). Analysis of endocardial cell morphology in RO-treated hearts at 5 dpci revealed clustered endocardial cells at the injury site (Fig. 3H), similar to our observations at 3 dpci (Fig. 2A). DMSO-treated hearts, however, exhibited a more orderly aligned injury endocardium (Fig. 3H), indicating that endocardial maturation had occurred in control but not in RO-treated hearts. Quantification of filopodia-like protrusions as one characteristic of the early immature wound endocardium (Fig. 2C,D) revealed a higher abundance in RO-treated hearts (Fig. 3H,I, Fig. S6A). Moreover, overactivation of the Notch pathway in Tg(UAS:NICD);ET33-mi60a hearts resulted in a reduced number of filopodia-like protrusions (Fig. 3H,I, Fig. S6A), suggesting a role for Notch in endocardial maturation. Additionally, cdh5 transcript levels, analysed as before (Fig. 2F), did not significantly differ after Notch signalling manipulation (Fig. S6B-E). Thus, these results indicate that a precise level of Notch activation is crucial for heart regeneration after cryoinjury and that Notch is involved in regulating endocardial maturation.

**Decreased Notch signalling affects endothelial, cardiovascular and wound healing processes**

To study the molecular changes resulting from Notch abrogation in the cryoinjured heart, we extracted RNA from the injured region of the ventricle after RO or DMSO treatment at 3 dpci (Fig. 4A). RNA-seq analysis identified 347 differentially expressed genes, 196 of which were upregulated and 151 downregulated (Fig. 4B, Table S6). Ingenuity-based gene ontology (GO) classification revealed that Notch signalling inhibition affected genes involved in various endothelial cell processes (Fig. 4C), suggesting a function for Notch in endocardial cells, since they are specialised endothelial cells and both cell types share structural and functional genes (Harris and Black, 2010). Furthermore, genes required in the cardiovascular system and for wound healing were dysregulated (Fig. 4C). GO assignments of differentially expressed genes to categories related to endothelial cells, the cardiovascular system and wound healing are indicated in Fig. S7A.

Notch inhibition dysregulated genes involved in the regulation of angiogenesis (vegfc, id1, efnb2a, egr1), endothelial integrity [cdln5b, heg (heg1)] and endothelial cell differentiation (klf2a, klf2b, aqp1a.1) (Fig. 4D). A subset of these (efnb2a, heg, klf2a, egr1, id1) are also endocardial genes (Mably et al., 2003; Grego-Bessa et al., 2007; Vermot et al., 2009; Zhao et al., 2011) and were expressed at the inner border of the injured heart (egr1, id1; Fig. S7B, C). Confirming the Notch signalling attenuation (Fig. S4A), RNA-seq detected the downregulation of efnb2a and bmp10, two Notch-dependent genes involved in chamber development (Grego-Bessa et al., 2007) (Fig. 4D). Three additional endocardial genes, heg, klf2a and klf2b, were upregulated upon Notch inhibition (Fig. 4D). heg regulates endothelial/endocardial integrity (Kleaveland et al., 2009) and growth of the zebrafish myocardium (Mably et al., 2003), klf2 gene endothelial expression is induced by shear forces and is related to a stretched, less migratory, differentiated endothelial phenotype (Dekker et al., 2002, 2006). klf2a and heg transcripts were present in a subset of GFP+ wound endocardial cells and also in the remote region (Fig. 4E,F), the latter being consistent with previous reports after ventricular resection (Kikuchi et al., 2011).

qPCR analysis confirmed that Notch inhibition increased heg, klf2a and klf2b expression (Fig. 4G) and that Notch overactivation attenuated heg expression (Fig. S7D). Moreover, heg and klf2a expression, which is normally restricted to non-chamber endocardium during cardiac development (DMSO, Fig. 4H) (Mably et al., 2003; Vermot et al., 2009), was expanded to the ventricular endocardium after Notch inhibition (RO, Fig. 4H), suggesting that Notch regulates these genes similarly during development and regeneration.

**Notch signalling inhibition alters the expression of inflammatory genes and leads to increased inflammatory cell abundance**

RNA-seq analysis revealed the involvement of Notch in cardiovascular and wound healing processes, which led us to study both more in detail. The expression of extracellular matrix (ECM)-degrading proteases [ctswa, ctswb.1 (ctsw2.1), mmp9] and hyaluronidase 2 (hyal2), which degrades hyaluronan (Chowdhury et al., 2013), was increased in Notch-diminished hearts (Fig. 5A). Notch inhibition (Fig. 5B) also led to increased expression of inflammatory markers and regulators ([pgs2b, pde7a, sgp1]) (Smith et al., 2003; Ogryzko et al., 2014) and the pro-inflammatory endothelial genes arg2 (Ryoo et al., 2008) and tnfrsf9a (CD137) (Olofsson et al., 2008; Teijeira et al., 2012) (Fig. 5B). This upregulation of inflammatory factors is consistent with the augmented pro tease gene expression because protease-induced low molecular weight ECM molecule fragments trigger pro-inflammatory signals and leukocyte recruitment (Franigoannis, 2008; Dobaczewski et al., 2010).
In zebrafish, injured cardiac tissue is infiltrated by immune cells (Schnabel et al., 2011; Wang et al., 2011; Han et al., 2014) but little is known about the timely regulation of inflammatory signals in the cryoinjured heart. qPCR analysis revealed that inflammatory and ECM degradation genes were strongly upregulated at 36 hpci, with levels declining thereafter to reach near-baseline levels at 7 dpci (Fig. 5C,D). ISH revealed ctssb.1, mmp9, and tnfrsf9a transcription at the inner injury border after cryoinjury (Fig. S8A,B). We detected inflammatory cells, indicated by l-plastin (lcp1) or mpeg1 expression (Herbomel et al., 1999; Ellett et al., 2011), mainly in the injury site and most of these macrophages were in close proximity to GFP+ endocardial cells in ET33-mi60a fish (Fig. 5E,F, Fig. S8C). We hypothesize that the early activated wound endocardium may be involved in the regulation of inflammatory cell recruitment, and investigated the function of Notch in this process.

High inflammatory gene expression (Fig. 5C,D) temporally coincided with the high number of l-plastin-expressing leukocytes at the inner injury border (Fig. S8D) and low Notch expression at 36 hpci (Fig. S3B). Consistent with the RNA-seq data (Fig. 5A,B), qPCR and ISH analyses revealed increased expression of mmp9, ctssb.1 and tnfrsf9a in regenerating hearts upon Notch inhibition at later stages (Fig. S8E-H). Moreover, an augmented number of wound endocardial cells expressed the pro-inflammatory gene tnfrsf9a in hearts after RO treatment, as compared with DMSO-treated hearts (Fig. 5G,H). Notch inhibition further caused elevated numbers of l-plastin+ and mpeg1+ macrophages associated with wound endocardial cells (Fig. 5I-K, Fig. S8I), and an increased abundance of l-plastin+ macrophages could be detected until 30 dpci after long-term Notch inhibition (Fig. 5L). These observations suggest that Notch...
Fig. 5. Notch signalling inhibition affects inflammatory gene expression and macrophage abundance. (A,B) RNA-seq analysis. Differential cardiac expression of genes related to ECM remodelling (A) and inflammation (B) in RO-treated fish at 3 dpci. (C,D) qPCR analysis of hearts with no cryoinjury (no ci) and at indicated time points following cryoinjury. Mean±s.d.; t-test, *P<0.05, **P<0.01, ***P<0.005. (E) FISH combined with IHC, showing l-plastin+ cells (arrowheads) in contact with GFP+ cells. l-plastin+ cell accumulations at the injury site locally coincide with high endocardial cell abundance (brackets). (F) The percentage of mpeg1-expressing or l-plastin-expressing inflammatory cells that contact (green) or do not contact (blue) endocardial cells at the injury site or remote region. Inflammatory cells at the outer epicardial region were not considered. (G) Intrafusal FISH combined with IHC. RO-treated hearts (regime indicated at top) show more GFP+ cells expressing tnfrsf9a. (H) Scatter plot showing percentage of tnfrsf9a+ endocardial cells. Mean±s.d.; t-test, *P<0.05. (I) mpeg1 FISH combined with IHC. RO treatment (regime in G) increased mpeg1+ macrophage abundance associated with the GFP+ endocardium. (J,K) Scatter plots of the number of mpeg1+ (J) or l-plastin+ (K) macrophages related to the area occupied by GFP+ cells. Only macrophages contacting GFP+ endocardial cells were considered. Mean±s.d.; t-test, *P<0.05. (L) MF20 IHC and l-plastin ISH on consecutive heart sections. RO treatment increased the numbers of l-plastin+ macrophages inside the MF20− injury site (red arrowheads), l-plastin+ macrophages in the outer region of the injury can be seen in both conditions (arrows). (M) Schematic showing time and intensity of the inflammatory response and Notch signalling activation at the injury site. Dotted lines delineate the injury site (is). Scale bars: 100 µm, except 50 µm in magnified views.
is required to restrict the inflammatory response in the injury site (Fig. 5M).

**Notch signalling regulates cardiomyocyte proliferation and differentiation**

We observed that endocardial activation and proliferation preceded myocardial regeneration (Fig. 1H-N). High-magnification 3D analysis showed that clusters of injury-adjacent cardiomyocytes were embedded in a dense endocardial network (Fig. 6A), and that myocardial cell protrusions, which are characteristic of migrating cardiomyocytes (Morikawa et al., 2015), were in close contact with endocardial cells (Fig. 6B). To investigate myocardial and endocardial interactions we analysed BrdU incorporation in Myocyte enhancer factor 2 (Me2f) wound-adjacent cardiomyocytes after Notch signalling manipulation at 7 dpci. Notch inhibition decreased cardiomyocyte proliferation (Fig. 5A,B), consistent with results obtained after ventricular resection (Zhao et al., 2014). Further, we observed that sustained Notch overactivation augmented cardiomyocyte proliferation (Fig. 6C-D), which differs from previous findings (Zhao et al., 2014). Analysis of Me2f cardiomyocyte density adjacent to the injury site at 7 dpci revealed higher cardiomyocyte numbers in Tg(UAS:NICD) than in control hearts (Fig. 6E,F), suggesting that cardiomyocytes accumulate in this region after Notch overactivation.

ISH against hand2 and nks2.5, hallmark transcription factors of dedifferentiated cardiomyocytes (Lepilina et al., 2006; Fig. 5C), indicated an expansion of dedifferentiated cardiomyocytes in Tg(UAS:NICD) hearts (Fig. 6G, Fig. 5D).

We next analysed myocardial genes that were differentially expressed in the RNA-seq, including the immediate early cardiac growth genes mycb and fosab (Fig. 6H). RNA-seq and ISH data showed that Notch inhibition decreased mycb levels in injury-adjacent cardiomyocytes (Fig. 6I), similarly to fosab expression (Fig. 6J). Both genes were shown to be expressed in dedifferentiated, proliferating cardiomyocytes in fish (Aguirre et al., 2014; Beauchemin et al., 2015), suggesting that changes in mycb and fosab expression might be associated with the reduction in cardiomyocyte proliferation in Notch-abrogated hearts. RNA-seq analysis indicated an upregulation of genes encoding sarcomere assembly and function proteins (Fig. 6H). During regeneration, dedifferentiating zebrafish and mouse cardiomyocytes disassemble the sarcomere for cell division to proceed (Jopling et al., 2010; Porrello et al., 2011), and high levels of sarcomeric proteins are characteristic of differentiated cardiomyocytes (O’Meara et al., 2015). Confirming the RNA-seq data, we detected fewer wound-adjacent cardiomyocytes showing decreased levels of myosin light chain kinase 3 (mylk3), which regulates sarcomere assembly (Seguchi et al., 2007), in hearts after RO treatment than in DMSO-treated hearts (Fig. 6K,L). Further, Notch overactivation decreased tcp expression in Tg(UAS:NICD) transgenic hearts (Fig. 6M). These results suggest that Notch signalling modulations interfere with sarcomeric gene expression and affect cardiomyocyte dedifferentiation.

**The early endocardial gene serpine1 is implicated in endocardial and myocardial proliferation**

To identify a potential early endocardial factor, the downregulation of which may depend on Notch signalling at later stages (Fig. 4D), we focused on plasminogen activator inhibitor 1 (serpine1). Secreted Serpine1 is the main physiological inhibitor of urokinase plasminogen activator (uPA) and tissue plasminogen activator (tPA), and thus inhibits fibrinolysis (Declercq and Gils, 2013).

Moreover, Serpine1 regulates endothelial cell proliferation (Ploplis et al., 2004), apoptosis (Balsara and Ploplis, 2008; Abderrahmani et al., 2012) and migration (Isogai et al., 2001) and is upregulated in the injured neonatal heart (Darezhereshki et al., 2015). Serpine1 expression was absent in the uninjured adult heart (Fig. S10A) but strongly upregulated early after cryoinjury (36 hpci, 3 dpci, Fig. 7A) and decreased at later stages (Fig. 7A, Fig. S10B). FISH combined with antibody staining detected a high number of serpine1-expressing GFP wound and wound-adjacent cardiac cells (Fig. 7C,D, Fig. S10C in ET33-mi60a fish at 24 hpci and 36 hpci (36.7±6.6% and 35.5±6.8%, respectively). The percentage of serpine1-expressing endocardial cells dramatically decreased at 3 dpci (14.4±5.6%; Fig. 7C,D) and remained low at 7 dpci (4.7±0.9%; Fig. 7C,D). Notch inhibition increased serpine1 expression levels (Fig. 7B, Fig. S10D) and indeed augmented the number of endocardial cells expressing serpine1 (Fig. 7E,F), indicating that serpine1 downregulation in the injury site endocardium requires Notch signalling at later stages of regeneration.

To investigate if this regulation could be a common mechanism in endothelial/endocardial cells we treated porcine aortic valve endothelial cells (PAVECs) with RO for 48 h. SERPINE1 expression was increased in Notch-abrogated PAVECs (Fig. 7G) in parallel with a marked downregulation of Notch targets (not shown). Also, RNA-seq data obtained in various mouse mutants with disrupted endocardial Notch signalling at different time points of development (Luxán et al., 2013; D’Amato et al., 2016) showed significantly increased Serpine1 expression (Fig. 7G), indicating that, similar to the zebrafish situation (Fig. 7B,E-G), Serpine1 expression is upregulated in mouse after Notch abrogation.

To investigate the function of Serpine1 in the cryoinjured endocardium, we treated fish with the inhibitor Tipilaxtinin (PAI-039, abbreviated as TP) (Fig. 7H), which blocks Serpine1 protease activity (Gorlatova et al., 2007; Daniel et al., 2015). TP treatment did not interfere with fibrotic tissue deposition (Fig. S10E-G) or endocardial activation, as indicated by the expression of adhl1a2 (Fig. S10H). However, we detected an augmented number of wound endocardial cells expressing Pena after TP treatment for 3 dpci (Fig. 7H,J), suggesting that endocardial proliferation at 3 dpci is linked to serpine1 downregulation in endocardial cells. Also, relative levels of cdh5 in wound endocardial cells were significantly higher (1.4±0.1; Fig. 7J,K) in TP-treated ET33-mi60a fish than in DMSO-treated controls (1.3±0.1; Fig. 7J,K) at 3 dpci, indicating that endocardial maturation had progressed further.

As Serpine1 is a secreted molecule, endocardial Serpine1 might also have non-cell-autonomous functions during heart regeneration. We examined the consequences of TP treatment on cardiomyocyte proliferation, and observed higher numbers of Pca-mi60a cardiomyocytes in TP-treated hearts (Fig. 7L,M), indicating that early Serpine1 abrogation augments cardiomyocyte proliferation. We next treated fish with TP or DMSO during the first days of regeneration (10 dpci), the time frame when we expected serpine1 expression to be active in the endocardium. Examination of TP- or DMSO-treated injured hearts at 22 dpci did not reveal any difference in injury site size (Fig. 7N,O), suggesting that early Serpine1 inhibition does not result in accelerated regeneration or in interference with collagen deposition or degradation.

In summary, these results reveal serpine1 as an early endocardial injury-response gene, the downregulation of which at later stages depends on Notch signalling. Moreover, Serpine1 is involved in the regulation of endocardium proliferation and maturation, and non-cell-autonomously influences myocardial proliferation.
Fig. 6. Notch signalling regulates cardiomyocyte proliferation. (A) ET33-mi60a;myl7:mRFP ventricle, volume rendering and optical section of a region of the inner injury border. Endocardial cells surround injury-adjacent cardiomyocytes (arrows) and precede into the wound. (B) IHC showing endocardial cells (GFP+) in contact with myocardial protrusions (tropomyosin+; blue arrowheads). (C,D) IHC against BrdU and Mef2 (C). Heat shock regime is indicated at the top. Scatter plot (D) of percentage of BrdU+ wound-adjacent cardiomyocytes (Mef2+; arrowheads between dotted lines). Mean±s.d.; t-test, **P<0.01. (E,F) Mef2 IHC showing a higher density of wound-adjacent cardiomyocytes (between the dotted lines) in Tg(UAS:NICD) than in control hearts (E), as quantified in F. Mean±s.d.; t-test, *P<0.05. (G) ISH showing higher numbers of nkx2.5+ wound-adjacent cardiomyocytes (between dotted lines) in Tg(UAS:NICD) hearts. (H) RNA-seq data showing differential myocardial gene expression in RO-treated hearts at 3 dpci. (I) ISH showing mycb expression in injury-adjacent cardiomyocytes (arrowheads) in DMSO-treated but not in RO-treated hearts (asterisks). (J) qPCR levels of fosab in the injured heart. Mean±s.d.; t-test, *P<0.05. LOF, loss of function. (K) MF20 IHC and mylk3 ISH on heart sections. RO treatment decreased the numbers of mylk3− cardiomyocytes adjacent to the injury site (arrowheads). (L) Scatter plot showing the percentage of mylk3− wound-adjacent (50 µm) cardiomyocytes. Mean±s.d.; t-test, **P<0.01. (M) qPCR analysis of mylk3 and tcap levels in the injured heart. Mean±s.d.; t-test, ***P<0.001. GOF, gain of function. Dotted lines delineate the injury site (is). Boxed areas are magnified in insets. Scale bars: 100 µm, except 25 µm in magnified views.
Fig. 7. serpine1 is upregulated early upon cryoinjury and responds to Notch in endocardial/endothelial cells. (A) qPCR analysis of serpine1 in hearts with no cryoinjury (no ci) and at indicated time points following cryoinjury. Mean±s.d.; t-test, *P<0.05. (B) serpine1 qPCR in the injured heart. Mean±s.d.; t-test, *P<0.05. (C) serpine1 FISH plus IHC. At 36 hpci, numerous wound and wound-adjacent GFP+ endocardial cells express serpine1. At 3 and 7 dpci, serpine1-expressing wound endocardial cells are less frequent. Boxed areas are magnified beneath. (D) Scatter plot of the percentage of serpine1+ cells among all wound and wound-adjacent (50 μm) GFP+ cells. Mean±s.d.; one-way ANOVA and Newman-Keuls test, ***P<0.005 (see Table S5). (E) serpine1 FISH combined with IHC. RO-treated hearts (regime indicated at top) show more GFP+ cells expressing serpine1 (arrowheads). (F) Scatter plot indicating the percentage of serpine1+ endocardial cells. Mean±s.d.; t-test, *P<0.05. (G) Log fold change (FC) of serpine1 showing increased expression as assessed by qPCR in RO-treated PAVECs, by RNA-seq in RO-treated zebrafish hearts and in various murine models of endocardial Notch disruption. (H) IF indicating Pcna+ and GFP+ cells after TP treatment (regime indicated at top). (I) Scatter plot of the relative number of Pcna+ cells among all wound and wound-adjacent GFP+ cells. Mean±s.d.; t-test, *P<0.05. (J) FISH combined with IHC showing that TP treatment increases cdh5 mRNA levels in GFP+ wound endocardium. (K) Scatter plot of relative red fluorescence intensity, comparing values of remote and wound endocardium (see Fig. 2F). Mean±s.d.; t-test, **P<0.01. (L) IF revealed more Pcna+ Mef2+ wound-adjacent cardiomyocytes (arrowheads) after TP treatment. (M) Scatter plot of the relative number of Pcna+ cells among all wound-adjacent (100 μm) Mef2+ cardiomyocytes. Mean±s.d.; t-test, **P<0.01. (N) AFOG-stained hearts, treated with DMSO or TP (treatment regime indicated at top). (O) Scatter plot indicating injury site size (mean±s.d.). Dotted lines delineate injury site. Scale bars: 100 μm in C,E,H,J,L; 200 μm in N; 20 μm in magnified views.
DISCUSSION

Endocardial dynamics at the injury site

In this report we provide the first 3D image analysis of the whole injured region of the zebrafish heart, and describe the dynamics and possible functions of the injured endocardium. Previous studies have reported morphological changes and the activation of wound-adjacent endocardial cells following ventricular resection (Kikuchi et al., 2011). Our results, using the cryoinjury model, reveal a highly dynamic endocardium during the first days of regeneration, with changes in endocardial cell morphology, behaviour and gene expression occurring at distinct phases of regeneration (Fig. 8).

Signals controlling endocardial dynamics

We identified Serpine1 as an early injury-induced molecule in the damaged endocardium (Fig. 8). Despite its inhibitory role in fibrinolysis (Declerck and Gils, 2013), Serpine1 inhibition did not interfere with fibrotic tissue deposition or resolution. We describe one function for Serpine1 as a negative regulator of proliferation and of cdh5 levels in wound endocardial cells (Fig. 8B). This could imply the involvement of Serpine1 in the maintenance of an initial activation state of the wound endocardium and is in line with its cell-autonomous role in endothelial cells, regulating proliferation or apoptosis (Bajou et al., 2008). Further, we identify Notch as an important player during wound endocardium maturation (Fig. 8), with similar functions in development and regeneration. This holds true for the augmented expression of the developmental genes heg and klf2a and the increased number of filopodia-presenting wound endocardial cells in Notch-inhibited hearts. During developmental angiogenesis, Notch blocks the migratory tip-cell fate of endothelial cells (Hellström et al., 2007; Lobov et al., 2007; Suchting et al., 2007) and Notch inhibition interferes with blood vessel maturation (Ehling et al., 2013).

Implication of endocardial signals in the inflammatory response and cardiomyocyte proliferation

We observed that early activated endocardium coincides with a high abundance of inflammatory cells and inflammatory gene expression. This raises the hypothesis that endocardial signals may regulate regenerative processes, and that endocardial maturation might be crucial for cardiac regeneration to progress from the inflammatory to the reparative phase (Chablais and Jazwinska, 2012a). During inflammation, vascular endothelial cells mediate the recruitment, adherence and passage of inflammatory cells (Pober and Sessa, 2007). The endocardium might possess this role in the regenerating heart as it responds to inflammatory signals (Kikuchi et al., 2011) and expresses cytokines (Fang et al., 2013). We extend these studies, showing that inflammatory macrophages are associated with wound endocardium. Also, Notch signalling abrogation resulted in increased wound-related endothelial and inflammatory gene expression and an increased abundance of macrophages. This anti-inflammatory role of Notch might be direct, by regulating endothelial inflammatory genes such as mfrs9a (Olofsson et al., 2008; Teijeira et al., 2012), or might be linked to the appearance and maturation of the endocardium upon Notch inhibition.

Cardiac injury induces the dedifferentiation and proliferation of existing cardiomyocytes, which peaks at 7 dpci (Kikuchi et al., 2010; Sallin et al., 2014; Bednarek et al., 2015), and endocardial signals are implicated in this process (Kikuchi et al., 2011; Zhao et al., 2014). Our results show that endocardial proliferation and

Fig. 8. Endocardial dynamics and signalling after cryoinjury. (A) The tip of the adult zebrafish ventricle in the uninjured situation (no ci), and at various time points after cryoinjury. Uninjured endocardium is depicted as a dark green monolayer and the myocardium in pink; the damaged tissue of the injury site (is) in purple and delineated by a dotted line, with the endocardium colonising the injury site in light green. (B) Injury endocardium is characterised by changes in morphology (filopodia, organisation), behaviour (proliferation, migration) and gene expression (cdh5, serpine1) at different time points of regeneration. Alterations in endocardial signals (Notch, Serpine1) affect these characteristics (arrows, lines) and interfere with inflammation attenuation (blue) and cardiomyocyte proliferation (red).
regeneration precede the regenerating myocardium. In addition, we revealed a possible non-cell-autonomous effect of Serpine1 and Notch signalling on cardiomyocyte proliferation. Secreted Serpine1 might directly signal to cardiomyocytes, preventing proliferative signals or controlling the degradation of specific ECM components, which are crucial for myocardial proliferation and regeneration (Trinh and Stainier, 2004; Mercer et al., 2013; Wang et al., 2013). The inverse correlation between high endocardial serpine1 expression and high cardiomyocyte proliferation (Kikuchi et al., 2010; Sallin et al., 2014; Bednarek et al., 2015) (Fig. 8B) at different stages of heart regeneration supports the hypothesis that serpine1 downregulation is one prerequisite for myocardial proliferation. Moreover, hearts with abrogated Notch signalling, where cardiomyocyte proliferation was decreased, also showed increased levels of serpine1. These observations suggest a Notch-mediated downregulation of serpine1 to regulate cardiomyocyte proliferation. Serpine1 expression is also upregulated in endocardial cells in the cryoinjured mouse heart (Darehzereshki et al., 2015) and might be involved in an evolutionarily conserved mechanism of cardiac repair. The inhibitory relationship between Notch and Serpine1 might be part of this mechanism, as supported by our observation that abrogated Notch signalling caused increased SERPINE1 expression in endothelial cells (PAVECs) and in mouse embryos. Future studies should investigate the regulatory effect of Notch on serpine1, and whether Serpine1 inhibition affects dedifferentiation of cardiomyocytes. Precise regulation of Serpine1 is crucial, as elevated Serpine1 levels increase cardiac fibrosis upon myocardial infarction in mice (Takehita et al., 2004), whereas Serpine1 deficiency leads to cardiac fibrosis (Moriwaki et al., 2004).

In our study, Notch overactivation led to increased BrdU incorporation by injury-adjacent cardiomyocytes (Fig. 8). This result appears to conflict with data from Zhao et al. (2014) showing decreased numbers of Pena+ cardiomyocytes upon Notch overactivation. We further observed the accumulation of dedifferentiated cardiomyocytes at the inner injury border, explaining why Notch overactivation impairs regeneration. We suggest two possible explanations. First, the dedifferentiated, proliferating state of cardiomyocytes might prevent their invasion into the injury. Second, cardiomyocytes might fail to enter the injury site due to altered ECM remodelling or endocardial organisation within the injury site. Our Notch inhibition data would support both possibilities, but this issue requires further investigation.

The results presented here reveal a highly dynamic endocardium after cryoinjury, with changes in cell behaviour, morphology and gene expression during regeneration. We identified serpine1 as an early endocardial injury-response gene and Notch signalling as a player later during regeneration. The maturation of the endocardium and the control of inflammatory cell infiltration require Notch signalling, and our data suggest that the endocardium promotes myocardial regeneration by providing Serpine1-dependent negative and Notch-dependent positive proliferative signals (Fig. 8). These findings demonstrate the importance of the endocardium in the regulation of the injury response and in regeneration upon cardiac insult. Future analysis of the specific endocardial signals orchestrating inflammation, fibrotic tissue deposition and cardiomyocyte renewal could contribute to the development of therapeutic applications for cardiac diseases.

MATERIALS AND METHODS
Zebrafish husbandry
Animal studies were approved by the CNIC Animal Experimentation Ethics Committee and by the Community of Madrid (ref. PROEX 118/15). Animal procedures conformed to EU Directive 2010/63/EU and Recommendation 2007/526/EC regarding the protection of animals used for experimental and scientific purposes, enforced in Spanish law under Real Decreto 53/2013. Zebrafish were raised and maintained under standard conditions (Kimmel et al., 1995). Cryoinjury was performed as described (González-Rosa and Mercader, 2012). Details of transgenic lines used are provided in the supplementary Materials and Methods.

Treatments
Adult zebrafish were injected intraperitoneally with 30 µl RO4929097 (S1575, Selleckchem; 600 µM in PBS) or DMSO as a control. Treatment regimens of each experiment are indicated in the corresponding figure. Embryos were incubated in fishwater containing DMSO or RO (50 µM). 30 µl Tipixantrin (PAI-039, S7922, Selleckchem; 1500 µM) or DMSO in PBS was injected intraperitoneally following the treatment regime indicated in the figures. See the supplementary Materials and Methods for further details.

Histology
Hearts were fixed in 4% paraformaldehyde (PFA) overnight, dehydrated, embedded in paraffin and sectioned at 7 µm. Serial sections were distributed on several slides, such that each slide contained sections of several levels of the heart. One or two slides of each heart were used for Acid Fuchsin-Orange G (AFOG) staining, immunohistochemistry (IHC) or in situ hybridisation (ISH) to obtain stained sections randomly distributed throughout the heart for unbiased quantification (see below). Primers used to generate RNA probes are listed in Table S7. IHC was performed as described (González-Rosa et al., 2011). For IHC on vibratome sections, hearts were fixed in 4% PFA overnight, washed with PBST (0.1% Tween in PBS) and embedded in 4% low melting point agarose. Sections of 70 µm were obtained and kept in PBST. For IHC, sections were incubated in blocking solution (5% BSA, 5% goat serum, PBS) for 1 h and then in primary antibody overnight at 4°C. After extensive washes with PBST, heart sections were incubated overnight with secondary antibody and FITC-coupled phallidin. Extensive washes and a 30 min incubation with DAPI followed. Sections were mounted in Fluormount (Sigma) for imaging. For details of antibodies and imaging see the supplementary Materials and Methods.

Cell culture
Isolation of porcine aortic valve endothelial cells (PAVECs) was carried out as described (Gould et al., 2010). Cells at passage five were seeded on gelatin-coated 6-well dishes (2.1×10^5 cells/well) and cultured in Dulbecco’s Modified Eagle’s Medium (MEM; Gibco) supplemented with fetal bovine serum (FBS; 10%), penicillin-streptomycin (1%) and bovine brain extract (20 ng/ml). After 24 h incubation, cells near confluence were serum starved (with 0.5% FBS) and treated with 10 µM RO or vehicle (DMSO) for 12, 36 or 48 h. After the corresponding incubation periods, cells were washed twice with PBS and collected for RNA extraction. Each time point was conducted in triplicate.

Gene expression analysis
For quantitative PCR (qPCR) and RNA sequencing (RNA-seq), hearts were dissected at different time points and apical portions of the three ventricles were pooled and (Fig. 4A) used for RNA extraction using Direct-zol RNA MiniPrep (Zymo Research). cDNA was synthesized with the SuperScript III First Strand Kit (Invitrogen). For qRT-PCR, three hearts were pooled, and the data represented here are from four to six samples. Primer sequences are provided in Tables S8 and S9. See the supplementary Materials and Methods for further details. For RNA-seq analysis, we used three pools, each of three apices from RO- or DMSO-treated fish. cDNA libraries were prepared with a TruSeq RNA Sample Preparation Kit v2 (Illumina) and were sequenced in a Genome Analyzer IIx Illumina sequencer using a 75 bp single-end elongation protocol. Sequenced reads were quality controlled and pre-processed using Cutadapt v1.6 to remove adaptor contaminants (Martin, 2011). The resulting reads were aligned and gene expression was quantified with RSEM v1.2.3 (Li and Dewey, 2011), using the Zv9_75
zebrafish reference genome. Differentially expressed genes were defined as those with altered expression levels with an adjusted P<0.05. RNA-seq data were analysed using Ingenuity Pathway Analysis Software (QIAGEN Bioinformatics).

Quantification and statistical analysis

For details of quantification and statistical analysis, including 3D volume, filopodia-like protrusions, fibrotic tissue, IF or ISH staining, BrdU incorporation, macrophage numbers and cardiomyocyte density, see the supplementary Materials and Methods. Sample numbers for all data are listed in Tables S1 and S2.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

J.M., D.G., A.G.-R., R.T.-C. performed experiments. J.M. and J.L.d.l.P. designed experiments, reviewed the data and wrote the manuscript. All authors reviewed the manuscript during its preparation.

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Data availability

RNA-seq data are deposited at NCBI Gene Expression Omnibus under accession number GSE68650.

Supplementary information

Supplementary information available online at http://dev.biologists.org/lookup/doi/10.1242/dev.143362.supplemental

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