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

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Summary statement

We describe, using 3D whole-mount imaging and functional analysis, the dynamics of the endocardium during zebrafish heart regeneration and the involvement of two endocardial-derived molecules, Serpine1 and Notch, on endocardial maturation, cardiomyocyte proliferation and inflammation.
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 the 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, in 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 heart and two key endocardial players, Serpine1 and Notch signalling, regulating crucial regenerative processes.
Introduction

The adult mammalian heart fails to regenerate after cardiac injury, due 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). A localized damage to the zebrafish heart, using the cryoinjury model induces processes similar to those in the infarcted mammalian heart: cell death, inflammatory cell infiltration and scar deposition. In fish, this scar is dissolved, and the injured tissue is replaced by new cardiomyocytes (Gonzalez-Rosa et al., 2011; Schnabel et al., 2011; Chablais and Jazwinska, 2012b). Most studies have focused on how cardiomyocyte dedifferentiation and proliferation is achieved to regenerate cardiac muscle (Kikuchi et al., 2010; Fang et al., 2013; Aguirre et al., 2014; Kikuchi, 2015), but how different cardiac tissues interact to orchestrate heart regeneration is poorly understood. Ventricular resection rapidly activates the epicardium and endocardium, and endocardial retinoic acid is required for cardiomyocyte proliferation (Kikuchi et al., 2011). However, the behaviour and function of the endocardium after cryoinjury has not been investigated.

Endocardial Notch signalling regulates cardiomyocyte proliferation, differentiation and patterning in a non-cell-autonomous manner during mouse cardiac chamber development (Grego-Bessa et al., 2007; Luxan et al., 2013; D’Amato et al., 2016). Notch function in the damaged adult mouse heart is unclear; it has been suggested to control fibrotic and regenerative repair in a pressure-overload model (Nemir et al., 2014), but is unable to promote cardiomyocyte proliferation after myocardial infarction (Felician et al., 2014).

Here, we study the behaviour and morphology of the endocardium after cryoinjury, using three dimensional (3D) confocal imaging to visualize the complete injured region. Our results revealed activated, proliferating and migrating endocardial cells at 3 days post cryoinjury (dpci), in contrast to the more mature less proliferative, coherent endocardial structure in the injury site at 7 dpci. Furthermore, we describe the consequences of Notch manipulation on endocardial morphology, attenuation of the inflammatory response and cardiomyocyte proliferation. We also establish Serpine1 as an early molecule of the injured endocardium with regulatory roles in cellular maturation and
proliferation. Our findings highlight the significance of endocardial signals regulating different processes during heart regeneration.

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 (lfng) (Poon et al., 2010). We used the cryoinjury model of heart regeneration that faithfully recapitulates mammalian ischemic injury (Chablais et al., 2011; Gonzalez-Rosa 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 size for all figures in Table S1 and S2). GFP+ cells expressed GFP mRNA (Fig. 1C), the ERG endothelial erythroblast-transformation-specific transcription factor (Figs. 1D,S1A) and the endocardial development gene nfatc1a (Figs. 1E,S1B) (Larson et al., 2004; Wong et al., 2012). This suggests that GFP+ cells in the wound endocardium 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(fli1a:GFP) (Lawson and Weinstein, 2002), presented GFP+ cells in the injury site (Fig. S1C).

We studied the wound endocardium dynamics by high-resolution 3D confocal imaging. Optical clearing of ET33-mi60a, myl7:mRFP hearts by CUBIC (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 hpci, a strongly reduced mRFP fluorescence at the injury site relative to non-injured tissue indicated the absence of myocardial cells (Fig. 1G, Movie S1). Despite the overall reduced GFP fluorescence, we detected spared GFP-labelled cells within the injury site (Fig. 1G, Movie S1). 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 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% (Fig. S1D). At 24 hpci, GFP+ cells were rare at the injury site, occupying 3.5±2.1% of the injured volume (Figs. 1G,L,S1D, Movie S1). By 36 hpci, the GFP+ volume increased slightly but significantly (8.5±3.8%; Figs. 1L,S1E, Movie S2). At 3 dpci, most hearts exhibited a higher occupation by GFP+ cells (15.5±8.9%; Fig. 1H,L, Movie S3). The proportion of the injured volume occupied by GFP+ cells progressively increased until 7 dpci (Figs. 1L,S1E, Movies S4,S5) and was stable at 9 dpci (47.1±13.7%, Fig. 1I, L, Movie S6). 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; Figs. 1J, L, S1E, Movie S7,S8). This may be due to the decrease of total size of the injury over this period of time (Fig. S1F), when cardiomyocytes had already begun to replace lost cardiac muscle (Figure 1J, 1 mpci, Figure S1E, 3 mpci), consistent with published data (Chablais 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 (Figure 1L,S1C), indicating that endocardial density is augmented at the injury site.

We next examined endocardial cell proliferation in ET33mi-60A hearts. GFP+ endocardial cells stained for proliferating cell nuclear antigen (PCNA) were very rare in uninjured hearts (Fig. S1G, 0.45±0.15 cells/heart section). At 24 hpci and 36 hpci, several GFP+ endocardial cells at the injury site presented PCNA-staining (Fig. 1M,N). At 3 dpci, we observed a massive proliferative response of GFP+ cells adjacent and (Fig. 1M,N) throughout the injury site (Fig. S1H). Endocardial proliferation decreased at 5 dpci (Figs. 1N,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 suggest...
that endocardial cell proliferation at 3 and 5 dpci contributes to the endocardial expansion from 3 dpci to 7 dpci (Fig. 1O). The observation that maximal endocardial proliferation (3dpci) 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), suggests that endocardial 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 endocardial cells at 24 hpci were similarly elongated (Fig. 2A). By contrast, we observed a dense and disorganized 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 were co-stained with phalloidin (Fig. 2B, 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 the expression of the endothelial cell-cell adhesion protein cadherin 5 (cdh5), 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 non-injured heart (Fig. S2B,C). We conducted fluorescent ISH (FISH) on ET33mi60A 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 (±0.08904) at 3 dpci (Fig. 2G); and even more at 7 dpci, (1.820±0.1424), indicating that injury endocardium maturation coincides with increasing levels of cdh5 expression (Fig. 2G). Also, more
mature injury endocardium at 7 dpci is 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 hypothesis, we cultured cryoinjured hearts and performed live imaging of the regenerating endocardium in ET33mi60A transgenic fish at around 2-3 dpci. We observed that endocardial cells changed their location and that individual cells migrated short distances within the injury site (Fig. 2H,I, Movies S9,S10). We also detected endocardial cells sending out filopodia (Fig. 2I, Movie S10). Overall, we show by morphological, gene expression and live imaging analysis a dynamic post-injury endocardium (Fig. 2J), and distinguish an early activated, proliferative endocardium (3 dpci) from a more mature, organized, 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 implication of Notch, a crucial endocardial cell signalling pathway during development and regeneration (Raya et al., 2003; Zhang et al., 2013; Luxan 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(fli1a:GFP) hearts (Figs. 3A,S3A,E). Notch1b expression was initially low (36 hpci, Fig. S3B), but strong notch1b, notch2, notch3 and lunatic fringe (lfng) transcription was evident in endocardial cells lining injury-adjacent cardiomyocytes and within the injury site at 3 and 7 dpci (Figs. 3B,S3C,D). FISH combined with immunofluorescence (IF) confirmed notch1b, notch2 and lfng expression by GFP+ wound-endocardial cells in ET33mi60A (Fig. 3C,D,E) and Tg(fli1a:GFP) (Fig. S3F) 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 the 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,
indicated by the increased amount of fibrotic tissue at 30 dpci (Fig. S4B,C), similarly to 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 as \( Tg(UAS:NICD) \)]. Heat shocks induced the expression of the Notch intracellular domain (NICD) (Scheer et al., 2001) and increased Notch target genes transcription in \( Tg(UAS:NICD) \) injured hearts (Fig. S4D). Differences in regeneration between in control and \( Tg(UAS:NICD) \) hearts were not evident until 33 dpci (Fig. S4E,F), presumably due to heat-shock-induced slowing of regeneration (Gemberling et al., 2013). At 90 dpci \( Tg(UAS:NICD) \) hearts retained more fibrotic tissue, comparing to 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 neither altered endocardial cell proliferation at 3 dpci (Figs. S5C-E). In \( ET33-mi60A \) transgenic fish GFP-expression was unaffected by RO-treatment or Notch 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 (Fig. 3F,G), which was similar in \( Tg(UAS:NICD);ET33-mi60A \) 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), similarly 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. 2A,B), revealed a higher abundance in RO-treated hearts (Figs. 3H,I,S6A). Moreover, the overactivation of the Notch pathway in \( Tg(UAS:NICD);ET33-mi60A \) hearts resulted in a reduced number of filopodia-like protrusions (Fig. 3H,I,S6A), suggesting a role for Notch in endocardial maturation. Additionally, \( cdh5 \) transcript levels analysed as before (Fig. 2), 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 up-regulated and 151 down-regulated (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 deregulated (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 deregulated the expression of genes involved in the regulation of angiogenesis (vegfc, id1, ephrinb2a, egr1), endothelial integrity (claudin5b, heg) and endothelial cell differentiation (klf2a, klf2b, aqp1a.1) (Fig. 4D). A subset of these (ephrinb2a, 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), the RNA-seq detected the down-regulation of ephrinb2a and bmp10, two Notch-dependent genes involved in chamber development (Grego-Bessa et al., 2007) (Fig. 4D). Three additional endocardial genes, heart of glass (heg) and krüppel like factors 2a and b (klf2a, klf2b), were up-regulated upon Notch inhibition (Fig. 4D). Heg regulates endothelial/endocardial integrity (Kleaveland et al., 2009) the growth of the zebrafish myocardium (Mably et al., 2003). Klf2 endothelial expression is induced by shear forces and is related to a stretched, less migratory, differentiated endothelial phenotype (Dekker et al., 2002; Dekker et al., 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; and (Mably et al., 2003; Vermot et al., 2009) was expanded to the ventricular endocardium after Notch inhibition (RO, Fig. 4H), suggesting that Notch regulates those 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 ECM degrading proteases (ctssba, ctssb.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 (ptgs2b, pde7a, sgpl1) (Smith et al., 2003; Ogryzko et al., 2014) and pro-inflammatory endothelial genes (Fig. 5B), arginase2 (arg2) (Ryoo et al., 2008) and tumour necrosis factor receptor superfamily member 9a (tnfrsf9a/CD137) (Olofsson et al., 2008; Teijeira et al., 2012) (Fig. 5B). This inflammatory molecules up-regulation is consistent with the augmented protease gene expression because protease-induced low-molecular-weight ECM molecule fragments, trigger pro-inflammatory signals and leukocyte recruitment (Frangogiannis, 2008; Dobaczewski et al., 2010).

In zebrafish, cardiac injured 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 up-regulated at 36 hpci, with levels declining thereafter to reach near-baseline levels at 7 dpcri
ISH displayed \textit{ctssb.1}, \textit{mmp9} and \textit{tnfrsf9a} transcription at the inner injury border after cryoinjury (Fig. S8A,B). We detected inflammatory cells, indicated by \textit{l-plastin} or \textit{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 \textit{ET33mi60A} fish (Fig. 5E,F,S8C). We hypothesize that the early-activated wound endocardium may be implicated in the regulation of inflammatory cell recruitment and investigated the function of Notch in this process.

High inflammatory gene expression temporally (Fig. 5C,D) coincided with the high number of \textit{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 \textit{mmp9},\textit{ctssb.1} and \textit{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 \textit{tnfrsf9a} in hearts after RO-treatment comparing to DMSO-treated hearts (Fig. 5G, H). Notch inhibition further caused elevated numbers of \textit{l-plastin}$^+$- and \textit{mpeg1}$^+$-macrophages associated with wound-endocardial cells (Figs. 5I-K,S8I) and increased abundance of \textit{l-plastin}$^+$-macrophages could be detected until 30 dpci after long-term Notch inhibition (Fig. 5L). These observations suggest the requirement of Notch to restrict the inflammatory response in the injury site (Fig. 5M).

\textbf{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 myocardial cell protrusions, 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 (\textit{mef2}$^+$) wound-adjacent cardiomyocytes after Notch signalling manipulation at 7 dpci. Notch inhibition decreased cardiomyocyte proliferation (Fig. S9A,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 is different to previous findings (Zhao et al., 2014). Analysis of mef2+ 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 nkx2.5, hallmark transcription factors of dedifferentiated cardiomyocytes (Lepilina et al., 2006), Fig. S9C) indicated an expansion of dedifferentiated cardiomyocytes in Tg(UAS:NICD) hearts (Figs. 7G,S9D).

We next analysed myocardial genes that were differentially expressed in the RNA-seq, including the immediate-early cardiac growth genes avian myelocytomatosis viral oncogene homolog b (myc-b) and FBJ murine osteosarcoma viral oncogene homolog Ab (fosab) (Fig. 6H). RNA-seq and ISH data showed that Notch inhibition decreased myc-b levels in injury-adjacent cardiomyocytes (Fig. 6I), similarly to fosab expression (Fig. 6J). Both genes were reported recently to be implicated in dedifferentiated, proliferating cardiomyocytes in fish (Aguirre et al., 2014) (Beauchemin et al., 2015), suggesting that changes in myc-b and fosab expression may be associated with the reduction in cardiomyocyte proliferation in Notch-abrogated hearts. RNA-seq analysis indicated an up-regulation 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 less 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-treated than in DMSO-treated hearts (Fig. 6K,L). Further, Notch overactivation decreased tcap 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 molecule, whose downregulation 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 (Declerck 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 becomes upregulated the injured neonatal heart (Darehzereshki et al., 2015), which led us to investigate on *serpine1*.

*Serpine1* 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, S10B). By conducting FISH combined with antibody staining, we detected indeed a high number of *serpine1*-expressing GFP+ wound- and wound-adjacent endocardial cells (36.7 % ±6.6% and 35.5 % ±6.8%; Figs. 7C,D,S10C) in ET33mi60A fish at 24 hpci and 36 hpci. 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 (qPCR, Figs. 7B,S10D) and indeed augmented the number of endocardial cells expressing *serpine1* (Fig. 7E,F), indicating that *serpine1* downregulation in the injury 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 (PAVEC) with RO for 48h. *SERPINE1* expression was increased in Notch-abrogated PAVEC (Fig. 7G) in parallel with a marked down-regulation 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 (Luxan et al., 2013; D’Amato et al., 2016) showed significantly increased *Serpine1* expression (Fig. 7G), indicating that similarly to the zebrafish situation (Fig. 7B,E-G) *Serpine1* expression is up-regulated after Notch abrogation.
To investigate the function of Serpine1 in the cryoinjured endocardium, we treated fish with the Serpine1 inhibitor tiplaxtinin (PAI-039, abbreviated as TP, Fig. 5J), 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, indicated by the expression of aldha1a2 (Fig. S10H). However we detected an augmented number of wound-endocardial cells expressing PCNA, after TP treatment for 3 dpci (Fig. 7H,I), suggesting that endocardial proliferation at 3 dpci is linked to serpine1 down-regulation in endocardial cells. Also, levels of cdh5 in wound-endocardial cells were significantly higher (1.4±0.1, Fig. 7J,K) of TP-treated ET33mi60A fish comparing to DMSO-treated fish (1.3 ±0.1, Fig. 7J) at 3 dpci indicating that endocardial maturation had progressed further. As Serpine1 is a secreted molecule, endocardial Serpine1 may also have non-cell autonomous functions during heart regeneration. We examined the consequences of TP-treatment on cardiomyocyte proliferation and observed higher numbers of PCNA+ 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 interferes with collagen deposition or degradation.

In summary, these results reveal serpine1 as an early endocardial injury-responsive gene, whose downregulation at later stages depends on Notch signalling. Moreover, Serpine1 is involved in the regulation of endocardium proliferation and maturation, and influencing non-cell-autonomously myocardial proliferation.
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 differences 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 (Fig. 8) and cdh5 levels of wound endocardial cells. 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 kl2a 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 (Hellstrom 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 on inflammatory response and cardiomyocyte proliferation

We observed that early activated endocardium coincides with high abundance of inflammatory cells and inflammatory gene expression. This raises the hypothesis that endocardial signals may regulate regenerative processes, and that endocardial maturation may 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 inflammatory cells passage (Pober and Sessa, 2007). The endocardium may 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 increased abundance of macrophages. This anti-inflammatory role of Notch might be direct, by regulating endothelial inflammatory genes, such as *tnfrsf9a* (Olofsson et al., 2008; Teijeira et al., 2012) or might be linked to the endocardial appearance and maturation 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 regeneration precedes the regenerating myocardium. In addition, we revealed a possible non-cell-autonomous effect of Serpine1 and Notch signalling on cardiomyocyte proliferation. Secreted Serpine1 may directly signal to cardiomyocytes, preventing proliferative signals or control 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. 7J) at different stages of heart regeneration supports the hypothesis that *serpine1* down-regulation 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 down-regulation of *serpine1* to regulate cardiomyocyte proliferation. Serpine1 expression upregulates also in endocardial cells in the cryoinjured mouse heart (Darehzereshki et al., 2015) and may be involved in an evolutionary conserved mechanism of cardiac repair. The inhibitory relationship between Notch and Serpine1 may be part of this mechanism, supported by our observation that abrogated Notch signalling caused increased *SERPINE1* expression in endothelial cells (PAVEC) and in mouse embryos. Future studies should investigate the regulatory effect of
Notch on *serpine1*, and whether Serpine1-inhibition affects dedifferentiation of cardiomyocytes. A precise regulation of Serpine1 is crucial, as elevated Serpine1 levels increased cardiac fibrosis upon myocardial infarction in mice (Takeshita et al., 2004), whereas *Serpine1*-deficiency led 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 PCNA-labelled cardiomyocytes upon Notch overactivation. We further observed 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 organization 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-responsive 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 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 (Gonzalez-Rosa and Mercader, 2012). Details on transgenic lines used are indicated in Supplementary Materials and Methods.

Treatments

Adult zebrafish were injected intraperitoneally with 30 μl of RO4929097 (S1575, selleckchem.com, 600 mmol/ml in PBS) or DMSO as a control. Treatment regimens of each experiments are indicated in the corresponding figure. Embryos were incubated in DMSO or RO (50 mmol/ml) containing fishwater. 30 μl of Tiplaxtinin (TP, PAI-039, S7922, selleckchem.com, 1500 mmol/ml) or DMSO in PBS were injected intraperitoneally following the treatment regime indicated in the figures. See Supplementary Materials and Methods for further details.

Histology

Hearts were fixed in 4% 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 hybridization (ISH) to obtain randomly, throughout the heart distributed, stained sections for unbiased quantification (see below). IHC was performed as described (Gonzalez-Rosa et al., 2011). For IHC on vibratome sections hearts were fixed in 4% PFA overnight, washed with PBST and embedded in 45% 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 1h and then after adding the first antibody, overnight at 4°C. After extensive
washes with PBST heart sections were incubated overnight with the secondary antibody and FITC-coupled phalloidin. Extensive washes and a 30min incubation with DAPI followed. Sections were mounted in Fluoromount (Sigma) for imaging. See Supplementary Materials and Methods for further details on antibodies.

**Cell culture**

Isolation of porcine aortic valve endothelial cells (PAVEC) was carried out as described (Gould et al., 2010). Cells at passage 5 were seeded on gelatin-coated 6-well dishes (2.1 x 10^5 cells/well), and cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with fetal bovine serum (10%), penicillin-streptomycin (1%) and bovine brain extract (20 ng/ml). After 24h incubation, cells near confluence were serum starved (with 0.5% FBS) and treated with 10μM γ-secretase inhibitor (RO4929097, Selleckchem) or vehicle (DMSO) for 12, 36 and 48 hours. After the corresponding incubation periods, cells were washed twice with PBS 1x 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. 4H) used for RNA extraction with Direct-zol™ RNA MiniPrep (Zymo Research). cDNA was synthesized with the SuperScript III First Strand kit (Invitrogen). For quantitative RT-PCR, 3 hearts were pooled, and the data represented here are from 4-6 samples. Primer sequences can be found in Table S8 and S9. For RNA sequencing analysis, we used three pools, each of three apexes from RO- or DMSO-treated fish. cDNA libraries were prepared with a TruSeq RNA Sample Preparation Kit v2 (Illumina, San Diego, CA), and were sequenced in a Genome Analyzer IIx Illumina sequencer using a 75bp 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. See Supplementary Materials and Methods for further details.
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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.

Competing interests

The authors declare no competing interests.

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Data availability

RNA-seq data are deposited in the NCBI GEO database under accession number GSE68650. The following link has been created to allow review of record GSE68650 while it remains in private status: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=ydwrnikwvhmxv&acc=GSE68650
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Fig. 1: The endocardium expands at the injury site

(A) *ET33-mi60a* transgenic heart, whole-mount views. Strong GFP-expression in the cryoinjured region of the ventricle. (B) *ET33mi-60a* heart section. Immunohistochemistry (IHC) shows GFP⁺ endocardial cells in an mf20⁻ area. (C) *ET33-mi60a* heart consecutive section to Fig. 1B, GFP ISH. (D-F) IHC only or combined with FISH. GFP⁺ cells express ERG (D), *nfatc1a* (E) and *Aldh1a2* (F). In (A-F) boxed areas are magnified on the right. (G-I) Volume rendering of *ET33mi-60A;myl7mRFP* injured ventricles (endocardium, green; myocardium, magenta). Boxed area is magnified on the right. (J) Volume rendering 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$_{\text{injured area}}$); and used as a mask to label GFP⁺ cells in this region (yellow) and determine their volume (V$_{\text{GFP cells}}$). (L) Scatter plot showing the relative volume occupied by GFP⁺ cells in the injury site (is) (red line=mean± s.d, One-way ANOVA and Newman-Keuls test, see Table S3. (M) IHC showing PCNA-labelled GFP⁺ endocardial nuclei (arrowheads). Boxed areas magnified below. (N) Scatter plot: percentage of PCNA⁺/GFP⁺ cells of GFP⁺ cells within and adjacent (50μm) to the injury site. (red line=mean± s.d, One-Way ANOVA and Newman-Keuls-test, *P<0.05, *** P<0.005, see Table S4). (O) Schematic: Injury endocardium proliferation precedes endocardial expansion at the injury site. (a, atrium; ba, bulbus arterious, vl, ventricular lumen). Dotted lines demarcate injured tissue. Scale bars: (A-C,G-J) 200μm, (D,E,F) 20μm, (M) 100μm; magnified views: (B,C) 50μm, (D,E,M) 20μm.
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 ET33mi-60A;myl7mRFP hearts. Remote region: elongated and coherent GFP+ cells. 1dpci: Individual GFP+ cells at injury site (is) (arrows). 3 dpci: Dense, clustered endocardial cells, present filopodia-like protrusions (arrowheads). 9 dpci: endocardial cells are more organized, aligned and mostly lack filopodia-like protrusions. (B) Vibratome section of ET33-mi60A heart, stained for GFP and phalloidin. Filopodia-like protrusions of wound-endocardial cells show phalloidin staining (arrowheads). (C) Volume rendering of part of the injured region from ET33mi-60A;myl7mRFP hearts. (D) Quantification of filopodia-like protrusions from comparable 3D-images (red line=mean ± s.d, t-test, *P< 0.05). (E) FISH combined with IF, high cdh5 expression (red) in GFP+ wound endocardial. Boxed areas magnified on the right. (F,G) cdh5 (red) fluorescent intensity measurements with ImageJ software: GFP+ endocardium was selected (yellow) in the remote or wound region. Scatter plot showing relative red fluorescence intensity comparing values of both regions (G; red line=mean± s.d, t-test, ** P< 0.01). (H,I) Confocal still pictures from time-lapse movies (Movies S9,S10) of wound endocardial cells in cultured ET33-mi60a;myl7mRFP hearts. The box in the schematic indicates the imaging region. Dimensions are indicated below. Endocardial cells move (H,I, red arrows) and change their position (H, blue arrow). 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 phases (9 dpci). cdh5 expression increases when regeneration proceeds. Scale bars: (A,B,D) 50μm, (E) 100μm, (H,I) 20μm.
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 the endocardium (arrows) and lining cardiomyocytes (arrowheads) of the injury site (is). (C-E) FISH for notch1b (C), notch2 (D), lfg (E) combined with IHC showing transcripts in GFP⁺ wound endocardial cells (arrowheads). (F) Volume rendering of injured ET33mi-60A;myl7mRFP ventricles after DMSO or RO treatment (indicated) and heat-shocked (HS) Tg(UAS:NICD);ET33mi60a;myl7;mRFP and control hearts. Treatments regimens are indicated. (G) Scatter plot presenting relative wound GFP⁺ cell volume. (Dotted line=mean±s.d., t-test). (H) Injury site: optical sections showing disorganization, clustered appearance of endocardial cells and more filopodia-like protrusions (arrowheads) after RO-treatment, and less filopodia-like-protrusion after HS in Tg(UAS:NICD);ET33mi60a;myl7;mRFP hearts. (I) Scatter plot: number of filopodia-like protrusions. For representative images used for quantification see Fig. S6A (dotted line=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: (A,B,C,D,E) 100μm; (F) 200μm; (H) 50μm; magnified views: (A,C,D, E) 20μm, (H) 25μm.
Fig. 4: Notch inhibition affects developmental and injury-related endocardial/endothelial genes

(A) RO-injection and RNA-extraction procedure from the ventricular apex (below the dotted line).
(B) RNA-seq: 347 genes differentially expressed in injured hearts after RO treatment (fold change >0.5; \( P<0.05 \)). (C) Ingenuity-based RNA-seq data analysis. The charts represent selected Ingenuity categories affected. (D) RNA-seq: Differential cardiac expression of endothelial genes at 3 dpcri in RO- or DMSO-treated fish (yellow: down-regulated, blue: up-regulated). (E,F) FISH against klf2a (E)
or *heg* (F) combined with IHC. Mosaic expression of both genes in wound and wound-adjacent GFP* endocardial cells (white arrowheads). Boxed areas magnified on the right. (G) qPCR analysis of *heg*, *klf2a* and *klf2b* expression in injured hearts (mean± s.d, t-test, *P*<0.05; **P**<0.01, ***P***<0.005). (H) ISH in embryos. RO treatment (regime is indicated) expanded ventricular expression of *heg* and *klf2a* (black arrowhead). Dotted lines delineate injury site (is). Scale bars: (E,F) 100µm; magnified views (E,F) 20µm.
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 (mean ± s.d., t-test *P<0.05; **P<0.01; ***P<0.005). (E) FISH combined with IHC, showing l-plastin+ cells (yellow arrowheads) in contact with GFP+ cells. l-plastin+ cell accumulations at the injury site locally coincides with high endocardial cell abundance (brackets). (F) Graph showing the percentage of mpeg1- or l-plastin–expressing inflammatory cells that contact with endocardial cells (green +) or not (blue -) at the injury site or remote region (indicated on the image by dotted lines). Inflammatory cells at the outer epicardial region were not considered. (G) tnfrsf9a FISH combined with IHC. RO-treated hearts (regime indicated) show more GFP+ cells expressing tnfrsf9a. (H) Scatter plots: percentage of tnfrsf9a+ endocardial cells (discontinuous line=mean ± s.d. t-test *P<0.05). (I) mpeg1 FISH combined with IHC. RO-treatment (regime in (G)) increased mpeg1+ macrophages abundance associated with the GFP+ endocardium. (J,K) Scatter plots: 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 (discontinuous line=mean ± s.d. t-test *P<0.05). (L) mf20 IHC and l-plastin ISH on consecutive heart sections. RO treatment increased numbers of l-plastin+ macrophages inside the mf20- injury site (is, red arrowheads). l-plastin+ macrophages in the outer region of the injury can be found 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: 100µm, magnified view 50µm.
Fig. 6: Notch signalling regulates cardiomyocyte proliferation.

(A) ET33mi-60A;myl7mRFP ventricle, volume rendering and optical section of a region of the inner injury border. Endocardial cells surround injury-adjacent cardiomyocytes (white 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. Heat-shock regime indicated. Scatter plot (D) percentage of BrdU⁺ wound-adjacent cardiomyocytes (mef2⁺) (arrowheads between dotted lines) (discontinuous line=mean ± s.d, t-test,**P<0.01). (E,F) Mef2 IHC: higher density of wound-adjacent cardiomyocytes (between the dotted lines) in Tg(UAS:NICD) than in control hearts. Quantification in (F) (mean ± s.d, t-test, *P<0.05). (G) ISH: higher numbers of nkh2.5⁺-wound-adjacent cardiomyocytes (between dotted lines) in Tg(UAS:NICD) hearts. (H) RNA-seq. Differential myocardial genes expression in RO-treated hearts, 3 dpci. (I) ISH showing myc-b expression in injury-adjacent cardiomyocytes (arrowheads) in DMSO- but not in RO-treated hearts (asterisks). (J) qPCR levels of fosab in the injured heart (mean±s.d, t-test, *P<0.05). (K) mf20 IHC and mylk3 ISH on heart sections. RO treatment decreased the numbers mylk3⁺-cardiomyocytes adjacent to the injury site (is, arrowheads). (L) Scatter plot: percentage of mylk3⁺ wound-adjacent (50 μm) cardiomyocytes (discontinuous line=mean ± s.d, t-test, **P<0.01). (M) qPCR levels of mylk3 and tcap in the injured heart (mean±s.d, t-test, ***P<0.001). Dotted lines delineate the injury site (is). Boxed areas are magnified in insets. Scale bars: 100μm; (C) and amplified views (C): 25μm
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 (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. 36 hpci: numerous wound and wound-adjacent GFP+ endocardial cells express serpine1. 3 and 7 dpci: serpine1-expressing wound-endocardial cells are less frequent. Boxed areas are magnified below. (D) Scatter plot: the percentage of serpine1+/GFP+ cells related to all wound and wound-adjacent (50 μm) GFP+ cells (red line=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) show more GFP+ cells expressing serpine1. (F) Scatter plots indicating the percentage of serpine1+ endocardial cells. (discontinuous line=mean ± s.d. t-test *P<0.05). (G) log fold change (FC) of Serpine1 showing increased expression by qPCR in RO-treated PAVEC, and by RNA-seq in RO-treated zebrafish hearts and in various murine models of endocardial Notch disruption. (H) IF indicating PCNA+/GFP+ cells after TP-treatment (regime is indicated). (I) Scatter plot: relative number of PCNA+/GFP+ cells related to all wound and wound-adjacent GFP+ cells (discontinuous line=mean±s.d, t-test, *P<0.05). (J) FISH combined with IHC: TP treatment increased cdh5-mRNA levels in GFP+ wound endocardium. (K) Scatter plot: relative red fluorescence intensity, comparing values of remote and wound endocardium (see Fig. 2F; discontinuous line=mean ± s.d, t-test, **P<0.01). (L) IF revealed more PCNA+/mef2+ wound-adjacent cardiomyocytes (arrowheads) after TP-treatment. (M) Scatter plot: Relative number of PCNA+/mef2+ cells related to all wound adjacent (100 μm) mef2+ cardiomyocytes (discontinuous line= mean ± s.d, t-test, **P<0.01). (N) AFOG stained hearts, treated with DMSO or TP (treatment regime is indicated). (O) Scatter plot indicating injury site size (discontinuous line=mean ± s.d). Dotted lines delineate injury site (is). Scale bars: (C,E,H,J,L) 100μm; (N) 200μm magnified views (C) 20μm.
Fig. 8: Endocardial dynamics after cryoinjury.

(A) Diagram depicting the tip of the adult zebrafish ventricle in uninjured situation (no ci), and at various time points after cryoinjury (24 hpci, 3, 9 dpci and 1 mpci). 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 dotted lines, and the endocardium colonizing the injury site in light green. (B) Injury endocardium (green) is characterized by differences in morphology (filopodia, cdh5, serpine1, Notch) and inflammation and cardiomyocyte proliferation in the adjacent tissue.
organization), behaviour (proliferation, migration) and gene expression (cdh5, serpine1) at different time points of regeneration. Alterations of endocardial signals (Notch, Serpine1) affect those characteristics (arrows, lines) and interfere with inflammation attenuation (blue) and cardiomyocyte proliferation (red).
Supplementary Information

Supplementary Material and Methods

Zebrafish transgenic lines

We used the following fish lines: WT AB strain, $ET(krt4:EGFP)^{sqet33-mi604} (ET33-mi604)$, $ET(krt4:EGFP)^{sqet33-1A} (ET33-1A)$ (Poon et al., 2010), $Tg(hsp70l:Gal4)^{kca4};Tg(UAS:myc-Notch1a-intra)^{kca3}$, [abbreviated here as $Tg(UAS:NICD)$] (Scheer et al., 2001), and $Tg(fli1a:GFP)y1$ (Lawson and Weinstein, 2002), $Tg(myl7:mRFP)$ (Rohr et al., 2008).

Treatments

Heat shocks to $Tg(UAS:NICD)$ fish were applied automatically as described (Munch et al., 2013). Regimes for heat shock applications of each experiments are indicated in the corresponding figure. For short-term experiments (1-3 dpci, 1-7 dpci) we applied 40 min heat shocks and for long-term experiments (1-33 dpci, 1-90 dpci) a 1-h heat shock. Control animals for these experiments were $Tg(hsp70l:Gal4)^{kca4}$ or $Tg(UAS:myc-Notch1a-intra)^{kca3}$ fish. BrdU (30 µl, 2.5 mg/ml in PBS) was administered intraperitoneally at 2 dpci (for endocardial proliferation analysis) or at 5 and 6 dpci (for myocardial proliferation analysis).

Histology

All antibodies used in our studies have been tested elsewhere. We used the following antibodies: GFP (1:100, Living Colors, rabbit, C#632592, and mouse, C#632381) (Gonzalez-Rosa et al., 2011), GFP (1:500, Aves, chicken, GFP-1010) (Zhang et al., 2013), BrdU (1:30, BD, ab6326) (Munch et al., 2013), Delta-4 (1:100, Santa Cruz Biotechnology, rabbit, sc-28915) (D’Amato et al., 2016), collagen type I (1: 100, DSHB, SP1.D8, mouse, C#SP1.D8, RRID:AB_528438) (Gonzalez-Rosa et al., 2011), myosin heavy chain (1:100,
MF20, DSHB, mouse, C#MF20RRID:AB_2147781) (Gonzalez-Rosa et al., 2011), ERG (1:200, Abcam, rabbit, ab110639) (Bednarek et al., 2015) and Mef-2 (1:100, Santa Cruz Biotechnology, rabbit, sc-313) (Bednarek et al., 2015) Phalloidin Conjugated to FITC (1:50, Sigma-Aldrich, P5282). For IHC against Delta-4 and collagen type-1 the signal was amplified with secondary antibodies coupled to horseradish peroxidase (1:100, Dako Cytomation, P0447-8) and tyramides coupled to Cy3 (1:100, TSA, Perkin Elmer, NEL744001KT) (Luxan et al., 2013). ISH was performed as described (Kanzler et al., 1998) using the following probes: atf3 (Chen et al., 2012), Ifng (Prince et al., 2001), hand2 (Yelon et al., 2000), nkd2.5 (Chen and Fishman, 1996), notch1b, notch2, notch3 (Westin and Lardelli, 1997), cdh5 (Larson et al., 2004), mmp9, nfatc1a, klf2a (Vermot et al., 2009) and l-plastin (Yoshinari et al., 2009). Primer sequences for new probes can be found in Table S7. For fluorescence ISH (FISH), we followed the same protocol (Kanzler et al., 1998), however we used a peroxidase-coupled antibody for DIG-labelled mRNA-probe detection. We developed the signal using tyramides coupled to Cy3 (TSA, Perkin Elmer, 1:200) and subsequently performed IHC after several washed with PBS. AFOG staining was as described previously (Kikuchi et al., 2011).

Imaging

Whole mount images were obtained with an Olympus DP71 camera fitted to a Leica stereomicroscope. For whole mount 3D imaging, hearts were fixed in 2% PFA overnight. Following 3 washes in PBS, hearts were incubated in CUBIC I (Susaki et al., 2014) at 37°C overnight. Using a Leica TCS SP-5 confocal microscope, we scanned 900 µm of whole hearts (Figs. 1G-J, 4D, S1E) taking z-stacks every 7 µm with a 10x objective or 200 µm with z-stacks every 3 µm for imaging with the 20x objective (Figs. 2A,2C,3C,4F,S5E). Images of ISH and immune-stained heart sections were taken with a DP71 camera fitted to an Olympus BX51 microscope. For confocal images of immune-stained heart sections, we used a Leica TCS SP-5 or Nikon A1-R confocal microscope.

Gene expression analysis

Power SYBR Green Master Mix (Applied Biosystems) was used for qPCR with the ABI PRISM 7900HT FAST Real-Time PCR System. Gene expression levels were calculated relative to elf1a or rpsm and then compared with gene activation in samples taken from non-injured hearts (wild-type gene expression analysis) from DMSO-treated samples (for RO treatment experiments) or from Tg(hsp70l:Gal4)kcnf or
Tg(UAS:myc-Notch1a-intra)kca3 heart samples (for Notch overactivation experiments). RNA-extraction and cDNA synthesis of PAVEC were conducted accordingly. Quantitative RT-PCR was performed as described before. Data shown represent the mean ± s.d. of three separate experiments. Gene expression levels were calculated relative to \textit{GADPH} and then compared with gene activation DMSO-treated samples.

**Quantification and statistical analysis**

Experiments were performed on 3-9 biological replicates to ensure adequate statistical power to detect specific effects. Detailed information on the sample size of each experiment can be found in Table S1 and Table S2.

For 3D volume rendering and volume quantification, we used IMARIS x64 software, with manual selection of the injured area (myl7:mRFP). A mask of this area was used to select the GFP+ endocardium within the injury site, and the volumes of both were calculated and compared (See Fig. 1K). Graphs represent values of individual hearts and means ± standard deviation (s.d.). Statistical significance was calculated with One-way ANOVA combined with the Newman-Keuls method for multiple comparisons. Filopodia like protrusions of the injury-induced endocardium (Figs. 2B,3 G) were quantified on randomly selected regions (513 µm x 513 µm x 10 µm) within the whole scanned region, 3-5 per heart. The graphs represent mean values of individual hearts and means ± s.d. Statistical significance was calculated with Student’s t-test.

The amount of fibrotic tissue on AFOG-stained heart sections, labelling fibrin and collagen deposition, was measured as follows. ImageJ/ Fiji (http://imagej.net/ImageJ) was used to determine the size of the injured area relative to the size of the ventricle on at least four sections taken at four different levels throughout the heart, so that the size of the injury site at different levels of the heart was represented. The mean value was then calculated. This allows estimation of the 3D volume of the injury site. For quantifications of immunofluorescent or ISH stainings, heart sections were selected randomly. It was assured that the injury site throughout the heart was represented by staining one slide containing sections of different levels of the heart (see Histology section). BrdU incorporation was estimated as the ratio of BrdU-labelled mef2+ cardiomyocytes to the total number of mef2+ cardiomyocytes flanking the injured area. We analysed 7-8 hearts and 4-8 sections per heart. Accordingly, endocardial cell proliferation and the percentage of endocardial cells expressing \textit{serpine1} were estimated using 4-6 hearts per time point and condition and analysing 4-6 sections per heart. For macrophage quantification, only \textit{l-plastin}- or \textit{mpeg1}-expressing cells
within the injury site but not in the outer epicardial region were considered. To estimate cardiomyocyte density, the number of mef2^+ cardiomyocytes adjacent to the injury site was divided by their area. Graphs represent values of individual hearts and means ± standard deviation. We expected normal distributions of the parameters analysed in our experiments, with similar variances in the experimental groups. Statistical significance was calculated with the two-tailed Student’s t-test or with One-way ANOVA combined with the Newman-Keuls method for multiple comparisons.
Figure S1_Münch et al
Fig. S1: Activated endocardium in the injury site

(A) IHC against ERG, GFP and mf20 on sections of ET33-mi60a transgenic hearts (7 dpci), showing GFP+ endocardial/endothelial cells within and lining the injury site (is) (white arrowheads). The boxed area is magnified in the right hand panel. (B) ISH against nfatc1a at 7 dpci, showing strong expression within the injury site (is). (C) IHC against GFP on sections of Tg(fli1a:GFP) transgenic hearts (3 dpci) showing endocardial cells at the injury site. The boxed area is magnified in the right hand panel. (D) Scatter plot showing the relative volume occupied by GFP+ cells in a selected region of the remote region and the injury site at 24 hpci. Values of endocardial volume at the injury site are also represented in Figure 1L (black line = mean ± s.d, t-test, ***P<0.005). (E) Volume rendering of injured ventricles of ET33mi-60A; myl7mRFP hearts, with endocardium labelled green and myocardium magenta, at indicated time points. Volume rendering movies are available in the expanded view section. (F) Scatter plot showing the evolution of injury-site volume after cryoinjury in ET33-mi60a; myl7:mRFP transgenic hearts, quantified with IMARIS. (G-I) IHC against GFP and PCNA on sections of ET33-mi60a transgenic hearts (no ci, 3 dpci, 5 dpci), showing almost any PCNA-expressing endocardial cell in the non-injured heart but high abundance of proliferating cells within the injury site (is) at 3 and 5 dpci (white arrowheads). The boxed area is magnified in the right hand panel. The dotted lines demarcate the injured tissue. Scale bars: (A) 50 µm; (B, E) 200 µm (C, G, H, I) 100 µm; magnified views: (A) 5 µm, (B) 100 µm, (C, G, I) 20 µm.
Fig. S2 The injury endocardium exhibits filopodia-like protrusions and high levels of cdh5

(A) Vibratome section of ET33-mi60A heart, immune-stained for GFP and with phalloidin (7 dpci). Filopodia-like protrusions of wound endocardial cells show phalloidin staining (A’, A’’, arrowheads) (B) cdh5 FISH combined with IF showing low levels of cdh5 levels (red) in GFP+ endocardial cells allover the ventricle. (C) ISH against cdh5 at 7 dpci, showing strong expression within the injury site (is, arrow) and weak expression in the remote region (arrowheads). Boxed areas are magnified below or on the right. (D, E) IHC against GFP and col1 on sections of ET33-mi60a transgenic hearts, showing low col1 signal at 3 dpci (B) and similar distribution of endocardial cells and col1 at the inner injury border (dotted line) at 7dpci (C, white arrowheads). Scale bars: (B, C, D) 200 µm; magnified views: (B, C) 50 µm.
Figure S3_Münch et al.
Fig. S3: Genes encoding Notch signalling elements are expressed in endocardial cells upon cryoinjury (A) IHC of dll4 and mf20, showing expression within the injury site (is) and surrounding adjacent cardiomyocytes. The boxed area is magnified in the panel below. (B) ISH for notch1b, showing low notch1b expression in the injury site and the remote region at 36 hpci. The dotted line demarcates the injury site (is). (C, D) ISH of lfg, notch2 and notch3 in regenerating hearts, showing expression adjacent to and within the injury site (is; 3 dpci, 7 dpci). Boxed areas are shown at higher magnification in the lower row. (E) IHC against dll4 and GFP on sections of Tg(fli1a:GFP) transgenic hearts (7 dpci), showing endocardial expression of dll4 (white arrowheads). The dotted line demarcates the injury site (is). (F) FISH against notch1b combined with IHC against GFP and mf20 on sections of Tg(fli1a:GFP) transgenic hearts (7 dpci) showing notch1b transcripts in endocardial cells (white arrowheads). The boxed area is magnified in the right hand panels. The dotted line demarcates the injury site (is). (Scale bars: (A, B) 200 µm; (C, D, E, F) 100 µm; magnified views: (A, B) 50 µm, (D, E, F) 20 µm.


**Figure S4_Münch et al.**
**Fig. S4: Notch signalling modulation impairs heart regeneration**

(A) Relative gene expression (qPCR) of the Notch target genes *dll4, her15*, and *her4*, showing reduced expression after RO treatment (indicated on top). * P<0.05; ** P<0.01; *** P<0.005. (B) Representative AFOG-stained sections from three levels of the hearts of fish treated with DMSO or RO for 30 dpci (treatment regime is indicated on top). Cardiac muscle: brown; fibrin: pink/orange; collagen: blue. (C) Scatter plot showing the amount of fibrotic tissue relative to ventricle size in hearts from fish treated with DMSO or RO (discontinuous line = mean ± s.d, t-test *P<0.05) (D) qPCR showing higher expression of *her4* and *her15* in injured hearts of transgenic *Tg(UAS:NICD)* fish compared than in the control at 3 dpci (heat shock regime is indicated on top (mean ± s.d, t-test, ***P<0.005). (E) AFOG-stained sections taken at 3 anatomical levels of WT and *Tg(UAS:NICD)* hearts at 33 dpci, showing similar regenerative capacity (heat shock regime is indicated on top). (F) Quantification of the progress of regeneration at 33 dpci (discontinuous line = mean± s.d, t-test, not significant). (G) AFOG-stained sections taken at 3 anatomical levels of WT and *Tg(UAS:NICD)* hearts at 90 dpci, showing failed regeneration (heat shock regime is indicated on top). (H) Quantification of the progress of regeneration at 90 dpci (discontinuous line = mean, t-test *P<0.05). Scale bars: 100 µm.
Fig. S5: Notch signalling modulation does not affect aldh1a2 expression or proliferation

(A, B) ISH for aldh1a2 on sections of hearts treated with DMSO or RO or from heat-shocked Tg(UAS:NICD) and control fish, showing no differences of expression. RO-treatment and heat shock regimes are indicated on top. (C) IHC against BrdU and GFP on heart sections of ET33-1a transgenic fish (3 dpci), showing BrdU incorporation by GFP+ endocardial cells adjacent to the injury site (is). (D) Quantification of BrdU+/GFP+ cell ratio in hearts of fish treated with DMSO or RO, indicating no difference in endocardial cell proliferation (mean ± s.d, t-test, not significant). (E) IHC against BrdU and GFP on heart sections of ET33-1a transgenic fish alone or crossed with Tg(UAS:NICD) at 3 dpci, showing BrdU incorporation by GFP+ endocardial cells adjacent to the injury site (is). (F) BrdU+/GFP+ cell ratio in hearts of ET33-1a and Tg(UAS:NICD); ET33-1a transgenic fish, indicating no difference in endocardial cell proliferation (mean ± s.d, t-test, not significant). (G) IHC for GFP and mf20 on sections of hearts treated with DMSO or RO or from heat-shocked Tg(UAS:NICD), showing no differences of expression. Boxed areas are magnified on the right. Hearts were treated for 3 days with RO, DMSO or heat shocks. Scale bars: (A) 100 µm
Figure S6_Münch et al. (2019) Development 146: doi:10.1242/dev.1443362: Supplementary information

Panel A: Image showing the effects of DMSO and RO on ET33-mi60a (GFP) over 5 dpci.

Panel B: Comparison of DMSO and RO treatments on CDH5 expression in ET33-mi60A at 7 dpci.

Panel C: Morphological changes in control and Tg(UAS:NICD) groups under different heat shock treatments.

Panel D: Graph showing the relative fluorescence intensity of CDH5 expression by GFP+ cells at the injury site for DMSO and RO.

Panel E: Graph illustrating CDH5 expression by GFP+ cells at the injury site for control and Tg(UAS:NICD) groups.
Fig. S6 Notch signalling modulation does no interfere with endocardial cdh5-expression

(A) Volume rendering of part of the injured region (513 µm x 513 µm x 10 µm) from ET33mi-60A; myl7mRFP hearts after treatment with RO or DMSO and from ET33mi-60A; myl7mRFP, Tg(UAS:NICD) and control hearts. Comparable 3D-images were used for quantification of filopodia-like protrusions. The graph of quantified filopodia is shown in Figure 3I. (B, C) FISH against cdh5 combined with IHC against GFP and mf20 on sections of ET33-mi60a transgenic hearts after treatment with DMOS or RO (7dpci) and of ET33mi-60A; Tg(UAS:NICD) and control hearts (3dpci). The treatment regime is indicated on top. GFP+ endocardial/endothelial cells at the injury site present similar levels of cdh5 expression in injured hearts after treatment with RO or DMSO and in ET33mi-60A; Tg(UAS:NICD) or control hearts. (D, E) Scatter plot showing relative red fluorescence intensity, comparing values of the endocardium of the remote region to the injury endocardium (see Figure 2D), in injured hearts (dotted line = mean± s.d, t-test, not significant). (Scale bars: (A) 50 µm. (B, C) 100 µm.
Figure S7_Münch et al.
Fig. S7: Notch signalling affects endocardial/endothelial gene expression

(A) Genes assigned to the three categories in (Figure 4I). Genes are ordered according to their level of differential expression (up-regulated upon RO-treatment, yellow; down-regulated, blue). The colour of small bars indicates the assigned Ingenuity categories (presented in the chart in Figure 4I). (B, C) ISH, showing egr1- and id1- expression at the inner injury border (yellow arrowheads). The boxed area is magnified in inserts. (D) qPCR analysis showing lower heg mRNA levels in Tg(UAS:NICD) transgenic injured ventricles than in control. (mean± s.d., t-test, *P<0.05). Dotted lines delineate the injury site (is). Scale bar: 100 µm.
Fig. S8: Notch inhibition affects ECM remodelling gene expression and inflammatory processes

(A) ISH for \textit{ctssb.1} and \textit{mmp9}, revealing high expression at the inner injury border at 36 hpci. (B) ISH against \textit{tnfrsf9a} on sections from an injured ventricle at 3 dpci, showing gene expression in cells with endocardial morphology within the injury site. (C) \textit{mpeg1}- FISH combined with IHC, showing \textit{mpeg}^+ cells (yellow arrowheads) in contact with GFP^+ cells. \textit{mpeg}^+ cell accumulations at the injury site (is) locally coincides with high endocardial cell abundance (brackets). (D) ISH on consecutive sections for \textit{l-plastin} showing high \textit{l-plastin} expression in the injury site at 36 hpci. (E) ISH against \textit{mmp9} (3 dpci), showing high numbers of positive cells within the injury site (is) of RO-treated hearts. (F) ISH against \textit{ctssb.1} (7 dpci), showing high numbers of positive cells within the injury site (is) of RO-treated hearts. (G) qPCR analysis showing increased \textit{ctssb.1} mRNA levels in injured ventricles at 7 dpci after RO-treatment. (mean± s.d., t-test, *P<0.05. (H) qPCR analysis of \textit{tnfrsf9a} expression in ventricles at 7 dpci after treatment with RO or DMSO mean± s.d., t-test, *P<0.05). (I) FISH against \textit{l-plastin} combined with IHC against GFP and \textit{mf20} on sections of \textit{ET33-mi60a} transgenic hearts (7 dpci) after RO- or DMSO-treatment. RO-treatment results in an increased abundance of \textit{l-plastin}^+ macrophages associated to the endocardium. Dotted lines delineate the injury site (is). Scale bars: 100 µm.
Fig. S9: Notch inhibition decreases cardiomyocyte proliferation

(A) IHC against BrdU and mef2 on heart sections (7 dpci), showing lower BrdU incorporation in mef2+ cells (white arrowheads) adjacent to the injury (between the dashed lines) after treatment with RO than after treatment with DMSO. (B) Scatter plot showing quantification of BrdU+ mef2+ cells in DMSO- and RO-treated hearts (discontinuous line = mean ± s.d., t-test, *P<0.05). (C) FISH against hand2 combined with IHC for mf20 showing, hand2-expression in cardiomyocytes. (D) ISH against hand2 at 7 dpci, showing higher numbers of injury-adjacent cardiomyocytes expressing these genes (between the dotted lines) in Tg(UAS:NICD) hearts than in control hearts. Dotted lines delineate the injury site (is). Scale bars: 100 μm; amplified views: 25 μm.

Figure S9_Münch et al.
Figure S10_Münch et al.
Fig. S10 Serpine1 is upregulated in the injury endocardium and its inhibition does not interfere with blood clot or collagen deposition. 

(A, B) ISH against serpine1, showing no expression in the uninjured heart, moderate levels at 7 dpci but almost no expression at 14 dpci. (C) FISH against serpine1 combined with IHC against GFP and mf20 on sections of ET33-mi60a transgenic hearts at 24 hpci. Serpine1 is strongly expressed in GFP+ endocardial cells adjacent and within the injury site but not in remote regions at 24 hpci. The boxed area is magnified on the right hand panel. (D) qPCR analysis showing higher serpine1 mRNA levels in injured ventricles at 7 dpci in RO-treated fish than in DMSO-treated fish (mean± s.d., t-test, *P<0.05). (E) AFOG stained TP- or DMSO treated hearts indicating similar fibrin deposition at the injury site (is) in both groups at 7dpci. (F) IHC for collagen 1 (coll1) and mef2 showing similar amount of collagen at the injury site upon TP or DMSO-treatment. (G) Scatter plot showing the area covered by collagen relative to the injury site area in hearts treated with TP or DMSO. (dotted line = mean± s.d, t-test, not significant). (H) ISH in hearts treated with TP or DMSO (regime is indicated on top) showing similar expression of aldhl2 in the injury endocardium. Dotted lines delineate the injury site (is). Scale bar: 100 µm; amplified view (C) 20 µm.

Supplemental Tables

Table S1: Sample numbers Figure 1-7 (Excell file attached)

Click here to Download Table S1
Table S2: Sample numbers Figure S1- S10 (Excell file attached)

Click here to Download Table S2

Table S3: Newman-Keuls Multiple Comparison Test for relative GFP\(^+\) cell volume at indicated time points (Figure 1L)

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Table S4: Newman-Keuls Multiple Comparison Test for relative GFP⁺ cell proliferation at indicated time points (Figure 1N)

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Table S5: Newman-Keuls Multiple Comparison Test for relative serpine1⁺/ GFP⁺ cells at indicated time points (Figure 7C)

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Table S6: RNA-seq data of RO- vs. DMSO-treated wild type regenerating hearts 3 dpci (Excell file attached)

Click here to Download Table S6
### Table S7: Primers used for RNA probe generation

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### Table S8: qPCR primers (*Danio rerio*)

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<td>CTTCATACATTGCTCTGAC</td>
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<tr>
<td>mylk3</td>
<td>AAGTTGAGTGCGAAGTCTGCTG</td>
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<td>rpm</td>
<td>GATTGGCGACGTCAAGAAC</td>
<td>CCAATCACAAGTTCTGCTG</td>
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<td>serpine1</td>
<td>GTCTATTCTAAAAGTTTCCAT</td>
<td>CTGAAAATGCTTCAAGGCC</td>
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<tr>
<td>sgpl1</td>
<td>CATTATATTAAAGTAAGAGACAA</td>
<td>CATCGATGCTCGAGAATG</td>
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<tr>
<td>tcpa</td>
<td>GGGACGAATCAATGCTTCAGG</td>
<td>CGTCACATAAAGTCCTTGACTATTT</td>
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<tr>
<td>tnfrsf9a</td>
<td>TACGGAAAACCTCAAGTCCTCA</td>
<td>TTTGAGTATTCCTACCCCCA</td>
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### Table S9: qPCR primers (*Sus scrofa*)

<table>
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<th>Gene</th>
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<tr>
<td>SERPINE1</td>
<td>TACACTGAGTTTCCCAACC</td>
<td>AATGAACATGCTCAGAGTG</td>
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<tr>
<td>GADPH</td>
<td>ACACCTACTTTCTACCTTCC</td>
<td>CAAATCCATTGTGCTCAGAG</td>
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</table>
Supplementary References


Supplementary Movies

**Movie S1:** IMARIS volume rendering of a whole mount *ET33-mi60a* (green); *Tg(myl7:mdsred)* (magenta) cryoinjured transgenic heart at 24 hpci.
**Movie S2:** IMARIS volume rendering of a whole mount *ET33-mi60a* (green); *Tg(myl7:mdsred)* (magenta) cryoinjured transgenic heart at 36 hpci.
**Movie S3:** IMARIS volume rendering of a whole mount *ET33-mi60a* (green); *Tg(myl7:mdsred)* (magenta) cryoinjured transgenic heart at 3 dpci.
**Movie S4:** IMARIS volume rendering of a whole mount *ET33-mi60a* (green); *Tg(myl7:mdsred)* (magenta) cryoinjured transgenic heart at 5 dpci.
Movie S5: IMARIS volume rendering of a whole mount ET33-mi60a (green); Tg(myl7:mdsred)(magenta) cryoinjured transgenic heart at 7 dpci.
**Movie S6**: IMARIS volume rendering of a whole mount *ET33-mi60a* (green); *Tg(myl7:mdsred)* (magenta) cryoinjured transgenic heart at 9 dpci.
**Movie S7**: IMARIS volume rendering of a whole mount *ET33-mi60a* (green); *Tg(myl7:mdsred)* (magenta) cryoinjured transgenic heart at 1 mpci.
**Movie S8:** IMARIS volume rendering of a whole mount *ET33-mi60a* (green); *Tg(myl7:mdsred)* (magenta) cryoinjured transgenic heart at 3 mpci.
**Movie S9:** Confocal 3D time-lapse movie of endocardial cells of a selected area within the injury site in *ET33-mi60a* (green) cryoinjured transgenic heart at around 56 hpci. Individual cells migrate and change their collocation. Still images can be found in Figures 2F.
**Movie S10:** Confocal 3D time-lapse movie of endocardial cells of a selected area within the injury site in *ET33-mi60a* (green) cryoinjured transgenic heart at around 56 hpci. Individual cells migrate and send out filopodia-like protrusions. Still images can be found in Figures 2G.