Tbx20 Induction Promotes Zebrafish Heart Regeneration by Inducing Cardiomyocyte Dedifferentiation and Endocardial Expansion

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Heart regeneration requires replenishment of lost cardiomyocytes (CMs) and cells of the endocardial lining. However, the signaling regulation and transcriptional control of myocardial dedifferentiation and endocardial activation are incompletely understood during cardiac regeneration. Here, we report that T-Box Transcription Factor 20 (Tbx20) is induced rapidly in the myocardial wound edge in response to various sources of cardiac damages in zebrafish. Inducing Tbx20 specifically in the adult myocardium promotes injury-induced CM proliferation through CM dedifferentiation, leading to loss of CM cellular contacts and re-expression of cardiac embryonic or fetal gene programs. Unexpectedly, we identify that myocardial Tbx20 induction activates the endocardium at the injury site with enhanced endocardial cell extension and proliferation, where it induces the endocardial Bone morphogenetic protein 6 (Bmp6) signaling. Pharmacologically inactivating endocardial Bmp6 signaling reduces expression of its targets, Id1 and Id2b, attenuating the increased endocardial regeneration in tbx20-overexpressing hearts. Altogether, our study demonstrates that Tbx20 induction promotes adult heart regeneration by inducing cardiomyocyte dedifferentiation as well as non-cell-autonomously enhancing endocardial cell regeneration.

Keywords: Tbx20, heart regeneration, cardiomyocyte dedifferentiation, endocardium, BMP signaling, zebrafish

INTRODUCTION

Adult mammalian hearts have limited regeneration capacity in response to cardiac damage. Injured hearts lose cardiac muscle and replace with fibrotic scar tissue, ultimately leading to arrhythmia and heart dysfunction (Xin et al., 2013; Tzahor and Poss, 2017). However, zebrafish and neonatal murine hearts exhibit increased regeneration capacity after various insults (Kikuchi and Poss, 2012; Li et al., 2015). Heart regeneration occurs through diverse mechanisms including activation of epicardial, myocardial, or endocardial tissues (Kikuchi and Poss, 2012). Although various mitogenic factors and signaling pathways have been identified to enhance heart regeneration (Kim et al., 2010; Heallen et al., 2013; D’Uva et al., 2015; Gemberling et al., 2015; Wang et al., 2015; Liu and Zhong, 2017; Mohamed et al., 2018; Singh et al., 2018), the signaling and transcriptional control of heart
regeneration by myocardial dedifferentiation and endocardial activation are largely unknown. Understanding injury-induced heart regeneration will provide therapeutic strategies to empower regenerative capacity to the diseased human heart.

T-box transcription factor 20 (Tbx20), a key cardiac transcriptional factor, is required for heart development and homeostasis (Greulich et al., 2011). In humans, the heterozygous nonsense or missense mutations of TBX20 are associated with diverse cardiac pathologies such as dilated cardiomyopathy, atrial septal defect, cardiac valve defects and tetralogy of Fallot (Kirk et al., 2007; Huang et al., 2017). Both tbx20-null zebrafish and mice are embryonic lethal and exhibit deleterious cardiovascular malformations with defects of CM proliferation and heart tube looping (Cai et al., 2013). Myocardial-specific Tbx20 ablation in adult mice leads to thinner ventricle wall and cardiomyopathy accompanied with arrhythmias (Shen et al., 2011; Sakabe et al., 2012). Conversely, inducible tbx20 overexpression in embryonic cardiomyocytes leads to increased CM proliferation and thickening of the myocardium in adult hearts (Chakraborty et al., 2013). Myocardial-specific tbx20 overexpression in zebrafish embryos also results in enlarged heart with both increased cardiac progenitor cell formation and the proliferation of differentiated CMs (Lu et al., 2017). Recent studies report that Tbx20 overexpression in adult mouse hearts after myocardial infarction increases CM proliferation in the injury border zone and improves cardiac function recovery (Xiang et al., 2016). Despite that previous studies demonstrate essential roles of Tbx20 transcription factor during heart development, injury repair and congenital heart disease, it is currently not understood whether and how endocardial cells respond to Tbx20 induction in the myocardium after cardiac damage, and the extent to which Tbx20 regulates CM dedifferentiation and proliferation during heart regeneration.

The heart develops through generation of CMs and tightly associated endocardial cells (Staudt and Stainier, 2012). Endocardial cells represent a subset of a larger endothelial cell pool (Harris and Black, 2010). During development, the endocardium is organized into arterial and venous subpopulations with comparable gene expression profiles (Staudt and Stainier, 2012). Endocardial differentiation and growth occur without an accretion of external cells, in a manner independent of vascular endothelial growth factor (VEGF) signaling (Dietrich et al., 2014). After cardiac injury, activated endocardium coincides with changes in cell morphology and gene expression (Kikuchi et al., 2011b). The activation and maturation of the endocardium require Notch signaling, which supports myocardial regeneration (Munch et al., 2017; Zhao et al., 2019). However, no signaling factor or molecular program has been shown to be essential for endocardial cell proliferation during zebrafish heart regeneration.

In this report, we have determined Bmp6 signaling as an early endocardial injury-response to myocardial Tbx20 induction, which promotes endocardial cell regeneration, a previously unrecognized mechanism. Tbx20 is also sufficient to induce injury-induced CM dedifferentiation, thus stimulate CM proliferation. Overall, our findings reveal novel roles and distinct mechanisms of myocardial Tbx20-mediated network in governing cardiac muscle production and endocardial cell proliferation during heart regeneration.

RESULTS

Tbx20 Is Induced in the Regenerating Zebrafish Heart Following Injury

To define the spatiotemporal expression pattern of T-Box Transcription Factor 20 (Tbx20) during adult heart regeneration, we first evaluated tbx20 expression during the window of zebrafish cardiac regeneration by in situ hybridization (ISH). In the uninjured adult heart, we detected faint tbx20 expression in the ventricles and atria (Figures 1A,B). By 3 dpa, tbx20 was strongly upregulated in the ventricular and atrial myocardium (Figures 1C,D). Specifically, more tbx20+ cells were accumulated in the injury border zone of the ventricle compared to the remote (uninjured) zone at 3 dpa and 7 dpa (Figures 1E-H). Furthermore, qPCR analyses validated the marked upregulation of tbx20 at the border zone of injured ventricles and the atrium at 1 dpa, 3 dpa and 7 dpa (Supplementary Figure S1A). The induction of tbx20 at the injury border zone and the atrial epicardium was also detectable at 5 days post cardiac cryoinjury (dpci), an independent injury approach (Supplementary Figures S1B–E). However, tbx20 induction at the atrial epicardium was hardly detectable at uninjured hearts (Figure 1B and Supplementary Figure S1D). As controls, tbx20 transcripts were not detectable in uninjured and injured hearts from 1 dpa to 7 dpa using tbx20 sense probes (Supplementary Figures S1F–M), confirming the specificity of tbx20 upregulation in the ventricle and the atrial epicardium after cardiac damage.

To unambiguously define the myocardial expression of tbx20, we generated a Tg(tbx20: moxGFP) transgenic zebrafish expressing monomeric oxidizing GFP (moxGFP) driven by a tbx20 promoter-based upstream region. We were able to detect CMs that expressed weak moxGFP in the ventricle of uninjured hearts after co-immunostaining for GFP and α-actinin (Z-disk marker) (Figure 1I and Supplementary Figure S1N). In the injured adult heart, extensive and stronger moxGFP signals were observed in the ventricles, as well as at the injury border zone by 7 dpa and 5 dpci, respectively, (Figure 1I and Supplementary Figure S1O). While moxGFP signals were hardly detectable in the epicardium in uninjured hearts (Supplementary Figures S1P, S2A), strong GFP signals colocalized with epicardial marker pan-cytokeratin (PKC) were detectable in the atrial epicardium of injured hearts (Supplementary Figures S1Q, S2B). These
results indicate the tbx20 expression is induced in the ventricular myocardium and the atrial epicardium after cardiac injury, consistent with ISH analyses.

Inducible tbx20 Overexpression in the Adult Myocardium Promotes CM Proliferation and Heart Regeneration

To investigate the biological functions of tbx20 during heart regeneration, we generated transgenic zebrafish, Tg(cmlc2:TetON-3G; cryaa:mCherry) carrying a CM-specific TetON-3G and a lens-specific marker mCherry, as well as Tg(TRE3G:tbx20-E2A-mCherry; cryaa:EGFP) containing a doxycycline (DOX)-inducible tbx20-E2A-mCherry and a lens-specific marker EGFP, hereafter referred to as TRE3G:tbx20 (Figure 2A). By crossing this two lines, we established a double transgenic zebrafish strain, Tg(cmlc2:TetON-3G, cryaa:mCherry; TER3G:tbx20-E2A-mCherry, cryaa:EGFP), referred to as TRE3G:tbx20CMOE, permitting conditional expression of tbx20 specifically in CMs induced by DOX (Figure 2A). After daily administration of adult zebrafish with DOX (50 mg/L) from the day before cardiac resection to the day of examination (Figure 2B), TRE3G:tbx20CMOE fish displayed mCherry fluorescence only in myocardium and no ectopic mCherry expression elsewhere (Supplementary Figures S3A–F). Lack of leaking expression of tbx20-E2A-mCherry in TRE3G:tbx20CMOE fish was further validated by our ISH analyses showing strong tbx20 induction in myocardium in DOX-treated TRE3G:tbx20CMOE fish (Figure 2C and Supplementary Figure S3I), but only weak signal in myocardium in TRE3G:tbx20CMOE fish without DOX treatment, which is comparable to that in DOX-treated TRE3G:tbx20 fish (Supplementary Figures S3G,H).

During zebrafish heart regeneration, newly formed CMs primarily come from the proliferation of pre-existing CMs (Jopling et al., 2010; Kikuchi and Poss, 2012). To assess whether tbx20 promotes CM proliferation during heart regeneration, TRE3G:tbx20CMOE and control TRE3G:tbx20 zebrafish were treated with DOX (Figure 2B), and subjected to ventricular apex resection next day. Heart sections at 7 dpa, a time point when CM proliferation peaks, (Wills et al., 2008; Wang et al., 2011), were immunostained with antibodies against proliferation marker PCNA and the CM nuclear marker Mef2. The results revealed that CM proliferation in injured TRE3G:tbx20CMOE hearts markedly increased by ∼61%, compared with that in control hearts (25.1 ± 1.4% versus 15.5 ± 1.3%) (Figures 2D,E). By contrast, tbx20 overexpression had no discernible effects on CM proliferation in uninjured adult hearts after 7 days of DOX treatment in which PCNA+Mef2+ CMs were not detectable in tbx20 overexpressing hearts.
FIGURE 2 | Myocardial tbx20 overexpression in adult hearts promotes CM proliferation and reduces fibrotic scars. (A) Schematic diagram of transgenes of the control line Tg(TRE3G:tbx20) and the inducible myocardial tbx20 overexpression line Tg(TRE3G:tbx20CMOE) with DOX treatment. (B) Experimental strategy employed to induce myocardial tbx20 overexpression during heart regeneration. (C) Representative images of ISH with tbx20-E2A-mCherry probe on 7 dpa heart sections from DOX treated Tg(TRE3G:tbx20) and Tg(TRE3G:tbx20CMOE) fish. Scale bar: 100 µm. (D) Upper panels: Representative confocal fluorescence images of heart sections from 7 dpa zebrafish immunostained for PCNA (green) and Mef2 (red). Insets showing high-magnification images of proliferating cardiomyocytes, arrowheads indicate PCNA+Mef2+ cells. Lower panels: Representative images of heart sections from 21 dpa fish stained with AFOG, muscle stained brown, collagen is blue and fibrin is red. The genotypes of fish were indicated above the images. Scale bar: 100 µm. (E) Quantification of CM proliferation at border zone and injury site on 7 dpa heart sections (n = 7 in each group). (F) Quantification of scar area at 21 dpa (n = 9 in each group). The fish in (C–F) were treated with DOX as indicated in (B). Each value in (E,F) represents mean ± SEM, **p < 0.01, ***p < 0.001.

and control hearts (Supplementary Figure S4). Collectively, these results demonstrate that myocardial overexpressing tbx20 stimulates injury-induced CM proliferation.

Given that increased tbx20 expression is able to enhance CM proliferation at injured sites at an early regeneration stage, we reasoned that long-term high transcription level of tbx20 reduced fibrotic scars and hastened wound healing. To test this possibility, heart regeneration of DOX-treated TRE3G:tbx20CMOE and TRE3G:tbx20 fish at 21 dpa were evaluated using Acid Fuchsin-Orange G (AFOG) staining of heart cryosections. Our study showed that hearts from TRE3G:tbx20CMOE fish were evidenced by contiguous cardiac muscle formation and reduced fibrotic
scars at the injured ventricle apex, whereas hearts from TRE3G:tbx20 fish remained variable of prominent scar tissues (Figures 2D,F).  

**Myocardial-Specific tbx20 Overexpression Enhances Injury-Induced CM Dedifferentiation**  
Cardiomyocyte dedifferentiation, a transition from mature state to immature state, is a mechanism to ensure subsequent CM proliferation that naturally occurs in response to cardiac injury in neonatal mouse and adult zebrafish (Jopling et al., 2010; Porrello et al., 2011; D’Uva et al., 2015). This process is characterized by disassembly of sarcomeric structure, loss of cell–cell adhesion and re-expression of cardiac embryonic, fetal or progenitor genes (Kubin et al., 2011; D’Uva et al., 2015). Since increased tbx20 expression enhances CM proliferation and cardiac regeneration (Figure 2), we asked whether tbx20 cardiac overexpression was capable of boosting CM dedifferentiation following cardiac injury. To test this idea, we determined morphological and molecular changes of CMs in DOX-treated control and TRE3G:tbx20CMOE hearts. We observed a marked reduction of a cell tight junction marker ZO-1 in α-actinin-marked CMs in the injury border zone in TRE3G:tbx20CMOE hearts compared to that in control hearts (Figures 3A–B,I). N-cadherin is a marker of cell–cell adhesion junction localized in the intercalated disks between neighboring CMs (Luo and Radice, 2003; Vite and Radice, 2014; Li et al., 2019). In injured hearts overexpressing tbx20 in CMs, we observed a reduction of N-cadherin in the border zone of the injured myocardium, indicating a loss of cell-cell contact between CMs (Figures 3C,D’ and Supplementary Figure S5). Notably, we observed CMs marked by cardiac troponin T (cTnT) in the wound edge exhibited greater extent of sarcomere disassembly that were devoid of myofibril striations in tbx20-overexpressing hearts than that in control hearts (Figures 3C–E).  

Concomitantly, q-PCR analyses showed that expression of CM dedifferentiation markers, including cardiac fetal markers, *alpha-smooth muscle actin (α-SMA)*, natriuretic peptide a (nppa) and natriuretic peptide b (nppb) (Dirkx et al., 2013; Man et al., 2018), as well as a progenitor cell marker runx1 (Poling et al., 2012; D’Uva et al., 2015; Wang et al., 2017) were significantly increased in DOX-treated TRE3G:tbx20CMOE hearts compared with control hearts (Figure 3F). We next assessed expression patterns of α-SMA (Figures 3G–I,L and Supplementary Figure S6) and Runx1 in injured hearts (Figures 3J,K,M) immunostained with CM marker α-actinin. We observed that α-SMA was markedly induced in the α-actinin-marked myocardial compact layer (Figure 3H and Supplementary Figures S6A, A-I-A-II”) and, some of α-SMA signal was detectable in the trabecular layer adjacent to the injury site (Figure 3H and Supplementary Figures S6A, A-II-A-II”) in DOX-treated TRE3G:tbx20CMOE injured hearts. In contrast, α-SMA re-expression was restricted to a small injury region in control hearts (Figure 3G). Furthermore, Runx1 was upregulated in more regenerating CMs in TRE3G:tbx20CMOE injured hearts than that in control wounded hearts (Figures 3J,K,M). Similarly, ISH analyses revealed the increased expression of α-SMA and nppb, as well as cardiac progenitor markers gata4, gata5 and hand2 in injured tbx20-overexpressing hearts in comparison to control hearts (Figure 3I and Supplementary Figures S7A–H). qPCR analyses validated the upregulation of gata4, gata5 and hand2 in TRE3G:tbx20CMOE hearts following ventricular apex resection (Supplementary Figure S7I). Taken together, our data demonstrated that enhanced cardiac tbx20 expression favors induction of cardiac fetal and progenitor gene programs, resulting in CM dedifferentiation and proliferation during regeneration.  

**Tbx20 Mediates Various Genetic Circuits Regulating Zebrafish Heart Regeneration**  
To decipher the molecular basis in response to cardiac injury with enhanced cardiac tbx20 expression, we analyzed gene expression profiles of the apical halves of the resected ventricles from DOX-treated TRE3G:tbx20CMOE and TRE3G:tbx20 zebrafish at 7 dpa (Figure 4A). We found that 1880 genes were differentially expressed in TRE3G:tbx20CMOE hearts (Log FC > 0.5, p-value < 0.05) compared to TRE3G:tbx20 hearts, in which 747 of them were upregulated and 1133 were downregulated (Supplementary Table S1).  

GO analyses of the upregulated gene subset revealed enhanced expression of functional categories including “cell growth”, “ECM function”, and “immune response” (Figure 4B and Supplementary Table S2). By qPCR and ISH, we validated the upregulation of cell cycle regulators (*cdk1, cdk4, ccnb1* and *cndl1*) (Figure 4C), the extracellular matrix (ECM)-type genes (*ctss2.1, nrg1, fgf2, vcan, vcanb* and *hbegfb*) (Figures 4D,G,H and Supplementary Table S3) and immune response regulators (*mpeg1.1, spiib, irf8, csf1ra, lrl1, socs1a, socs3b*) in regenerating hearts overexpressing tbx20 (Figure 4E and Supplementary Table S3). These results indicate that myocardial induction of tbx20 participates in heart regeneration not only by upregulating expression of cell cycle regulators, but also modulating genes regulating ECM function and immune response.  

GO analyses of downregulated genes revealed an enrichment of factors involved in sarcomere organization, including sarcomere formation genes (*cnmlc1, desma, myom1b, tmt1c, tmtnt2a*) and sarcomere assembling factors (*mylk2, mylk3, asb2b*) (Supplementary Table S3). We further verified down regulations of *cnmlc1, tmtnt2a, asb2b* and *mylk3* in regenerating hearts from DOX-treated TRE3G:tbx20CMOE fish by qPCR and ISH analyses (Figures 4F,I,J). These findings indicate that the regenerating hearts overexpressing tbx20 caused a reduction of genes regulating sarcomere formation and assembly to favor CM dedifferentiation (Figures 3B,D), consistent with our dedifferentiation experiments (Figure 3).  

**Myocardial tbx20 Overexpression Induces Endocardial Activation and Regeneration**  
Previous studies revealed that there is a highly dynamic endocardium during cardiac regeneration, including changes in cell morphology, behavior and gene expression (Kikuchi
et al., 2011b; Munch et al., 2017). As enhanced expression of *tbx20* in CMs augments heart regeneration after injury (Figure 2), we wondered if CM-specific *tbx20* overexpression also affects dynamics of endocardium in injured hearts. To test this possibility, we evaluated the impact of CM-specific *tbx20* overexpression on the behaviors of endocardium in injured hearts. Vascular endothelial growth receptor 2 (*Vegfr2*/Kdr/Flk) and ETS-family transcriptional factor *Fli1* are well-known endothelial/endocardial cell markers in the cardiovascular field and can be used for visualizing endocardial cell morphology and nuclei, respectively, during zebrafish heart regeneration (Munch et al., 2017; Sanchez-Iranzo et al., 2018; Zhao et al., 2019). *Tg(TRE3G:tbx20; flk:GFP)* and *Tg(TRE3G:tbx20^CMOE; flk:GFP)* were generated by crossing the *Tg(flk:GFP)* line and *Tg(TRE3G:tbx20)* line, respectively. We damaged heart tissues using cryoinjury methods rather than ventricular apex resections, because the retained wound tissue following cryoinjury allows us to visualize the revascularization process and the dynamic of endocardium (Marin-Juez et al., 2016; Munch et al., 2017). DOX-treated *Tg(TRE3G:tbx20^CMOE; flk:GFP)* animals and *Tg(TRE3G:tbx20; flk:GFP)* control fish were subjected to cryoinjury (Figure 5A).
The injured hearts were immunostained with cTnT antibody that labels CMs and GFP antibody that recognizes flk:GFP-marked endocardial cells (Figure 5A). Previous studies report that endocardial expansion within the injury site is mediated through extension and migration of existing endocardial cells from the uninjured site (Munch et al., 2017). In the remote (uninjured) region of the tbx20-overexpressing and control heart, a coherent network of flk:GFP-marked endocardial cells surrounded cardiac muscles labeled by cTnT (Figures 5B,B-I,C,C-I). Within the injury site, we observed that a population of disorganized endocardial cells extended from the uninjured site and displayed cell protrusions, suggestive of migration (arrows in Figures 5B-II,C-II; Munch et al., 2017). Remarkably, Tbx20-overexpression hearts in DOX-treated Tg(TRE3G:tbx20CMOE; flk:GFP) animals exhibited a marked increase in endocardial cells labeled by flk:GFP extending the injury site (Figures 5C,C-II). Quantification analyses indicated the proportion of the injured area occupied by flk:GFP+ cells was increased in tbx20-overexpressing hearts (35.2 ± 2.5%) compared to that in controls (21.2 ± 2.2%) (Figure 5F). These findings suggest that myocardial tbx20 promotes extension and migration of endocardial cells into the injury site. We also appraised the endocardial cell proliferation adjacent to and within the cryoinjury site from DOX-treated TRE3G:tbx20CMOE hearts at 5 dpci by co-immunostaining for endothelial/endocardial cell nuclear marker Fli1 and cell proliferation marker PCNA.
were subjected to double immunostaining of GFP antibody and Tbx20 overexpression promotes endocardial cell migration and proliferation during heart regeneration.

Next, we assessed whether the endocardium is activated in myocardial Tbx20 overexpressing hearts. Nfatc1 is required for endocardial development in the heart and serves as a specific marker for endocardial activation after cardiac injury (Wong et al., 2012; Munch et al., 2017). ISH analyses revealed a striking upregulation of nfatc1 at the injury site at 5 dpci in DOX-treated TRE3G:tbx20CMOE hearts (Figures 5H,I). In consistent, re-survey of our RNA-seq data revealed an increase of two other endocardial markers, aldehyde dehydrogenase 1 family member a2 (aldh1a2) and lepbin (lepb), in DOX-treated TRE3G:tbx20CMOE hearts (Supplementary Table S3). Previous studies report that the Retinoic Acid synthesis enzyme Aldh1a2 and the secreted regulator of energy homeostasis protein Lepb are induced in the endocardium after cardiac injury, indicative of endocardial activation (Kikuchi et al., 2011b; Kang et al., 2016). We also validated the upregulation of aldhl1a2 and lepb at endocardial cells in the injured region in myocardial tbx20 overexpressing hearts by ISH analyses (Figures 5J–M). Altogether, these results suggest that inducible tbx20 overexpression in the adult myocardium contributes to endocardial cell migration and regeneration through endocardial cell activation, revealing crosstalk between the myocardium and endocardial cells during regeneration.

Tbx20 Induction Augments Endocardial Bmp6 Signaling During Heart Regeneration

To identify the molecular signaling involved in activating the endocardial regeneration program in the injured hearts with CM-specific tbx20 overexpression, we searched differentially expressed genes participating in endocardial/endothelial activation in our RNA-seq/GO analysis data generated from control and tbx20-overexpressing hearts. We found a profound upregulation of BMP signaling, including bmp6 ligand and its downstream targets, inhibitor of DNA-binding proteins (id1, id2a, and id2b) in myocardial tbx20 overexpressing hearts following ventricular injury (Figures 6A,B and Supplementary Table S3). ISH analyses revealed that the expression of bmp6, id1 and id2b was marked induced in the injury border zone and the inside of the wound endocardium at 5 dpci hearts from DOX-treated TRE3G:tbx20CMOE fish in comparison to TRE3G:tbx20 control fish (Figures 6C–I). To determine the identity of bmp6+ cells in the injury site of hearts, we performed fluorescence in situ hybridization (FISH) experiments using bmp6 or id2b antisense-mRNA probes on DOX-treated Tg(TRE3G:tbx20CMOE;flk:GFP) hearts and Tg(TRE3G:tbx20;flk:GFP) control hearts following cryoinjury (Figure 6J). These fluorescence hybridization hearts were subjected to double immunostaining of GFP antibody recognizing the flk:GFP transgenic endocardium and α-actinin antibody labeling cardiac muscle at 5dpi (Figure 6J). We found that bmp6 transcripts were located predominantly in flk:GFP-marked endocardial cells in tbx20-overexpressing and control hearts (arrowheads in Figures 6K,K-I,L,L-1,L-1'); however, only a few number of non-endocardial cells expressed bmp6 in tbx20-overexpressing hearts (arrows in Figures 6L-L-1'). Quantification analyses indicated a significant increase of bmp6+ Flk-GFP+ cells in tbx20-overexpressing hearts (35.7 ± 2.7%), in comparison with control hearts (33.6 ± 2.9%) (Figure 6M). Similarly, expression of id2b, a Bmp6 downstream target, overlapped with flk:GFP in endocardial cells in both DOX-treated TRE3G:tbx20CMOE hearts (arrowheads in Figures 6O,0-I,O-I') and TRE3G:tbx20 control hearts (arrowheads in Figures 6N,N-I,N-I'). id2b transcripts were only detectable in a few non-endocardial cells in tbx20-overexpressing hearts (arrows in Figures 6O-O-I,O-I'). Importantly, myocardial tbx20 overexpression resulted in a significant increase in id2b+ Flk-GFP+ endocardial cells at the cardiac injury region (52.5 ± 3.5%) compared to that in TRE3G:tbx20 control hearts (30.2 ± 2.5%) (Figure 6P). In contrast, expression of bmp3 or bmp10 was not significantly upregulated in tbx20 overexpressing wound hearts (Supplementary Figure S8). Collectively, these findings indicated that Bmp6/Id2b signaling is activated mostly in the endocardium in response to Tbx20 induction following cardiac injury.

To test whether the increase of endocardial regeneration caused by myocardial tbx20 overexpression is Bmp6 signaling-dependent, two highly specific BMP signaling inhibitors, LDN-193189 and K02288, which selectively interferes with BMP type I Alk2, Alk3 receptors (Sanvitale et al., 2013; Palencia-Desai et al., 2015), were used to assess the effect of Bmp6 signaling on Tbx20-mediated endocardial regeneration. LDN-193189 or K02288 was intraperitoneally injected every 24 h starting at 2 dpci. We found that BMP inhibitor-treatments diminished id1 and id2b expression at cryoinjury sites in DOX-treated TRE3G:tbx20CMOE animals (Figures 7A–F) but not TRE3G:tbx20 control animals (Supplementary Figure S9), suggesting that upregulation of Bmp6 signaling is Tbx20-dependent following injury. Moreover, co-immunostaining with anti-PCNA and anti-Flk1 antibodies revealed that endocardial cell proliferation adjacent to and within the cryoinjury site in LDN-193189- or K02288-treated TRE3G:tbx20 control hearts were comparable to that in vehicle-treated control hearts (vehicle: 7.8 ± 1.1%; LDN-193189: 6.4 ± 1.0%; K02288: 6.7 ± 1.4%) (Figures 7G–L,M). On the contrary, the increased endocardial cell proliferation was significantly suppressed (vehicle: 19.0 ± 1.0%; LDN-193189: 5.5 ± 1.0%; K02288: 5.5 ± 0.8%) in wounded TRE3G:tbx20CMOE hearts treated with LDN-193189 or K02288 inhibitors (Figures 7J–L,M). These findings suggest that CM-specific tbx20 overexpression induces endocardial cell proliferation, at least in part, by upregulating Bmp6 signaling.

DISCUSSION

Based on our findings, we propose a working model for Tbx20-dependent transcriptional network in governing heart
A

DOX treatment

Immunostaining (GFP/cTnT & PCNA/Fli1)

Croinjury

TRE3G:tbx20; flk:GFP

TRE3G:tbx20^CDE; flk:GFP

B

C

D

E

F

G

TRE3G:tbx20

TRE3G:tbx20^CDE

nfatc1

aldh1a2

lepb

H

J

L

I

K

M

FiguRe 5 | Continued
regeneration using zebrafish heart injury model (Figure 7N). Following heart injury, tbx20 is strongly induced in the myocardial wound region and the atrial epicardium. Myocardial-induced tbx20 upregulates expression of embryonic or fetal genes as well as cell-cycle regulators, promoting CM proliferation via dedifferentiation. We find that Tbx20 induction also plays a key role in endocardial cell migration and regeneration through upregulating endocardial Bmp6 signaling after cardiac damage. Thus, this putative Tbx20-mediated transcriptional program would coordinate two distinct mechanisms of zebrafish heart regeneration.

Because adult hearts are least likely to exist cardiac stem cells (Kretzschmar et al., 2018; He et al., 2019), myocardial regeneration occurs via stem-cell independent mechanisms, in which resident CMs in the injured heart undergo dedifferentiation prior to their proliferation (Jopling et al., 2010; Kikuchi et al., 2010; Senyo et al., 2013). Our study indicates that inducible tbx20 overexpression in adult zebrafish hearts enhance CM proliferation and myocardial regeneration, similar to that observed in mice by Xiang et al. (2016). Mechanistically, we have demonstrated that Tbx20 promotes injury-induced CM proliferation via dedifferentiation through mediating cellular changes and molecular dedifferentiation circuits. Specifically, CMs at the wound border zone express a myocardial fetal marker α-SMA and a stem cell marker Runx1 in injured zebrafish hearts. Both α-SMA and Runx1 were discovered originally as CM dedifferentiation markers in human patients suffered from myocardial infarction (Kubin et al., 2011). Thus, CM dedifferentiation following cardiac injury is a conserved mechanism, defining zebrafish as a model system to study injury-induced CM dedifferentiation during regeneration. Interestingly, we find that tbx20 induction is not only localized to the injured myocardium, but also detectable in the atrial epicardium following ventricle injury. During development, tbx20 is expressed in epicardial cells surrounding the atrioventricular canal groove (Yamagishi et al., 2004; Boogerd et al., 2018), but the function of epicardial tbx20 is still unclear. Previous studies indicate that epicardium is required for heart regeneration in zebrafish (Kikuchi et al., 2011a; Wang et al., 2015). It will be important to test whether atrial tbx20+ epicardial cells contribute to injury-induced CM proliferation, and how atrial epicardial activation relates to ventricle regeneration.

BMP signaling plays pivotal functions via diverse mechanisms involving vascular, myocardial, endocardial and mesenchymal tissues during cardiovascular development (Morrell et al., 2016). We determined that Bmp6 signaling in the endocardium is an early injury-response to myocardial Tbx20 induction, which promotes endocardial cell regeneration. Precedent for this type of crosstalk between myocardium and endocardium exists in the context during heart development. For example, Bmp2 and Bmp4 function in the myocardium is required for the epithelial-mesenchymal transformation (EMT) within the endocardium, leading to the formation of endocardial cushions and valves (Ma et al., 2005; Choi et al., 2007). During zebrafish heart regeneration, our RNA-seq analyses revealed upregulation of multiple bmp ligands in tbx20-overexpressing hearts, consistent with part of BMP regulation following cardiac injury (Wu et al., 2016). However, the cellular sources of secreted bmp ligands remain unclear in injured hearts. In this study, we determined that bmp6 and id2b are primarily upregulated in the endocardium following diverse cardiac damages, while their expression was not detectable in uninjured WT hearts, revealing Bmp6 as a specific BMP signal in the endocardium in response to cardiac injury. Previous studies report that Notch signaling and Retinoic Acid (RA) pathways are activated in the wound endocardium (Kikuchi et al., 2011b; Munch et al., 2017). However, both Notch and RA pathways in the endocardium signal to the myocardium to stimulate CM regeneration (Kikuchi et al., 2011b; Munch et al., 2017; Zhao et al., 2019). Our data suggest that Bmp6 signaling in the endocardium promotes endocardial cell regeneration in response to myocardial Tbx20 induction. In uninjured mouse hearts, Tbx20-mediated increases in CM proliferation are due to activation of Bmp2 and P13K signaling in the myocardium (Chakraborty et al., 2013). Thus, the influence of Tbx20 on distinct Bmp ligands in myocardial cells or the endocardium appears to be context dependent. We have also observed the increased immune response genes in Tbx20-overexpressing hearts in RNA-seq analyses. Immune response participates in heart injury and repair (Lai et al., 2019). Previous studies report that immune cells, such as macrophages, play important roles in the process of inflammation, scar resolution and wound healing, which in turn stimulates myocardial regeneration and repair (Lai et al., 2019). It will be interesting to test whether Tbx20 directly or indirectly regulates immune response genes during cardiac regeneration, and whether Bmp6/Id2b signaling might be activated in immune cells such as macrophages following cardiac injury.
FIGURE 6 | Continued
Tbx20 and Heart Regeneration

FIGURE 6 | Myocardial Tbx20 mediates endocardial regeneration by activating Bmp6 signaling. (A,B) Myocardial tbx20 overexpression upregulates BMP target genes id1 and id2b as well as the BMP ligand bmp6 after heart resection (A) or cryoinjury (B), respectively. Data from three biological replicates. Mean ± SEM, **p < 0.01, ***p < 0.001. (C–E) Representative ISH images of heart sections from DOX-treated uninjured Tg(TRE3G:tbx20) (C), 5 dpci Tg(TRE3G:tbx20) (D) and Tg(TRE3G:tbx20;CMOE) zebrafish (E) with bmp6 probe. Red arrowheads indicate endocardium with bmp6 signal. (F–I) Representative ISH images of heart sections from 5 dpci DOX treated Tg(TRE3G:tbx20) and Tg(TRE3G:tbx20;CMOE) fish with id1 (F,G) and id2b (H,I) probe. Red arrowheads indicate endocardium with id1 (G) and id2b (I) signal in the injury site. (J) Experimental procedures for DOX treatment, cryoinjury, FISH combined with immunostaining experiments. (K,L) Representative images of FISH analysis of bmp6 (red) combined with immunostaining for GFP and α-actinin (blue) on heart sections from 5 dpci Tg(TRE3G:tbx20;flk:GFP) (K) and Tg(TRE3G:tbx20;CMOE;flk:GFP) (L) zebrafish. Boxed areas indicate locations of the magnified and channel-separated panels below. White arrowheads point to GFP+ cells with bmp6 transcripts in border zone and injury site (K-I,K'-I',L-I,L'-I'), yellow arrows in (L-I) and (L-I') indicate non-endocardial cells with bmp6 transcripts. (M) Dotted diagram indicates the percentage of bmp6+ endocardial cells in injury site from 5 dpci DOX treated Tg(TRE3G:tbx20) (K, n = 5) and Tg(TRE3G:tbx20;CMOE) (L, n = 6) fish. Mean ± SEM, **p < 0.01. (N,O) Representative images of FISH analysis of id2b (red) combined with immunostaining for GFP and α-actinin (blue) on heart sections from 5 dpci Tg(TRE3G:tbx20;flk:GFP) (N) and Tg(TRE3G:tbx20;CMOE;flk:GFP) zebrafish (O). Boxed areas indicate locations of the magnified and channel-separated panels below. White arrowheads point to GFP+ cells with id2b transcripts in border zone and injury site (N-I,N'-I,O-I,O'-I'), yellow arrows in (O-I) and (O'-I) indicate non-endocardial cells with id2b transcripts. (P) Dotted diagram indicates the percentage of id2b+ endocardial cells in injury sites from 5 dpci DOX treated Tg(TRE3G:tbx20) (N, n = 5) and Tg(TRE3G:tbx20;CMOE) (O, n = 5) fish. Mean ± SEM, ***p < 0.001. Dashed lines delineate the injured area. Scale bar: 100 µm (C-I,K-L,N,O), 20 µm (K-I'-L',N-I'-O'-I').

FIGURE 7 | Inhibition of Bmp6 signaling restricts endocardial cell proliferation activated by myocardial tbx20 overexpression. (A–F) Representative ISH images showing expression of id1 (A–C) and id2b (D–F) in DOX treated Tg(TRE3G:tbx20;CMOE) fish at 5 dpci after vehicle or inhibitors treatment. Red arrowheads indicate endocardium with id1 (A) and id2b (D) signal in the injury site. (G–L) Representative confocal fluorescence images of heart sections immunostained for Fli1 (green) and PCNA (red) from vehicle, LDN-193189 and K02288 treated control (G–I) and myocardial tbx20 overexpressing fish (J–L) at 5 dpci. Boxed areas indicate locations of the magnified insets. Arrowheads point to the Fli1+PCNA+ proliferating endocardial cells. (M) Scatter plot showing the percentage of Fli1+ cell proliferation ratio in the border zone and injury site from (G–L). The values are mean ± S.E.M. Two-way ANOVA followed with Tukey’s multiple comparison test. n.s.: none significance, **p < 0.01. (N) Model of myocardial tbx20 function during zebrafish heart regeneration: In CMs, myocardial tbx20 overexpression promotes CM dedifferentiation and proliferation following injury. In addition, myocardial tbx20 promotes endocardial regenerative progress by enhancing its proliferation via partially through Bmp6 signaling. Dashed lines delineate the injured area. Scale bar: 100 µm.
Although adult zebrafish can efficiently regenerate their hearts in response to different forms of damage, there are some intrinsic disadvantages to the model system. For instances, proliferative CMs in zebrafish are mononuclear and diploid, whereas most adult human CMs become polyploid that restraints CM proliferation (Xin et al., 2013; Tzahor and Poss, 2017). Although CM renewal is extremely low in uninjured mammalian hearts, a small number of proliferative CMs can be detectable in the injury border zone of murine hearts after myocardial infarction (Malliaras et al., 2013; Xiang et al., 2016; Xie et al., 2020). We believe that specific factors and mechanisms obtained from zebrafish regeneration study should be confirmed in mammal system such as mouse before exploring potential therapeutics in humans. Tbx20 plays a conserved role in promoting injury-induced CM regeneration in zebrafish and mouse (Xiang et al., 2016), raising a possibility that Tbx20 might be a target to be explored in the medical context. For instance, Tbx20 administration at local injury regions might be potential strategy to improve repair capacity in the wound human heart. Overall, understanding of the mechanisms underlying heart regeneration in model systems will provide inspiration for the regeneration intervention in humans.

**MATERIALS AND METHODS**

**Zebrafish Cardiac Injury**

All animal work used in this study were approved by the Animal Care Committee of Fudan University. We bred wild-type zebrafish, or zebrafish carrying Animal Care Committee of Fudan University. We bred wild-type zebrafish before use. All animal work used in this study were approved by the Animal Care Committee of Fudan University. We bred wild-type zebrafish, or zebrafish carrying Animal Care Committee of Fudan University. We bred wild-type zebrafish before use. Overall, understanding of the mechanisms underlying heart regeneration in model systems will provide inspiration for the regeneration intervention in humans.

**Generation of Tg(tbx20:moxGFP), Tg(cryaa:mCherry; cmic2:TetON-3G) and Tg(cryaa:EGFP; TRE3G:tbx20-E2A-mCherry) Zebrafish**

A 7.3-kb promoter of tbx20 was amplified from zebrafish genome and cloned upstream of moxGFP in plasmid pT2KXI8DA1 in plasmid to replace xenopus ef1α to EGFP (Costantini et al., 2015). To generate the transgenic line, Tol2 mRNA and the plasmid were co-injection into one-cell stage fertilized eggs.

The cmic2-TetON-3G cassette was first constructed by cloning TetON-3G from pCMV-Tet3G vector (Clontech, #z1164n) downstream of a 3.0-kb cmic2 promoter. In order to identify the transgenic animals by lens fluorescence, the Cryaa-mCherry cassette from plasmid pT2-hsp70l-dnMEK1 (Liu and Zhong, 2017) was then cloned downstream of the cmic2-TetON-3G cassette in the opposite orientation. The entire construct was franked by two copies of the chicken β-globin insulators at upstream and one human 3′ β-globin insulator was also included between the two cassettes.

The TRE3G-tbx20-E2A-mCherry cassette was first constructed by cloning TRE3G sequence from pTRE3G-BI vector (Clontech, #z1164n) upstream of the assembled tbx20-E2A-mCherry fusion gene. mCherry fragment in Cryaa-mCherry cassette described above was replaced by EGFP using NEB HIFI Assembly Master Mix (NEB, #E2621S). Cryaa-EGFP cassette was then cloned downstream of the TRE3G-tbx20-E2A-mCherry cassette in the opposite orientation. The entire construct was insulmed as same as cryaa:mCherry;cmic2:TetON-3G (see above paragraph). These constructs were injected into one-cell-staged embryos, respectively, using Tol2 transposase-mediated transgenesis techniques (Suster et al., 2009).

**Immunostaining, ISH, AFOG and Histology Analyses**

Zebrafish hearts were dissected and fixed in 4% paraformaldehyde (PFA) solution at 4% overnight before embedded in OCT (Thermo Fisher Scientific). 10 μm cryosections were used in all histological analyses, in situ hybridization (ISH) and immunostaining. The primers used to generate RNA probes were listed in Supplementary Table S4. ISH and Acid Fushin-Orange G (AFOG) analyses were performed as described, the quantification of the scar area was performed using Fiji software (Poss et al., 2002). The TSA-Plus fluorescence system (Perkin Elmer, #NEL752001KT) were used for fluorescence in situ hybridization (FISH) analyses according to the manufacturer’s instructions. Primary and secondary antibody staining were performed according to the standard protocol, except for PCNA staining which required heat-induced antigen retrieval (Lepilina et al., 2006). All primary antibodies were incubated at 4% overnight and secondary antibodies were incubated at room temperature for 2 hrs. Primary antibodies used in this study include GFP (Invitrogen, #A21311, 1:200), GFP (Aves Labs, #GFP-1010, 1:500), α-actin (Sigma-Aldrich, #A7732, 1:250), PCK (Abcam, #ab86734, 1:200), PCNA (Sigma-Aldrich, #P8825, 1:300), Me2 (Santa Cruz Biotechnology, #sc-313, 1:75), ZO-1 (Invitrogen, #339188, 1:200), α-SMA (Genetex, #GTX124505, 1:250), cTnT (DSHB, #CT3,1:200), N-cadherin (Abcam, #76011, 1:250), Runx1 (Abcam, #ab92336, 1:250) and Fli1 (Abcam, #ab133485, 1:250). Secondary antibodies used in this study include goat anti-mouse Alexa Fluor 647 (Invitrogen, #A21236), goat anti-mouse Alexa Fluor 488 (Invitrogen, #A28175), goat anti-rabbit Alexa Fluor 594 (Invitrogen, #A11037), goat anti-rabbit Alexa Fluor 647 (Invitrogen, #A11011), goat anti-chicken Alexa Fluor 488 (Invitrogen, #A-11039) and goat anti-rabbit Alexa Fluor 488 (Invitrogen, #A11034) with concentration of 1:1000.

AFOG images were taken using an Olympus DP80 microscope. ISH images were taken using a Nikon microscope. FISH and immunofluorescence images were taken using a ZEISS LSM880 confocal microscope. For quantitative analyses of immunofluorescence images, sections containing the largest wounds were selected and manually counted. A defined region
injected intraperitoneally every 24 h with 10 µM in DMSO to a final concentration of 50 mM. Adult zebrafish were treated with 50 mg/L DOX from the day before heart injury.

Doxycycline (DOX) (Sigma-Aldrich, #d9891) was dissolved in ddH2O at 50 mg/ml as stock solution. Adult fish were daily treated with 50 mg/L DOX from the day before heart injury. LDN-193189 (Selleck, #S73507) was dissolved in PBS to a final concentration of 50 mM, K02288 (Selleck, #S7359) was dissolved in DMSO to a final concentration of 50 mM. Adult zebrafish was injected intraperitoneally every 24 h with 10 µL LDN-193189 (50 µM in PBS), K02288 (50 µM in PBS) or vehicle (0.1% DMSO in PBS) from 2 dpci to 4 dpci.

**Gene Expression Analysis**

For RNA-seq analysis, 7 dpa hearts were dissected, and the apical portions of 6-8 ventricles were collected in each group. RNA samples were extracted using TRIzol Reagent (Invitrogen, #15596018). Next generation sequencing library construction and sequencing was performed by GENEWIZ Company, Suzhou on an Illumina HiSeq sequencer. Reads from the sequenced samples were qualified and aligned to zebrafish transcriptome (Ensembl genebuild GRCz10.86) using Hisat2 (v2.0.1). Differentially expressed genes were analyzed using the DESeq Bioconductor package, genes identified with altered expression levels with a Benjamini and Hochberg adjusted p-value < 0.05 were retained. GO-TermFinder was used to define Gene Ontology terms. For quantitative RT-PCR analysis (qPCR), RNA samples were extracted from ventricular apices, border zone and atrium at specific time points, respectively, 6-8 samples were pooled together for each group, cDNA was synthesized using the SuperScript III First-Strand Synthesis System (Invitrogen, #18080051) following the manufacturer’s instructions. q-PCR was performed on a Q7 Real-Time PCR System (Applied Biosystems) using SYBR Green ROX dye (Applied Biosystems, #A25742). Primers used are listed in Supplementary Table S4.

**Small Molecule Treatments**

All quantitative analyses of immunofluorescence images and assessment of stained images were performed in blinded fashion. GraphPad Prism 8 was used for data analyzing. Statistical comparisons between two groups were analyzed by Student's t-test. For samples with more than two independent groups, ANOVA followed by Tukey’s multiple comparisons test were performed. All statistical tests were calculated when normality was detected in ventricular epicardium. Dotted line demarcates the edge of the wound area. Scale bar: 100 µm.

**Statistical Analysis**

All quantitative analyses of immunofluorescence images and assessment of stained images were performed in blinded fashion. GraphPad Prism 8 was used for data analyzing. Statistical comparisons between two groups were analyzed by Student's t-test. For samples with more than two independent groups, ANOVA followed by Tukey’s multiple comparisons test were performed. All statistical tests were calculated when normality was detected in ventricular epicardium. Dotted line demarcates the edge of the wound area. Scale bar: 100 µm.

**DATA AVAILABILITY STATEMENT**

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/geo/, GSE144831.

**ETHICS STATEMENT**

The animal study was reviewed and approved by the Animal Care Committee of Fudan University.

**AUTHOR CONTRIBUTIONS**

YF, KL, and TZ designed the study. YF and KL performed the experiments. YF, KL, PS, JS, WT, and TZ analyzed the experimental data. YF and TZ wrote the manuscript. WT and TZ edited the manuscript. All authors contributed to the article and approved the submitted version.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.00738/full#supplementary-material
observed in the zebrafish fin, skin or muscle after DOX treatment (D). Scale bar: 250 μm. (E,F) Images of whole mount hearts of the transgenic fish at 7 dpa treated with DOX in the brightfield (E) or mCherry channel (F). mCherry signal was not detected in the outflow tract (asterisk) and the injured site (arrow) from Tg(TRE3G:txb20CMOE) hearts. Scale bar: 500 μm. (G–I)ISH analysis of txb20 overexpression using txb20-E24-mCherry probe on 7 dpa heart sections from Tg(TRE3G:txb20) zebrafish treated with DOX (G). Tg(TRE3G:txb20CMOE) zebrafish treated with (I) or without DOX solution (H). Scale bar: 100 μm.

**FIGURE S5** | Confocal fluorescence images of injured ventricle sections from Tg(TRE3G:txb20) (A) and Tg(TRE3G:txb20CMOE) (B) after 7 days of DOX treatment co-stained with PCNA (red) and Mef2 (green) antibody. Scale bar: 100 μm.

**FIGURE S6** | Myocardial txb20 overexpression resulted in loss of cell-cell contact in the border zone myocardium. (A,B) Representative confocal fluorescence images of injured ventricle sections from Tg(TRE3G:txb20) (A) and Tg(TRE3G:txb20CMOE) (B) zebrafish co-stained with antibodies against cTNT (green) and N-cadherin (red). DAPI was used to stain nuclei. Boxed areas are locations of magnified images in Figures 3C,D. Scale bar: 100 μm.

**TABLE S1** | FPKM values of differentially expressed genes in control and myocardial txb20 overexpressing hearts at 7 dpa.

**TABLE S2** | Gene Ontology analysis of differentially expressed genes in control and myocardial txb20 overexpressing hearts at 7 dpa.

**TABLE S3** | Primers used in this study.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.