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Histone deacetylase is required for the activation of Wnt/β-catenin signaling crucial for heart valve formation in zebrafish embryos

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ABSTRACT

During vertebrate heart valve formation, Wnt/β-catenin signaling induces BMP signals in atrioventricular canal (AVC) myocardial cells and underlying AVC endocardial cells then undergo endothelial-mesenchymal transdifferentiation (EMT) by receiving this BMP signals. Histone deacetylases (HDACs) have been implicated in numerous developmental processes by regulating gene expression. However, their specific roles in controlling heart valve development are largely unexplored. To investigate the role of HDACs in vertebrate heart valve formation, we treated zebrafish embryos with trichostatin A (TSA), an inhibitor of class I and II HDACs, from 36 to 48 h post-fertilization (hpf) during which heart looping and valve formation occur. Following TSA treatment, abnormal linear heart tube development was observed. In these embryos, expression of AVC myocardial *bmp4* and AVC endocardial *notch1b* genes was markedly reduced with subsequent failure of EMT in the AVC endocardial cells. However, LiCl-mediated activation of Wnt/β-catenin signaling was able to rescue defective heart tube formation, *bmp4* and *notch1b* expression, and EMT in the AVC region. Taken together, our results demonstrated that HDAC activity plays a pivotal role in vertebrate heart tube formation by activating Wnt/β-catenin signaling which induces *bmp4* expression in AVC myocardial cells.

1. Introduction

Heart development starts with a simple tube consisting of an outer layer of myocardial cells separated from an inner layer of endocardial cells by an extracellular matrix (ECM) known as cardiac jelly. Heart valve formation depends on signaling networks between myocardial and endocardial cell layers across an elaborate extracellular matrix involving BMP, Wnt/β-catenin, and Notch signaling [1,2]. During heart tube looping, Wnt/β-catenin signaling induces *bmp4* and *tbx2b* expression in the future atrioventricular canal (AVC) myocardial cells [3]. Subsequently, a subset of these AVC endocardial cells receives BMP signals from the overlying myocardial cells and delaminate, transdifferentiate, and migrate into the cardiac jelly. Populations of these cells then expand and form a discrete swelling referred to as a cardiac cushion [1,4,5]. The cardiac cushion formation is characterized by the endothelial-mesenchymal transdifferentiation (EMT) of AVC endocardial cells [1,5]. During further maturation of the heart, the cardiac cushion undergoes extensive remodeling to give rise to the heart valve through a poorly understood mechanism.

Recently, chromatin remodeling via posttranslational histone modification was found to function in a genome-wide manner and contributes to an extensive range of biological functions including heart development [6]. Acetylation and deacetylation of core histones in chromatin are the most important types of histone modification and essential for many biological processes. Generally, histone deacetylases (HDACs) repress transcription by removing acetyl groups from conserved lysine residues within the N-terminal tails of histones H3 and H4. In contrast, histone acetyltransferases (HATs) induce transcriptional activation by adding acetyl groups [7,8]. Recent studies have indicated that HATs also contribute to transcription repression while HDACs may influence transcriptional activation [9,10]. HDACs are categorized into four classes (I–IV). Among the class I members (HDAC1, 2, 3, and 8), HDAC1, 2, and 3 regulate cardiomyocyte differentiation and heart muscle lipid metabolism [6,11,12]. Among class II members (HDAC4, 5, 6, 7, 9, and 10), HDAC5 and 9 are functionally

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redundant in the regulation of heart growth. Thus, double knockout mice developed ventricular septal defects and thin-walled myocardium. Very recently, defective heart valve formation caused by the over-activated nucleus HDAC5 activity in zebrafish *bungee* (*bng*) mutant was reported [13]. HDAC4 and 6 are likely to be unnecessary for heart development [6,14]. The roles of the class III (SIRT1 to 7) and class IV (HDAC11) HDACs in heart development have not been reported. Therefore, specific functions of HDACs in regulating heart valve development still remain largely unexplored.

In zebrafish, heart looping starts at 36 h post-fertilization (hpf) and functional valves are almost completely formed by 48 hpf although valve development is completed around 55 hpf [15–17]. To understand the roles of HDACs in epigenetic regulation of vertebrate heart valve development, we treated zebrafish embryos with trichostatin A (TSA), a well-known inhibitor for classes I and II HDAC families, from 36 to 48 hpf [18,19]. In contrast to the reported negative effect of HDAC5 on heart valve formation [13], our data indicated that HDACs have essential roles in this developmental process by activating Wnt/β-catenin signaling, which induces AVC myocardial *bmp4* and subsequent AVC endocardial *notch1b* expression.

2. Materials and methods

2.1. Chemicals and reagents

TSA was purchased from Calbiochem (Darmstadt, Germany). valproic acid (VPA) and LiCl were obtained from Sigma (St. Louis, MO). Antibodies against human H3, acetylated H3K9, and acetylated K3K18 were from Cell Signaling Technology (Danvers, MA).

2.2. Zebrafish strains and maintenance

Zebrafish were maintained as previously described [22]. The AB strain was used as a wild-type control. A transgenic line expressing enhanced green fluorescent protein (EGFP) under the control of a *cmlc2* promoter, *Tg(cmlc2:EGFP)* [20], was used to visualize the embryonic heart. To detect AVC endocardial cells, the *Tg(flk1:EGFP)* transgenic line [21] expressing EGFP under the control of a *flk1* promoter was used. All embryos were incubated at 28.5 °C until use.

2.3. Treatment of chemicals to zebrafish embryos

To inhibit DHAC activity, the zebrafish embryos were treated with TSA (0.25, 0.5, and 1.0 μM) or valproic acid (50 μM) from 36 to 48 hpf. To activate Wnt/β-catenin signaling, the embryos were treated with LiCl (0.1 and 0.15 M) with or without TSA (0.25, 0.5 and 1.0 μM) from 36 to 48 hpf.

2.4. Whole mount in situ hybridization

Digoxigenin-labelled antisense RNA specific for *cmlc2, tbx5, cdh5, notch1b, bmp4, tbx2b, versican a* (*vcan a*), and *has2* were prepared and used for *in situ* RNA hybridization as previously described [23]. Zebrafish embryos were subjected to *in situ* hybridization at 48 hpf and were then digested with proteinase K. Hybridization and washing were carried out at 65–70 °C. To detect the hybridized RNA probes, the embryos were incubated with an anti-digoxigenun Fab fragment conjugated to alkaline phosphatase (Roche Applied Science, Indianapolis, IN). Color detection reaction utilized BCIP/NBT as alkaline phosphatase substrate (Roche Applied Science).

2.5. Fluorescence immunostaining

Zebrafish embryos were immunostained with a mouse monoclonal antibody against zn5 (1:500; Hybridoma Bank, IA) and a 594-conjugated anti-mouse IgG antibody (1:1000; Molecular Probes, Grand Island, NY). Alexa 594-conjugated secondary antibodies (1:500, Molecular Probes) were used for primary antibody binding detection. Images of the immunostained hearts were acquired using a Zeiss LSM5 Pascal confocal microscope.

2.6. Western blotting

Zebrafish embryos were lysed in a triple-detergent lysis buffer (50 mM Tris–Cl, 150 mM NaCl, 0.1% SDS, 100 μg/ml PMSF, 1 μg/ml Aprorinin, 1% NP-40). The cell lysates were centrifuged (12,000×*g*) for 20 min at 4 °C and total protein concentrations were determined using a Bio-Rad protein assay kit. Denatured protein samples were subjected to Western blot analysis and immune-reactive bands were detected with ECL PLUS Western Blotting Detection Reagents (Amersham Biosciences, Piscataway, NJ).

3. Results and discussion

3.1. Heart defects caused by TSA are correlated with increased histone acetylation

When *Tg(cmlc2:EGFP)* transgenic zebrafish embryos were treated with TSA (an inhibitor of class I and II HDACs) from 36 to 48 hpf (the period during which heart looping and valve formation occur), defective heart development was observed (Fig. 1A and B). In contrast to the normal S-shaped hearts of control embryos, TSA treatment induced linear heart tube formation in a dose-dependent manner (Fig. 1B, right panel). Similar results were also obtained from embryos treated with VPA, another HDAC inhibitor (Supplementary Fig. 1). To verify whether the heart valve defect arising in TSA-treated embryos was caused by inhibition of HDACs, changes in the acetylation of histones H3 Lys-9 (H3K9) and H3K18 were analyzed. Acetylation of H3K9 and H3K18 was also increased by TSA treatment in a dose-dependent manner (Fig. 1C), suggesting that the heart valve defects might be caused by HDAC inhibition. These results indicate that the normal activity of class I and II HDACs, which are inhibited by TSA and VPA, may play a crucial role in heart development.

3.2. Differentiation of heart chamber myocardial and endocardial cells is not affected by TSA

To examine whether abnormal heart formation in the TSA-treated zebrafish embryos was caused by defective differentiation of myocardial and endocardial cells throughout the heart chamber, expression of myocardial (*cmlc2*) and endocardial (*cdh5*) markers [20,24] was analyzed. Expression of *cmlc2* and *cdh5* throughout the heart chamber in TSA-treated embryos was comparable to that in the untreated control embryos (Supplementary Fig. 2). Similarly, expression of *vmhc*, which encodes a ventricle-specific myosin heavy chain, was also unaffected in the TSA-treated embryos (data not shown). These observations imply that the defective heart valve formation observed in TSA-treated embryos was not caused by a failure of myocardiocytes and endocardiocytes to differentiate in either the atrium or ventricle.

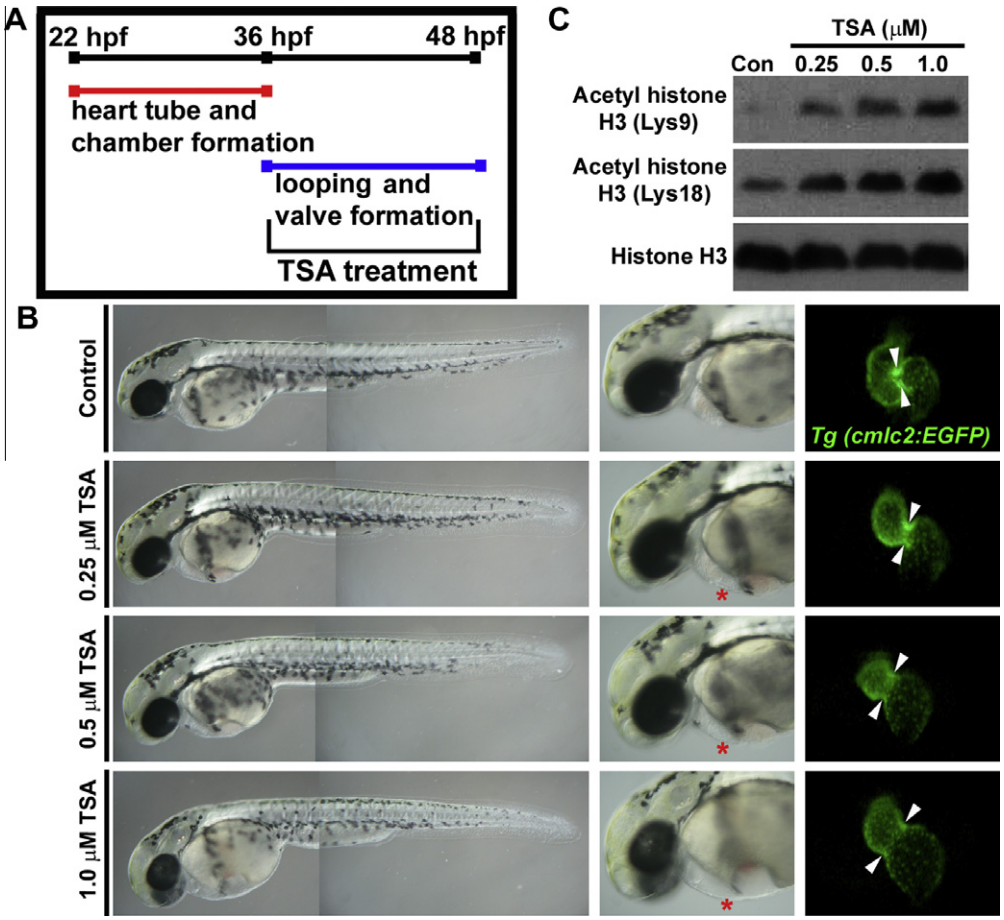

Fig. 1. Effect of TSA on embryonic heart development and histone acetylation. (A) Time course of TSA treatment during zebrafish heart development. (B) Effects of TSA on heart looping and valve formation in 48 hpf *Tg(cmlc2:EGFP)* zebrafish embryos. Morphology of embryonic hearts (middle panels) and heart looping in the AVC (right panels). Cardiac edema induced by TSA and the heart valve regions are indicated by asterisks and arrowheads, respectively. Lateral view; the dorsal side is to the right. (C) Dose-dependent effect of TSA (0.25, 0.5, and 1.0 μM) on the acetylation of histone H3 in 48 hpf zebrafish embryos was analyzed by Western blotting. For preparing the protein samples, 26 embryos per group were pooled and lysed. Each protein sample (50 μg) was subjected to Western blotting.

3.3. TSA treatment reduces the expression of genes required for heart valve formation in the AVC

It has been reported that heart valve formation depends on signaling between endocardial and overlying myocardial cell layers in the AVC [1]. To verify whether defective cell differentiation in the AVC was responsible for abnormal heart development in TSA-treated zebrafish embryos, expression of AVC myocardial and endocardial markers was analyzed. Expression of AVC myocardial *bmp4* and *tbx2b*, two genes that play a crucial role in the initiation of heart valve formation [1,3,25], was clearly decreased by TSA in a dose-dependent manner (Fig. 2A–F). Similarly, expression of *notch1b* and *NFATc1* in the AVC endocardial cells [26,27] was also markedly decreased by TSA treatment (Fig. 2G–L). It is well known that ECM in cardiac jelly plays an important role in facilitating signaling that initiates the differentiation of AVC endocardial cells [1]. In cardiac jelly, glycosaminoglycan hyaluronic acid exists as a hydrated gel that expands the extracellular space, regulates ligand availability, and interacts with numerous ECM components including the proteoglycan versican, a major constituent of cardiac jelly [1]. In contrast to the control embryos, expression of *vcan a* and *hyaluronan synthase* 2 (*has2*) marking AVC myocardial and endocardial cells, respectively [28], was greatly decreased or absent in the TSA-treated embryos (Fig. 2M–R). Taken together, these observations indicate that HDAC activity is required for the expression of essential genes that regulate the differentiation of AVC myocardial and endocardial cells during heart valve formation.

3.4. Activation of Wnt/β-catenin signaling by LiCl can rescue heart valve defects caused by TSA treatment

Wnt/β-catenin signaling is upstream of myocardial *bmp4* that is required for initiating EMT in AVC endocardial cells [1,3]. To verify whether reduced Wnt/β-catenin signaling is responsible for decreased AVC myocardial *bmp4* expression following TSA treatment, zebrafish embryos were co-treated with LiCl and TSA. LiCl treatment alone (from 36 to 48 hpf) disturbed neither the normal formation of S-shaped heart tube (Fig. 3B and F) nor the expression of myocardial *bmp4* or endocardial *notch1b* in the AVC (Fig. 3C, D, G and H). LiCl treatment rescued TSA-induced defective S-shaped heart looping in a dose-dependent manner (Fig. 3J, N and R) with restoring AVC myocardial *bmp4* (Fig. 3K, O and S) and endocardial *notch1b* expression (Fig. 3L, P and T). Similarly, LiCl also rescued cardiac edema and heart looping defects in VPA-treated embryos (Supplementary Fig. 1). These findings indicate that HDAC is required for the activation of Wnt/β-catenin signaling which plays an important role in the initial stage of heart valve formation [1,3].

3.5. Activation of Wnt/β-catenin signaling by LiCl can restore AVC endocardial cell EMT in TSA-treated embryos

We next tested whether failure of the AVC endocardial cells to undergo EMT in the TSA-treated embryos was responsible for defective heart valve formation. For this, we used *Tg(flk1:EGFP)*

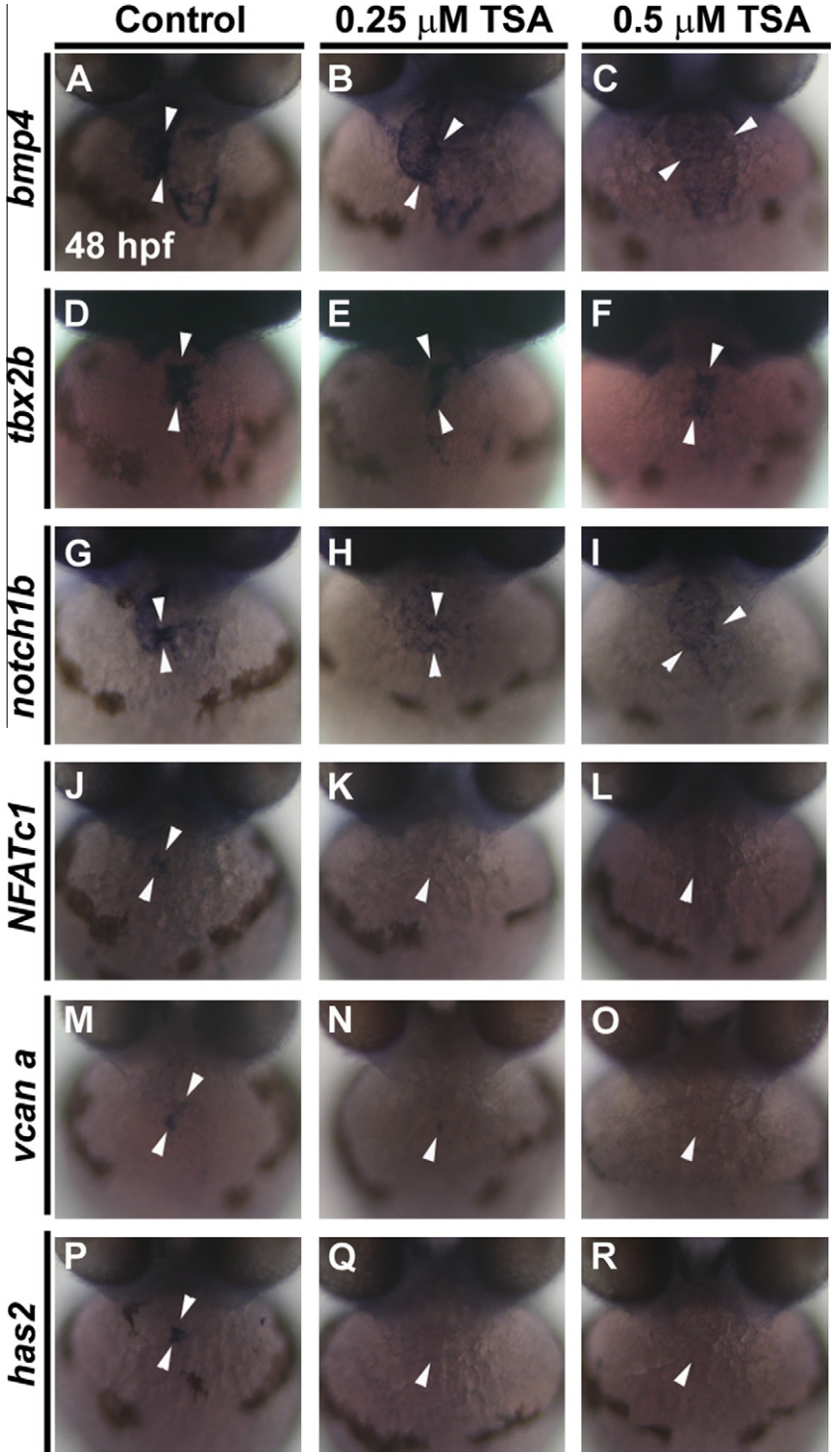

Fig. 2. Effect of TSA on the expression of the AVC myocardial and AVC endocardial marker genes. Expression of *bmp4* (A–C) and *tbx2b* (D–F) in AVC myocardial cells. Expression of *notch1b* (G–I) and *NFATc1* (J–L) in AVC endocardial cells. Expression of *vcan a* in AVC myocardial cells (M–O) and *has2* in AVC endocardial cells (P–R). All embryos were collected at 48 hpf and subjected to analyses. The AVC regions are indicated by arrowheads. Head-on views.

transgenic zebrafish embryos to visualize EMT changes in the AVC endocardial cells. It has been reported that a subset of endocardial cells undergoing EMT in the AVC as well as all myocardial cells can be positively stained with a zn5 antibody which recognizes a Dm-grasp cell-surface adhesion molecule belonging to the immunoglobulin superfamily in zebrafish [29].

In contrast to the untreated control transgenic embryos in which several GFP-positive AVC endocardial cells undergoing EMT were recognized by the anti-zn5 antibody (Fig. 4A), TSA treatment decreased the number of AVC endocardial cells positive for zn5 staining (Fig. 4B), demonstrating that TSA inhibits EMT in AVC endocardial cells. This observation is in agreement with the decreased expression of AVC endocardial *notch1b* observed in TSA-treated embryos (Fig. 2G–I). Therefore, we further investigated whether Wnt/β-catenin signaling activated by LiCl treatment can restore EMT in the TSA-treated embryos. Although LiCl treatment alone did not appear to alter the number of AVC endocardial cells positive for zn5 staining (Fig. 4C), LiCl increased the number

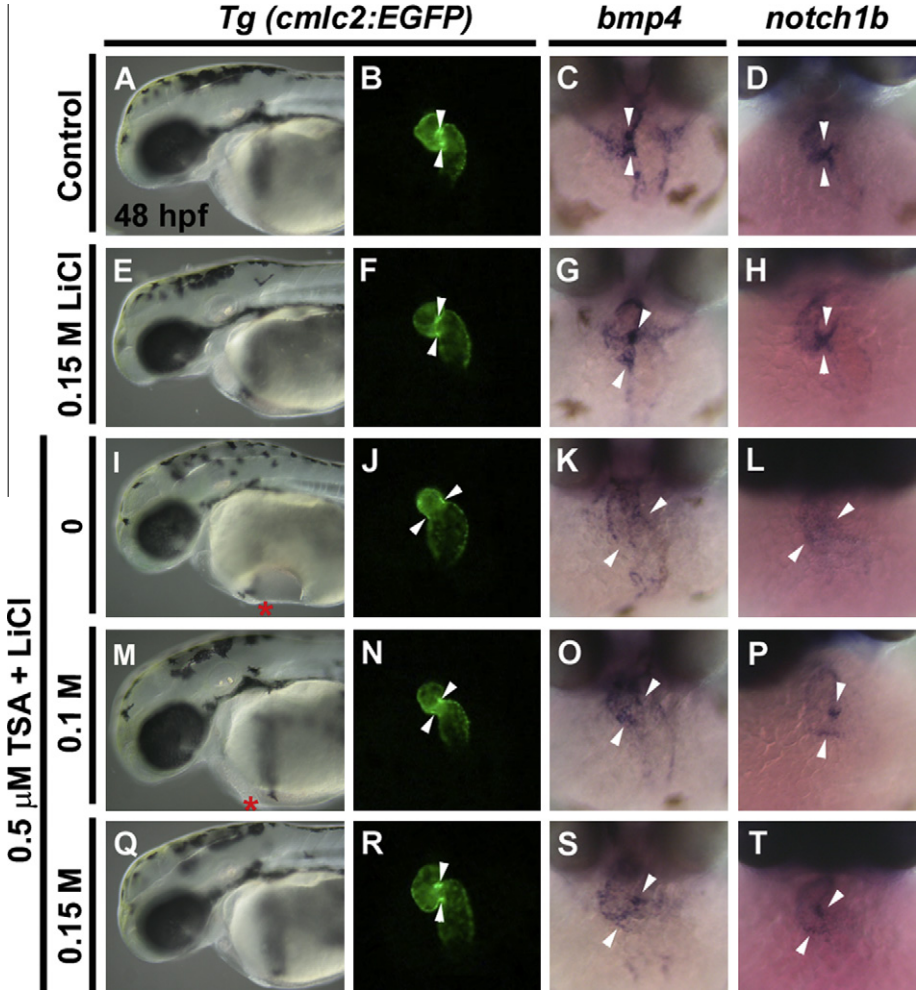

Fig. 3. LiCl restores expression of AVC marker genes and heart looping in TSA-treated embryos. (A, B, E, F, I, J, M, N, Q, and R) To visualize changes in heart morphology, *Tg(cmlc2:EGFP)* zebrafish embryos were used. (C, D, G, H, K, L, O, P, S, and T) For *in situ* hybridization, non-transgenic wild type embryos were used. (A, E, I, M, and Q) Effect of TSA and/or LiCl on embryonic heart development. Severe or mild cardiac edema is indicated by an asterisk. (B, F, J, N, and R) Heart looping in the AVC region is indicated by arrowheads. (C, G, K, O, and S) Expression of *bmp4* in the AVC myocardial cells is denoted by arrowheads. (D, H, L, P, and T) Expression of *notch1b* in the AVC endocardial cells is marked by arrowheads. All embryos were collected at 48 hpf and subjected to analyses. (A, E, I, M, and Q) Lateral view; the dorsal side is to the right. (B–D, F and G, L-L, N–P, and R–T) Head-on views.

of AVC endocardial cells positive for zn5 in TSA-treated embryos (Fig. 4D). This observation confirms that inhibition of HDACs by TSA downregulated Wnt//β-catenin signaling required for EMT in AVC endocardial cells. Similarly, LiCl treatment also restored S-shaped heart valve formation in VPA-treated embryos (Supplementary Fig. 1). Taken together, these data indicate that HDACs are required for activating Wnt/β-catenin signaling crucial for the induction of myocardial *BMP4* and subsequent endocardial *Notch1b* signaling through which EMT, an initial event that leads to S-shaped heart valve formation, can be promoted in AVC endocardial cells.

Gene silencing is associated with histone deacetylation while the activation of gene expression is affected by histone acetylation [7,8]. Therefore, different levels of HDAC activity have been implicated in the regulation of numerous embryonic developmental processes. However, histone acetylation or deacetylation can either activate or repress gene expression depending on the cellular context [7–10]. In fact, loss of Rpd3/HDAC1 activity in *Drosophila* results in embryonic lethality with abnormal segmentation [30]. Similarly, development of sea urchin and starfish embryos is arrested at the late blastula and gastrula stage, respectively, by TSA treatment [31,32]. TSA also induces embryonic lethality in *Xenopus* with several defects arising in the head and tail regions [33], suggesting a requirement for HDACs during normal embryonic development. HDAC1 and HDAC3 have also been implicated in neurogenesis and the development of craniofacial cartilage, pectoral fin, liver, and pancreas in zebrafish [34–37]. Nevertheless, the differential roles of specific HDACs in heart valve development are still poorly understood.

Heart valve formation is initiated by myocardial BMP signals [1,3]. After receiving these signals, underlying AVC endocardial cells start to undergo EMT necessary for cardiac cushion formation with increasing *notch1b* expression. Recently, it has been reported that failure of embryonic heart valve formation in zebrafish *bng* mutants is caused by enhanced HDAC5 activity with subsequent decreased expression of *notch1b* in AVC endocardial cells [13]. However, the expression of AVC myocardial *bmp4*, a gene upstream of AVC endocardial *notch1b*, is not affected by this mutation, indicating that HDAC5 only plays a negative role in EMT during heart valve formation. On the other hand, our data indicate that other HDACs aside from HDAC5 might positively regulate Wnt/β-catenin signaling upstream of AVC myocardial *bmp4* during heart valve development.

In conclusion, our study showed that TSA-sensitive HDAC activity may play an important role in the initial step of heart valve formation. This would be achieved through the activation

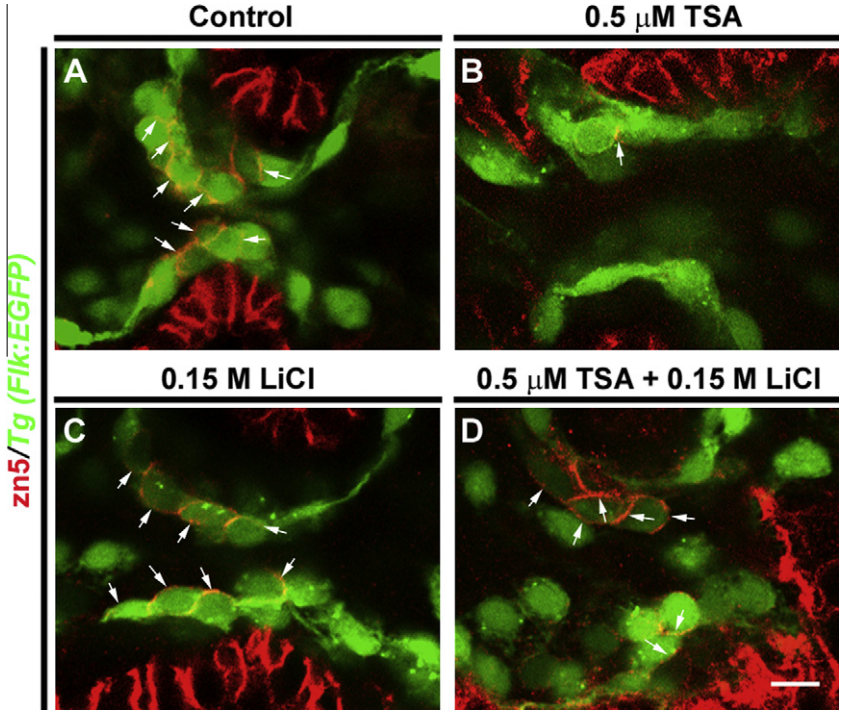

Fig. 4. LiCl rescues defective AVC endocardial EMT in TSA-treated embryos. *Tg(flk1:EGFP)* zebrafish embryos were used to visualize all endocardial cells (green). Myocardial cells were recognized by staining with zn5 antibody (red). AVC endocardial cells undergoing EMT were recognized by GFP expression and zn5 antibody staining. (A) Control embryos. (B) Embryos treated with TSA. (C) Embryos treated with LiCl. (D) Embryos co-treated with LiCl and TSA. AVC endocardial cells undergoing EMT are indicated by arrows. All embryos were collected at 48 hpf and subjected to analyses. Scale bar, 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Wnt/β-catenin signaling required for increased AVC myocardial *bmp4* expression. However, characterization of specific HDACs and detailed mechanisms involved in the regulation of Wnt/β-catenin signaling still need to be elucidated.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.05.098.

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