MicroRNA-23 Restricts Cardiac Valve Formation by Inhibiting Has2 and Extracellular Hyaluronic Acid Production

Anne Karine Lagendijk, Marie Jose Goumans, Silja Barbara Burkhard, Jeroen Bakkers

Rationale: Since their discovery almost 20 years ago, microRNAs have been shown to perform essential roles during tissue development and disease. Although roles for microRNAs in the myocardium during embryo development and cardiac disease have been demonstrated, very little is known about their role in the endocardium or during cardiac valve formation.

Objective: To study the role of microRNAs in cardiac valve formation.

Methods and Results: We show that zebrafish dicer mutant embryos, lacking mature miRNAs, form excessive endocardial cushions. By screening miRNAs expressed in the heart, we found that miR-23 is both necessary and sufficient for restricting the number of endocardial cells that differentiate into endocardial cushion cells. In addition, in mouse endothelial cells, miR-23 inhibited a transforming growth factor-β-induced endothelial-to-mesenchymal transition. By in silico screening of expression data with predicted miR-23 target sites combined with in vivo testing, we identified hyaluronic acid synthase 2 (Has2), Icat, and Tmem2 as novel direct targets of miR-23. Finally, we demonstrate that the upregulation of Has2, an extracellular remodeling enzyme required for endocardial cushion and valve formation, is responsible for the excessive endocardial cushion cell differentiation in dicer mutants.

Conclusions: MiR-23 in the embryonic heart is required to restrict endocardial cushion formation by inhibiting Has2 expression and extracellular hyaluronic acid production. (Circ Res. 2011;109:00-00.)

Key Words: microRNA ■ endocardial cushions ■ cardiac valves ■ zebrafish ■ TGF-beta ■ Has2

The development of valve structures occurs in distinguishable phases, which are highly conserved.1 The first event during valve development is the induction of endocardial cushions (ECs) within the atrioventricular (AV) canal (AVC) and outflow tract of the primitive heart tube. It has been well established that bone morphogenetic protein (BMP), a member of the transforming growth factor (TGF)-β superfamily, is the major myocardial signal that initiates EC formation from human to zebrafish.2–5 Compromised BMP signaling results in downregulation of multiple pathways including TGF-β, Has2 (hyaluronic acid synthase 2), and Notch1, as well as the transcription factors Snail1 and Twist1.2,4 The importance of regulating Has2 expression in the endocardium has been exemplified by the observation that in Has2 deficient mice the cardiac jelly does not expand and ECs fail to form.6 Has2 is responsible for the production of hyaluronic acid (HA), one of the ECM components of cardiac jelly.6 HA production leads to expansion of the extracellular space because it binds salt and water and induces PI3K and ErbB signaling (reviewed by B. Toole7).

Despite differences in the cellular processes that precede valve formation the molecular signals regulating valve formation (eg, Notch, NFAT, ErbB, and TGF-β signaling) have been conserved between annamites and zebrafish.8–11 MicroRNAs are a class of 21- to 25-nucleotide single-stranded noncoding RNAs transcribed from DNA but not translated into protein. Instead, miRNAs interact with messenger RNAs in the cytosol and regulate their final output at the protein level.12 Dicer, an RNase III endonuclease, processes hairpin-like pre-miRNAs into double-stranded miRNA molecules. MiRNA function during organogenesis has been studied mainly by generating conditional Dicer knockout mouse models because conventional Dicer mutant mice are embryonic lethal.13 Conditional depletion of Dicer from the endocardium has been previously performed, using the Tie2-Cre line (which excises Dicer from all hematopoietic and endothelial cells); however, the status of the endocardium in these animals was not reported.13

We report excessive EC formation in dicer null mutants accompanied by a strong increase in cardiac jelly. We found that the loss of miR-23 was responsible for the observed endocardial defects in dicer knockout embryos by regulating
Has2 expression and HA production. Together, our data suggest the existence of a negative miR-23–Has2 feedback mechanism to control EC size in the AVC.

Methods

Fish Lines
Fish were kept under standard conditions. Dicer allele used in this study is dicer<sup>Plk68</sup>.<sup>14</sup> To generate transgenic MZ<sub>dicer</sub> lines, we performed dicer mutant germ cell transplantations into Tg(kdr-l-GFP) embryos as previously described.<sup>15</sup>

Immunohistochemistry
Immunohistochemistry was carried out as previously described.<sup>16</sup> Mouse anti-GFP (Santa Cruz Biotechnology) and mouse anti-zn8 (Dm-grasp, DSHB) were applied 1:200. F-actin was stained with phallloidin-TRITC (Sigma; 1:100). Dm-grasp staining was quantified by manual counting using Volocity software (Improvision). The ratio of Dm-grasp–positive endocardial cells was calculated by the number of Dm-grasp–positive endocardial cells divided by the total number of GFP-positive endocardial cells per heart. We used myocardial Dm-grasp staining as a reference outline of the heart. Statistical significance was determined by a t test (Microsoft Excel). Results are expressed as mean±SEM. ImageJ software was applied to remove myocardial Dm-grasp expression for 3D reconstructions shown in Figures 1, 2, 4, and 7.

In Situ Hybridization
In situ hybridization was carried out as previously described.<sup>17</sup> Embryos were cleared in methanol and mounted in benzylbenzoat/ benzylalcohol (2:1) before pictures were taken.

MiRNA in situ hybridizations were performed as described by Kloosterman et al.<sup>18</sup> Modifications to this protocol were as follows. Overnight fixing in 4% PFA was followed by an additional overnight fixation in 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) to prevent diffusion of the signal.<sup>19</sup> We used an LNA-modified miR-23a probe (1:500 Exonq), sequence 5’-tggaatccctggcaatgtgat-3’, DIG modified on both ends.

Morpholino and RNA Injections
Morphpolo oligonucleotides (MOs; Gene Tools) were dissolved in water to 5 mmol/L (miR-23a MO) and 1 mmol/L (p53 MO and Has2 MO). For injection, MOs were diluted to concentrations between 0.2 mmol/L and 1 mmol/L, of which 1 mL was injected at the one cell stage. In a dose-response experiment, the most optimal concentration for the miR-23 MO was determined as 0.5 mmol/L. Capped zebrafish Has2 RNA was prepared with the SP6 Message Machine kit (Ambion) and injected at 50 ng/μL. MiRNA mimics for miR-430, miR-23, and miR-27 were all injected at 200 ng/μL. Morpholino and miRNA mimic sequences are provided in the Online Supplement Materials and Methods at http://circres.ahajournals.org.

Quantitative Reverse Transcriptase–PCRs
Total RNA from mouse embryonic endothelial cells (MEECs) was isolated using TRIzol Reagent (Invitrogen). cDNA was transcribed using Superscript III reverse transcriptase (Invitrogen). Reverse transcriptase (RT)-PCRs were performed using RedTaq polymerase (Sigma).

Sequences of qRT-PCR primers and detailed protocol are provided in the Online Supplement Materials and Methods at http://circres.ahajournals.org. Results are expressed as mean fold change ±SEM. Statistical significance was determined by a t test (Microsoft Excel).

HA Staining
Staining was performed on parafin sections as described previously.<sup>20</sup> Serum-free medium of HEK293 cells expressing a neurocan-alkaline phosphatase fusion protein was a generous gift from Uwe Rauch, University of Lund.

GFP Silencing Assays
We use a full-length tmem2 3’-UTR (full length 3’-UTR for Has2 and icat prevented stable GFP expression). In addition, a 111 bp, 165 bp, and 348 bp PCR fragment from the zebrafish Has2 3’ UTR, tmem2 3’UTR, and icat 3’ UTR, respectively, was amplified, each containing the predicted miR-23 target sites. Primer sequences used and cloning strategy are provided in the Online Supplement Materials and Methods at http://circres.ahajournals.org.

Cell Culture
MEECs were cultured as previously described.<sup>21</sup> MEECs were transfected with 50 μmol/L miR-23 mimics duplex oligos (IDT), using oligofectin, following the manufacturer’s protocol (Invitro-

Figure 1. MiRNAs are required to restrict endocardial cushion differentiation. A and B, left, Hyaluronic acid (HA) staining (blue) at 2 dpf in wild-type (A) and MZ<sub>dicer</sub>+430 (B) embryos counterstained with neutral red. A and B, right, HA staining in ventricle wall (boxed in A and B). Myocardial cell is indicated as m; endocardial cell, e. Restricted endocardial expression of Has2 in wild-type endocardial cushions (C) and ectopic Has2 expression in MZ<sub>dicer</sub> mutant chambers (D). E and F, Three-dimensional projections of endocardial-specific Dm-grasp expression (yellow) marks endocardial cushion cells.
pericardial edema was evident in miR-23 morphant (n = 18/18 Figure 1B) compared with wild-type embryos (Figure 1A).

**Ectopic Expression of EC Markers in MZdicer Embryos**

We analyzed patterning of myocardial AVC differentiation in MZdicer+430 mutant embryos. *Nppa* (natriuretic peptide precursor type A) is expressed in ventricular and atrial working myocardial cells, whereas *bmp4* (bone morphogenetic protein 4) shows a reciprocal expression pattern, restricted to the AVC myocardium. Spatial expression patterns of *nppa* and *bmp4* were unaffected in MZdicer+430 mutants (Online Figure I), demonstrating correct patterning of the myocardium.

Next, we analyzed differentiation of the endocardium. Expression of *nfatc1* (nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1) in MZdicer+430 mutant embryos demonstrated the presence of an endocardial lining throughout the heart tube (Online Figure I). Normally,

**Results**

**Cardiac Jelly and ECs of Dicer Mutants Are Structurally Altered**

To study miRNA function during heart development, we generated wild-type females carrying a homozygous *dicer* mutant germline by germ cell transplantation. Embryos derived from such females (lacking all maternal and zygotic *dicer* [MZdicer]), were injected with miR-430 to clear the embryo from maternal mRNA transcripts and thereby rescue early gastrulation defects. At 3 days after fertilization (dpf), pericardial edema was evident in MZdicer+430 mutant embryos (Online Figure I). However, ventricular and atrial chamber differentiation occurred normally, and both chambers maintained contractile capabilities (Online Figure I). The size of the heart and cardiac looping, however, were impaired in MZdicer+430 mutant embryos (Online Figure I). We consistently observed lumen narrowing in both the atrium and the ventricle accompanied by increased space between the endocardium and myocardium, where the cardiac jelly resides (Online Figure I). Because the extracellular glycosaminoglycan HA is a major component of the cardiac jelly, we analyzed HA levels in the heart tissue. We detected excessive deposition of HA between the endocardium and the myocardium, indicating an excessive production of cardiac jelly (Online Figure I). We consistently observed lumen narrowing in both the atrium and the ventricle accompanied by increased space between the endocardium and myocardium, where the cardiac jelly resides (Online Figure I). Because the extracellular glycosaminoglycan HA is a major component of the cardiac jelly, we analyzed HA levels in the heart tissue. We detected excessive deposition of HA between the endocardium and the myocardium, indicating an excessive production of cardiac jelly components in MZdicer mutants (n = 18/18 Figure 1B) compared with wild-type embryos (Figure 1A).

**Western Blot Analysis**

MEECs were lysed in sample buffer containing 10% SDS, 20% glycerol, 0.1% bromophenol blue, 5% β-mercapto-ethanol, and Tris HCl, pH 6.8. Homogenates were size-fractionated on 10% PAGE gels and transferred to Hybond PVDF membranes. Membranes were incubated with the anti–α-smooth muscle actin (SMA) (1:2000; Sigma) and anti-Pecam-1 (1:1000; Santa Cruz) followed by incubation with a horseradish peroxidase–labeled secondary antibody.

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a small population of endocardial cells located at the AVC form ECs, marked by Has2 expression (Figure 1C). Consistent with the increased levels of HA in the cardiac jelly in MZdicer+430 mutants, we found that Has2 expression was no longer restricted to AVC endocardium but was expanded into the endocardium lining the chambers (n=37/39, Figure 1D). Ectopic Has2 expression was observed in both chambers but was most prominent in the ventricle. For osteopontin or secreted phosphoprotein 1 (Spp1), another marker for differentiated ECs, low levels were observed in wild-type ECs, whereas ectopic and high levels of Spp1 were observed in MZdicer mutant embryos (Online Figure I). Formation of the ECs in zebrafish is accompanied by endocardial cell-shape changes, from squamous to cuboidal, and expression of the cell adhesion molecule, Dm-grasp/Alcam.8 We examined spatial Dm-grasp expression in wild-type and MZdicer+430 mutant embryos carrying the Tg(kdr-l:GFP) transgene, in which all endothelial cells are labeled by GFP. In wild-type embryos, Dm-grasp was expressed in all myocardial cells and endocardial cells located in the AV region (Online Figure II). In MZdicer+430 mutant embryos, however, we observed ectopic Dm-grasp expression in endocardial cells aligning the chambers with preference for the ventricle (Online Figure II). On quantification of the ratio of endocardial cells that express Dm-grasp, we found a 2-fold increase of Dm-grasp–positive endocardial cells in MZdicer+430 mutants (Dm-grasp expression index in wild-type, 0.21±0.01, n=6, and in MZdicer+430, 0.41±0.03, n=7; P<0.01) (Online Figure II). To analyze the structure of the endocardial cushions, we made 3D reconstructions of the endocardial Dm-Grasp expression by omitting its myocardial expression. In the 3D reconstruction of endocardial Dm-Grasp in a wild-type heart, compact superior and inferior cushion structures were observed (Figure 1E). In MZdicer+430 mutants, Dm-Grasp–expressing cells did not form separated cushions but instead formed a large structure composed of both AVC and chamber endocardial cells (Figure 1F). In addition to the ectopic Dm-grasp expression, we also observed a change in endocardial cell shape. In wild-type embryos, AVC endocardial cells are cuboidal with cytoplasmic protrusions, whereas chamber endocardial cells are flat and squamous (Online Figure II). Interestingly, in MZdicer+430 mutant embryos, chamber endocardial cells were like wild-type AVC cells, also cuboidal with cytoplasmic projections (Online Figure II). Furthermore, we observed in MZdicer+430 mutant embryos (n=3/15) endothelial cells that resided in the extracellular space of the cardiac jelly (Online Figure II). We never observed such cells in wild-type hearts (n=0/10). Together, these results reveal that a loss of miRNAs has a significant impact on regional endocardial differentiation during AV valve formation independent of myocardial patterning.

**Loss of MiR-23 Results in Enhanced EC Formation**

To identify the mechanism responsible for the observed ectopic expression of Has2 and remodeling of ECs in MZdicer+430 mutants, we searched for miRNAs that are expressed in the heart. We injected antisense MO, targeting the mature sequence of 9 miRNA families for which cardiac expression had been reported23 (for a complete list, see Online Supplement Experimental Procedures). MO-injected embryos were examined morphologically at 2 and 3 dpf for blood pooling on the yolk and thickness of the cardiac jelly and by in situ hybridization for Has2 expression (data not shown). Only the injection of a miR-23 MO resulted in cardiac defects similar to what we observed in MZdicer+430 mutants (described further below). There are 5 copies of miR-23 family members in the zebrafish genome, all of which reside in polycistronic clusters together with miR-24 and miR-27 family members (Online Figure III). The MO designed to target miR-23 family members targets all 5 members, resulting in an efficient knockdown (Figure 2A through 2C). Interestingly, on miR-23 knockdown, we observed enhanced expression of Has2 at 2dpf (n=21/24) and 3dpf (n=27/33) (Figure 2D through 2G). Besides an increase in Has2 expression level, we also observed ectopic Has2 expression in the chambers, although this expansion was less pronounced compared with MZdicer+430 mutant embryos. Concomitant with the enhanced Has2 expression, we observed a thickening of the cardiac jelly containing HA fibers in miR-23 morphants (n=8) (Figure 2H and 2I). Injection of p53 MOs, which has no reported phenotype,24 did not alter Has2 (n=16/16) or HA expression (n=5/5) (Online Figure IV). Patterning of myocardial cells in the AVC appeared normal on loss of miR-23 (Online Figure IV). In agreement with our previous observations in MZdicer+430 mutant embryos, we observed a significant increase in endocardial Dm-grasp expression in miR-23 knockdown embryos (Dm-grasp expression index in miR-23 MO, 0.36±0.02, versus 0.21±0.01 in wild-type embryos; P<0.01) (Figure 2J through 2L). These results demonstrate that the loss of miR-23 results in endocardial defects, which are comparable to those observed in MZdicer+430 mutants.

**Reexpressing MiR-23 Rescues Ectopic EC Differentiation in MZdicer Mutants**

By in situ hybridization, we observed expression of miR-23 starts at 2 dpf. Interestingly, expression of miR-23 was confined to the ECs (n=20/25, Figure 3A through 3D) and was still detected in the adult valve leaflets (n=3) (Figure 3E). To address whether a loss of miR-23 was indeed causative for the observed ectopic expression of Has2 and Dm-grasp in MZdicer+430 mutant embryos, we coinjected MZdicer embryos not only with miR-430 but also with an miR-23 mimic. Reintroducing miR-23 in MZdicer+430 mutant embryos resulted in a clear reduction (8/23) or loss (15/23) of Has2 expression (Figure 4A and 4B and Online Figure IV). Injection of miR-27 mimics or mutated miR-23 mimics, however, did not rescue ectopic Has2 expression (Online Figure IV). Reduction in Has2 expression on miR-23 overexpression was accompanied by a strong reduction in cardiac jelly size and HA production (n=6/6) (Figure 4C and 4D). In addition, miR-23 mimic injection in MZdicer+430 mutant embryos led to a rescue of the ratio of endocardial cells expressing Dm-grasp to wild-type values (MZdicer+430+23, 0.22±0.02, versus wild-type, 0.21±0.01) (Figure 4E through 4G). Together, these results demonstrate that miR-23 exerts a restricting effect on
MiR-23 Inhibits TGF-β–Induced Endothelial-to-Mesenchymal Transition in Mouse Endothelial Cells

In mouse embryos and chick embryos, EC cells undergo a TGF-β–induced endothelial-to-mesenchymal transition (Endo-MT), which requires HA production by Has2. To examine the role of miR-23 during TGF-β–induced Endo-MT, we used cultured MEEC. TGF-β stimulation of MEEC induced the expression of α-smooth muscle actin (α-SMA), a protein expressed by mesenchymal and fibroblast-like cells (Figure 5A). In addition, other Endo-MT–related genes such as Snail1, Has2, and fibronectin, were also upregulated in MEECs after treatment with TGF-β (Figure 5B). Platelet endothelial cell adhesion molecule-1 (Pecam-1) expression conversely decreased, indicative of a reduction in endothelial cell-cell interactions and a loss of endothelial cell morphology (Figure 5A). TGF-β–treated cells exhibited a fibroblast-like morphology compared with their rounded, cobblestone appearance in the absence of TGF-β (Figure 5C and 5D). In addition, loss of endothelial intercellular connections results in remodeling of the actin cytoskeleton. During Endo-MT, cortical f-actin translocates to intracellular stress fibers of transformed mesenchymal cells (Figure 5G and 5H). Transfection of MEECs with miR-23 mimics before the TGF-β stimulation prevented upregulation of α-SMA, Snail1, Has2, and TGF-β–induced factor (Tgf) (Figure 5A and 5B). Furthermore, miR-23–transfected MEECs did not obtain a fibroblast-like morphology, and f-actin remained at the cortical surface of the cells (Figure 5E, 5F, 5I, and 5J). Together, these results demonstrate that in mouse ECs, ectopic miR-23 inhibits TGF-β–induced Endo-MT.

Identification of Novel MiR-23 Target Genes

To identify putative miR-23 target-genes that would explain how miR-23 might exert its regulation of cardiac valve formation, we set up an in silico screen. We identified genes specifically expressed in mouse embryonic hearts at E10.5. Next, we computationally selected those genes that also contained miR-23 recognition sequences defined by strict target prediction criteria using TargetScan software, resulting in 122 candidate genes (Online Table I). Since we observed that miR-23 inhibits TGF-β–induced EMT in mouse endothelial cells, we selected those candidate genes that are expressed during early stages of EMT. By RT-PCR analysis on unstimulated and TGF-β–stimulated MEECs, we identified 48 candidate genes that are upregulated during TGF-β–induced EMT (Figure 6A and Online Table I). To identify which of these cardiac genes are true miR-23 target genes, we selected 15 candidate genes for which the zebrafish homologue also contains an miR-23 binding site. We amplified them each individually downstream of an eGFP coding sequence for use in a GFP silencing assay. We only observed strong silencing of the GFP signal with miR-23 mimics when the Has2, icat, and tmem2 3’UTR sequences were present (Figure 6B through G, Online Figure V, and Online Table I). Silencing of these GFP-3’UTR mRNAs was miR-23–dependent, because no silencing was observed on coinjection of a mutated miR-23 mimic (Figure 6H through J) and mutating 2 nucleotides within the seed sequence of miR-23 target sites (Online Figure V). Together, these data reveal that miR-23 targets several transcripts expressed in the embryonic heart.

Ectopic Has2 Expression in MZdicer Mutants Is Responsible Excessive EC Formation

Because we identified Has2 as an miR-23 target gene and Has2 expression was upregulated both in MZdicer mutant and miR-23 knockdown hearts, we investigated its expression in more detail. We observed that in the linear heart tube of zebrafish embryos, Has2 was expressed in few endocardial cells already at 30 hpf (Online Figure VI). This early endocardial Has2 expression precedes the previously described Dm-Grasp expression and cellular changes initiating EC formation at 36 hpf. To address whether ectopic Has2 can induce Dm-Grasp expression in the endocardium, synthetic Has2 mRNA was injected into 1-cell stage embryos. As a consequence of ectopic
Has2 expression, we observed thickening of the cardiac jelly, which stained positive for HA (n=8/8) (Figure 7A). In addition, injection of Has2 mRNA into wild-type embryos significantly induced Dm-grasp expression throughout the endocardium (wild-type, 0.22±0.01, versus Has2 RNA, 0.71±0.05; P<0.01) (Figure 7B, B’, and 7G). We subsequently investigated whether Has2 is required to induce Dm-grasp expression in ECs of wild-type embryos by injecting previously characterized Has2 morpholinos. We observed in Has2 MO-injected embryos retrograde blood flow from the ventricle back to the atrium, suggestive for compromised EC formation. When we examined Dm-grasp expression in Has2 knockdown embryos, we observed a near complete loss of Dm-grasp expression in the endocardium (Figure 7G and Online Figure VII) (wild-type, 0.22±0.01, versus Has2 MO, 0.01±0.01; P<0.01). In addition, endocardial cells located in the AVC remained squamous (Online Figure VII).

Finally, we examined whether Has2 was required for the enhanced cardiac jelly production and ectopic Dm-Grasp expression observed in miR-23 knockdown and MZdicer mutant embryos. Therefore, the Has2 MO was either co-injected with the miR-23 MO in wild-type embryos or co-injected with the miR-430 mimic in MZdicer mutant embryos. In both cases, the Has2 knockdown caused a reduction in cardiac jelly and HA production (miR-23+ Has2 MO, n=6/6; MZdicer+430+ Has2MO, n=5/5) (Figure 7C, 7E, 1B, and 2I). In addition, the Has2 knockdown in MZdicer mutant or in miR-23 knockdown embryos caused a significant reduction in the ratio of endocardial cells expressing Dm-grasp (miR-23 MO, 0.36±0.02, versus 0.14±0.02 in miR-23+Has2 MO co-injected embryos; MZdicer+430, 0.41±0.03, versus MZdicer+430+Has2 MO, 0.04±0.03; P<0.01) (Figure 7D and 7D’, 7F and 7F’, 7G, 1F, 2K and 2K’). In summary, these results demonstrate that Has2 is required and sufficient to induce Dm-Grasp expression in the endocardium and that the ectopic Has2 activity observed in MZdicer mutant hearts is the most likely cause of the excessive number of Dm-Grasp-positive EC cells in MZdicer mutant hearts.

Discussion
To summarize, we have characterized the endocardial defect in dicer mutant zebrafish embryos and thereby identified excessive EC formation caused by a loss of miR-23 activity. We conclude that miR-23 is required to restrict EC formation by restricting Has2 expression and HA production. Unexpectedly, both miR-23 and its target Has2 are expressed in EC cells, suggesting the existence of an intrinsic negative feedback mechanism in the ECs that controls the size of the ECs (see model in Online Figure VIII).

The first visually recognizable process during AV valve formation in the zebrafish heart is a change in cell shape of endocardial cells in the AV region.8 The transition from squamous to cuboidal cells is first apparent at 36 hpf and is accompanied by expression of the cell adhesion molecule Dm-grasp/Alcam. We showed that endocardial Has2 expression is already detected at 30 hpf and thereby precedes the endocardial cell shape changes and Dm-grasp expression, suggesting a role for Has2 in this process. Indeed, we observed that in Has2
knockdown embryos, Dm-grasp expression was nearly lost, whereas ectopic expression of Has2 induced Dm-grasp expression. At 2 dpf, the superior and inferior ECs become apparent in the AVC, and at this stage, miR-23 expression was first observed in the ECs. The level of endocardial miR-23 expression increased over time and was still present in the valve leaflets of the adult heart. Simultaneously with the increase in miR-23 expression in the ECs, the level of Has2 expression declines resulting in very low expression at 3 dpf (Online Figure VI). At 3 dpf, Dm-grasp expression is downregulated and the ECs start to form valve leaflets through a process of invagination.8,10 Interestingly, we observed that in embryos lacking miR-23, the downregulation of Has2 expression in ECs at 3 dpf did not occur. Because we found that Has2 is a direct target for miR-23, it suggests that miR-23 in the ECs is required to downregulate Has2 expression during the process of valve formation, resulting in a negative feedback mechanism (Online Figure VIII). Consistently, in dicer mutant or miR-23 knockdown embryos, recognizable valve leaflets were never formed. Instead, the endocardial cells remained cuboidal-shaped and loosely attached to each other, resulting in endocardial ruptures and allowing blood cells to invade the cardiac jelly (A.L. and J.B., unpublished data).

Although cardiac expression of miR-23 was restricted to the ECs, we observed ectopic Has2 and Dm-grasp expression in endocardial cells outside of the AVC in MZ dicer mutant and miR-23 knockdown embryos. Possibly, this non–cell-

Figure 5. MiR-23 inhibits TGF-β-induced Endo-MT in mouse embryonic endothelial cells. A, Western blot analysis for α-SMA (top) and Pecam-1 (middle) protein expression in unstimulated MEECs or MEECs stimulated with 1 or 5 ng of TGF-β and/or miR-23. GAPDH protein levels were used as a loading control (bottom). B, Quantitative RT-PCR analysis of Snail1, fibronectin, Has2, and Tgif expression relative to unstimulated MEECs (n=3 experimental replicates). Error bars represent mean±SEM; t test analyses resulted in statistically significant differences, with *P<0.01 and #P<0.05. C through F, Bright-field images (×40) showing fibroblast-like transformation on TGF-β stimulation. G through J, Phalloidin staining of f-actin (red). Cortical f-actin in unstimulated MEECs (G) translocates to stress fibers of mesenchymal cells on TGF-β stimulation (H). F-actin remained at the cortical surface of the cells when transfected with miR-23 mimics (I and J).
autonomous role for miR-23 is explained by the autoregulatory loop between Has2 and its product extracellular HA. In epithelial cells, extracellular HA stimulates the PI3K pathway through the CD44 receptor, and activation of the PI3K pathway results in increased Has2 expression and extracellular HA production.26–27 In addition, the HA/CD44/ErbB2 interaction induces β-catenin activation, which results in the induction of Has2 expression.28–31

In the avian and mammalian heart, endocardial cells overlaying the ECs undergo Endo-MT, which can be induced by TGF-β and requires Has2 activity.1,6 Our data demonstrate that miR-23 inhibits the TGF-β–induced Endo-MT in mouse embryonic endothelial cells, suggesting a conserved role for miR-23 in restricting EC formation and highlighting the need for future investigations into the role of miR-23 during mammalian valve formation.

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Disclosures

References
Novelty and significance

What Is Known?

- Atrioventricular valves are formed from the endocardial cushions located in the atrioventricular canal of the vertebrate embryonic heart.
- Transforming growth factor signals originating from the atrioventricular canal myocardium induce endocardial cushion formation.
- Hyaluronic acid synthase 2 is required for endocardial cushion and valve formation.
- The zebrafish embryo is an excellent model to identify and characterize essential regulators of valve formation.

What New Information Does This Article Contribute?

- MicroRNA-23, a small noncoding RNA, is present in the embryonic and adult atrioventricular valves of the zebrafish heart.
- MicroRNA-23 is required in the endocardium to restrict the number of endocardial cells that differentiate and contribute to the cardiac valves.
- Hyaluronic acid synthase 2 is a direct target of microRNA-23 and is sufficient and required to induce endocardial cushion differentiation.
- Mechanistically, microRNA-23 provides a negative feedback loop to restrict extracellular matrix production by inhibiting hyaluronic acid synthase 2 activities.

Cardiac malformations present at birth are a major class of clinically significant congenital defects that arise during embryonic development, but currently there is very limited understanding of their origin. For improved treatment and prevention, a better understanding of the causes of congenital heart malformations is required. Most frequently, the cardiac malformations identified in the young affect the functionality of the valves (aortic, pulmonary, mitral, or tricuspid). It has been well documented that the cardiac valves originate from the endocardium that locally forms endocardial cushions; however, there is limited understanding of the signals that regulate and in particular restrict this process to these local regions. In the present study, we used the zebrafish model to identify and characterize a small, noncoding RNA molecule (microRNA-23) that is essential to restrict the endocardial differentiation process to the valve-forming region of the atrioventricular canal. Because microRNA-23 expression is confined to the endocardial cushions, we propose the presence of a negative feedback mechanism within the differentiating endocardium to prevent ectopic differentiation outside the valve-forming region. These novel findings pose new insights into the molecular and genetic regulation of endocardial cushions and thereby valve formation during embryonic development.
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Supplemental Experimental Procedures

Genotyping dicer mutant allele
The following nested PCR primers were used for dicer genotyping;
Forward_1 5’-gcagttagtcttagggaagc-3’
Reverse_1 5’-agtgcatagttgcaatctcaag-3’
Forward_2 5’-tggtattgaaagtgtacattgc-3’
Reverse_2 5’-catcagtttagcattcctgtagtg-3’
Reverse_2 was used for subsequent sequencing.

Morpholino and RNA injections
Morpholino sequences:

<table>
<thead>
<tr>
<th>MiRNA</th>
<th>MO sequence</th>
<th>Expression in heart</th>
</tr>
</thead>
<tbody>
<tr>
<td>MiR-21</td>
<td>GCCAACACCAGTCTGATAAGCTA</td>
<td>Valves</td>
</tr>
<tr>
<td>MiR-23</td>
<td>GGAATCCCTGGCAATGTGAT</td>
<td>Valves</td>
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<tr>
<td>MiR-24</td>
<td>CTGTTCCTGCTGAACGTGAGCCA</td>
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<tr>
<td>MiR-126</td>
<td>GCATTATTACTCAGGACGTG</td>
<td>Whole heart</td>
</tr>
<tr>
<td>MiR-138</td>
<td>GGCCTGCTTACACAACACCAGCT</td>
<td>Outflow tract</td>
</tr>
<tr>
<td>MiR-143</td>
<td>GAGCTACAGTGCTTCATCTCA</td>
<td>Whole heart</td>
</tr>
<tr>
<td>MiR-145</td>
<td>GGGATTCCCTGGGAAAACGTGGAC</td>
<td>Outflow tract</td>
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<tr>
<td>MiR-150</td>
<td>CACTGGTACAGGATTTGGGAGA</td>
<td>Valves</td>
</tr>
<tr>
<td>MiR-499</td>
<td>TAAACATCAGGACTGCTTCAA</td>
<td>Whole heart</td>
</tr>
</tbody>
</table>

*Has2* morpholino was described previously \(^1\).

miRNA mimics
Since the mature miRNA sequences of zebrafish miR-23a and miR-23b are identical apart from a single base at the 3’end we assumed that the miR-23a morpholino used here will also target the mature miR-23b sequence.
For miRNA mimic injection we designed RNA duplex oligos (IDT) having a 2 nucleotides overhang on both ends and a single nucleotide mismatch at the 3’ end of the star sequence to ensure RISC loading of the guide strand. RNA sequences were as follows (mismatch bases in star sequence are underlined).
MiR-430a guide 5’-UAAGUGCUAUAUUUGUUGGGTA-3’
MiR-430a star 5’-CCCCAACAAAUAGCACUATT-3’
MiR-23a guide 5’-AUCACAUUGCCAGGGAUUUCCT-3’
MiR-23a star 5’-GAAAUCCCUGGCAUUGUGUTT-3’
MiR-23a mismatch guide 5’-AUCGCGUUGCCAGGGAUUUCCT-3’
MiR-23a mismatch star 5’-GAAAUCCCUGGCAACGCGUUTT-3’
MiR-27 guide 5’-UUCACAGUGGCUAAGUUCCGC-3’
MiR-27 star 5’-GGAACUUAGCCACUGUGUATT-3’

The last 4 bases at the 3’end of the mature miRNA sequence of the 5 zebrafish miR-27 copies vary. Therefore we designed the miR-27 guide sequence as such that the last 3 nucleotides would be represented by the most common nucleotide present at the corresponding position of all 5 copies. All miRNA duplexes were diluted to a concentration of 200 ng/µl of which 1nl was injected at the one cell stage.

**In situ hybridization**

_in situ_ hybridization was carried out as previously described. Embryos were cleared in methanol and mounted in benzylbenzoaat/ benzylalcohol (2:1) before pictures were taken. Contrast enhancement on whole picture was performed using Adobe Photoshop 7.0 software. A _nppa_ probe was generated from a 400 bp zebrafish _nppa_ fragment cloned into a PCRII vector (Invitrogen). A _nfatc1_ was generated from Image clone 7258781 containing a 900 bp EST fragment of zebrafish _nfatc1_.

MiRNA _in situ_ hybridizations were performed as described by Kloosterman et al. Modifications to this protocol were as follows. Overnight fixing in 4% PFA was followed by an additional overnight fixation in 1-ethyl-3-(3-dimethylaminopropyl) carboidiimide (EDC) to prevent diffusion of the signal. We used a LNA modified miR-23a probe (1:500 Exiqon), sequence 5’-tggaaatccctggcaatgtgat-3’, DIG modified on both ends.

**Plastic sectioning**

Embryos were mounted in Technovit 8100 (Kulzer). Sections of 7 µm thickness were cut with a microtome (Reichert-Jung 2050). Counterstaining was done using 0.05% neutral red, staining all nuclei. Sections were mounted in Pertex.

**RNA isolation and Northern blotting**

Total RNA from embryos was isolated using TRIzol Reagent (Invitrogen). For Northern Blotting RNA was separated on 12,5% polyacrylamide gels. DIG labeled
(3’end) LNA and DNA probes complementary to miR-23a (LNA probe, Exiqon) and 5S RNA (DNA probe: ATCGGACGAGATCGGGCGTA) respectively, were used for overnight hybridizations at 60°C. The complete Northern Blot procedure was performed as described 5.

**Quantitative reverse transcriptase (RT)-PCRs**

Primer sequences for qRT-PCR were as follows:

- **gapdh** forward: TCAACAGCAACTCCCACTCTT
- **gapdh** reverse: ACCCTGTTGCTGTAGCCGTAT
- **has2** forward: ACGGTGGGATGATGTCTTGGC
- **has2** reverse: ATGATCCACCCACACAAAGCAT
- **fibronectin** forward: ATCACAGTAGTTGCGGCAGGA
- **fibronectin** reverse: TGTCATAGTCAATGCCAGGCT
- **snail1** forward: ACACTGGTGAGAAGCCATTCT
- **snail1** reverse: TCTTCACATCCGAGTGGGTTT
- **tgf** forward: TCCACCAGACCTCAACCAGGA
- **tgf** reverse: AGTTTGCCCTGAAGCTCCATC

For quantitative RT-PCRs all cDNA samples were adjusted to 500ng/µl. A standard curve was defined for each primer pair using a cDNA dilution series (1:1 (500 ng/µl), 1:10 (50 ng/µl), 1:100 (5 ng/µl), 1:1000 (0.5 ng/µl)). We amplified 2 ng of each experimental cDNA sample in triplicate using SYBR green supermix (BioRad). To analyse amplicon content we applied the ΔCt-method using the housekeeping gene **gapdh** as a reference. Fold changes in expression levels between cDNA samples were normalized against expression changes in **gapdh**. To ensure comparability of different primer pairs, expression levels were also normalized against the primer efficiency calculated from the standard curve using MyIQ software (BioRad). Results are expressed as mean fold change ± s.e.m. Statistical significance was determined by a t-test (Microsoft Excel).

**GFP silencing assays**

Carried out as previously described 6.

Primer sequences for amplification of miR-23 target sites

- **has2** UTR forward: 5’-ggattccCACCAAAGATTCTCTATTTTCA-3’
The PCR fragments were cloned into pCS2 (Clontech), containing the eGFP cDNA sequence between restriction sites BamHI and ClaI (pCS2-eGFP). UTR fragments were cloned downstream of eGFP between EcoRI and XbaI (restriction enzyme recognition sequences are underlined in previous primer sequences) resulting in reporter construct.

RFP RNA, transcribed from pCS2-RPF plasmid, was co-injected as a control. Mutations in the miR-23 seed sequences were introduced by site directed mutagenesis using high-fidelity Phusion DNA polymerase (Finnzymes). Primer sequences used are provided in supplementary Materials and Methods.

Capped RNA was prepared with the SP6 Message Machine kit (Ambion). GFP and RFP expression levels were inspected at 1dpf using a Leica MZFLIII microscope and photographed with a digital camera. For GFP and RPF panels the red and green levels respectively were adjusted to 0 using Adobe Photoshop 7.0 software.

Primer sequences (mutated bases are underlined):

**has2** UTR reverse: 5'-gctctagaTGCTGTACATCACAGAGACAA-3'

**tmem2** UTR forward: 5'-cggaattccgGTGAGCCTTTTACTAGATGTAATC-3'

**tmem2** UTR reverse: 5'-gctctagaAACCACAATCACGCAAGAC-3'

**icat** UTR forward: 5'-cggaattccgATTTGACTGAATGGCGTCAG-3'

**icat** UTR reverse: 5'-gctctagaCATTTAAGTCAAGGGACGAC-3'

Primer sequences (mutated bases are underlined):

**has2** UTR-mut forward:
5'-CTTGCCCTATCTGACCAAAATGCGCGATAAGGAGCTGGATTTGTC-3'

**has2** UTR-mut reverse:
5'-GACAAATCCAGCTCCTATTGAGCATTGTGCAGATAGGGAAG-3'

**tmem2** UTR-site1 mut forward:
5'-GTGAGCCTTTTACTAGATGCGCGATAAGGAGCTGGATTTGTC-3'

**tmem2** UTR-site1 mut reverse:
5'-GCTGGTTGTGTTGTTGATTGGCATCTAGTAAAGGCTCAC-3'

**tmem2** UTR-site2 mut forward:
5'-CCAGCAAATGAATGCCAGTTTATTTATTTTGGC-3'

**tmem2** UTR-site2 mut reverse:
5'-GCCAAAATAAATTACGTTCATGAGTTGGCATTTGCTCAG-3'

**tmem2** UTR-site3 mut forward:
5'-GGACTTAAGTATTTGTTATGCCAGTTTGTGCAGATAGGGAAG-3'

**tmem2** UTR-site3 mut reverse:
5'-GGACTTAAGTATTTTGTAGTCCAAGGAGCTGGATTTGTC-3'
5’-GACAAATACATCCACTGGCATAACAAATACTTAAGTCC-3’

*icat* UTR-site1 mut forward:
5’-CTCGCTTGAGCTGTTAAACTATTTAATGCCAAATGCTTTTT-3’

*icat* UTR-site1 mut reverse:
5’-AAAAGCAATTGGGATTTAAATAGTTTAACAGCTCAAGCGAG-3’

*icat* UTR-site2 mut forward:
5’-GTCAAGGTTTCTTGATCCATGCCAACCGTCGGATTATCTCAC-3’

*icat* UTR-site2 mut reverse:
5’-GTGAGATAATCAGCGTGGCATGGACAAAAAACCCTTGAC-3’
References


Online Figure I: Loss of miRNA function does not alter myocardial patterning but does induce ectopic differentiation of endocardial cushion cells.

(a) Live images of a wild-type (top) and MZdicer+430 mutant embryo (bottom) at 3dpf. (b,c) Hematoxylin and eosin staining on transversal sections of a wild-type (b) and MZdicer+430 mutant heart (c). Ventricle (V), atrium (A). Myocardial cell (black arrow), endocardial cell (black arrowhead). Blood cells occupy the atrial lumen of the wild-type heart.

(d-i) Myocardial expression of the myosin genes cmlc2 (d,e), vmhc (f,g) and amhc (h,i) at 2dpf in wild-type (d,f,h) and MZdicer+430 mutant (e,g,i) embryos. (j,k) Patterning of the myocardium in working myocardium (nppa expression in j and k) and AVC myocardium (no nppa expression, indicated with black arrowheads in j and k) was not affected in MZdicer+430 mutant embryos. (l,m) Myocardial bmp4 expression at 2dpf in wild-type (l) and MZdicer+430 mutant (m) embryos. Black arrowheads indicate the AV boundary and dotted lines outline the heart tube; the arterial pole (outflow) is to the top and the venous pole (inflow) to the bottom.

(n-q) Endocardial expression of nfatc1 (n,o) and spp1 (p,q) at 2dpf. In wild-type EC cells (p) spp1 was very low while in MZdicer+430 mutant embryos (q) spp1 expression was increased and at ectopic places.
Online Figure II: Ectopic Dm-grasp expression in accompanied by aberrant endocardial cell morphology in MZdicer mutants.

(a-d’). Immunofluorescence staining for Dm-grasp (red, myocardium and endocardial cushion cells) in Tg(kdr-l:GFP) hearts (green, endocardium) of wild-type (a-b’) and MZdicer+430 (c-d’) embryos at 2dpf. Ventricle (V), Atrium (A). Panels b, b’, d and d’ show single confocal images of the ventricle with the AVC to the right. AVC (white arrowheads in a and c). Endocardial cushion cells located at the AVC (white arrows in b’ and d’). (e) Ratio of Dm-grasp expressing endocardial cells in wild-type (n=6) and MZdicer+430 (n=7) embryos at 2dpf. Error bars represent means ± s.e.m. T-test analyses resulted in statistically significant differences with p<0.01 (*). (Q-R’)

(f) In wild-type hearts endocardial cells positioned in the ECs (Dm-grasp positive, indicated with white asterisk) appeared cuboidal, while neighboring endocardial cells located in the chambers appeared squamous (white arrows in f). (g,h) MZdicer+430 ventricular endocardial cells appeared cuboidal and made extensive cytoplasmic protrusions (white arrowheads in g and h) into the cardiac jelly. (i) 3D projection of MZdicer+430 mutant endocardium showing a group of cells located outside the endocardial lining (white arrowhead); ventricle (V), atrium (A). (j) Individual cell (white arrowhead) from MZdicer heart shown in i located in the cardiac jelly between the endocardium (green cells) and myocardium (red cells). (k) Phalloidin staining for f-actin (red) on Tg(kdr-l:GFP) (green) MZdicer+430 mutant embryo counterstained for DAPI (blue) at 3dpf. White arrowheads indicate two clusters of f-actin positive cells located in within the cardiac jelly.
Online Figure III: Schematic representation of zebrafish miR-23-24-27 clusters.
(a) In the zebrafish genome 5 miR-23-24-27 clusters can be identified positioned on different chromosomes. (b) RNA sequences of mature miRNAs residing in all miR-23-24-27 clusters. Sequence variation amongst copies occurs in the last 4 bases at the 3’end.

### mature miRNA sequences

- miR-23a: AUCACAUGCCAGGGAAUCCA
- miR-23b: AUCACAUGCCAGGGAAUACCA
- miR-24a: UGGCUCAGUUCACAGGAACAG
- miR-24b: UGGCUCAGUUCACAGGAACCG
- miR-27a: UUCACAGUGGCUAAAGUUCGCU
- miR-27b: UUCACAGUGGCUAAAGUUCUGCA
- miR-27c: UUCACAGUGGCUAAAGUUCUGC
- miR-27d: UUCACAGUGGCUAAAGUUCUCA
- miR-27e: UUCACAGUGGCUAAAGUUCAGU
Online Figure IV: MiR-23 is not required for myocardial patterning but can rescue abnormalities in endocardial patterning.

(a,b) Brightfield images of a miR-23 knock-down (a) and a p53 knock-down (b) embryo at 2dpf. (c) In situ hybridization showing wild-type levels of has2 expression in p53 knock-down embryo at 2dpf. (d) Hyaluronic acid (HA) staining (blue) at 2dpf in p53 morphant counter stained with neutral red. (e) HA staining in ventricle wall (boxed in d) of p53 knock-down embryo. Myocardial cell (m), endocardial cell (e). (f,i) Myocardial expression of cmcl2 (f,h) and bmp4 (g,i) at 2dpf in wild-type (f,g) and miR-23 knock-down embryos (h,i). Black arrowheads indicate restricted bmp4 expression in the AVC. (j-m) Has2 expression in wild-type ECs (j) compared to ectopic expression in MZdicer+430 mutants (k), MZdicer+430 mutants injected with miR-27 mimics (l) and MZdicer+430 mutants injected with mutated miR-23 mimics (m). Black arrowheads indicate the AV boundary and dotted lines outline the heart tube; the arterial pole (outflow) is to the top and the venous pole (inflow) to the bottom. All panels show has2 expression at 2dpf.
Online Figure V: miR-23 interacts with specific sequences within the zebrafish has2, icat and tmem2 3′UTRs.

(a) Schematic representation of has2, icat and tmem2 3′UTRs. Location of miR-23 seed sequences in red and blue (minimal miR-23 seed sequence green in b). Results of miR-23 silencing assays of 3′UTR regions (between lines) indicated by − (no silencing) and + (silenced). (b) Conservation of miR-23 seed sequence in has2 UTR (blue in a). Injection of synthetic mRNA containing the coding region for GFP and the 3′UTR sequences of zebrafish has2 (c,d), icat (e-h) and tmem2 (i-n) with a single putative target site mutated. The synthetic mRNA was injected alone (c,e,g,i,k,m) and in combination with miR-23 mimic (d,f,h,j,l,n). No effective silencing of the GFP was observed when a wild-type miR-23 mimic was co-injected upon mutation all the individual putative target sites. mRNA encoding RFP (red) was always co-injected as an injection control.
Online Figure VI: Has2 expression precedes miR-23 in the endocardial cushions.
(a) Combined in situ hybridization for has2 (blue) and cmlc2 (red) in a wild-type embryo at 30hpf already showing AVC restricted has2 expression. (b) MiR-23 is not expressed in the heart at 30hpf but can only be detected in the tail (black arrow). (c) Low levels of has2 expression in the ECs of a wild-type embryo at 3dpf. (d) Prominent miR-23 expression in the ECs of a wild-type embryo at 3dpf. Black arrowheads indicate the AVC ECs. White arrowheads indicate the OFT ECs.
Online Figure VII: Has2 loss of function prevents induction of AVC endocardial cell shape changes.

(a-d) Single confocal images of immunofluorescence staining for Dm-grasp (red) in Tg(kdr-l:GFP) hearts (green, endocardium) of wild-type (a,d) and has2 knock-down embryos (c,d) at 2dpf. (a,b) Wild-type EC cells are cuboidal and Dm-grasp positive (boxed in a, white arrowhead in b). The cardiac jelly enlarges at the wild-type AVC (asteriks in b). (c,d) Upon has2 knock-down endocardial cells at the AVC remained squamous and are Dm-grasp negative (boxed in c, white arrowhead in d).
**Online Figure VIII: Model for regulation of Has2 by miR-23 in ECs**

(a) Cartoon showing regulation of Has2 expression by both positive and negative feedback mechanisms. (b,c) Proposed mechanism how miR-23 restricts EC formation to the AVC region. (b) In the AVC signals from the myocardium, such as Tgf-β growth factors (blue arrows), induce has2 expression in the endocardial cells and thereby enhance Has2 enzyme activity (represented by solid curved line). Has2 produces extracellular HA (blue ribbons) resulting in the activation of endocardial cushion cells and Dm-Grasp expression (indicated by red cell outlines). MiR-23 is expressed in the ECs, where it provides a negative feedback mechanism for Has2 activity. (c) Without miR-23 expression this negative regulation on Has2 activity is lost and as a consequence Has2 activity will increase resulting in more HA production and increase of the cardiac jelly. HA above a threshold (dashed line) induces Dm-Grasp expression (indicated by red cell outlines) and cell shape changes. Since Has2 activity is also regulated via an auto-regulatory and positive feedback mechanism (see discussion for explanation) Has2 expression and its activity will increase even more and expand outside the AV canal region. e, endocardium, m, myocardium.