PRSS23 is essential for the Snail-dependent endothelial-to-mesenchymal transition during valvulogenesis in zebrafish

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Aims
Cardiac valve disease is a common cause of congenital heart failure. Cardiac valve development requires a complex regulation of assorted protease activities. Nevertheless, the regulation of these proteases during atrioventricular (AV) valve formation is poorly understood. Previously, PRSS23, a novel vascular protease, is shown to be highly expressed at the AV canal during murine heart development; however, its function remains unknown. In this study, we sought to characterize the functional role of PRSS23 during cardiac valve formation.

Methods and results
We used a transgenic zebrafish line with fluorescently labelled vasculature as a tool to study the function of PRSS23. We first cloned the zebrafish prss23 and confirmed its sequence conservation with other vertebrate orthologues. Expression of prss23 was detected in the ventricle, atrium, and AV canal during zebrafish embryonic development. We found that morpholino knockdown of Prss23 inhibited the endothelial-to-mesenchymal transition (EndoMT) at the AV canal. Moreover, in human aortic endothelial cell-based assays, PRSS23 knockdown by short-hairpin RNA not only repressed the transforming growth factor-β-induced EndoMT, but also reduced Snail transcription, suggesting that Snail signalling is downstream of PRSS23 during EndoMT. We further demonstrated that human PRSS23 and SNAIL could rescue the prss23 morpholino-induced AV canal defect in zebrafish embryos, indicating that the function of PRSS23 in valvulogenesis is evolutionarily conserved.

Conclusion
We demonstrated for the first time that the initiation of EndoMT in valvulogenesis depends on PRSS23-Snail signalling and that the functional role of PRSS23 during AV valve formation is evolutionarily conserved.

Keywords
PRSS23 • Snail • EndoMT • Cardiac valve formation • Zebrafish

1. Introduction
It has been estimated that 5% of the children produced from live births may carry a congenital valve deformation and that one-quarter of all cardiovascular malformations are related to defects in the cardiac valve.1 2 Specifically, 10–20% of all congenital heart diseases are caused by defects in the atrioventricular (AV) canal. Thus, a better understanding of the molecular mechanisms that regulate cardiac valve formation is important for both clinicians and developmental biologists.

The molecular cues that modulate early valvulogenesis have been studied extensively. During embryonic development, the primitive heart is composed of two layers: the outer myocardium and the inner endocardium.3 The endocardium cushion formation is an important multi-step process during cardiac valve morphogenesis.4 Timely signals instigate the AV endothelial cells to undergo endothelial-to-mesenchymal transition (EndoMT), followed by continuous proliferation and invasion into the cardiac jelly. These steps are tightly regulated by a complex signalling network between the endocardium and the myocardium.4 5 Among the known factors involved in this process, bone morphogenetic protein (BMP), transforming growth factor-β (TGF-β), and Notch are known to play important roles in regulating cardiac valve formation at the AV canal.6 The downstream signalling of TGF-β, Notch, and BMP at the...
correct time induce the expression of Snail family proteins. In turn, Snail represses endothelial marker expression (such as VE-Cadherin and CD31) while promoting mesenchymal marker expression [such as α-smooth muscle actin (α-SMA)] during the EndoMT process.

These specific cells and tissue interactions are expected to enlist a wide array of protease actions. However, owing to the restrictions of the mouse model system and the frequent embryonic lethality in its knockout approach, the identification and regulation of the proteases involved in AV valve formation are still poorly understood.

The zebrafish has recently become a powerful model system for studying cardiac development. Zebrafish embryos are transparent and their early development does not depend entirely on a working cardiovascular system. Unlike humans, the zebrafish heart has only two chambers: one atrium and one ventricle. Nevertheless, many studies have revealed that the core molecular mechanisms driving zebrafish cardiac valve formation are remarkably similar to those found in mammalian hearts. In the zebrafish, the first stage of cardiac valvulogenesis is endothelial cells undergoing the EndoMT process at the AV canal, which occurs at ~36 h after fertilization (h.p.f.). Next, the endocardial cells at the AV canal proliferate and extend into the cardiac jelly to form the superior cushion at ~80 h.p.f. Confocal data have revealed that the cardiac cushion starts its morphogenetic rearrangement at ~105 h.p.f. to form the valve leaflets, indicating that the entire course of valvulogenesis can be tracked within 5 days in live zebrafish.

PRSS23 is a novel serine protease and its expression can be detected in endothelial cells and multiple organs in developing foetuses and adults. In previous studies, PRSS23 is involved in tissue remodelling during ovarian follicular atresia and is essential for oestrogen-dependent proliferation in breast cancer. Interestingly, the human foetal heart has a relatively high level of PRSS23 expression, and its expression is also up-regulated during mouse cardiac development. Specifically, mouse PRSS23 expression can be detected at the AV canal between stages E10.5 and E11. However, the exact role of PRSS23 during cardiac development remains unknown.

Here, we showed that PRSS23 is conserved among vertebrates and is essential for the cardiac valve formation. The deficiency of PRSS23 in zebrafish embryos inhibited the initiation of the EndoMT, which resulted in AV canal malformation. Additionally, in the human aortic endothelial cell-based assay, knockdown of PRSS23 significantly reduced the TGF-β-induced Snail expression and suppressed the endothelial cells ability to undergo the EndoMT. Our data further demonstrated that human PRSS23 and SNAIL were captured using a stereomicroscope Nikon SMZ1500 equipped with a CCD camera (DS-Fi1; Nikon) and the NIS-Elements image software (Nikon).

2.2 Identification and cloning of zebrafish PRSS23

For the identification of zebrafish Prss23, BLAST searches were performed using the human PRSS23 amino acid sequence (NM_007173.4). This search found a predicted Prss23 sequence (XM_687292) from NCBI. The zebrafish prss23 PCR fragment was cloned into the pcS2+ flag vector (Addgene). The mutant forms of zprss23 were generated from the wild-type plasmid by a Quickchange™ site-direct mutagenesis kit (Stratagene).

2.3 Whole-mount in situ hybridization

Whole-mount in situ hybridization (ISH) was performed as previously described. The probes pss23, bmp4, and notch1b were PCR amplified and cloned directly into the pGEM-T-easy vector (Promega). Images were captured using a stereomicroscope Nikon SMZ1500 equipped with a CCD camera (DS-Fi1; Nikon) and the NIS-Elements image software (Nikon).

2.4 Morpholino knockdown and DNA rescued

For knockdown assays, morpholino antisense oligos (MOs; Gene Tools LLC) were injected at the one- to two-cell stage. Zebrafish pss23, human SNAIL, and human PRSS23 sequence DNA were cloned into the pcS2-flag vector and injected into one-cell stage embryos. The mutant forms of zprss23 were generated from the wild-type plasmid by the Quickchange™ site-direct mutagenesis kit (Stratagene). The embryos were anaesthetized with an anaesthetic mixture of 65 p.p.m. MS-222 (Sigma) and 65 p.p.m. isoflurane (Baxter).

2.5 Culture of human aortic endothelial cells

Human aortic endothelial cells (HAECs, passage 4–7; Gibco) were grown in M200 supplemented with LSGS (Gibco). The TGF-β2 treatment assay was followed as previously described. The phosphorylated SMAD2 (pSMAD2) level was measured 1 h after TGF-β2 (R&D) treatment, and the SNAIL expression level was measured 1 day after TGF-β2 treatment. For EndoMT verification, HAECs were stained for detection of CD31 and α-SMA expression 7 days after TGF-β2 treatment.

2.6 RNAi interference

Gene silencing was performed using the GIPZ lentiviral shRNAmir system (Open Biosystems). The short-hairpin RNAs (shRNAs) were introduced into the cells as previously described.

2.7 Immunohistochemistry and immunocytochemistry

Immunohistochemistry and Immunocytochemistry were performed following a previously published protocol. Images were acquired using the Nikon A1R and the Carl Zeiss LSM 510 META confocal imaging systems.

2.8 Whole-mount BrdU incorporation assay

The whole-mount BrdU incorporation assay was performed following a previously published protocol. Images were acquired using the Nikon A1R confocal imaging systems.

2.9 Real-time quantitative polymerase chain reaction

Total RNA of zebrafish embryos and HAECs were isolated with the TRIzol reagent (Invitrogen). The cDNA was generated from total RNA using oligo dT (Invitrogen) and the Superscript III (Invitrogen). The transcription levels of target genes were measured using SYBR Green Master Mix (Applied Biosystems). Data were analysed using the ABI 7500 System SDS Software. All real-time quantitative polymerase chain reaction
reaction (RT–qPCR) products were cloned into the pGEM-T-easy vector (Promega) to verify sequences.

2.10 Western blot analysis
Total protein was extracted from HAECs and zebrafish using RIPA lysis buffer (Roche) containing 1 mM PMSF, 1× complete mini (Roche), 0.1 mM Na3VO4, 10 mM NaF, and 20 mM β-glycerophosphate. The proteins were separated by SDS–polyacrylamide gel electrophoresis and electrically transferred to a polyvinylidene difluoride membrane (Millipore). Blots were visualized by ECL (GE health). A semi-quantitative analysis of the results was performed using the Image J software.

2.11 Transfection and co-immunoprecipitation
Cells were transfected with relevant vectors using jetPE™ transfection reagent (Polyplus transfection) according to the manufacturer’s instructions. Cells were lysed in extraction buffer at 48 h after transfection. The collected protein complexes proteins were resolved on SDS–PAGE and analysed by western blot.

2.12 Promoter analysis
Snail promoter sequences of human, mouse, and zebrafish were downloaded from the ensembl database. These sequences were uploaded to the Cartwheel website (http://cartwheel.caltech.edu/), a framework for genomic sequence analysis, and were analysed by the FRII software.

2.13 Image processing and statistical analysis
To improve clarity, images were adjusted for brightness and/or contrast in Adobe Photoshop. The statistical significance of the differences between two sample populations was determined by Student’s t-test in Microsoft Excel.

3. Results

3.1 Cardiac expression of the conserved prss23 gene during zebrafish development
By comparing the annotated homologene gene and protein sequences in the NCBI GenBank, EMBL, and DDBT databases, the zebrafish orthologue of prss23 mRNA (XM_687292) was identified. The predicted zebrafish Prss23 protein sequence shared an ≏54% identity with its human (NM_007173.4), mouse (NM_029614), and chicken (XM_417210) orthologues (Supplementary material online, Figure S1A and B). The Prss23 protein contained one conserved hydrophobic domain and one trypsin-like domain that could be well aligned to S1A and S1 orthologues among other peptidase S1 family proteins, which implies that PRSS23 is evolutionarily conserved (Supplementary material online, Figure S1C).

Next, the expression profile of prss23 during zebrafish development was analysed by RT–qPCR. Its expression level was detected to be retained after splicing, leading to a reduced level of accurate mRNA transcription. Furthermore, the RT–qPCR data showed that the prss23 mRNA level in the prss23_MO2 group was reduced by 63% compared with the level observed in the prss23_5MIS2 group. These data showed that prss23_MO2 can effectively bind to the exon/intron boundary to block zprss23 mRNA maturation. These results confirmed that the two specific MOs could effectively knockdown zPrss23 translation in vivo.

3.2 Zebrafish prss23 mRNA is successfully targeted using the morpholino knockdown approach
To evaluate the loss of Prss23 function at early stages of development, two prss23 MOs were designed to generate Prss23 deficiency in zebrafish embryos; prss23_5MO1 targets the ATG start codon, while prss23_5MO2 targets the boundary of intron1/ exon2. Additionally, we used reciprocal five-mismatched morpholino oligonucleotides (5MIs), prss23_5MIS1 and prss23_5MIS2, as controls. The effectiveness of these morpholinos was confirmed either by western blot or by RT–qPCR analysis (Supplementary material online, Figure S3). We first designed a plasmid including the human cytomegalovirus promoter, the zprss23 5′ UTR sequences (−310 to +24) and EGFP without the start site sequence. When this plasmid was co-injected with the prss23_5MIS1 into zebrafish embryos at the one-cell stage, EGFP expression was observed in the embryos. When the plasmid and prss23_MO1 were co-injected into embryos, the immunoblot of whole-embryo extracts revealed a loss of EGFP signal (Supplementary material online, Figure S3B). These data suggested that prss23_MO1 can successfully and specifically bind to the start site to inhibit the Prss23 protein translation. For prss23_MO2, knockdown effectiveness was confirmed by reverse transcription polymerase chain reaction and RT–qPCR (Supplementary material online, Figure S3Q). The injection of prss23_MO2 caused the intron to be retained after splicing, leading to a reduced level of accurate mRNA transcription. Furthermore, the RT–qPCR data showed that the prss23 mRNA level in the prss23_MO2 group was reduced by greater than two-fold compared with the control (71.6% vs. 32.9%). Furthermore, the co-injection of prss23_MO1 and pCS-zprss23 cDNA plasmid could significantly...
reduce the malformation rate from 61.1 to 26.7%. Consistent with the MO1 results, co-injection of prss23_MO2 and the pCS-prss23 cDNA plasmid could also effectively reduce the AV canal malformation rate from 71.6 to 38.5%. These data indicated that the Prss23 knockdown-induced AV defect could be rescued, which further validates the specificity of Prss23 on cardiac valve formation. In addition, the co-injection of mutant forms of prss23 cDNAs with a dysfunctional catalytic triad and prss23_MOs did not effectively rescue the AV canal malformation (Supplementary material online, Figure S5), implying that the enzymatic function of Prss23 could be involved in valvulogenesis. Taken together, our results suggested that Prss23 is required for cardiac valve development.

### 3.4 PRSS23 is required for EndoMT initiation during cardiac valve formation

To investigate how Prss23 is involved in cardiac valve development, we assessed the expression pattern and levels of several well-known AV canal-specific markers, including bmp4, notch1b, and has2 in the presence or absence of Prss23 morpholinos. As baseline controls,
normal notch1b expression (Figure 2A) was detected primarily in the AV myocardium at 48 h.p.f., while bmp4 and has2 expression (Figures 2C and Supplementary material online, Figure S6A) was evident in the AV endocardium in the 5MIS control group. In contrast, in the prss23_MO group, displaced expression of both bmp4 and notch1b was detected throughout the ventricular myocardium and endocardium, while ectopic notch1b expression extended further into the distal atrium endocardium (Figure 2D and B). However, Prss23 knockdown did not affect the has2 expression pattern (Supplementary material online, Figure S6B). The penetrance analysis derived from the above-mentioned ISH assays showed that the ectopic pattern of notch1b expression increased from 36.4 to 66.7% in the absence of Prss23 and that the rate of abnormal patterning of bmp4 expression increased from 19.2 to 69.2% in the prss23_MO group compared with the control prss23_SIMS group (Figure 2E). Additionally, the expression levels of hey2 and sps1, two well-known Notch1 downstream genes, were not affected by prss23_MOs (Supplementary material online, Figure S6D).

It has been shown that cardiac valve formation is initiated by BMP4, which is secreted by the AV myocardium to activate the AV endocardium for subsequent transformation. As a result, endothelial cells up-regulate Notch1 expression to undergo EndoMT.24 Because our data demonstrated that prss23 morpholino knockdown disrupted the programmed expression patterns of notch1b and bmp4, we postulated that Prss23 might be involved in the AV canal’s EndoMT during heart valve development. To verify this hypothesis, we performed an immunohistochemical check on the expression of the endocardial cushion marker, Dm-grasp, in Prss23-deficient embryos during cardiac valve formation.13 We inspected and compared the 72 h.p.f. hearts from the control embryos with the prss23 morphants. In the control hearts, we found that the AV canal endothelial cells that were Dm-grasp positive would accumulate at the AV canal as previously described (Figure 2F–I).13 However, in the Prss23 knockdown hearts, the endothelial cells formed a single layer at the AV canal, while reduced Dm-grasp signal was detected in the endocardial cushion (Figure 2J–M). In addition, we investigated the cell proliferation by in situ whole-mount BrdU assay and found that Prss23 knockdown significantly reduced endothelial cell proliferation at the AV canal (Supplementary material online, Figure S7). Taken together, our results suggested that the loss of Prss23 blocks the initiation of EndoMT at the AV canal during zebrafish heart valve formation.

Table 1 Phenotype frequencies of AV canal malformation at 72 h.p.f.

<table>
<thead>
<tr>
<th>Injection</th>
<th>Malformation of AV Canal</th>
<th>Normal</th>
<th>Total live embryos (repeat experiment)</th>
</tr>
</thead>
<tbody>
<tr>
<td>prss23_MO1 (5 ng)</td>
<td>61.1 ± 3.1%</td>
<td>38.9 ± 3.1%</td>
<td>184 (4)</td>
</tr>
<tr>
<td>prss23_SIMIS1 (5 ng)</td>
<td>30.9 ± 6.7%</td>
<td>69.1 ± 6.7%</td>
<td>188 (4)</td>
</tr>
<tr>
<td>Rescue (prss23_MO1 5 ng+pCS-zprss23 200 pg)</td>
<td>26.7 ± 7.1%</td>
<td>73.3 ± 7.1%</td>
<td>175 (3)</td>
</tr>
<tr>
<td>prss23_MO2 (5 ng)</td>
<td>71.6 ± 9.4%</td>
<td>28.4 ± 9.4%</td>
<td>284 (4)</td>
</tr>
<tr>
<td>prss23_SIMIS2 (5 ng)</td>
<td>32.9 ± 5.1%**</td>
<td>67.1 ± 5.1%**</td>
<td>193 (4)</td>
</tr>
<tr>
<td>Rescue (prss23_MO2 5 ng+pCS-zprss23 200 pg)</td>
<td>38.5 ± 2.9%**</td>
<td>61.5 ± 2.9%**</td>
<td>157 (5)</td>
</tr>
</tbody>
</table>

The frequency of malformation or normal phenotypes observed under the morpholino treatment was calculated as the number of zebrafish embryos with the indicated phenotype divided by the number of live embryos in each experiment. The malformation of the AV canal was defined as an abnormal EGFP signal in AV canal endothelial cells that failed to pattern properly. The mean ± SEM was calculated from three to five experimental replicates.

**Compared with prss23_MO1, P < 0.01.

3.5 Knockdown of PRSS23 suppresses TGF-β2-induced EndoMT in human aortic endothelial cells

Our in vivo findings revealed that Prss23 knockdown in zebrafish might repress initiation of EndoMT, resulting in AV canal malformation. To verify this finding, we switched to a primary cell-based assay system for HAECs. It has been shown that TGF-β stimulation can induce endothelial cells to undergo EndoMT.25 and Bartram et al.19 has reported that TGF-β2 is important for valve formation during heart development. On the basis of these findings, we chose TGF-β2 to induce EndoMT in HAECs for the subsequent analysis. To examine the loss-of-function effect, we generated two lentiviral shRNAs to specifically knockdown PRSS23 in HAECs. The efficiency of shRNA knockdown was validated by RT–qPCR, and Prss23 mRNA expression under shRNA1 and shRNA2 treatment was reduced to 13.8 and 6.2%, respectively, compared with the non-silencing control (NSC) treatment (Supplementary material online, Figure S8A). Moreover, immunoblot assays further validated that shRNA1 and shRNA2 could reduce PRSS23 protein level down to 56.1 and 36.3%, respectively, compared with the control (Supplementary material online, Figure S8B). In conclusion, either shRNA could effectively reduce the endogenous PRSS23 expression of HAECs at both the mRNA and protein levels.

Next, we proceeded to examine TGF-β2-induced EndoMT with or without PRSS23 knockdown in HAECs by immunostaining as previously described.26 In comparison with the EBM2 medium-only group (Figure 3A), TGF-β2 treatment in the NSC group could induce EndoMT as expected; the spindle-shaped HAECs displayed a strong reduction in CD31 expression and a significant increase in α-SMA expression (Figure 3D). However, TGF-β2 treatment with PRSS23 knockdown could not induce EndoMT. The HAECs still displayed a high level of the CD31 (red signals in Figure 3E and F), while α-SMA expression remained at the medium-only levels (Figure 3B–C vs. E–F). To further analyse the role of PRSS23 in EndoMT, we counted the number of α-SMA-positive cells (Figure 3G). In the NSC group, after 7 days of TGF-β2 treatment, the α-SMA-positive cells made up 9.9% of all cells. However, in the PRSS23 shRNA1 and the shRNA2 groups, the α-SMA-positive cells represented only 4.3 and 1.9% of all cells, respectively. Consistently, the mRNA expression of VE-cadherin significantly decreased by 37% and α-SMA expression significantly increased by 20.3-fold, compared with medium-only controls.
In contrast, mRNA expression of VE-cadherin and that of α-SMA were not affected in the presence of PRSS23 shRNA1 and shRNA2, respectively (Figure 3H). Taken together, these data strongly indicated that a loss of PRSS23 would significantly inhibit TGF-β2-induced EndoMT in HAECs.

3.6 PRSS23 is required for Snail transcription under TGF-β2 stimulation

After we confirmed that PRSS23 was involved in TGF-β2-induced EndoMT, we began to explore the role of PRSS23 in TGF-β2-dependent signalling during EndoMT using immunoblot assays. During TGF-β2-induced EndoMT, the downstream effectors, such as Smads, are phosphorylated to relay activation signals to Snail, which is a well-known regulator of EndoMT. We thus investigated whether the expression levels of pSMADs are altered in the presence of PRSS23 shRNAs. After TGF-β2 treatment for 1 h, the expression level of pSMAD2 in HAECs displayed a significant greater than three-fold increase in the NSC control group (Figure 3I). However, in the PRSS23 knockdown groups, SMAD2 was also phosphorylated after TGF-β2 stimulation. Furthermore, the immunocytochemical staining data revealed that the nuclear translocation of pSMAD2 was unaffected. Either in the NSC or in the PRSS23 knockdown groups, pSMAD2 could be detected in the HAEC nucleus after TGF-β2 treatment for 1 h (Supplementary material online, Figure S9). These data suggested that PRSS23 may not regulate EndoMT via the SMAD2-dependent pathway.

Next, we investigated whether there was an association between PRSS23 and Snail expression in TGF-β2-induced EndoMT. One day after stimulation with TGF-β2, the protein expression level of SNAIL significantly increased 6.3-fold, compared with the medium-only controls. In contrast, the protein expression levels of SNAIL showed only limited increase in the presence of PRSS23 shRNA1 and shRNA2 (Figure 3J). The same phenomenon was also verified at the mRNA level (Figure 3H), which strongly supports the observation at the protein level. Incidentally, we found that SNAIL expression would display a transient increase profile, in which its expression level peaked at 24 h with a significant 9-fold increase (Supplementary material online, Figure S10A). On the other hand, PRSS23 expression level was quickly increased 3.5-fold and sustained at the same level under TGF-β2 stimulation (Supplementary material online, Figure S10B), which is different from the SNAIL expression profile. According to a previous yeast two hybrid study, PRSS23

(Figure 3H). In contrast, mRNA expression of VE-cadherin and that of α-SMA were not affected in the presence of PRSS23 shRNA1 and shRNA2, respectively (Figure 3H). Taken together, these data strongly indicated that a loss of PRSS23 would significantly inhibit TGF-β2-induced EndoMT in HAECs.
might interact with TCF12. Recently, Lee et al.\(^\text{28}\) further showed that TCF12 is involved in the epithelial-to-mesenchymal transition process. We thus moved to investigate the relationship between PRSS23 and TCF12 by immunostaining and co-immunoprecipitation (Co-IP) assays. Our data first revealed that PRSS23 and TCF12 were co-localized in the nuclei of HAECs (Supplementary material online, Figure S11A), and their physical interaction was also confirmed with the Co-IP assay (Supplementary material online, Figure S11B). Furthermore, analysis of the Snail promoter sequence revealed a conserved region including the E-box, which is a conserved TCF12 binding site across the vertebrates (Supplementary material online, Figure S12).

Together, these data implied that the loss of PRSS23 would inhibit Snail transcription to suppress the EndoMT process induced by TGF-β2 stimulation through a TCF12-dependent pathway.

### 3.7 Human Snail could rescue the AV canal defects caused by prss23 knockdown in zebrafish

TGF-β2-induced EndoMT is an essential and ubiquitous cellular process conserved throughout evolution and such transition is critical to valve formation during embryonic development. Therefore, we...
wanted to explore whether human PRSS23 could substitute for its zebrafish orthologue to initiate Snail-dependent EndoMT during heart development. We approached this question by performing rescue experiments with human PRSS23 and SNAIL in zebrafish prss23 morphants. After injecting the morpholinos, we again observed the endocardial defect described in Figure 1, wherein endothelial cells diffused and spread beyond the AV canal (Figure 4A and D). Upon co-injection of human PRSS23 and the prss23 MOs, the endothelial cells at the AV canal appeared to accumulate in a pattern similar to the normal developing heart (Figure 4B and E). Similarly, the co-injection of human SNAIL could also rescue the prss23 MO-induced defect and displayed profound accumulation of endocardial cells at the site of the AV canal (Figure 4C and F). The snail expression level was decreased by 40 and 34% with prss23 MO1 and prss23 MO2 treatments in comparison with the prss23_5MISs while injection of human PRSS23 could increase the zebrafish snail expression in Prss23 morphants (Figure 4H). The penetrance data of the rescue phenotype showed that the co-injection of human PRSS23 or SNAIL cDNA in pCS2 vectors significantly reduced the AV canal defects in prss23 morphants (Figure 4G). The percentage of embryos

Figure 4  Human PRSS23 and Snail could rescue the AV canal malformation induced by prss23 morpholino injection in zebrafish. (A–F) Ventral view of zebrafish embryonic hearts at 72 h.p.f. in Tg (flk1: EGFP) s843. Yellow box highlights the AV canal region. The arrowheads indicate the abnormal reduction of endothelial aggregation caused by prss23 MO1 and prss23 MO2 injection (A and D). White arrows indicate the recovered pattern of endothelial cell aggregation in the presence of pCS_hPRSS23 (B and E) and pCS_hSNAIL (C and F). A, atrium; while V, ventricle. The scale bar represents 50 μm. (G) Quantification of human PRSS23- and SNAIL-rescued embryos in the prss23 morphants. (H) The mRNA expression levels of zebrafish snail under each condition were determined by RT–qPCR. Error bars represent the mean ± SEM. Each experiment was repeated more than three times. *P <0.05; **P < 0.01; n is the total number of live embryos.
showing prss23_MO1-induced AV canal defects was significantly reduced from 61.1 to 40.9% with human PRSS23 and down to 40.2% with human SNAIL. In contrast, the percentage of embryos showing AV canal defects under the influence of prss23_MO2 could be partially reduced from 71.6 to 49.7% with human PRSS23 and down to 53.7% with human SNAIL. These data strongly suggested that PRSS23 could modulate the EndoMT via Snail during cardiac valve formation and that the functional role of PRSS23 in cardiac valve formation is evolutionarily conserved.

### 4. Discussion

In this study, we first identified the zebrafish orthologue of human PRSS23 and confirmed its expression at the AV canal during valvulogenesis. We then demonstrated that zebrafish Prss23 is required for the initiation of EndoMT during cardiac valve formation in vivo. The reduction of PRSS23 expression repressed SNAIL expression and disrupted TGF-β2-induced EndoMT in vitro. Moreover, human PRSS23 and SNAIL could rescue the prss23 morpholino-induced defects at the AV canal during zebrafish development. Together, our data revealed that PRSS23 has an essential and conserved role in the EndoMT process via Snail signaling during vertebrate valvulogenesis. A model scheme that summarizes the potential role of PRSS23 in the initiation of EndoMT during cardiac valve formation is shown in Figure 5.

Cardiac valve development in zebrafish requires the multitude of specific cellular processes, including the initiation of EndoMT, cell proliferation, EndoMT migration, and ECM remodelling. During the initiation step, the myocardium secretes BMP and TGF-β. The TGF-β signal leads to SMAD2 phosphorylation in the endocardium, which promotes Snail expression. Our data indicated that PRSS23 may modulate Snail transcription to promote EndoMT initiation and that PRSS23 is required in the endocardial cells to drive the cells to undergo EndoMT.

PRSS23 is required for cardiac valve formation
stimulation. However, the induction of α-SMA expression was observed in only 10% of HAECs under TGF-β2 treatment in our study, which is less than the value observed in the foetal endothelial cell-based assay reported previously. 26 We infer that foetal endothelial cells have higher potential to undergo EndoMT than adult endothelial cells.

Interestingly, human SNAIL and PRSS23 could rescue the AV canal defect caused by prss23_MOs in zebrafish and overexpression of human PRSS23 could increase zebrafish snail expression in Prss23 morphants. Such data demonstrate that the functional role of PRSS23 on EndoMT during cardiac valve formation is both critical and well conserved among the vertebrate. In order to test whether Notch1 could rescue snail transcription following Prss23 knockdown, we have attempted to use zebrafish notch1 to rescue the Prss23 morphants. Unfortunately, in the presence of extra notch1, we observed severe deformation in >50% of the embryos, which subsequently died before the assay time point at 48 h.p.f. (data not shown). Thus, whether Notch1 could rescue Snail transcription following Prss23 knockdown remains unclear at this time.

It is noteworthy that our data indicated that PRSS23 did not affect pSmad2 at the protein level; instead, we found that PRSS23 inhibited Snail transcription under TGF-β2 stimulation and PRSS23 expression level was increased under TGF-β2 treatment. On the basis of the data shown in our current and previous studies, PRSS23 could interact with TCF12, 27 while the latter has been shown to be involved in heart development. 35 Moreover, study of Gata4-deficient mice demonstrates that when endothelial cells fail to undergo EndoMT during cardiac valve formation, the expression level of TCF12 is down-regulated. 35 TCF12, along with TCF3 and TCF4, is a member of the E-protein family that can bind to a consensus E-BOX (CANNTG) site to regulate downstream genes 36 involved in epithelial-to-mesenchymal transition. 28,36 It shall be noted that E-protein family can regulate Snail expression. 37,38 Taken together, these evidences suggest that PRSS23 might interact with TCF12 to regulate Snail expression during EndoMT.

In summary, our data strongly suggest that PRSS23 is essential for the initiation of EndoMT via Snail during cardiac valvulogenesis and that this PRSS23-dependent mechanism for valve formation is evolutionarily conserved. The understanding of how PRSS23 modulates Snail transcription to initiate EndoMT during mammalian valve formation will enhance our knowledge of human heart development, with the potential to develop diagnostic tools that can screen for congenital cardiac valve diseases.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

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