Krox20 defines a subpopulation of cardiac neural crest cells contributing to arterial valves and bicuspid aortic valve

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ABSTRACT

Although cardiac neural crest cells are required at early stages of arterial valve development, their contribution during valvular leaflet maturation remains poorly understood. Here, we show in mouse models that neural crest cells from pre-otic and post-otic regions make distinct contributions to the arterial valve leaflets. Genetic fate-mapping analysis of Krox20-expressing neural crest cells shows a large contribution to the borders and the interleaflet triangles of the arterial valves. Loss of Krox20 function results in hyperplastic aortic valve and partially penetrant bicuspid aortic valve formation. Similar defects are observed in neural crest Krox20-deficient embryos. Genetic lineage tracing in Krox20−/− mutant mice shows that endothelial-derived cells are normal, whereas neural crest-derived cells are abnormally increased in number and misplaced in the valve leaflets. In contrast, genetic ablation of Krox20-expressing cells is not sufficient to cause an aortic valve defect, suggesting that adjacent cells can compensate this depletion. Our findings demonstrate a crucial role for Krox20 in arterial valve development and reveal that an excess of neural crest cells may be associated with bicuspid aortic valve.

KEY WORDS: Neural crest, Cardiac development, Krox20, Mouse, Genetics, Bicuspid aortic valve, Egr2

INTRODUCTION

Heart valve development occurs through an epithelial-to-mesenchymal transition (EMT) process (Eisenberg and Markwald, 1995; Person et al., 2005). The development of arterial (or semilunar) valves is differentiated from that of atrioventricular valves. Loss of Krox20 function results in alteration of the antero-posterior positional identity of r3 and r5, which may require treatment and/or surgery (Fedak et al., 2002). The BAV is often isolated but may be also associated with other congenital malformations, including coarctation of the aorta, interruption of the aortic arch, patent ductus arteriosus or isolated ventricular septal defect, suggesting a common etiology between these cardiac defects (Siu and Silversides, 2010). Although usually asymptomatic at birth, BAV predisposes to valvular dysfunctions, such as aortic stenosis and/or insufficiency, which may require treatment and/or surgery (Fedak et al., 2002; Garg, 2006). Despite the frequency and potential morbidity of BAV, our understanding of the mechanism underlying this congenital malformation remains limited.

We recently reported that the zinc-finger transcription factor Krox20 (Egr2 – Mouse Genome Informatics) is a crucial activator of fibrillar Col1a1 and Col3a1 genes during valve development (Odelin et al., 2014). Here, we provide evidence that post-otic NCCs expressing Krox20 migrate through the 3rd pharyngeal arch to later invest murine arterial valves. Previous studies have shown that Krox20 is transcribed in r3 and r5, but not in other part of the neural tube, and that its deletion results in alteration of the antero-posterior positional identity of r3 and r5 (Schneider-Maunoury et al., 1993; Wilkinson et al., 1989). Using various transgenic mouse lines, we compared neural crest origins and showed segregated distribution of their derivatives in the arterial valve leaflets. Loss of Krox20 function results in abnormal development of the aortic valve, which in 30% generates a BAV phenotype. Similar defects are observed in neural crest Krox20-deficient embryos. Genetic fate-mapping analysis in Krox20−/− showed that additional neural crest-derived cells are found in the leaflets of the aortic valve. In addition, we used different transgenic mice to examine the contribution of pre-otic and post-otic NCCs in aortic valve development. Our study identifies an essential role for Krox20-expressing NCCs during arterial valve formation and disease.

RESULTS

Homozygous disruption of Krox20 results in bicuspid aortic valves

We have already shown that Krox20 mutant mice have aortic valve dysfunction associated with disorganization of the extracellular
matrix (Odelin et al., 2014). Here, we expand our analysis to the morphology of the valves from embryonic day (E) 12.5 to E18.5 (Fig. 1 and Fig. S1). No anomaly was detected at E12.5 (n = 18) or E14-E15.5 (n = 14) (Fig. 1A-D, I). At 18.5, all wild-type (n = 32) embryos observed had normal tricuspid aortic valves comprising two coronary (RC and LC) and one non-coronary (NC) valve leaflets (Table 1; Fig. 1E). In contrast, all (22/22) Krox20lacZ/lacZ (termed Krox20−/−) fetuses had hypertrophic aortic valve leaflets and 27% of these (6/22) had developed bicuspid aortic valve (BAV) at E18.5 (Table 1; Fig. 1F). Of note, pulmonary valves and the great arteries were normal in both wild-type and Krox20−/− embryos.

To characterize the type of BAV, we performed three-dimensional (3D) reconstructions of the aortic valve in wild-type and mutant fetuses. 3D reconstructions demonstrated that the phenotype of BAV in Krox20−/− resulted from the fusion of the non-coronary leaflet with either the right or the left coronary leaflets (R-N or L-N) (Fig. 1G, H). We also observed a raphe between the non-coronary and the right coronary leaflets in 33% of the BAVs in Krox20−/− (Table 1 and Fig. S1). Consistent with our previous observation (Odelin et al., 2014), quantification of the 3D volume or the measurement of the relative surface area showed that mutant valves were 1.8 times larger than control valves (Fig. 1J, K; P = 0.0002, Student’s t-test). The total number of mutant valvular cells was increased by 30% over that of the controls (Fig. 1L, P = 0.014, Student’s t-test). Interestingly, quantifications of these parameters in distinct leaflets revealed no significant differences between coronary and non-coronary leaflets in Krox20−/− aortic valve (Fig. S1). Together, these results suggest that lack of Krox20 perturbs the development of the aortic valve, and it is associated with bicuspid phenotype.

**Table 1. Aortic valve anomalies in Krox20−/− and conditional embryos at E18.5**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>Hypertrophic AoV</th>
<th>Bicuspid AoV</th>
<th>Bicuspid with raphe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>32</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Krox20−/−</td>
<td>22</td>
<td>6 (27%)</td>
<td>0</td>
<td>2/6</td>
</tr>
<tr>
<td>Wnt1-Cre</td>
<td>29</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Wnt1-Cre; Krox20flox/flox</td>
<td>19</td>
<td>2 (10.5%)</td>
<td>0</td>
<td>2/2</td>
</tr>
<tr>
<td>Tie2-Cre; Krox20flox/flox</td>
<td>20</td>
<td>2 (10.5%)</td>
<td>0</td>
<td>0/2</td>
</tr>
<tr>
<td>Krox20flox/+.</td>
<td>14</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Krox20flox/−</td>
<td>13</td>
<td>1 (8%)</td>
<td>0</td>
<td>0/1</td>
</tr>
</tbody>
</table>

n indicates number of scored embryos; AoV; aortic valve.

**Fig. 1. Krox20 mutant mice display bicuspid aortic valves.** (A-F) Cross-sectional Hematoxylin and Eosin-stained images through the aortic valve leaflets of wild-type (A,C,E) and Krox20lacZ/lacZ (B,D,F) littermate. At E12.5 (n=18) and E15.5 (n=14), no obvious difference is seen between wild-type (A,C) and Krox20lacZ/lacZ (B,D) arterial valves. (I) At E15.5, the relative surface of the aortic valve leaflet per section is similar in wild-type and mutant embryos. (E,F) At E18.5, three aortic valve leaflets (asterisks) are observed in the wild-type embryo (E), whereas abnormal aortic valve with thickened and unequal-sized leaflets are detected in the mutant (F). Asterisks indicate valvular leaflets of the aortic valve. (G,H) Three-dimensional (3D) reconstructions of histological sections at E18.5. Wild-type embryo (G) has an aortic valve with three leaflets, each with three commissures, whereas a Krox20−/− embryo (H) demonstrates an abnormal aortic valve with two leaflets. (I) The relative surface area of the aortic valve leaflet per section is significantly increased in the mutant compared with controls. (K) Quantification of the average volume of the aortic valve leaflet displays that mutant valves are 1.8 times larger than control valves. (L) The mean of total nuclei per leaflet shows a significant increase in the mutant aortic valve. All data were calculated from n=6 embryos for each genotype. Data are mean±s.e.m. (*P<0.05, Student’s t-test). Left coronary (LC, blue), right coronary (RC, green), non-coronary (NC, purple) and fused left coronary-non coronary (LC-NC, red) leaflets. Scale bars: 100 μm.

**Fate-mapping of Krox20-derived cells in the arterial valves**

At E9.5, Krox20 expression is detected in r3 and r5, but also in NCCs originating from r5 (Fig. S2) (Ghislain et al., 2003; Schneider-Maunoury et al., 1993; Wilkinson et al., 1989). We used Wnt1-Cre and Krox20Cre mice to perform a genetic lineage tracing of NCC populations during arterial valve formation. At E12.5 and E15.5, distinct valve leaflets were apparent, and
Wnt1-Cre-labeled cells were mostly present in the coronary leaflets of the aortic valve (Fig. 2A,B). At E18.5, all three aortic valve leaflets had contributions from NCC, even if fewer Wnt1-Cre-labeled cells were observed in the non-coronary compared with the coronary leaflets (Fig. 2C). Many NCC-derived cells were also detected at the insertion zone (attachment point) of the valve leaflets of Wnt1-Cre;Rosa<sup>mtG/TmG/+</sup> embryos (Fig. 2B,C, arrowheads). At this stage, the left and right leaflets of the pulmonary valve contained NCC-derived cells, whereas the anterior leaflet had very few NCC (Fig. 2D). To genetically fate-map the Krox20-expressing cells, we used a Krox20<sup>Cre</sup> allele together with the RosatdTomato reporter line (Madisen et al., 2010; Voiculescu et al., 2000). At E13.5, the three aortic leaflets contained Krox20-derived cells, although the non-coronary leaflet contained very few Tomato-positive cells (Fig. 2E). However, a substantial contribution to aortic valve structure was observed at E15.5 and E18.5 (Fig. 2F,G). Similar to Wnt1-Cre-labeled cells, Krox20-derived cells were located at the insertion zone of the valve leaflets (Fig. 2F,G; arrowheads). Krox20-expressing cells contribute also to the pulmonary valve leaflets, with a reduced contribution to the anterior leaflet (Fig. 2H). Using the Z/AP reporter line, we examined the lineage of cells that expressed Krox20 in E17.5 Krox20<sup>Cre/+</sup> embryos. Valvulography confirmed a large contribution of Krox20-Cre-labeled cells at the borders and in the interleaflet triangles (RΔN, RΔL and LΔN) of the arterial valves (Fig. 2I-K). Endogenous expression of Krox20 mRNA and proteins had been seen in a subset of mesenchymal cells of the aortic valve after E12.5 stage (Odelin et al., 2014). At E18.5, Krox20 was expressed at the border of the aortic valve leaflets (Fig. S3). Immunostaining revealed several double Tomato/Krox20-positive cells in the aortic valve of Krox20<sup>Cre/+;</sup> Rosa<sup>mtG/+;TmG/+</sup> embryos (Fig. S3). To examine the origin of these valvular cells, we performed further co-immunostaining in Wnt1-Cre;Rosa<sup>mtG/+</sup> and Tie2-Cre;Rosa<sup>mtG/+</sup> embryos. At E13.5 and later, Krox20 protein expression was detected in both NCC- and endothelial-derived cells (Fig. S3), suggesting different origins of Krox20-expressing cells during arterial valve development. These experiments demonstrate that Krox20 is expressed in a subset of NCCs that make a substantial contribution to the arterial valves.

**Fig. 2. Concordance of Krox20-Cre and neural crest labeling in the arterial valve.** (A-D) Immunofluorescence staining of the arterial valves of Wnt1-Cre;Rosa<sup>mtG/+</sup> embryos. The cell membrane-localized green fluorescence (GFP) reporter protein in Cre recombinase-expressing cells. GFP-reporter protein is in green and Pecam is labeled in purple. Immunofluorescence reveals expression of the reported protein (membrane-GFP; green) in the forming leaflets at E12.5 (A). (B,C) At E15.5 and E18.5, the GFP-positive cells are found in the three leaflets of the aortic valve. Contribution of NCCs is more abundant in both coronary leaflets than in the non-coronary leaflet. (D) The right (R) and left (L) leaflets of the pulmonary valve contain NCC-derived cells (Tomato; red), whereas the anterior (A) leaflet contains very few NCCs. (E-H) Immunofluorescence staining on the aortic valve of Krox20<sup>Cre/+</sup>;RosatdTomato embryos. Tomato-reporter is in red and Pecam is labeled in green. Immunofluorescence reveals expression of the reported protein (Tomato; red) in a few cells at the base of the leaflets (arrowheads) at E13.5 (E). (F,G) By E13.5, Krox20-Cre-labeled cells are located the insertion zone of the valve leaflets and along each commissure. (H) The right and left leaflets of the pulmonary valve contain Krox20-derived cells; there are a few Krox20-Cre-labeled cells present in the anterior leaflet. (I,J) E17.5 hearts from Krox20<sup>Cre/+</sup>;Z/AP embryos were stained for alkaline phosphatase and treated to be transparent. Expression is detected in the insertion zone (attachments) of the aortic (AoV) and pulmonary (PV) valve leaflets, and in the interleaflet triangle regions (arrowheads). (K) Three- and two-dimensional representations of the arterial valve leaflets. The red lines correspond to the parabolic hinge of each leaflet. The interleaflet triangle is shown in yellow. LC, left coronary leaflet; NC, non-coronary leaflet; RC, right coronary leaflet; RΔN, right-non-coronary interleaflet triangle; RΔL, right-left interleaflet triangle. Scale bars: 100 μm.
Deletion of Krox20 in neural crest cells results in aortic valve defects

To investigate the role of Krox20 in NCCs that contribute to arterial valve development, we used the Krox20flox/flox allele together with the Wnt1-Cre line. Wnt1-Cre;Krox20flox/flox embryos were observed at the expected Mendelian ratio and newborn mice were viable. Because the Wnt1-Cre line was reported to cause developmental phenotype by ectopic activation of Wnt signaling (Lewis et al., 2013), we checked whether heterozygous Wnt1-Cre embryos had any valve defect. At E18.5, our observation revealed no valve defect in this genetic context (n=29; Table 1). However, examination of Wnt1-Cre;Krox20flox/flox fetuses at E18.5 revealed morphological anomalies of the aortic valve, including hyperplasic (19/19) and bicuspid (2/19) leaflets (Table 1; Fig. 3A,B). The phenotype of BAV observed in Wnt1-Cre;Krox20flox/flox resulted from the fusion between the non-coronary and the right coronary leaflets (Fig. 3A-D). To further assess cardiac function, we performed echocardiography on 1-month-old mice. Pulse-wave Doppler analysis of the aorta showed flow reversal in wild-type and mutant embryos at E18.5, spanning a 180°-wave Doppler analysis from the aorta in 1-month-old wild-type (E) and mutant littermates (Fig. 3G,H). Quantification of total number of aortic valve leaflets showed a significant increase of mesenchymal cell number in Wnt1-Cre;Krox20flox/flox and Krox20flox/flox compared with wild-type littermates (Fig. 3I). Suggesting that this defect is a proliferative-independent process. We next assessed whether there was a deficiency in apoptosis. At E13.5 and E17.5, counting of caspase 3-positive cells did not show any significant difference between wild-type (n=3 at each stage) and Krox20flox/flox (n=3) embryos (Fig. S4), indicating that cell death is not affected in the aortic valves of the mutant embryos.

Additional neural crest cell derivatives in Krox20-/- is associated with bicuspid aortic valve

To examine further the mechanism underlying aortic valve enlargement, we performed a fate-mapping of endothelial- (Tie2-Cre;Rosaflox/Tomato) and neural crest- (Wnt1-Cre;Rosaflox/Tomato)
derivatives in wild-type and Krox20-null mutant embryos. At E18.5, Tie2-Cre-expressing endothelial cells were found at the same number in the aortic valve leaflets of Krox20−/− and wild-type embryos (Fig. 4A-C). However, the number of neural crest-derived cells (Wnt1-Cre) was significantly increased (≥1.8 fold; P<0.05) in the aortic valve leaflets of Krox20−/− compared with wild-type littermates (Fig. 4D-F). At E18.5, quantification of Tomato-positive cells in the distinct leaflets showed additional neural crest-derived cells in the right and left coronary leaflets as well as in the non-coronary leaflet of Krox20−/− aortic valve (Fig. S5). These data indicate that the abnormal aortic valve in Krox20−/− embryos is associated with an increased number of neural crest-derived cells located in the valve leaflets.

We further examined the contribution of Krox20-derived cells using Krox20Cre;RosatdTomato reporter mice. As Krox20flox/flox is a null allele, we analyzed the contribution of Krox20-Cre labeled cells in a heterozygous background (Voiculescu et al., 2000). To avoid the presence of two copies of the Cre gene in the homozygotes, leading to a higher initial expression relative to heterozygotes, we used Krox20Cre/lacZ compound mutant embryos. At E18.5, Krox20-null mutant embryos displayed higher number of Krox20-derived cells (Fig. 4G-I). Interestingly, the augmentation of Wnt1-Cre- and Krox20-Cre-labeled cell number was similar in the Krox20−/− mutant embryos (compare Fig. 4F with 4I). Further analysis revealed singular accumulation of Krox20-derivatives in the non-coronary leaflet, although the coronary leaflets shown also moderate increase (Fig. S5). To assess whether this augmentation was associated with higher proliferation, we counted phospho-histone H3 (PHH3) and Tomato double-positive cells in the three reported mice (Fig. 4J-L). Our results showed no defect in proliferation of valvular cells, confirming that higher numbers of neural crest-derived cells in Krox20−/− valve leaflets is not due to excessive proliferation in the valve tissue. Together, these results suggest that accumulation of Krox20-Cre-labeled cells is caused by an earlier defective event.

As Krox20 expression was detected in few endothelial-derived cells of the forming aortic valve (Fig. S3), we examined whether endothelial-specific deletion of Krox20 can cause abnormal aortic valve development (Table 1; Fig. S6). At E18.5, anatomic and morphometric analyses of Tie2-Cre;Krox20flox/flox embryos shown no difference in the volume and thickness of the aortic valve leaflets when compared with control valves (Fig. S6). Surprisingly, a bicuspid was observed in 10% of the Tie2-Cre;Krox20flox/flox embryos (2/20) (Table 1). 3D reconstructions displayed two equal-sized leaflets without associated raphe (Fig. S6). These data suggest that BAV can also be observed when endothelial-specific deletion of Krox20 occurs; however, the type of bifoliate valves was different from those observed in Wnt1-Cre;Krox20flox/flox embryos, without enlargement or excess cell numbers in the leaflets (Fig. S6).

**Krox20-expressing neural crest cells contribute to the aortic valve leaflets**

Krox20 controls and coordinates the formation of r3 and r5 territories (Schneider-Maunoury et al., 1993; Voiculescu et al., 2001).

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**Fig. 4. Abnormal contribution of neural crest-derived cells in Krox20-null mice.** (A,B,D,E,G,H) Fate-mapping of the endothelial, neural crest and Krox20 lineages in the aortic valves of wild-type (WT, A,D), heterozygous (G) and Krox20-null (B,E,H) embryos at E18.5. (A-C) Immunofluorescence staining using Tie2-Cre;RosatdTomato reporter mice demonstrates normal contribution of endothelial cells in wild-type (A) and Krox20−/− (B) littermate embryos. (C) Quantification confirms equivalent number of endothelial cells in wild-type (n=5) and mutant (n=5) embryos. (D-F) Immunofluorescence staining using Wnt1-Cre;RosatdTomato reporter mice shows an increased in the number of neural crest derivatives in the absence of Krox20. (F) Quantification of neural crest-derived cells demonstrates significant increase in Krox20−/− (n=5) compared with wild-type (n=5) littermate embryos. (G-I) Immunofluorescence staining using Krox20Cre;RosatdTomato reporter shows an increased in the number of Krox20-Cre-labeled cells in aortic valve leaflets of compound Krox20flox/flox mutants compared with heterozygote Krox20flox/+ embryos. (I) Quantification of Krox20-Cre-labeled cells confirms the significant increase in Krox20-derived cells in Krox20flox/flox (n=4) compared with Krox2flox/+ (n=4) littermate embryos. (J-L) Proliferation of Tomato-positive cells in the aortic valves evaluated using anti-phospho-Histone H3 (p-H3). The mitotic index is the percentage of total p-H3/Tomato-positive cells in 10 separate sections for three individuals of each genotype. The mitotic index shows no significant difference in Tie2-Cre;RosatdTomato+, Wnt1-Cre;RosatdTomato+ and Krox2-Cre;RosatdTomato+ reporter mice in wild-type and mutant embryos. Data are means±s.e.m. (*P<0.05, Student’s t-test). LC, left coronary leaflet; NC, non-coronary leaflet; RC, right coronary leaflet. Scale bars: 100 μm.
Interestingly, Krox20 expression is detected in NCCs migrating from r5 towards the 3rd pharyngeal arch (Fig. S2). However, this expression is transient as detection of the Krox20 transcripts is low by E9.5 (Fig. S2). Comparison of Krox20-expressing cells with the WntI-positive population showed that both populations coincide (Fig. S7). To follow the fate of the r5-derived NCCs in the absence of a functional Krox20 protein, we used the compound mutant embryos Krox20Cre/flox, which carry only one copy of the Cre gene (Taillebourg et al., 2002; Voiculescu et al., 2000). At E9.5, lacZ reporter gene activity in Krox20Cre/flox;R26R embryos was detected in r3 and r5, and the derivatives of r5 neural crest that migrate toward the 3rd pharyngeal arch (Fig. 5A,B). Consistently, at E10.5, Tomato-positive cells were observed in the cardiac outflow tract of Krox20Cre;Rosa26Tomato embryos (Fig. 5C). Subsequently, Krox20-Cre-labeled cells were detected in all leaflets of Krox20Cre; R26R aortic valve (Fig. 5D-F). In Krox20Cre/+;R26R embryos, a higher number of migrating NCCs were detected in the pharyngeal region at E9.5 (Fig. 5G,H). At E10.5, we found more Krox20-Cre-labeled cells in the outflow tract of homoygous than heterozygous embryos (Fig. 5I), consistent with additional Krox20-Cre-labeled cells detected in the aortic valve leaflets at later stages (Fig. 5J-L). At E13.5, quantification revealed an increased Tomato-positive cell number in aortic valve leaflets of Krox20Cre/+;Rosa26Tomato embryos compared with Krox20Cre/+;Rosa26Tomato heterozygous littermates, which was particularly obvious for the non-coronary leaflet (Fig. S8). Interestingly, in a homozygous background, Krox20-Cre labeled cells appeared misplaced within the leaflets (compared Fig. 5K,E), and due to the hyperplasia of the non-coronary leaflet, its sinus looked smaller in the mutant compared with controls (Fig. 5L, arrowhead). To further examine whether loss of Krox20 function in its own lineage can lead to aortic valve defect, we analyzed Krox20Cre/flox mutant embryos. Analysis of Krox20Cre/flox hypomorphic mutants revealed morphological anomalies of the aortic valve, including hyperplasic (13/13) and bicuspid (1/13) leaflets (Table 1), suggesting that reduction of Krox20 expression in Krox20-expressing cells results to similar valve phenotype observed for Wnt1-Cre;Krox20flox/flox mutants.

To investigate the importance of the Krox20-Cre-labeled subpopulation of NCCs to aortic valve morphogenesis, we performed their genetic ablation in the mouse. For this purpose, we used the Krox20GFP(DT) allele, in which the A chain of the diphereria toxin (DT) is placed downstream of the floxed GFP sequence (Vermolen et al., 2003), where the DT is activated only upon Cre-mediated recombination (Fig. 5). Krox20GFP(DT)+/+ mice were crossed with a Wnt1-Cre mouse to obtain a specific depletion of Krox20 derivatives and to avoid the early lethality linked to the

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**Fig. 5. Rhombomere 5 derivatives are affected in Krox20 mutant mice.** (A,B,G,H) X-gal staining on whole-mount embryos at E9.5 (A and G, right views; B and H, dorsal views), showing expression of the Rosa26lacZ (R26R) reporter gene in heterozygous (A,B; Krox20Cre+/+) and compound homozygous mutant (G,H; Krox20Cre/flox) mice. Transcriptional expression of Krox20 is detected in rhombomeres (r) 3 and r5, and in neural crest cells (NCCs, arrowheads). (C-F,I-L) Detection of the Tomato reporter protein in Krox20Cre+;Rosa26Tomato and Krox20Cre/flox;Rosa26Tomato embryos. At E10.5, Tomato-positive cells (arrowheads in C,I) are observed into the cardiac outflow tract of both heterozygous (C) and homozygous mutant (I) embryos. Note the increased number of Tomato-positive cells in the outflow tract of Krox20-null embryos (J-L). At E15.5, tomato-positive cells are significantly increased in Krox20-null (J-L) compared with heterozygous (D-F) embryos. Note the enlargement of the aortic valve leaflets in the Krox20-null embryos. Pecam is labeled in green. Arrowhead in L indicates valvar sinus. (M,N) Targeted expression of diphereria toxin using Wnt1-Cre;Krox20GFP(DT)+/+ embryos. (M) At E17.5, the total number of nuclei per section of aortic valve leaflets reveals no major difference between Wnt1-Cre;Krox20GFP(DT)+/+ (n=4) and Krox20GFP(DT)+/+ (n=5) embryos. (N) The relative surface area of the leaflets is indistinguishable between the Krox20GFP(DT)+/+ and Wnt1-Cre;Krox20GFP(DT)+/+ embryos. LC, left coronary leaflet; NC, non-coronary leaflet; OFT, outflow tract; RC, right coronary leaflet; RV, right ventricle. Scale bars: 200 μm in A,B,G,H; 100 μm in C-F,I-L.
At E17.5, the mesenchymal cells and relative surface were counted in this condition, the elimination of these cells occurred around E9.5. Previous studies have identified a cis-acting enhancer element 

**The Krox20 NCE element marks the NCC population that contributes to aortic valve leaflets**

Previous studies have identified a cis-acting enhancer element (NCE) located upstream of the Krox20 gene that can recapitulate its neural crest pattern in transgenic mice (Ghislain et al., 2003, 2002).

Fig. 6. Analysis of the NCE element of Krox20 during arterial valve development. (A) Schematic representation showing the genomic region of Krox20 and the 31/-23.5 Krox20/lacZ transgenic construct containing a lacZ in-frame insertion in Krox20. Restriction enzyme sites used to clone the genomic fragment are shown. Distances are in kb and indicate the position relative to the start site of transcription of Krox20. The conserved sequences and putative binding sites for Sox10 (black circles) and Krox20 (gray circles) are indicated. (B-D) In vivo analysis of the transgenic line -31/-23.5 Krox20/lacZ at E9.5 (B), E10.5 (C) and E13.5 (D). (B) At E9.5, β-galactosidase (β-gal) activity is detected in migratory neural crest cells (NCCs). (C) At E10.5, β-gal-positive cells (arrowhead) are observed in the outflow tract. (D) Transverse section through the outflow tract cushions at E13.5 shows β-gal-positive cells in the arterial valve leaflets. (E-G) Rhombomere (r) 4 fate mapping was performed on b1r4-Cre; Rosa-EYFP transgenic embryos (r7). (E) At E9.5, b1r4-Cre-labeled cells are specifically detected in the r4 and the 2nd pharyngeal arch (ba). (F) At E12.5, section staining shows b1r4-Cre-labeled cells at the top of the interventricular septum (IVS). (G) At E17.5, a small number of b1r4-Cre-labeled cells are detected in the aortic valve. Note the presence of YFP-positive cells in the left coronary artery. αSMA (red) marks the smooth muscle cells. Nuclei are labeled with DAPI in blue. (H) Model depicting the contribution of a NCC population in normal aortic valve development (H) and its disruption in Krox20−/− embryos (I). (J) Distribution of NCCs migrating from rhombomere (r) 4 (b1r4-Cre;Rosa-EYFP; green) and r5 (Krox20Cre;RosaFrtTomato; red) in wild-type (WT) embryos. (I) In loss of Krox20 function mutants, an excess of Krox20-derived neural crest cells leads to the swelling of the aortic valve leaflets. As a consequence, commissural fusion of the aortic valve leaflet occurs. AoV: aortic valve; ba1: 1st pharyngeal arch; IVS, interventricular septum; LA, left atrium; LCA, left coronary artery; LC, left coronary leaflet; LV, left ventricle; NC, non-coronary leaflet; OFT, outflow tract; ov, otic vesicle; PV, pulmonary valve; RA, right atrium; RC, right coronary artery; RCA, right coronary artery; RV, right ventricle. Scale bars: 200 μm in B,C; 50 μm in D.

One of the constructs studied, carrying sequences between -31 kb and -23.5 kb relative to the transcription start site of Krox20 (-31/-23.5 Krox20/lacZ) was active in the neural crest (Fig. 6A). We used the -31/-23.5 Krox20/lacZ transgene to examine β-galactosidase pattern at different stages. At E9.5, X-gal staining detected β-gal-negative cells in the arterial valve leaflets. At E10.5, β-gal staining showed that β-galactosidase-positive cells migrated from the r5 towards the 3rd pharyngeal arch region (Fig. 6B). By E10.5, reflecting the stability of the β-galactosidase protein, X-gal-stained cells were detected in the outflow tract and the arterial valve leaflets (Fig. 6C,D). These data identified the population of Krox20-expressing cells that are involved in arterial valve formation.

As our data indicate that r5 provides a population of migratory NCCs that contributes to the arterial valve leaflets, we asked whether a more cranial rhombomere such as r4 was also responsible for neural crest contribution to the arterial valves. We used the previously reported b1r4-Cre;R26R-EYFP transgenic mouse embryos to analyse r4-derived NCC contribution (Di Bonito et al., 2013). At E9.5, YFP-positive cells were detected in the r4 and the NCCs migrating towards the 2nd pharyngeal arch (Fig. 6E). At E12.5, EYFP-positive cells were detected at the top of the interventricular septum of the heart (Fig. 6F). By E13.5, reflecting the stability of the β-galactosidase protein, X-gal-stained cells were detected in the outflow tract and the arterial valve leaflets (Fig. 6G). These results suggest that the contribution of pre-otic NCCs to the cardiac valves is distinct from that of post-otic NCCs, and that r5 constitutes the main source of NCC population that contribute to the arterial valves (Fig. 6H).
crest that located in the neural folds spanning from the middle of the otic placode to the caudal border of somite 3, corresponding to rhombomeres (r) 6, 7 and 8 (Kirby, 2007). Studies in chick and mouse embryos have shown that cardiac NCCs contribute to the arterial valves (de Lange et al., 2004; Jiang et al., 2000; Waldo et al., 1998). More recently, Arima and colleagues (2012) have identified the contribution of pre-otic cranial neural crest to developing coronary arteries and adjacent cardiac tissues (Arima et al., 2012).

In this study, quail-chick chimera experiments made with neural crest between the midbrain and pre-otic hindbrain (r1-r5) showed the presence of quail-positive cells primarily in the interventricular septum but not in the arterial valves. Pre-otic neural crest ablation resulted in defects in coronary arteries, but not in valve defects, indicating that, at least in the chick embryo, neural crest cells derived from r1 to r5 are not crucial for arterial valve formation (Arima et al., 2012). Lack of direct evidence has led many investigators to question which neural crest region contributes to valve development. Our present findings provide direct evidence by identifying a specific subpopulation of NCCs derived from r5 as a source of arterial valve.

Previous studies have identified expression of Krox20 in r3 and r5, and shown that its expression is important for patterning of the hindbrain (Ghislain et al., 2003; Schneider-Maunoury et al., 1993; Voiculescu et al., 2001). In the neural crest, Krox20 expression is mainly restricted to cells migrating lateral to r5 towards the 3rd pharyngeal arch and presumably originating from r5 (Schneider-Maunoury et al., 1993). In addition, a Krox20 neural crest-specific element (NCE) has been characterized that drives expression of the reporter lacZ gene in NCCs emigrating from r5 in transgenic mice (Ghislain et al., 2003, 2002). Our lineage-tracing analysis showed that Krox20Cre-labeled cells are detected in outflow tract cushions and subsequently in the aortic valve, which reproduced the profile of the −31/−23.5 Krox20/lacZ (NCE) transgene, suggesting that NCCs emigrating from r5 contribute to arterial valve formation.

Interestingly, a study in the chick suggested that Krox20 expression in pharyngeal neural crest does not correlate with rhombomeric segmentation, and that there may be intrinsic differences in regulation between the r5 and r6 Krox20-expressing population (Nieto et al., 1995). The same study demonstrated that Krox20 expression occurs in neural crest precursors not only in r5, but also in r6, and that both of these precursor populations contribute to the stream of Krox20-expressing NCCs (Nieto et al., 1995). Detailed analysis of the fate of prospective r5 cells in Krox20 mutant mice using molecular markers demonstrated that a large part of r5 cells acquire even-numbered rhombomere characters in Krox20-null embryos (Voiculescu et al., 2001). Our lineage-tracing analysis in compound Krox20Cre/Sox10 derivative embryos revealed an increase in the number of migrating NCCs, suggesting that acquisition of r6 identity disturbs the neural crest migration. Furthermore, the increased number of migrating NCCs is likely to contribute to the hyperplasia of the aortic valves observed in Krox20 mutant. These findings lead us to speculate that neural crest arising from r5 provides important information for valve development.

The identification of the NCE transcriptional enhancer as a regulatory element expressed in r5-derived NCCs raises the question how is Krox20 expression regulated in these cells? The −31/−23.5 Krox20/lacZ transgene reporter used in our study to mark the NCCs that contribute to the arterial valves comprises a 1 kb fragment (~26.5 kb to ~25.5 kb) that contains Krox20-binding sites necessary for its autoregulation in the NCCs (Ghislain et al., 2003). Interestingly, the same fragment contains two adjacent, head-to-head, HMG box-binding sites for two members of the HMG box group expressed in the pre- and post-migratory NCCs: Sox10 and Sox9. Ghislain et al. (2003) have reported that Sox10 is a crest-specific factor essential for Krox20 autoregulation in NCCs (Ghislain et al., 2003). Although several studies have reported expression of Sox10 in the valve and a contribution of Sox10-Cre labeled cells to the heart, no cardiac defects have yet been reported in Sox10 mutant embryos (Montero et al., 2002; Simon et al., 2012). Interestingly, a double outlet right ventricle, which occurs when outflow tract septation is malformed, has been reported in one patient carrying a deletion at the SOX10 locus (Bondurand et al., 2007). Together, these data suggest that Sox10 is an important regulator of migrating NCCs, including the subpopulation of neural crest expressing Krox20.

In human patients, the most frequent BAV subtypes result from fusion of either the left coronary and right coronary leaflets (R-L) or the right coronary and non-coronary leaflets (R-N) (Sievers and Schmidtko, 2007). A recent study using mouse and Syrian hamster as animal models suggested that R-N and R-L BAVs could have distinct embryological origins; R-N BAVs would result from defective development of the outflow tract cushions, whereas R-L BAVs would result from abnormal septation of proximal outflow tract region associated with defective NCC behavior (Fernández et al., 2009). However, the precise role of NCCs in the formation of the arterial valves remained unclear until a recent study showed that the aggregation of NCCs, once they enter the outflow tract cushions, is important to define how the arterial leaflets form after outflow tract septation (Phillips et al., 2013). Thus, the position of NCCs is crucial for the pattern of aortic valve leaflets. Deletion of Krox20 results in R-N or L-N subtypes, suggesting abnormal development of outflow tract cushions. However, we never observed abnormal great arteries in Krox20−/− embryos, and the BAV phenotype is only detected after E15.5. These findings suggest that fusion between two leaflets may occur late in valve development. Although the phenotype in the Krox20−/− null and Krox20Cre/+ hypomorphic embryos is likely to result from a combination of requirement for Krox20 in neural crest- and endothelial-derived cells, we always observed an enlargement of the arterial valve similar to those observed in the Wnt1-Cre;Krox20lox/lox embryos. Our lineage-tracing analysis shows that this enlargement is caused by addition of Krox20 derivatives. Additional Krox20-Cre-labeled cells appear misplaced in the leaflet, which may explain the phenotype of bifoliate valves. Our previous study demonstrated that Krox20 is a key regulator of fibrillar Colla1 and Cola1 genes (Odelin et al., 2014). In addition, we have detected a reduction of collagen fibers and an increase of proteoglycan proteins in Krox20−/− aortic valves. Thus, the hyperplasia observed in Krox20 mutants is associated with a myxomatous-like phenotype. Therefore, modification of the extracellular matrix at the insertion zone of the arterial valve leaflets would lead to a widening and loss of delimitation of these insertion zones at the time of the excavation phase. The insertion zone would no longer be dense enough to maintain the endothelium close to the aortic wall, leading to the fusion of two layers during valvular maturation. Thus, an odd maturation of the valve could lead to the abnormal development of the aortic valve, such as BAV. Interestingly, commissural fusion of the native aortic valve leaflets occurs frequently in individuals supported by a continuous axial flow left ventricular assist device (Gallen et al., 2012; Mudd et al., 2008), suggesting that fusion between two
leaflets can happen during adulthood depending on environmental events affecting flow in the arterial valves.

Gene expression and lineage-tracing analyses revealed endogenous expression of Krox20 in the forming aortic valve (Odelin et al., 2014). Here, we confirm that very few neural crest- and endothelial-derived cells express Krox20 in the aortic valve at E18.5 (Fig. S3). Interestingly, mice with neural crest-specific deletion of Krox20 have less BAV (10.5%) than Krox20−/− mice (27%), suggesting additional contributions (Table 1). Endothelial-specific deletion of Krox20 leads to BAV phenotype (10%) (Table 1). However, our morphometric analysis demonstrates that aortic valve leaflets are not hyperplastic in this context and BAV are formed by equally sized leaflets (Fig. S6). Collectively, these findings indicate contribution of Krox20-expressing endothelial cells during arterial valve development; however, additional studies are needed to further clarify the function of Krox20 in the endothelial cells. In conclusion, our data highlight the role of NCCs in aortic valve disease and provide a new model to understand the pathophysiology of the bicuspid phenotype.

MATERIALS AND METHODS

Mice

All animal procedures were carried out under protocols approved by a national appointed ethical committee for animal experimentation (Ministère de l’Education Nationale, de l’Enseignement Supérieur et de la Recherche; Authorization N° 2931-2015113016228473). The Krox20fluc, Krox20ppSf (DT), Krox20lus and Krox20cre alleles have been previously described (Schneider-Maunoury et al., 1993; Taillebourg et al., 2002; Vermeren et al., 2003; Voiculescu et al., 2000). The Gr(ROSA)26Sor1neoDse (R26R), Gr(ROSA)26Sor1mTmGal/ACTBαMTomato GFP mEFP/Cre (RosatdTomato-GFP), Gr(ROSA)26Sor1mPEGmEFP/Cre (RosatdTomato-GFP) and ZAPI transgenic lines have been previously described (Lobe et al., 1999; Madisen et al., 2007; Soriano, 1999; Srinivas et al., 2001). The Tg(Tek-Cre)Ywa (Tie2-Cre), Wnt1-Cre and bI1r4-Cre mice have been previously described (Di Bonito et al., 2013; Jiang et al., 2000; Kisanuki et al., 2001). Krox20fluc−/− or Krox20lacZ+cK mice were maintained on a C57BL/6 background and inter-crossed with Tie2-Cre, Wnt1-Cre or Krox20αα to generate heterozygous offspring that were obtained at expected Mendelian ratios. The ~31 kb to ~23.5 kb Krox20lacZ transgene has been previously described by Ghislain et al. (2003). Transgenic mice were generated after revitalization of frozen eggs. After echocardiography analysis, adult Wnt1-Cre;Krox20fluc− mice were sacrificed via intraperitoneal injection of sodium pentobarbital (0.5 ml). Tissue sections were collected at embryonic day (E) 9.5 to E18.5, counting evidence of a vaginal plug as E0.5.

Histological and immunostaining

Standard histological procedures were used (Ryckebusch et al., 2010). Heart tissues from Krox20−/− or Krox20αα and littermate controls were fixed in neutral-buffered 4% paraformaldehyde in PBS, rinsed, dehydrated, paraffin-embedded and tissue sections cut at 8 μm. Sections were stained with Harris’ Hematoxylin and Eosin (Sigma). AP2x (1:500, clone 3B5 DHSB), αSMA (1:500, Sigma, F3777), CD31 (1:100, Pharamingen, 01951A), GFP (1:500, Invitrogen, A11122), Krox20 (1:100, Covance, PRP-236P), and phospho-histone H3 (1:100, Millipore, 06-570) immunofluorescence were performed using Alexa fluorescent dye-conjugated secondary antibodies (Life Technologies) at 1:500. Nuclei were labeled with DAPI.

In situ hybridization and X-gal staining

X-gal staining and in situ hybridization were performed on whole-mount embryos or 12 μm frozen sections as described previously (Odelin et al., 2014). For each experiment, a minimum of three embryos of each genotype were observed. Embryos were examined using an AxioZoom.V16 (Zeiss) and photographed with an AxioCam digital camera (Zen 2011, Zeiss).

Quantification of valve anomalies

Quantification of valve thickness: the leaflets of the valves were used over a minimum depth of 100 μm using a DM5000 Leica microscope with LAS software. ImageJ software was used to measure the surface of the valve and the number of nuclei labeled by DAPI. Measurements were performed on at least ten different sections per leaflet. The values were averaged. A minimum number of six animals were used per genotype.

Three-dimensional (3D) reconstructions: Fiji software was used to make the 3D reconstructions presented. At E18.5, images of 20-30 8 μm paraffin sections were manually aligned to generate 3D reconstruction. Volumetric measurements of the aortic valve are presented as the average of six reconstructions of each genotype.

Echocardiography

In vivo valve structure and function of Wnt1-Cre;Krox20fluc− mice were evaluated using a high-frequency scanner (Vevo2100 VisualSonics) as previously described (Odelin et al., 2014). Briefly, 1-month-old mice were anesthetized with 1-2% isoflurane inhalation and placed on a heated platform to maintain temperature during the analysis. Two-dimensional imaging was recorded with a 22-55 MHz transducer (MS550D) to capture long- and short-axis projections with guided M-Mode, B-Mode and color and pulsed-wave Doppler. Doppler interrogation was performed on the arterial valve outflow in the parasternal long-axis view to assess aortic flow, using a sample volume toggle to optimize the angle of interrogation. Aortic insufficiency was defined as valve incompetence with reversal of flow in diastole.

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Competing interests

The authors declare no competing or financial interests.

Author contributions


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Supplementary information

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