A Retinoic Acid Responsive *Hoxa3* Transgene Expressed in Embryonic Pharyngeal Endoderm, Cardiac Neural Crest and a Subdomain of the Second Heart Field

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**Abstract**

A transgenic mouse line harbouring a β-galactosidase reporter gene controlled by the proximal 2 kb promoter of *Hoxa3* was previously generated to investigate the regulatory cues governing *Hoxa3* expression in the mouse. Examination of transgenic embryos from embryonic day (E) 8.0 to E15.5 revealed regionally restricted reporter activity in the developing heart. Indeed, transgene expression specifically delineated cells from three distinct lineages: a subpopulation of the second heart field contributing to outflow tract myocardium, the cardiac neural crest cells and the pharyngeal endoderm. Manipulation of the Retinoic Acid (RA) signaling pathway showed that RA is required for correct expression of the transgene. Therefore, this transgenic line may serve as a cardiosensor line of particular interest for further analysis of outflow tract development.

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**Introduction**

Cardiac development requires specification, proliferation, migration and differentiation of progenitor cells from diverse tissues of the embryo [1]. Myocardial progenitor cells in the anterior splanchnic mesoderm are destined to form the left ventricle and contribute to the formation of the atrio-ventricular canal and the atria. These cells characterize the first heart field (FHF) as opposed to a population of cardiac progenitor cells defining the second heart field (SHF) [2,3]. SHF proliferating progenitor cells are located in the pharyngeal mesoderm lying medial to the FHF. Initially, FHF cells, which differentiate at embryonic day (E)7.5, form the primitive heart tube while subsequent addition of SHF cells at both anterior and posterior poles lead to elongation and looping of the forming heart and contribute to right ventricular, outflow tract (OFT) and atrial myocardium [4,5,6,7]. SHF cells express genes including *Fgf8, Foxh1, Tbx1, Ihl1* and *Mnx2.5* of which inactivation leads to defects in the development of SHF progenitors and subsequently of the OFT [5,8,9,10,11].

Neural crest cells are multipotent stem cells that originate from the dorsal neural tube and give rise to various structures such as nerves, ganglia, cartilages, bones and connective tissue [12,13]. Cardiac neural crest cells are a subdivision of the cranial crest originating from the level of the otic placode to the caudal border of somite 3, corresponding to rhombomeres 6, 7 and 8 [12,14,15]. Cells of the neural crest migrate to the third, fourth and sixth pharyngeal arches (PA), where they are largely devoted to glandular and vascular development. Cardiac neural crest cells play an important role in patterning the aortic arch arteries and form the smooth muscle tunics of the great arteries. The migration patterns of neural crest cells in mammalian species have been identified by fate-mapping studies with gene expression markers for neural crest cells [16,17,18]. Thus, a subset of the cardiac neural crest cells migrates between the aortic sac and the pharyngeal endoderm and infiltrates the cardiac outflow cushions [16,17,18]. Ablation and quail-chick chimera experiments showed that cardiac neural crest cells are absolutely required to form the aorticopulmonary septum dividing the cardiac arterial pole into systemic and pulmonary circulations [12].

The morphogenesis of the arterial pole (outflow tract) of the heart is a complex process that is defective in many congenital heart defects and depends on the interaction between cardiac neural crest and SHF cells after formation of the primitive heart tube [12,19,20]. Indeed, addition of SHF derived cells and migration of cardiac neural crest cells into the OFT temporally overlap (embryonic days 9.5–10.5) [4,21]. Recent data have suggested that a cross-talk between these two cell populations is
crucial for normal OFT development [22,23]. First, ablation of cardiac neural crest results in failure of the OFT to lengthen by addition of myocardial progenitors from the SHF [19,20]. Second, loss of Tbx1 in pharyngeal mesoderm (SHF) can negatively impact on cardiac neural crest cells [11]. Pharyngeal endoderm has been implicated as a third player in development of the OFT. Indeed, a study showed that Sonic Hedgehog produced by pharyngeal endodermal cells has a direct or indirect action on cardiac neural crest and SHF cells survival, respectively [22]. These data suggest that normal OFT morphogenesis depends on an intricate interplay between cardiac neural crest, SHF and pharyngeal endoderm.

**Hox** genes encode a class of transcription factors that play an important role in patterning vertebral axis development [24,25]. For example, the axial identity of the hindbrain neural crest is controlled by a combinatorial pattern of **Hox** gene expression [26]. Among the **Hox** family, **Hoxa3** is expressed in the hindbrain neural tube in rhombomeres 5 and 6 [27]. The neural crest cells populating the third pharyngeal arch originate from rhombomeres 5, 6 and 7 [28]. These cardiac neural crest cells express **Hoxa3** as does the endodermal epithelium of the third pharyngeal pouch [29]. In **Hoxa3** null mutant embryos, the neural crest cell population fails to induce differentiation of surrounding arch and pouch tissues leading to abnormal development of the third arch artery, and defects in the thymus, parathyroid gland and carotid body [30,31]. Interestingly, a recent study has shown that **Hoxa3** is also expressed in a sub-population of SHF cells that contributes to the OFT myocardium [32].

A classical approach to gain insight into regulatory mechanisms controlling **Hox** gene expression is to dissect the flanking regions and test for activity in transgenic embryos. To document the expression pattern provided by the proximal 2 kb of the **Hoxa3** promoter in transgenic mice, we generated **Hoxa3-lacZ** transgenic embryos that revealed β-galactosidase expression in specific territories including particular hindbrain, ganglionic and branchial compartments development of which relies on **Hoxa3** activity [33].

**Results**

The **Hoxa3-lacZ** transgene is active in the pharyngeal region and cardiac neural crest cells

The expression profile of the proximal 2 kb promoter of **Hoxa3** was previously examined both in transgenic mouse line and in transient transgenic embryos. This transgene recapitulates a subset of the **Hoxa3** gene expression in specific territories including particular hindbrain, ganglionic and branchial compartments development of which relies on **Hoxa3** activity [33].

After four backcrosses in an outbred, CD-1 mouse genetic background, the transgenic line derived from the construct (HSTG3, 33) exhibited a slightly narrowed expression pattern in whole mount stained embryos with respect to our initial record (Figure 1). At embryonic day (E) 9.5 X-gal staining of **Hoxa3-lacZ** transgenic embryos revealed detectable β-galactosidase activity at the dorsal midline of the neural tube, in lateral plate mesoderm, in otic vesicles and in the pharyngeal region (Figure 1A). This pattern was similar at later stages including E9.5 (Figure 1B), E10.5 (Figure 1C) and E11.5 (not shown). Focusing on the pharyngeal region, β-galactosidase activity was confined in the pharyngeal endoderm and developing pharyngeal arches (PAs) at E9.5 (Figure 1D and not shown). By E9.5 and E10.5, whole mount stained embryos exhibited β-galactosidase activity in PA3, 4 and 6 (Figure 1E, F). Since pharyngeal tissue provides different cellular progenitors crucial for outflow tract (OFT) development, we extended our analysis of **Hoxa3-lacZ** transgene expression during cardiac development.

We first stained whole transgenic hearts at different developmental stages (Figure 2), β-galactosidase activity was essentially detected in the arterial pole (outflow tract) of E9.5 (Figure 2A), E10.5 (Figure 2B), E12.5 (Figure 2C) and E15.5 (Figure 2D) transgenic hearts. Since both SHF and cardiac neural crest cells contribute to OFT development during cardiogenesis, we investigated the nature of β-galactosidase-positive cells in this region. As previously reported, mesenchymal cells in the 3rd and 4th PAs expressed the **Hoxa3-lacZ** reporter at E9.5 [33] and E10.5 (Figure 3). Histological sections from E10.5 transgenic embryos confirmed clear β-galactosidase expression in PA3, 4 and 6 (Figure 3A) and OFT mesenchyme (Figure 3E). Of note, at E15.5, X-gal labeled cells were found associated with vessels deriving from these three caudal PAs (Figure 2D). These observations suggest that X-gal-labeled cells are cardiac neural crest cells. In addition to surface ectoderm, neural crest cells including the cardiac neural crest cell population are a prominent site of AP-2α expression [34]. Therefore, we used AP-2α as a specific marker of the migratory cardiac neural crest cell population that populate PA3 to 6 and the OFT of the heart [34,35]. Immunostaining for AP-2α on transgenic sections at E9.5 and E10.5 showed that X-gal labeled ectodermal cells expressed AP-2α transcription factor as expected (Figure 3B, arrowheads). In addition, β-galactosidase–expressing mesenchymal cells of PA3-6 also exhibited nuclear staining for AP-2α (Figure 3B) suggesting that these cells are neural crest–derived cells. At E10.5, the aortic sac (AS) expressed the transgene on whole mount heart (Figure 2B, asterisk), and on histological sections X-gal labeled cells were detected in the pharyngeal endoderm and AS (Figure 3C). AP-2α-expressing cardiac neural crest cells could be detected in the region between the pharyngeal endoderm and the AS, and, in contrast to X-gal–positive cells of the pharyngeal endoderm, β-galactosidase–expressing cells populating the AS were AP-2α positive (Figure 3D). This pattern matches to that previously reported for independent mouse neural crest lineage markers [16,35,36,37].

Expression of AP-2α is mostly extinguished by E11.5 when the cardiac neural crest cells have migrated into the OFT of the developing heart [35] whereas the AP-2α lineag is detected in the OFT cushion in AP-2α-Cre; R26R mouse embryos [37]. Cardiac neural crest cells undergo extensive cell rearrangements during the formation of the aortico-pulmonary septum in the OFT which require N-cadherin [38]. We therefore analyzed N-cadherin expression in wild-type and transgenic embryos at E9.5 and E10.5 as an alternative marker of cardiac neural crest cells (Figure
At E9.5, cardiac neural crest cells have not yet migrated into the OFT, whereas N-cadherin was already expressed in OFT, atrial and ventricular myocardium (Figure S1A, C). At E10.5, cardiac neural crest cells migrated into the distal end of the OFT, as demonstrated by detection of N-cadherin in this region, although at lower levels compared to myocardial expression (Figure S1B, D) [38]. Immunostaining for N-cadherin on E10.5 transgenic hearts demonstrated that β-galactosidase activity colocalized with the N-cadherin protein in the OFT mesenchyme (Figure 3E, F), suggesting that these transgenic cells represent cardiac neural crest cells migrating into the OFT. In conclusion, the Hoxa3-lacZ transgene was expressed in migratory cardiac neural crest cells of the caudal PAs (3, 4 and 6) and in cardiac neural crest derived mesenchymal cells that populate the cushions in the OFT between E9.5 and E10.5, prior to formation of the aorticopulmonary septum.

Since cardiac neural crest cells differentiate into smooth muscle cells (SMC) during formation of the aorticopulmonary septum, we tested whether differentiated SMC were also β-galactosidase-positive in the cushions (inset Figure 2B). By E11.5, reporter activity was observed in the OFT of whole mount stained hearts (not shown). Histological sections confirmed X-gal labeled cells in the cushions (Figure 4A, asterisks). Immunostaining for smooth muscle α-actin (α-SMA) demonstrated that β-galactosidase-positive cells in mesenchyme expressed α-SMA (Figure 4B). Indeed, those β-galactosidase- and α-SMA-positive cells were negative for the marker cardiac actin (c-actin) on adjacent sections (Figure 4C) that is normally expressed by myocardial cells that penetrate the septum from the myocardial wall and are also α-SMA-positive (Figure 4B, C), as previously observed [39]. By E13.5, the aorta and pulmonary trunk contained X-gal labeled cells (Figure 2C). On histological sections, β-galactosidase-positive cells were detected in the aortic and pulmonary trunks as well as in the ductus arteriosus derived from the left 6th aortic arch artery (Figure 4D) and co-expressed α-SMA on adjacent sections (Figure 4E). At E13.5, the mesenchymal outlet septum formed by fusion of the OFT cushions exhibited β-galactosidase activity (Figure 4F, 4G, arrowhead). These cardiac neural crest derived mesenchymal cells were previously demonstrated to be derived from the AP-2α lineage [16,37]. These cushions were in the process of extensive muscularization as revealed by c-actin staining (Figure 4G). Scattered β-galactosidase/c-actin-double positive cells were observed in the aortic trunk but not in the ductus arteriosus where β-galactosidase-positive cells were not stained for c-actin (Figure 4G). Together, the pattern of transgene expression in the PAs and arterial pole of the heart recapitulated a subset of the cardiac neural crest migratory route [16,18,35,37].

Expression of the transgene in a subdomain of the SHF and OFT myocardium

SHF progenitor cells are characterized by the expression of Isl1 [5], Nkx2.5 [9], Fgf8, Fgf10 [4], and Tbx1 [11], and cells that have transcribed these genes form the arterial pole of the heart. The Mef2C SHF enhancer (Mef2c-AHF-lacZ) [40] and the Mlc1v-nlacZ-24 transgene [4] are also expressed in the SHF. Cell tracing and explant culture experiments have shown that SHF progenitor cells contribute to the inflow and outflow tracts [4,5,6,7]. As mentioned above, Hoxa3-lacZ transgenic embryos exhibited X-gal staining in the forming posterior PAs and pharyngeal endoderm at E8.5 (Figure 1D, arrowhead). At E9.5, on whole mount stained heart, transgene expression was detected in the distal region of the OFT (Figure 2A). Histological sections showed that X-gal-labeled cells...
were restricted to a subdomain of the SHF, whereas the pharyngeal endodermal cells displayed broad β-gal staining (Figure S1G). Between E10 and E10.5, we observed increased X-gal staining in the OFT (Figure 2B) as well as in pharyngeal mesoderm caudal to this region (Figure 1F, arrow). Sections confirmed reporter activity in OFT myocardium (Figure 5A, B) as well as in a sub-region of the SHF (Figure 5B). To better characterize the identity of the X-gal labeled cells in the SHF and OFT myocardium, we performed immunostaining for Isl1 protein and c-actin, as a marker of differentiated cardiomyocytes, in E9.5 and E10.5 transgenic embryos. Isl1 protein is used as a pan-marker of SHF cells as well as cardiomyocytes of the distal OFT [9,41]. Results showed that X-gal labeled cells were either single positive for Isl1 or double Isl1/c-actin positive (Figure 5C-F, and Figure S1H). Taken together, these results showed that the transgene is expressed in the anterior SHF, in differentiating as well as recently differentiated cardiomyocytes of the distal portion of the OFT. The changing position of X-gal labeled cells during OFT formation therefore provided a “chase”, marking cells which previously transcribed the Hoxa3-lacZ transgene. Similarly, in Mf2C-AHF-lacZ [40] and Mbx1-nlacZ24 [4] transgenic embryos, β-galactosidase expression was more extended in the SHF and included the OFT and right ventricle at E9.5 and E9.5 (see below, Discussion). In accordance with restricted staining of the SHF by Hoxa3-lacZ cells, the inflow tract, atria and right ventricle were negative for β-galactosidase activity between E9.5 and E15.5.

Therefore, at E10.5, the Hoxa3-lacZ transgene is activated in both cardiac neural crest and SHF-derived myocardial cells at a crucial time of OFT development.

**Hoxa3-lacZ activity is sensitive to Retinoic Acid signaling**

Retinoic Acid (RA) is the active derivative of vitamin A that plays crucial roles in various steps of cardiovascular development [42,43]. In addition, a recent study has shown that expression of Hoxa3 in a sub-population of SHF cells depends on RA signaling [32]. We therefore analyzed the responsiveness of Hoxa3-lacZ transgene to changes in RA signaling in transgenic embryos.

To visualize the Hoxa3-lacZ expression pattern in the absence of RA signaling, we generated embryos harboring the Hoxa3-lacZ transgene but deficient for retinaldehyde dehydrogenase 2 (Raldh2−/−), the enzyme that catalyzes the second oxidative step in RA biosynthesis [44]. As previously reported, Raldh2−/− embryos failed to undergo heart looping and have impaired atrial and sinus venous development compared to wild-type hearts [45] (Figure 6A, B). In contrast to wild-type Hoxa3-lacZ transgenics, Raldh2−/− Hoxa3-lacZ embryos lacked β-galactosidase activity in the entire embryo except for faint staining in the pharyngeal region at E8.5 (compare Figure 6A and 6B) and E9.5 (Figure 6C, D). Hoxa3-lacZ expression was thus abrogated under RA deficiency showing that transgene-expressing cells are regulated by RA signaling.

Because cardiac patterning is sensitive to administration of exogenous RA at early stage (E6.75–E8.5) [46,47,48,49], we have also examined the effect of exogenous RA on transgene expression in the embryo at a stage when cardiac neural crest and SHF cells are responsive to RA [46,47,48,49]. In contrast to Hoxa3-lacZ control E7.5 embryos treated with vehicle only (Figure 6E), those treated with exogenous RA exhibited an up-regulation of Hoxa3-lacZ in the heart field at E8 (Figure 6F, G), confirming that the transgene is sensitive to an increase in RA dosage.

**Discussion**

In this study, we characterize transgenic embryos harboring a reporter transgene controlled by the proximal 2 kb of the Hoxa3 gene promoter that is expressed in several tissues required for OFT development and depends on the RA signaling pathway. Using X-gal staining and immunostaining for markers of distinct cardiac lineages, we demonstrated that the transgene exhibited β-galactosidase activity in the PAs and the OFT during cardiogenesis. General feature of our analysis may be relevant to the tripartite interactions between cardiac neural crest, SHF and pharyngeal endodermal cells during cardiac development in general and OFT morphogenesis in particular. First, the transgene is expressed in the pharyngeal endoderm from E9.5 to E11.5. Second, the transgene is activated in the migratory cardiac neural crest cells of the PAs and those that populate the OFT and participate in its septation. Third, a subdomain of SHF progenitor cells expressed the transgene at E9.5 and, most importantly, at E10.5, SHF-derived cells in the OFT further displayed transgene expression. Finally, we observed that transgene activity is lost in Raldh2−/− mutant embryos while it is precociously and highly up-regulated in all-trans-RA-treated embryos.**

**Hoxa3-lacZ expression in cardiac neural crest cells, SHF and OFT myocardium**

Our study showed Hoxa3-lacZ expression in migratory cardiac neural crest cells that invade the 3rd, 4th and 6th PAs [28], OFT,
smooth muscle cells of the cushions and aortic and pulmonary trunk as well as in those contributing to the developing 3rd, 4th and 6th pharyngeal arteries. In all these territories, β-galactosidase activity colocalized with cardiac neural crest markers such as AP-2α and N-cadherin. Interestingly, the expression pattern of Hoxa3-lacZ in the cardiovascular system partially recapitulated that previously reported for transgenic lines expressing lacZ in cardiac neural crest under the control of the proximal 650-bp of the Connexin43 gene promoter [18] as well as of those obtained upon Cre/lox-mediated induction involving the neural crest specific Pax3, Wnt1 or human tissue plasminogen activator (Ht-PA) promoters [16,17,18,50]. Our results are consistent with previous studies showing that Hoxa3 expression is detected in the neural crest specific Pox3, Wnt1 or human tissue plasminogen activator (Ht-PA) promoters [16,17,18,50]. Our results are consistent with previous studies showing that Hoxa3 expression is detected in the neural crest specific Pox3, Wnt1 or human tissue plasminogen activator (Ht-PA) promoters [16,17,18,50]. Our results are consistent with previous studies showing that Hoxa3 expression is detected in the neural crest specific Pox3, Wnt1 or human tissue plasminogen activator (Ht-PA) promoters [16,17,18,50].

In addition to being expressed in the cardiac neural crest that populate the OFT, the Hoxa3-lacZ reporter is also active in a sub-domain of the SHF and myocardial cells of the OFT. This expression pattern in SHF progenitors and SHF derived-myocardial cells is in sharp contrast with other previously described SHF reporter lines [4,5,40]. Indeed, the Mlc1v-nlacZ-24 and Mef2C-AHF-lacZ reporter transgenes both mark a larger portion of the SHF, and subsequently identify cells that are found in both OFT and right ventricular myocardium [4,7,40]. The labeling of these progenitor cells occurred essentially at the time (i.e. E7.5) of anatomic appearance of the SHF [4,40]. Conversely, Hoxa3-lacZ expression was delayed by 24h since Hoxa3-lacZ positive cells were detectable by E8.5 and only in a sub-domain of the SHF. Thus, it only partially overlaps with that of the Mef2C-AHF-lacZ or Mlc1v-nlacZ-24 lines (Figure 7). However by E10.5, Hoxa3-lacZ transgene expression was extended in the SHF and

Figure 3. lacZ-positive cells in the pharyngeal arches, aortic sac and OFT identify migratory neural crest cells. Sagittal sections of E10.5 transgenic embryos at the level of the 3rd, 4th and 6th pharyngeal arches (A, B), aortic sac (C, D), and outflow tract (E, F). Ap2α (B, D) and N-cadherin (F) immunohistochemistry on X-gal-stained adjacent sections demonstrates that lacZ-positive cells in the pharyngeal arches (B) as well as those populating the space between the pharyngeal endoderm and aortic sac (D) and outflow tract mesenchyme (F) are cardiac neural crest cells. Arrowheads and arrows (B) indicate the pharyngeal ectoderm and AP2α LacZ cells in pharyngeal arch ectoderm and mesoderm, respectively. Arrows and arrowheads (C, D) show the pharyngeal endoderm and AP2α LacZ cells, respectively. AS, aortic sac; OFT, outflow tract. RA, right atrium 3, 4, and 6 indicate the 3rd, 4th and 6th pharyngeal arches, respectively. Scale bars = 100 μm.

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OFT myocardium, overlapping with Mef2C-AHF-lacZ and Mlc1v-nlacZ-24 transgene activities, except for its exclusion from the right ventricle (Figure 7). Thus, in our transgenic model, the initial SHF progenitors deployed to the heart tube at both poles to generate myocardial cells of the atria and right ventricle are negative for Hoxa3-lacZ (Figure 7). Subsequently (i.e. at E10-10.5) the entire OFT myocardium (also labeled by Mlc1v-nlacZ-24 and Mef2C-AHF-lacZ) expresses Hoxa3-lacZ concurrently with cardiac neural crest cells populating the OFT. This cardiac neural crest contribution would bring cells needed in the OFT to interact with the underlying myocardium for correct OFT development at E9.5–10.5 [16]. It has been reported that the presence of cardiac neural crest cells in the caudal pharynx is crucial for the addition of SHF to the OFT myocardium [19,20,52,53], but to date, no gene and/or reporter transgene were reported to be expressed in the underlying pharyngeal endoderm, cardiac neural crest cells and SHF–derived myocardium during OFT development. For the first time here, we describe a transgenic line (Hoxa3-lacZ) which therefore appears attractive to investigate potential “tripartite” interactions between these cell populations crucial for OFT development.

The Mlc1v-nlacZ-24 and Mef2C-AHF-lacZ transgenes show bona fide expression patterns with respect to the Fgf10 and Mef2C enhancer elements from which they are derived respectively [4,40]. A recent study shows Hoxa3 expression in a sub-population of SHF cells that contribute to OFT myocardium [32]. Thus, the Hoxa3-lacZ transgene is likely to reflect a specific activity of Hoxa3 in SHF cells.

Hoxa3-lacZ and RA signaling

Several lines of evidence show that the expression of some Hox genes from paralogous group 1 is dependent on RA signaling [54,55]. Moreover, the study by Bertrand et al. (2011) [32] shows that Hoxa3 expression in the SHF is dependent on RA signaling. Here, we showed that RA deficiency negatively affected the Hoxa3-lacZ in all the territories in which it is active including the PAs, OFT and pharyngeal endoderm whereas exogenous RA induced its precocious activation and further enhanced its activity in cardiac progenitors. These data therefore demonstrate that Hoxa3-lacZ is sensitive to RA dosage. The Hoxa3-lacZ transgene was made up of the proximal 2 kb of the Hoxa3 gene promoter [33]. Careful sequence analysis allowed predicting a highly conserved DR5-RARE element, which could mediate this RA responsiveness (Figure S2). Alternatively, it may be possible that this Hoxa3 promoter region is indirectly regulated by RA signaling via an RA sensitive Hoxa3 transcriptional modulator.

RA deficiency alters gene expression in the SHF and induces a posterior expansion of markers of the SHF, including Tbx1, Fgf8, Esl1 and the Mlc1v-nlacZ-24 reporter transgene [48,49]. This is in sharp contrast with the results reported here for Hoxa3-
lacZRaldh2-/- embryos. However, the sensitivity of the transgene to RA is consistent with the down regulation of Hoxa3 reported in the hindbrain and pharyngeal region of Raldh22/2 embryos [56]. A recent report on the fate of RA-activated embryonic cell lineages revealed that among many tissues, migratory cardiac neural crest cells of PA3 to 6, pharyngeal endoderm, atrial chamber, OFT, aortic arches, and dorsal aorta are responsive to RA signaling [57]. Hence, the loss of Hoxa3-lacZ transgene expression in Raldh2-/- embryos at E8.5 and E9.5 may result from cell death and/or failure in the development of cardiac neural crest cells fated to populate PA 3, 4 and 6. Indeed, increased apoptosis was observed in the hindbrain and neural crest cells of Raldh2-/- embryos [56], and a loss of X-gal staining in these tissues was reported at E8.5 and E9 in Raldh2-/- embryos harbouring RARE-Cre and R26R transgenes [57].

Hoxa3 expression has been reported to be altered in RA-exposed embryos [58,59]. Indeed, RA-exposed embryos exhibited precocious and increased Hoxa3 expression within its normal expression domain and expression in the hindbrain was shifted rostrally. Among the developmental defects reported in these RA-treated fetuses, transposition of the great arteries or stenosis of the carotid artery, interrupted aortic arch, double outlet right ventricle, truncus arteriosus with a high ventral septal defect and insertion of the internal and external left carotid arteries directly in the aortic arch, were reported [58]. These defects are reminiscent of those observed in several genetic models of congenital heart diseases affecting SHF progenitors and cardiac neural crest cell development [22,23]. The precocious or ectopic activation of Hoxa3 in RA-treated embryos may reflect the way changes in the combinatorial Hox code affect cardiac development [32,58,60,61]. RA may thus act as a global morphogen involved in coordinated Hox gene expression in cardiac progenitor cells, as it does for embryo axial structures.

Conclusion
We have described a mouse reporter transgene which delineates three crucial cell lineages for the OFT development. In contrast to the cardiosensor lines reported until now, this Hoxa3-lacZ reporter
transgenic embryos. Lateral views of E8.5 (A, B), E9.5 (C, D), and embryos (A, C), ß-galactosidase activity is lost in indicates the otic vesicle. HT, heart tube. Scale bars = 100 (F, G, arrowheads). Arrows (F, G) indicate the cardiac crescent. Asterisk embryos display a precocious ß-galactosidase upregulation in the SHF doi:10.1371/journal.pone.0027624.g006

Figure 6. RA signaling affects Hoxa3-lacZ expression in transgenic embryos. Lateral views of E8.5 (A, B), E9.5 (C, D), and frontal views of E8 (E,F,G) embryos. In contrast to control Hoxa3-lacZ embryos (A, C), ß-galactosidase activity is lost in Hoxa3-lacZ; Raldh2−/− embryos (B, D). Hoxa3–lacZ embryos treated with vehicle (DMSO) show no activation of the transgene (E) whereas RA-treated transgenic embryos display a precocious ß-galactosidase upregulation in the SHF (F, G, arrowheads). Arrows (F, G) indicate the cardiac crescent. Asterisk indicates the otic vesicle. HT, heart tube. Scale bars = 100 µm. doi:10.1371/journal.pone.0027624.g006

is the first that is expressed in cardiac neural crest cells, SHF-derived OFT myocardium and pharyngeal endoderm at the period of OFT morphogenesis. The other reported lines mark either cardiac neural crest cells [16,17,18,50,62] or the OFT myocardium [4,40] but not both cell populations. This line appears as an alternative tool for further studies of interactions between cardiac neural crest cells, OFT myocardial cells and pharyngeal endoderm in specific genetic systems and for understanding the etiology of congenital heart defect.

Materials and Methods

Ethics statement

All animal work has been conducted according to relevant national and international guidelines and approved by the “Comité d’éthique pour l’expérience animale, Université catholique de Louvain, Louvain-la-Neuve” (approval number # 053001).

Mouse lines

The reporter transgene we generated, referred to as Hoxa3-lacZ, consists of the Escherichia coli lacZ gene fused to a 2-kb fragment of the Hoxa3 promoter extending up to the ATG codon of the Hoxa3 reading frame (Figure S2). The detailed transgene construction and mouse transgenesis have been described previously [33].

The Raldh2 mutant line was described by Niederreither et al. (1999)[44]. To assess the expression Hoxa3-lacZ in Retinoic Acid deficient background, males hemizygote for Hoxa3-lacZ and heterozygote for Raldh2 (Hoxa3-lacZ; Raldh2−/+ ) were crossed with heterozygote female Raldh2−/+ to generate E8.5 and E9.5 Hoxa3- lacZ; Raldh2−/− embryos.

X-gal staining and immunohistochemistry

To document the expression pattern of the reporter, embryos from E7.5 until E17.5 were analysed both morphologically (whole mount stained) and histologically. At least three transgenic embryos of each stage were analysed according to Theiler’s nomenclature. For lacZ staining, embryos obtained between E7.5 and E17.5 were fixed in 4% paraformaldehyde for 30 to 90 min at 4°C. They were then washed twice in 1% phosphate buffer saline (PBS) at room temperature for 20 min and stained overnight at 30°C in 40 mg/ml X-gal, 200 mM K3Fe(CN)6, 200 mM K4Fe(CN)6, 1M MgCl2, 1xPBS. For histological analyses, embryos to be sectioned were fixed overnight in 4% paraformaldehyde at 4°C, stained as above, dehydrated and embedded in paraffin (or stored in 70% ethanol until use), 6–8 µm thick sections were counterstained with cosin.

For immunohistochemistry, sections from E9.5–13.5 dissected embryos were deparaffinized in xylene, rehydrated and finally washed in 1xPBS. Following antigen retrieval in 10 mM citric acid (pH 6.0), sections were blocked with 5% goat serum in 1xPBS for 1 hour at room temperature and then incubated with various primary antibodies. Endogenous peroxidases samples were inhibited with 3% H2O2 in water for 10–15 min and washed in 1xPBS. For the detection of cardiac actin (c-actin), Isl-1, N-cadherin, AP-2z, PECAM-1 and z-smooth muscle actin (z-SMA) expression, the following antibodies were diluted in a solution of 0.1% Triton X-100/5% goat serum/PBS and used at 4°C O/N at the dilutions shown: anti-mouse c-actin (1:400, A2547 Sigma), anti-rat N-cadherin (1:400, DSHB clone MND2) and anti-mouse Is1 (1:100, DSHB, clone 39.4D5), AP-2z (1/100, DSHB clone 3B5) and mouse anti z-SMA (clone 1A sigma, 1/1000). The primary antibodies were detected with biotinylated second antibody goat anti-rabbit, goat anti-mouse, or goat-anti-rabbit (1:200, Vector Laboratories) followed by avidin-biotin binding (Vectastatin ABC kit, Vector Laboratories) and application of 3,3-diaminobenzidene (Vector Laboratories). After desired color intensity was achieved, reaction was stopped by extensive wash in distilled water. The samples were then dehydrated through increasing ethanol (70%, 95% and 100%), immersed in xylene and mounted in DEPEX (EMS).

For immunofluorescence, sections were treated with sodium borohydride (1 mg/ml) on ice to eliminate the epifluorescence due to fixative, and blocked in a solution of 0.1% Triton X-100/5% donkey serum/1xPBS. The following antibodies were used at the dilutions shown: c-actin (mouse, 1:200, A2547 Sigma), anti-Isl-1 (mouse, 1: 100, DSHB), anti-N-cadherin, (rat, 1: 50, DSHB). Following overnight incubation at 4°C and washes in 1xPBS, fluorescently labeled second antibodies (Molecular Probes) were used at a 1:250 dilution to detect primary antibodies: 555-conjugated donkey anti-mouse for c-actin, Alexa 488-conjugated donkey anti-rabbit for N-cadherin. Sections were washed in PBS, mounted in Prolong with DAPI (Invitrogen) and imaged on a fluorescent microscope for analysis.

Retinoic Acid Treatment of Embryos

Retinoic Acid treatment of embryos was done as previously described by Ryckebusch et al. (2006)[48]. All-trans-RA (Sigma)
was dissolved in DMSO and diluted at 20 mg/ml. At E7.5, the Hoxa3-lacZ transgenic mice were given a single intra peritoneal injection of the RA solution (65 mg/kg) or control DMSO. Control and treated embryos were harvested at E7.5, E8 and E8.5 and stained for β-galactosidase activity as described above.

Supporting Information

Figure S1 Cardiac expression of N-cadherin, cardiac Actin and Islet-1 at E9.5 and E10.5. Expression of N-cadherin in the heart of E9.5 (A) and 10.5 (B) transgenic embryos, with corresponding magnification of the outflow tract region (C and D, respectively). Cardiac neural crest cells lack N-cadherin expression at E9.5 (arrowhead in C), while it is upregulated at E10.5 (arrowheads in D). (E) At E9.5, cardiac actin is excluded from SHF progenitor cells (arrowheads). Arrowhead in (F) indicates the posterior limit of differentiated cells positive for c-actin in the outflow tract at E10.5. (G) lacZ expressing cells are present in the pharyngeal endoderm (arrow) and in a region that expresses Isl1 (H, arrowheads) in addition to the pharyngeal endoderm and outflow tract proper at E9.5. OFT, outflow tract; PE, pharyngeal endoderm; RA, right atria; RV, right ventricle. Scale bar = 100 μm.

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Figure 7. Schematic view of lacZ expression pattern in of Mlc1v-nlacZ24, Hoxa3-lacZ and Wnt1Cre;R26R cardiogenesis lines. Mlc1v-nlacZ24 (A, D, G), Hoxa3-lacZ (B, E, H) and Wnt1Cre;R26R (C, F, I). At E8.5 Mlc1v-nlacZ-24 are activated in the second heart field, developing OFT and heart tube (A), while Hoxa3-lacZ is activated in PE and barely detected in the SHF (B). At this stage the heart is devoid of migrating cardiac neural crest cells (C). At E9.5 the SHF progenitors, OFT and RV myocardium are labeled in Mlc1v-nlacZ-24 (D). The PE, CNC (dots in the caudal pharynx) and a subdomain of the SHF express Hoxa3-lacZ (E). Cardiac neural crest cells are also labeled in Wnt1cre;R26R bigenic embryos at E9.5 (F). At E10.5, Mlc1v-nlacZ-24 transgenic hearts identify SHF, OFT and RV (G). In addition to the anterior portion of SHF, PE, Hoxa3-lacZ transgene activity identifies cardiac neural crest and myocardial cells of the OFT, whereas the RV remains negative for lacZ staining (E). Cardiac neural crest cells similarly invades the OFT in Wnt1cre;R26R bigenic embryos at E10.5 (I). Hoxa3-lacZ clearly appears as an intermediary reporter line that display hybrid expression pattern reminiscent to that of Mlc1v-nlacZ24 and Wnt1cre;R26R bigenic. Blue and pink colors indicate lacZ-positive and -negative cells, respectively. Black and red arrowheads designate the pharyngeal endoderm (PE) and second heart field (SHF), respectively. A, atria; HT, heart tube; Oft, outflow tract; RA, right atria; RV, right ventricle.

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The Asp178-Val179 2-kb genomic fragment from the mouse Hoxa3 locus was fused to the E. coli lacZ coding sequence to generate the reporter transgene. The sequence of the 5' moiety of the promoter is shown and contains a predicted DR5 RARE site (underlined bold italics). Asp178 and XbaI restriction sites are shown in bold case.

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Author Contributions
Conceived and designed the experiments: NYSGD JJP SZ RR. Performed the experiments: NYSGD SR NB. Analyzed the data: NYSGD NB JJP SZ RR. Contributed reagents/materials/analysis tools: NYSGD SR NB. Wrote the paper: NYSGD SZ RR.

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