Rotation of the Myocardial Wall of the Outflow Tract Is Implicated in the Normal Positioning of the Great Arteries

Fanny Bajolle,* Stéphane Zaffran,* Robert G. Kelly, Juliette Hadchouel, Damien Bonnet, Nigel A. Brown, Margaret E. Buckingham

Abstract—Congenital heart defects frequently involve a failure of outflow tract (OFT) formation during development. We analyzed the remodeling of the OFT, using the y96-Myf5-nlacZ-16 transgene, which marks a subpopulation of myocardial cells of the pulmonary trunk. Expression analyses of reporter transcript and protein suggest that the myocardial wall of the OFT rotates before and during the formation of the great arteries. Rotational movement was confirmed by Di-I injection experiments with cultured embryos. We subsequently examined the expression of the transgene in mouse models for OFT defects. In hearts with persistent truncus arteriosus (PTA), double outlet right ventricle (DORV), or transposition of the great arteries, rotation of the myocardial wall of the OFT is arrested or fails to initiate. This is observed in Splotch (Pax3) mutants with PTA or DORV and may be a result of defects in neural crest migration, known to affect OFT septation. However, in Pitx2c mutant embryos, where cardiac neural crest cells are present in the heart, PTA and DORV are again associated with a rotation defect. This is also seen in Pitx2c mutants, which have transposition of the great arteries. Because Pitx2c is involved in left–right signaling, these results suggest that embryonic laterality affects rotation of the myocardial wall during OFT maturation. We propose that failure of normal rotation of OFT myocardium may underlie major forms of congenital heart disease. (Circ Res. 2006;98:421-428.)

Key Words: heart ■ outflow tract ■ Splotch ■ Pitx2 ■ transposition of the great arteries

Congenital heart defects are observed in 0.8% of children in developed countries and are responsible for more than 20% of spontaneous abortions and 10% of all stillbirths.1,2 Many of these defects are caused by abnormal development of the outflow region (also called conotruncal region) of the heart, leading to malformations such as persistent truncus arteriosus (PTA), double outlet right ventricle (DORV), or transposition of the great arteries (TGA). Experiments on avian and mammalian embryos have shown that myocardium of the outflow tract (OFT) is derived from pharyngeal mesoderm.3–5 In the mouse, this has been described as the anterior heart field,6 which also contributes myocardium to the right ventricle7 and is itself part of a larger second heart field.8 In the chick embryo, it has been shown that the vascular smooth muscle tunic of the aorta and pulmonary trunk, in addition to myocardium of the outflow region, is derived from pharyngeal mesoderm.8 Moreover, its ablation leads to OFT defects such as tetralogy of Fallot (TOF) and pulmonary atresia.9

As development proceeds, the single OFT undergoes remodeling into separate pulmonary and aortic arteries. This process involves interactions between diverse cell types, including myocardium, endocardium, and neural crest cells.10 Endocardial cells respond to signals from the overlying myocardium and undergo an epithelial-to-mesenchymal transformation to form the conotruncal cushions.11 Neural crest cells, which play an essential role in the normal septation of the heart,12 invade the extracellular matrix of the cushions and participate in aortico-pulmonary septation. In the chick, cardiac neural crest ablation leads to OFT defects including TOF, PTA, DORV, and interrupted aortic arch.13 In the mouse, Splotch mutations, known to result from disruption or deletion of the Pax3 gene, give rise to a similar spectrum of conotruncal defects, secondary to a neural crest cell migration defect.14–16 Recently, cardiac neural crest ablation has been shown to affect formation of OFT myocardium from pharyngeal mesoderm.17 Pitx2c mutant mice also have conotruncal defects.18 Pitx2c confers embryonic left–right signaling to asymmetrically developing organs.19 Pitx2c is expressed asymmetrically in pharyngeal mesoderm and has been implicated in patterning of OFT myocardium.20,21
Classic morphological studies of the developing chick heart have shown torsion of the OFT, which, when physically interrupted, leads to malposition of the great arteries.\textsuperscript{22} Radioactive tattoos of the outflow region further establish that rotation occurs during OFT morphogenesis in the chick embryo.\textsuperscript{23} Previous studies on human embryos suggested that the OFT undergoes rotation during its remodeling.\textsuperscript{24} Computation of distances and angles between major anatomical landmarks, in particular the axis of the semilunar valves, showed that the junction of the OFT and the great arteries undergoes a rapid rotation in a counterclockwise direction, facing downstream, between Carnegie stages 15 and 19.\textsuperscript{24} In addition, the angle of the aortic to pulmonary valve axis, relative to the inferior surface of congenitally malformed hearts, suggested that TOF, DORV, and TGA may result from an arrested rotation of the outflow region at the base of the great arteries.\textsuperscript{25,26}

We now report a counterclockwise rotation of OFT myocardium in the mouse embryo and examine this phenomenon in mice with OPT defects. A transgenic line, y96-Myf5-nlacZ-16,\textsuperscript{27} in which β-galactosidase activity marks a part of OPT myocardium, permits visualization of OPT rotation before, and during, the septation of this region. Di-I labeling experiments in cultured mouse embryos confirm this rotation. Comparison of transgene expression in Splotch and Pitx2\textsuperscript{c} mutant embryos, which have conotruncal defects, suggests that the abnormal position of the great arteries results from a premature arrest or failure to initiate OPT rotation. These results support the hypothesis that a spectrum of cardiac anomalies with abnormally positioned great arteries may arise from a perturbation of myocardial rotation, in addition to abnormal OPT septation caused by neural crest cell defects. These observations have implications for the understanding of conotruncal abnormalities in human congenital heart defects.

## Materials and Methods

### Mice and Genotyping

The transgenic line y96-Myf5-nlacZ-16 (96-16) has been previously described.\textsuperscript{23} The transgene consists of YAC DNA containing a ∼96-kb genomic fragment upstream of the mouse Myf5 gene followed by an n lacZ reporter. Heterozygous 96-16 (C57BL/6) and Splotch (Sp, C57BL/6, Jackson Laboratories) mice were interbred to obtain Sp mutants carrying the transgene. The transgene interferes with normal splicing of intron 3 and leads to 4 aberrantly spliced mRNAs with exon 4 deleted.\textsuperscript{28} Sp mutant embryos were identified by PCR using primers through exon 4: Sp1/exon4, TTTCGCTAAAGAGCTGGAAAGAATGCG; Sp2/exon4, TCCTCAGGATGGCGGTGATAAAGTCAACAC. The same procedure of interbreeding was performed with the 96-16 and Pitx2\textsuperscript{c} (C57BL/6×CBA, F1) mice. The Pitx2\textsuperscript{c} neon allele has a deletion of the majority of exon 4 including all coding sequences within this exon.\textsuperscript{29} Pitx2\textsuperscript{c} mutant embryos were identified by PCR using primers for exon 3 and the neo sequence: 118/exon3, CTAATATCAGCTACCTGGTCCGTACCTC; 119/exon3, CTGGAAATGATGCGGTATGCTGCACCTC; and 126/neo, CGACGACCAGAGCAACAGACTAGCT.

### Analysis of Transgene Expression

Embryos were staged taking embryonic day (E) 0.5 as the morning of the vaginal plug. Dissection, X-gal staining, and in situ hybridization using an n lacZ antisense probe were performed as described previously.\textsuperscript{6} Embryos were examined using a Leica ZM20 stereomicroscope or Zeiss Axioshot microscope and photographed with a digital camera (AxioCam-Zeiss).

### Embryo Sections and Immunohistochemistry

Corrosion casts: E17.5 embryos were isolated and the heart exposed by a thoracic incision. Batson’s number 17 acrylic (Polysciences) was injected into right and left ventricles until the great arteries were filled. After hardening overnight in distilled water at 4°C, tissues were removed with Maceration Solution at 50°C for 24 hours without a shaking. All samples were mounted on stubs and then sputter-coated with gold. Samples were observed on a Zeiss SM940 scanning electron microscope.

### Di-I Injection and Mouse Embryo Culture

Di-I labeling at E9.5 was initially performed levolaterally, through the yolk sac, amnion, and pericardial wall, with injection into the myocardium of the OPT, on the left, midway between the aortic sac and the dextral bend in the heart tube, as previously described.\textsuperscript{4} Two embryos were euthanized immediately to verify the injection site, and the rest cultured for 24 hours. Of 25 live embryos, 8 showed counterclockwise spread of the dye. In a second series of experiments, the technique was refined to permit more precise localization of smaller amounts of dye, by dissecting a “window” through the yolk sac, opening the amnion and pericardium. This method can lead to growth impairment. However, in 10 live embryos with labeled cells, 3 showed counterclockwise relocation of the labeled cells. In this series, 4 embryos were euthanized immediately to verify the injection site.

### Corrosion Cast and Scanning Electron Microscopy

Corrosion casts: E17.5 embryos were isolated and the heart exposed by a thoracic incision. Batson’s number 17 acrylic (Polysciences) was injected into right and left ventricles until the great arteries were filled. After hardening overnight in distilled water at 4°C, tissues were removed with Maceration Solution at 50°C for 24 hours without a shaking. All samples were mounted on stubs and then sputter-coated with gold. Samples were observed on a Zeiss SM940 scanning electron microscope.

### Results

The y96-Myf5-nlacZ-16 Transgenic Line Provides a Marker of Myocardium at the Base of the Pulmonary Trunk

The transgenic mouse line y96-Myf5-nlacZ-16 (96-16), in which an n lacZ reporter gene is under the control of 96 kb of genomic DNA upstream of the myogenic regulatory gene Myf5, expresses the transgene at sites of skeletal muscle formation, as expected, but also shows ectopic expression in the OPT of the heart attributable to an integration site effect (Figure 1a). At E9.5, expression is observed in the dorsal (or inferior) wall of the OPT and contiguous pharyngeal mesoderm in the dorsal wall of the pericardial cavity (Figure 1a). In the tubular OPT at E10.5, X-gal staining predominates in myocardial cells of the dorsal wall (Figure 1b, 1b’, and 1c). By E12.5 (Figure 1d), transgene expression is restricted to the pulmonary trunk, to give a sharp boundary at the base of this vessel at E15.5 (Figure 1e and 1e’). At all stages, β-galactosidase colocalizes with cardiac myosin heavy chain (Figure 1c and data not shown), indicating expression in the myocardium and not the adjacent smooth muscle of the vessel.
Labeling continues to be detectable, although weaker, at E18.5 (data not shown). \( \beta \)-Galactosidase–positive cells are not detected in the valves or septum (data not shown). These observations suggest that the 96-16 transgenic line provides a marker of myocardium at the base of the pulmonary trunk, which can be followed during development of the OFT region.

Rotation of OFT Myocardium

The expression profile of the 96-16 transgene between E9.5 and E12.5 in OFT myocardium shows a counterclockwise rotation during formation of the great arteries. \( \beta \)-Galactosidase activity, is initially stronger in the right-hand side of the dorsal OFT wall, as seen in whole mount (Figure 2a) and sections (Figure 2e). Subsequently, \( \beta \)-galactosidase–positive cells are not present in the smooth muscle wall of the pulmonary trunk (pt) or aorta (ao) but are in the adjacent myocardium (m).
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ventrally, showing that these cells have now changed position 24 hours of embryo culture, labeled cells are observed more exteriorization (Figure 3c) (see Materials and Methods). After 24 hours of embryo culture, labeled cells are observed more

positive cells are located dorsally (Figure 2b and 2f) and then in the left-hand and ventral part of the tubular OFT (Figure 2c and 2g), before becoming largely confined to the base of the pulmonary trunk (Figure 2d and 2h), as this separates from the aorta during individualization of the great arteries from the OFT. The expression pattern of the transgene is presented schematically in Figure 2a’ through 2d’. These observations suggest that the myocardial wall of the OFT rotates during great artery development and that the 96-16 transgene marks the myocardiom component which will contribute to the base of the pulmonary trunk throughout this process. To show that this result does not reflect de novo transgene expression, we analyzed nlacZ transcripts by in situ hybridization between E9.5 and E12.5 (Figure 2i through 2l). nlacZ transcripts are observed at E9.5 and E10.5 (Figure 2i and 2j) but are not detectable subsequently (Figure 2k and 2l), whereas β-galactosidase activity is still present (Figure 2c, 2d, 2g, and 2h), reflecting the stability of the β-galactosidase protein compared with that of its mRNA. The changing position of X-gal–labeled cells during great artery formation therefore provides a “chase,” marking the cells that previously transcribed the transgene. A direct demonstration of OFT rotation comes from Di-I labeling experiments where dye was injected into myocardial cells on the left-hand side of the OFT at E9.5 either through the yolk sac (Figure 3a) or after exteriorization (Figure 3c) (see Materials and Methods). After 24 hours of embryo culture, labeled cells are observed more ventrally, showing that these cells have now changed position (Figure 3b and 3d). This result was observed on 11 of 35 live labeled embryos. Protein and transcript expression from the

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PS indicates pulmonary stenosis.

transgene, together with Di-I labeling experiments, are all consistent with a counterclockwise rotation of the OFT.

Defects in OFT Rotation in Splotch Embryos

To gain insight into the role of myocardial rotation in OFT remodeling, we examined the expression of the 96-16 transgene in mouse mutants in which this process is defective. Splotch embryos develop defects in the OFT, including PTA and DORV (Table and Figure 4). These conotruncal defects result from failure of the cardiac neural crest to colonize the developing heart in the absence of Pax3,14,16 In Splotch mutant hearts with PTA at E14.5, the transgene is expressed in the left-hand part of the myocardium at the base of the single outflow vessel (Figure 4b and 4b’), whereas normally at this stage β-galactosidase activity is concentrated in a ventral location at the base of the pulmonary trunk (Figure 4a and 4a’). In Splotch mutant hearts with DORV at E15.5, a rotation defect is again observed with β-galactosidase–positive cells present in the left-hand side of the outflow region, instead of ventrally (Figure 4d compared with 4c). At E11.5, during OFT septation, the transgene is normally expressed on the left side of the OFT (Figure 4e), whereas in the Splotch mutant, β-galactosidase activity is observed dorsally (Figure 4f, arrowhead), as in wild-type hearts at E10.5 (see Figure 2f). These observations suggest that rotation of the myocardial wall of the OFT is affected at early stages in hearts, giving rise to DORV or PTA and is therefore associated with abnormal positioning of the great arteries and failure of OFT septation.

Defects of OFT Rotation in Pitx26c Embryos

All Pitx26c mutants have OFT anomalies with a majority characterized by abnormal positioning of the great arteries (93%), such as TGA or DORV, whereas 7% present PTA (Table). At E12.5, the aorta is normally positioned dorsally to the pulmonary trunk (Figure 5a), whereas in Pitx26c mutants with DORV, the aorta is positioned ventrally (Figure 5b). As in the case of Splotch mutants with PTA, we observed 96-16 expression in the left-hand side of the OFT in Pitx26c mutant hearts with the same defect (Figure 5c and 5d), reinforcing the notion that PTA is associated with a rotation defect.

Among the types of malposition of the great arteries observed in Pitx26c mutants, 70% of embryos have TGA (Table and Figure 6b). Lateral views show that in these cases, the pulmonary trunk is positioned dorsally to the aorta instead.
of spiraling around it (Figure 6a and 6b). The pulmonary trunk normally emerges from the right ventricle (Figure 6c), whereas in Pitx2c mutant hearts with TGA, the pulmonary trunk is connected to the left ventricle (Figure 6d). This is referred to as ventriculo-arterial discordance. At E15.5, transgene expression is normally detected at the base of the pulmonary trunk, which lies ventral to the aorta (Figure 6e and 6e’). In contrast, in TGA hearts, transgene expression is maintained in myocardium at the base of the pulmonary trunk, now positioned dorso-laterally (Figure 6f and 6f’), also indicative of a rotation defect.

Discussion

Analysis of the expression profile of reporter protein compared with transcript from the y96-Myf5-nlacZ-16 (96-16) transgene, specifically expressed in myocardium at the base of the pulmonary trunk, suggested that the OFT rotates during development of the great arteries. Rotation of the myocardial wall of the OFT was demonstrated by Di-I injection experiments in cultured mouse embryos. Observations of 96-16 transgene expression in Splotch mice with PTA or DORV reveal an arrest of OFT rotation associated with a septation defect. This is also observed in Pitx2c mutant hearts which have similar malformations. In addition, we show that Pitx2c mutants displaying TGA have a failure of OFT rotation. Thus, the expression profile of the 96-16 transgene in Splotch and Pitx2c mutants demonstrates that rotation of the myocardial wall of the OFT is disturbed in conotruncal defects such as PTA, DORV, and TGA, suggesting a crucial role for myocardial rotation in positioning of the great arteries.

At early stages of OFT development, expression of the 96-16 transgene colocalizes with myocardial markers, and β-galactosidase continues to be present at the base of the pulmonary trunk.
pulmonary trunk at late fetal stages, after nlacZ transcripts have ceased to be detectable. No β-galactosidase–positive cells are detected in the smooth muscle of the pulmonary trunk. This, together with the sharp boundary of transgene expression at the base of this vessel, is consistent with a retraction of the myocardium as the smooth muscle tunic develops. Further support for rotation of OFT myocardium in the mouse comes from a clonal analysis, which showed that the junction of the OFT and great arteries undergoes a rotational movement between Carnegie stages 15 and 19. Moreover, measurements on embryos with conotruncal defects suggested that this rotation is prematurely arrested at different developmental stages according to the type of malformation.25,26 Our study provides the first direct evidence for such counterclockwise rotation of the OFT in mammalian embryos. The rotation of OFT myocardium in the mouse embryo, as visualized with the 96-16 transgene, begins at E9.5, equivalent to Carnegie stage 11, whereas rotation has been documented from Carnegie stage 15 in humans. This may reflect the sensitivity of transgene detection compared with physical measurements. Most of the myocardium has been added to the mouse OFT by E9.5, suggesting that the OFT first elongates and then rotates.

Rotation of the myocardial wall of the OFT is integrated into the remodeling process of the outflow region, which is intimately linked to the influx of neural crest. Splotch mutant mice develop OFT defects, including PTA and DORY, as a result of reduced colonization of the OFT by cardiac neural crest cells. Our study demonstrates that rotation of the OFT myocardial wall is prematurely arrested in Splotch embryos. This precedes OFT septation, a process dependent on neural crest cells that guide the organization of the endocardial cushions. Because we have never detected Pax3 expression in OFT myocardium, including in Pax3−/− mice in which the heart is β-galactosidase negative, the reduction of cardiac neural crest in Splotch mutant embryos may indirectly influence the process of OFT rotation. Moreover, rotation is observed from E9.5, when neural crest cells first invade this region. The rotation defect supports the conclusion that OFT development requires extensive cross-talk between neural crest and myocardial cells. However, this is not the only factor that leads to defective OFT rotation associated with PTA, because it is also seen in the 7% of hearts with this malformation in Pitx2−/− mutants where cardiac neural crest migration appears to occur normally. These findings show that abnormal septation, secondary to a neural crest cell defect, is not the only cause of PTA, which we show is associated with a rotation defect.

Ablation of cardiac neural crest in the chick embryo induces a large spectrum of malformations affecting the OFT region but not TGA. This malformation probably has multiple causes, as evidenced by the Perlecan mouse, which has an extracellular matrix defect, or by retinoic acid treatments, which induce endocardial cushion defects. As seen here for Pitx2−/− mutations in genes that affect left–right asymmetry, such as cryptic or type IIB activin receptor can also lead to TGA. Our observations on Pitx2−/− mutant embryos suggest that this malformation can be induced by a laterality defect affecting rotation of the myocardial wall of the OFT. This may result from an earlier effect on OFT myocardial precursors in the anterior heart field, where cells express Pitx2c and are perturbed in its absence. Left–right signaling may also exert its effect via the myocardium itself, because misexpression of Pitx2 in the embryonic heart
correlates with abnormal OFT development. Whatever the underlying cause, our results suggest that arrested rotation of OFT myocardium is related to TGA in the absence of Pitx2c. The fact that this phenomenon is also associated with PTA and DORV probably reflects underlying complexity in the cell populations and stages affected in the Pitx2c mutant, leading to a spectrum of abnormalities during OFT remodeling.

Rotation of the myocardium at the base of the OFT is probably essential to achieve normal positioning of the great arteries with respect to each other at the ventriculo-arterial junction. Indeed, the spiraling movement of the aortico-pulmonary septum, which generates specific ventriculo-arterial connections, may result from the rotation of the myocardial wall of the OFT (Figure 7). Understanding the molecular and genetic regulation of OFT rotation, as visualized here for the first time in the mouse, should provide important new insights into congenital heart defects affecting ventricular septation and great artery development.

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