Circulation Research

American Heart Association®

*Learn and Live*sm

Cardiovascular Defects Associated With Abnormalities in Midline Development in the Loop-tail Mouse Mutant

Deborah J. Henderson, Simon J. Conway, Nicholas D. E. Greene, Dianne Gerrelli, Jennifer N. Murdoch, Robert H. Anderson and Andrew J. Copp

The online version of this article, along with updated information and services, is located on the World Wide Web at:

http://circres.ahajournals.org/cgi/content/full/89/1/6

Cardiovascular Defects Associated With Abnormalities in Midline Development in the *Loop-tail* Mouse Mutant

Deborah J. Henderson,* Simon J. Conway,* Nicholas D.E. Greene,* Dianne Gerrelli,
Jennifer N. Murdoch, Robert H. Anderson, Andrew J. Copp

Abstract—*Loop-tail* (*Lp*) is a naturally occurring mouse mutant that develops severe neural tube defects. In this study, we describe complex cardiovascular defects in *Lp* homozygotes, which include double-outlet right ventricle, with obligatory perimembranous ventricular septal defects, and double-sided aortic arch, with associated abnormalities in the aortic arch arteries. Outflow tract and aortic arch defects are often related to abnormalities in the cardiac neural crest, but using molecular and anatomic markers, we show that neural crest migration is normal in *Lp/Lp* embryos. On the other hand, the heart fails to loop normally in *Lp/Lp* embryos, in association with incomplete axial rotation and reduced cervical flexion. As a consequence, the ventricular loop is shifted posteromedially relative to its position in wild-type embryos. This suggests that the observed cardiac alignment defects in the *Lp* mutant may be secondary to failure of neural tube closure and incomplete axial rotation. Double-sided aortic arch is a rare finding among mouse models. In humans, it is usually an isolated malformation, only rarely occurring in combination with other cardiac defects. We suggest that the double-sided arch arises as a primary defect in the *Lp* mutant, unrelated to the alignment defects, perhaps reflecting a role for the (as-yet-unknown) *Lp* gene in maintenance/regression of the aortic arch system. (*Circ Res*. 2001;89:6-12.)

Key Words: congenital heart defects ■ mouse mutants ■ double-outlet right ventricle ■ double-sided aortic arch ■ midline defects

Cardiovascular defects are the most common cause of congenital disease in humans, occurring in almost 1% of newborns.[1] Septation and alignment defects make up the largest single group of cardiac malformations, including ventricular and atrial septal defects, tetralogy of Fallot, and double-outlet right ventricle. The developmental origin of these defects involves disruption of early embryonic events, including cardiac looping and neural crest cell migration. During cardiac looping, the primordial midline heart tube is remodeled into an asymmetric structure that brings the left and right ventricular chambers into alignment with the outflow vessels and atria. To achieve concordance of the ventriculoarterial connections, however, the cardiac neural crest is required, which is involved in septation of the outflow tract to yield separate aortic and pulmonary channels. The aorta is then remodeled so that the initially symmetrical aortic arch becomes left-sided, as the right side regresses.

In view of the importance of midline events in early cardiac morphogenesis (looping, immigration of neural crest cells, and remodeling of the original symmetrical structure), it is not surprising that an association is evident between midline defects of noncardiac structures and cardiac defects of sep-

tation and alignment. Midline defects and cardiovascular abnormalities coexist in humans with Opitz syndrome and Jarcho-Levin syndrome.[2,3] Although these rare associations are indicative of a developmental link between midline development and cardiac defects, they are not accessible for experimental analysis. An alternative approach is to use the many mouse genetic mutants that exhibit early abnormalities of axial development. *Loop-tail* (*Lp*) is a naturally occurring mouse mutant[4] that provides a model for the human neural tube defect craniorhachischisis. *Lp/Lp* embryos fail to initiate closure of the neural tube in the cervical region (so-called closure 1), whereas the forebrain neural tube appears to close relatively normally.[5] This results in a severe abnormality in which the neural tube is open from the midbrain to the base of the spine. *Lp/Lp* embryos also have somite defects and abnormalities in axial rotation.[6] A small percentage of cases exhibit gastroschisis, in which the ventral body wall fails to close correctly, resulting in herniation of the abdominal contents.[7] Hence, *Lp* homozygotes exhibit a series of midline developmental defects. For this reason, we decided to examine the development of the cardiovascular system in the *Lp* mouse. We describe complex cardiovascular defects in *Lp/Lp*

Comparison of Cardiovascular Malformations Detected and Laterality Status of +/+, *Lp*/+ and *Lp/Lp* Embryos

Cardiovascular Malformation	Genotype of Fetuses, No. Exhibiting Defect/Total No. Examined		
	+/+	*Lp*/+	*Lp/Lp*
Double-outlet right ventricle	0/3	0/6	18/18
Ventricular septal defect	0/3	0/6	18/18
Aortopulmonary window	0/3	0/6	3/18
Double-sided aortic arch	0/3	0/6	7/9†
Right-sided aortic arch	0/3	0/3	2/9†
Coarctation (in ink-injected fetuses*)	0/8	0/28	13/33
Laterality of embryos			
Embryo turned to right	3/3	3/3	25/25
Heart looped to right	3/3	3/3	25/25

*Some of these fetuses were sectioned and also included in data for double-outlet right ventricle and ventricular septal defect.

†Includes three *Lp/Lp* fetuses examined at E18.5.

embryos, supporting the idea that abnormalities in the development of the embryonic midline and cardiac alignment defects might be causally related.

Materials and Methods

Mouse Strains and Embryos

The LPT/Le inbred strain, which carries the *Lp* mutation, was obtained from Jackson Laboratories (Bar Harbor, Maine) and has now been bred to F117. Mice were bred and genotyped as described previously[5,8] to generate litters containing *Lp/Lp*, *Lp*/+, and +/+ embryos. Embryos were dissected from the uterus and processed for histological staining and immunocytochemistry as described previously.[6,9] The α-smooth muscle actin antibody was obtained from Sigma Chemical Co (clone 1A4).

Analysis of Embryonic Vasculature

Embryos were explanted from the uterus into fresh DMEM containing 10% FCS. The yolk sac and amnion were opened, and the umbilical cord was left attached. India ink was injected either into the umbilical artery or the left ventricle. The heart was allowed to continue beating until carbon particles in the ink had been distributed throughout the heart and vasculature. Embryos were then fixed by immersion in cold 4% paraformaldehyde, cleared in a 2:1 mixture of benzyl alcohol and benzyl benzoate, and then either dissected further and photographed or embedded in paraffin wax for serial sectioning.

Whole-Mount In Situ Hybridization

The preparation of probes for *cadherin 6* and *RhoB* have been described previously.[10,11] The *erbB3* probe was a gift from Dr C. Birchmeier (Max-Delbruck-Center for Molecular Medicine, Berlin, Germany). *Pitx2c* and *lefty-2* were obtained from Prof N. Brown (St. Georges Hospital Medical School, London, UK). The methodology for the whole-mount in situ hybridization of Wilkinson[12] was followed, with minor modifications as described previously.[11]

Results

Lp Mutants Develop Double-Outlet Right Ventricle

Histological examination at embryonic day (E)13.5 revealed a spectrum of cardiovascular defects in *Lp/Lp* fetuses, whereas *Lp*/+ and +/+ fetuses had no apparent defects of the cardiovascular system (Table). The most commonly observed defect was double-outlet right ventricle, which occurred in all
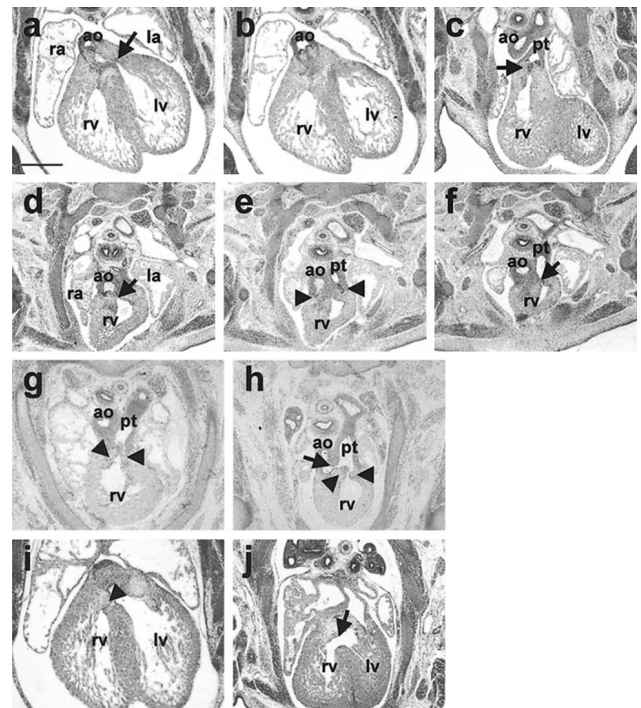

Figure 1. Cardiac alignment defects in *Lp/Lp* fetuses at E13.5. Transverse sections of +/+ (a through c, g, and i) and *Lp/Lp* (d through f, h, and j) fetuses were stained with hematoxylin and eosin. a through c, Sections of +/+ fetus showing concordant ventriculoarterial connections, with the aorta (ao) arising from the left ventricle (lv) (arrow in a) and the pulmonary trunk (pt) arising from the right ventricle (rv) (arrow in c). d through f, Sections of *Lp/Lp* littermate showing double-outlet rv, with both the ao (arrow in d) and the pt (arrow in f) continuing to exit from the rv. The aortic and pulmonary valves appear at the same level in the *Lp/Lp* fetus (arrowheads in panel e). g, Normal separation of the ao and pt in +/+ fetus, showing the leaflets of the pulmonary valve (arrowheads). The aortic valve leaflets are not visible, lying more caudally. h, Common arterial valve in *Lp/Lp* fetus, showing that the ao and pt are fed through a common valve exiting the rv (arrowheads). The proximal part of the trunks are common (arrow). i, Normal ventricular septation in the +/+ fetus, showing fusion of the interventricular septum with the proximal part of the outflow tract cushions (arrowhead). j, Perimembranous ventricular septal defect in *Lp/Lp* fetus (arrow). la indicates left atrium; ra, right atrium. Bar=470 μm (a through i) and 380 μm (j).

Lp/Lp fetuses examined (Figures 1d through 1f). In wild-type fetuses, the ventriculoarterial connections are concordant (Figures 1a and 1c), with the aorta arising from the left ventricle and the pulmonary trunk retaining its original connection with the right ventricle (arrows in Figures 1a and 1c). In contrast, in all *Lp/Lp* fetuses examined, both arterial trunks retain their origin from the right ventricle (Figures 1d through 1f). Furthermore, in the normal embryo, the aortic valve, developing in the left ventricle, comes into fibrous continuity with the mitral valve, whereas the pulmonary valve is supported at a more cranial level by a free-standing infundibular sleeve. The arterial trunks then spiral around one another as they ascend into the mediastinum. In contrast, the arterial valves in both the aorta and pulmonary trunk appear at the same level in *Lp/Lp* fetuses (arrowheads in Figure 1e), being separate in most fetuses (n=15) but appearing as a common valve (arrowheads in Figure 1h; compare with

Figure 1g), which guards the entrance to a common arterial trunk exclusively supported by the infundibular musculature of the right ventricle, in the remainder of the fetuses (n=3). A ventricular septal defect is an obligatory part of the pattern of circulation in the *Lp/Lp* embryos, in which both arterial trunks, or a common trunk, arise from the right ventricle, being necessary to permit the exit of blood from the left ventricle (compare Figures 1i and 1j).

Double-Sided Aortic Arch and Associated Aortic Arch Abnormalities in *Lp/Lp* Fetuses

In addition to the abnormalities of ventriculoarterial connections in *Lp*, we also noted major abnormalities in the arrangement of the aortic arches. Four of six *Lp/Lp* fetuses showed persistence of the right arch at E13.5, in addition to the normal left-sided arch, resulting in a double-sided aortic arch (Table and Figure 2b). This formed a vascular ring with the arches of the aorta enclosing completely the trachea and esophagus (arrows in Figure 2c). In the remaining (2 of 6) *Lp/Lp* fetuses, the right side of the aortic arch had persisted abnormally, but the left side had partially regressed, resulting in a right-sided aortic arch with a retroesophageal left subclavian artery (arrow in Figure 2d). In contrast, *Lp/+* and *+/+* always exhibited regression of the right side of the aortic arch and maintenance of the left arch (Figure 2a). Sectioning of three *Lp/Lp* fetuses at E18.5 confirmed the persistence of the double-sided aortic arch throughout gestation (data not shown).

Ink injections into the umbilical artery or directly into the left ventricle were carried out to examine further the development of the aortic arch and its associated arteries in the *Lp* mutant (n=33). This revealed narrowing or, in severe cases, interruption of the left aortic arch in ≈40% (13 of 33) *Lp/Lp* fetuses (compare Figures 2f and 2e).

Cardiac Neural Crest Cell Migration Is Normal in *Lp* Mutants

Cardiac neural crest anomalies have been described previously in other mouse mutants, and in humans, in association with outflow tract and aortic arch abnormalities.[13–15] *Lp/Lp* embryos were collected at E10.5 of gestation, when neural crest cells are migrating toward the cardiac outflow tract, and subjected to whole-mount in situ hybridization using digoxigenin-labeled riboprobes for the neural crest markers *RhoB*, *cadherin 6*, and *erbB3*. Robust staining of each neural crest marker could be seen in the region where the cardiac neural crest cells migrate toward and through the third, fourth, and sixth branchial arches, in both the wild-type and *Lp/Lp* embryos (arrows in Figures 3a and 3b and data not shown). The cranial and dorsal root ganglia were also normally sized and positioned in *Lp/Lp* compared with their wild-type littermates (Figures 3a and 3b). Sectioning of these embryos at the level of the heart supported this finding, showing that the developing cranial and dorsal root ganglia were appropriately sized and positioned despite the widely splayed open neural tube (Figures 3c and 3d). These data suggest that there are no marked abnormalities in neural crest cell migration in *Lp/Lp* embryos. Because of the abnormalities in development of the ventricular outlet of the heart, it
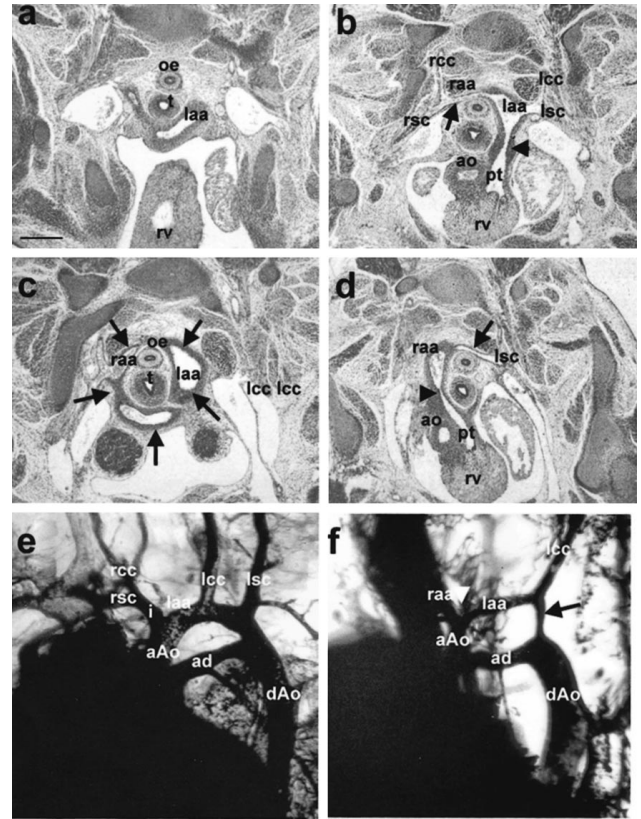

Figure 2. Aortic arch abnormalities in *Lp/Lp* fetuses. There was hematoxylin and eosin staining of transverse sections of +/+ (a) and *Lp/Lp* (b through d) fetuses at E13.5 and ink injections into the fetal vasculature of +/+ (e) and *Lp/Lp* (f) fetuses at E15.5. a, Normal left-sided aortic arch (laa) in +/+ fetus. b, Double-sided aortic arch in *Lp/Lp* fetus, showing persistence of the right side of the arch (arrow). The arterial duct (ad) (ductus arteriosus) is marked by an arrowhead, and the origins of the left and right common carotid (lcc and rcc, respectively) and subclavian (lsc and rsc, respectively) arteries can be seen. c, Vascular ring in *Lp/Lp* fetus, with the esophagus (oe) and trachea (t) completely enclosed within the arches of the ao (arrows). d, Right-sided aortic arch (raa) in *Lp/Lp* fetus, with a right-sided ad (arrowhead). A retroesophageal lsc (arrow) can be seen arising from the right-sided arch. e, Vasculature of +/+ fetus showing the normal laa with its associated arch arteries and pt. The regressed right side of the arch has given rise to the innominate (brachiocephalic) artery (i) from which the rcc and rsc arise. The ad joins the pt and the descending segment of the aortic arch (dAo). The lcc and lsc arteries can be seen arising from the aortic arch. f, Vasculature of *Lp/Lp* fetus showing the left side of the aortic arch system. The raa and laa are approximately equal (arrowhead). The laa is abnormally narrow, with severe narrowing or interruption in the region proximal to the ad (arrow). In addition, the lsc artery is completely absent (compare with panel e). aAo indicates ascending portion of the aorta. Bar=290 μm (a through d) and 100 μm (e and f).

was important to determine whether there might be a specific abnormality in the cardiac neural crest cells migrating into the developing outflow tract. Most gene expression markers for migrating neural crest cells are switched off as the cells enter the environment of the cardiac outflow tract. Therefore, the expression of α-smooth muscle actin, a marker for neural crest cells within the outflow tract,[16] was examined in the outflow tract cushions of wild-type and *Lp/Lp* embryos at E11.5 and was found to be equivalent between the two
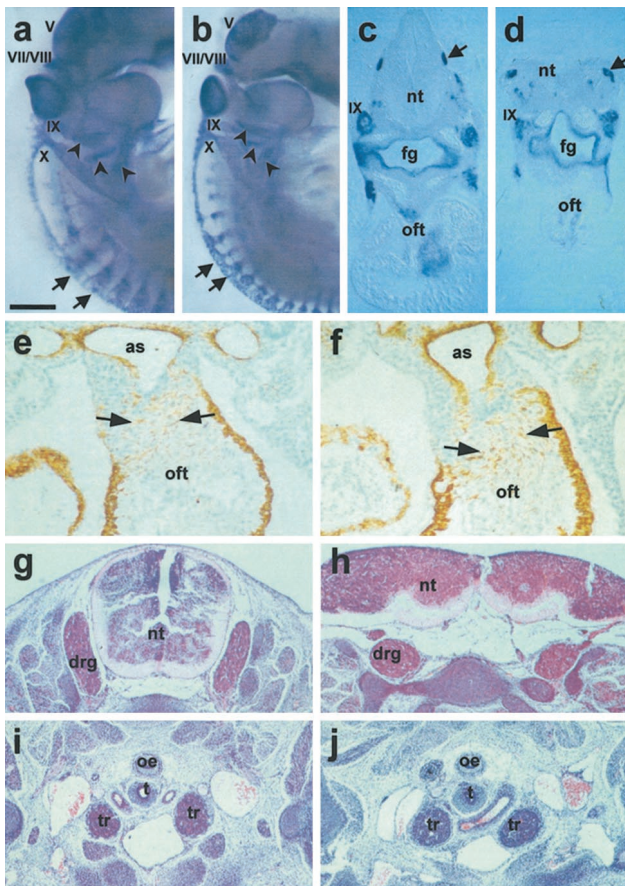
Figure 3. Neural crest derivatives in *Lp/Lp*. Whole-mount in situ hybridization for *erbB3* mRNA (a through d), immunocytochemistry for α-smooth muscle actin (e and f), and hematoxylin and eosin staining of sectioned fetuses (g through j) are shown. a and b, *ErbB3* expression in migrating neural crest cells of *Lp/+* (a) and *Lp/Lp* (b) littermate at E10.5. Robust neural crest cell migration can be observed in aortic arches 3, 4, and 6 in both embryos (arrowheads). Expression can also be seen in the developing dorsal root ganglia (drg, arrows) and in the trigeminal (V), facioacoustic (VII/VIII), glossopharyngeal (IX), and vagal (X) cranial ganglia. c and d, Transverse sections of the embryos in panels a and b showing that the drg (arrows) and glossopharyngeal ganglia (IX) are comparable between the *Lp/Lp* embryo (d) and its *Lp/+* littermate (c). Differences in the sections at the level of the heart reflect the altered positioning of the heart with respect to the rest of the body in the *Lp/Lp* embryo. e and f, Localization of α-smooth muscle actin–positive neural crest cells (arrows) within the outflow tract (oft) cushions of *Lp/+* (e) and *Lp/Lp* (f) embryos at E11.5, showing equivalent numbers in both embryos. g and h, The drg can clearly be seen in the +/+ fetus (g) at E13.5 and also appear to be normally sized in the *Lp/Lp* fetus (h). i and j, Thymus rudiments (tr) in the +/+ fetus (i) at E13.5 are comparable to those in the *Lp/Lp* fetus (j). nt indicates neural tube; as, aortic sac; and fg, foregut. Bar=400 μm (a and b), 250 μm (c and d), 100 μm (e and f), and 300 μm (g through j).

genotypes (Figures 3e and 3f), strongly suggesting that neural crest cell population of the outflow tract is normal in *Lp/Lp* embryos. Finally, formation of the dorsal root ganglia is dependent on neural crest migration, and at the level of the cardiac outflow tract, the dorsal root ganglia appeared to be normally sized in *Lp/Lp* fetuses at E13.5 compared with their wild-type littermates (Figures 3g and 3h). The thymic rudiments, which are thought to be specifically of cardiac neural crest origin,[17,18] also appeared to be normally sized and

positioned (Figures 3i and 3j). These data suggest that there is no abnormality in neural crest cell migration or differentiation in *Lp/Lp* fetuses.

Abnormal Heart Looping Associated With Axial Rotation Defects in *Lp/Lp* Embryos

An alternative explanation for the cardiovascular abnormalities in *Lp* is a disturbance of heart looping, inasmuch as this has been associated with double-outlet right ventricle.[19–21] Examination of embryos at E8.5, as the heart is just beginning to loop, revealed that although looping always occurs to the right in *Lp/Lp* embryos, as in wild-type littermates, there are marked abnormalities in the looping process. In wild-type embryos, the base of the ventricular loop lies 90° to the midline, but in *Lp/Lp* embryos, it is rotated clockwise and displaced to the right (see dotted lines in Figure 4a). This is still visible at E9.5 and E10.5 (Figures 4b through 4e).

Although the heart appears to be displaced in relation to the orientation of the embryonic head in *Lp/Lp* embryos at E9.5 and E10.5, it remains in the same orientation as the forelimb buds (Figures 4d and 4e), by virtue of the incomplete axial rotation that characterizes *Lp/Lp* embryos.[7] Hence, the head and the trunk are misaligned in *Lp/Lp* embryos, which might result in misalignment of the outflow vessels with respect to the ventricular chambers, leading to the development of double-outlet right ventricle.

Lp/Lp embryos also exhibit abnormalities in cervical flexure. At E8.5, compared with their wild-type littermates, *Lp/Lp* embryos have reduced cervical flexure (Figure 4a). This continues to be marked throughout development; at each stage, considerably more of the first and second branchial arches are visible in a frontal view of *Lp/Lp* embryos than of *Lp/+* and +/+ littermates (large arrow in Figures 4a and 5b). Side views at E9.5 and E13.5 confirm that cervical flexure is reduced in *Lp/Lp* embryos compared with their wild-type littermates (Figures 5a and 5c).

Apparently Normal Left-Right Axis Formation in *Lp/Lp* Embryos

As a consequence of the failure in regression of the right side of the aortic arch system, we looked for evidence of laterality defects in *Lp/Lp* embryos. All *Lp/Lp* embryos (n=25) turned to the right side, as in the wild-type embryos, and the heart looped to the right (Table). Moreover, in every case examined (n=18), the lungs were normal, with four lobes on the right and one on the left, and there were both morphologically left and right atrial appendages (Figures 6a and 6b), suggesting that pulmonary and/or atrial appendage isomerism was not a feature of *Lp/Lp* fetuses. Finally, we examined the expression of the genes *Pitx2c* and *lefty-2* at E8.5, the stage at which the left-right axis is being specified. *Pitx2c* was expressed symmetrically in the forebrain of +/+, *Lp/+*, and *Lp/Lp* embryos, whereas in the heart, *Pitx2c* exhibited markedly asymmetric expression. The left sinus horn, which becomes incorporated into the left ventricular groove as the coronary sinus, was strongly positive for *Pitx2c* transcripts in all the genotypes (Figures 6c and 6d). Examination of *lefty-2* expression at E8.5 revealed asymmetric localization of transcripts in the left lateral plate mesoderm (data not shown), with
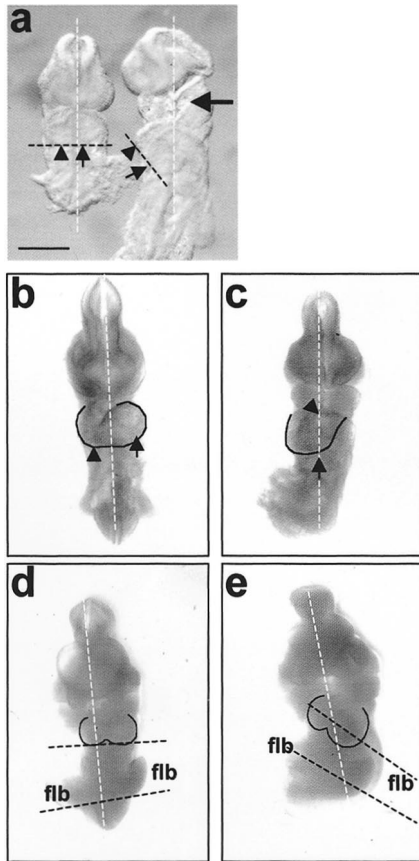
Figure 4. Abnormal heart looping in *Lp/Lp* embryos. a through e, Frontal whole-embryo views of +/+ and *Lp/Lp* embryos at E8.5 (a), E9.5 (b and c), and E10.5 (d and e). Panels b and d are +/+, and panels c and e are *Lp/Lp*. In panels b through d, the ventricles are outlined. White dotted lines indicate the embryonic midline. a, In +/+ embryos, the heart has looped at E8.5, and the ventricular loop is positioned on the left side of the midline (see arrow). The outflow tract and presumptive right ventricle are positioned to the right of the midline (arrowhead). Dotted lines show that the base of the ventricles lies approximately perpendicular to the embryonic midline. In *Lp/Lp* stage-matched littermates, the heart is abnormally looped, such that the ventricular loop is displaced caudally and to the right of the midline (see arrow). The outflow tract and presumptive right ventricle are also displaced (arrowhead), resulting in clockwise rotation of the base of the ventricles (dotted line). Abnormal cervical flexure also results in exposure of the developing first branchial arch (large arrow) in the *Lp/Lp* embryo. The caudal part of both embryos has been removed to facilitate the viewing of the heart. b, At E9.5, the ventricular chamber is beginning to be septated in the +/+ embryo, with the left (arrow) and right (arrowhead) ventricle positioned to the left and right sides of the midline, respectively. c, In the *Lp/Lp* embryo at E9.5, the developing left ventricle remains displaced toward the midline (arrow). The right ventricle is hidden from view, behind the left ventricle (arrowhead). Again, reduced cervical flexure results in exposure of the first and second branchial arches. d, In +/+ embryos at E10.5, the base of the ventricles is approximately parallel to the base of the forelimb buds (flb, dotted lines). e, In *Lp/Lp* embryos at E10.5, the base of the ventricles and the flb are also parallel to one another (dotted lines) but are rotated clockwise relative to the embryonic midline. Bar=465 μm (a), 500 μm (b and c), and 690 μm (d and e).

no discernible differences in expression pattern between +/+, *Lp/+*, and *Lp/Lp* embryos. These findings effectively rule out laterality defects as a cause of the malformations seen in the *Lp* mutant.
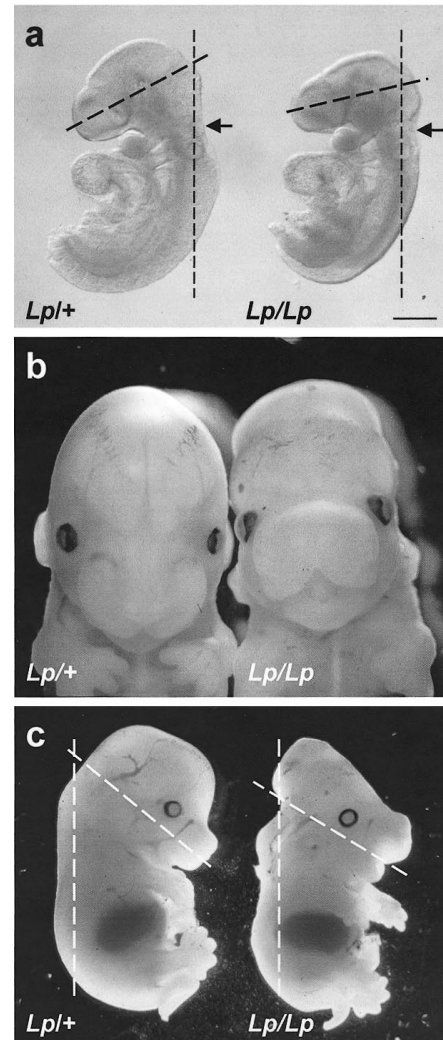

Figure 5. Reduced cervical flexure in *Lp/Lp* embryos and fetuses. Whole-embryo views at E9.5 (a) and E13.5 (b and c). a, Side views of +/+ and *Lp/Lp* embryos at E9.5 showing the reduced cervical flexure (arrows) in the mutant embryos. The dotted lines show the angle of flexure. b, Frontal view of *Lp/+* and *Lp/Lp* fetuses at E13.5, showing that the abnormality in cervical flexure is retained into the fetal period, such that the snout of the *Lp/Lp* fetus appears to be tilted upward compared with that of the *Lp/+* fetus. c, Side view of fetuses shown in panel b, showing that the angle of cervical flexure is still reduced at E13.5 in the *Lp/Lp* fetus (dotted lines). Bar=400 μm (a), 1.6 mm (b), and 1 mm (c).

Discussion

Cardiovascular defects have not previously been reported in the *Lp* mouse mutant, which has been studied mainly as a model of severe neural tube closure defects. We describe a range of complex cardiovascular defects in *Lp* homozygotes, including a defect of cardiac alignment, double-outlet right ventricle, and structural abnormalities of the aorta, including double-sided aortic arch.

Looping Disturbances as a Cause of Cardiac Alignment Defects

Double-outlet right ventricle, accompanied by an obligatory perimembranous ventricular septal defect, was found in all the *Lp/Lp* fetuses examined. This defect has been associated
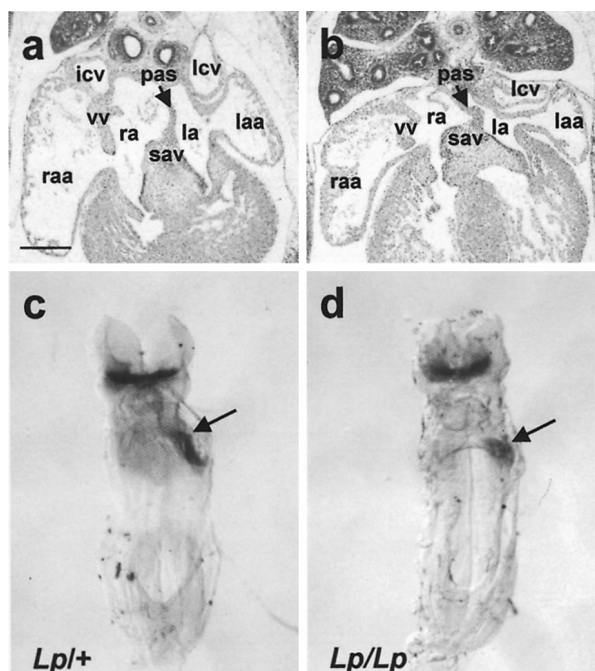
Figure 6. Normal laterality in *Lp/Lp* mutants. Hematoxylin and eosin staining of transverse sections through the hearts of +/+ and *Lp/Lp* sectioned fetuses at E13.5 (a and b) and whole-mount in situ hybridization using digoxigenin-labeled riboprobes for *Pitx2c* in *Lp*/+ (c) and *Lp/Lp* (d) embryos at E8.5 are shown. a, In the +/+ fetus, the normal configuration of the atria and atrial appendages can be seen, with the valves of the inferior caval vein (icv) appearing in the right atrium. The primary atrial septum (pas) is fused with the superior atrioventricular endocardial cushion (arrow). b, In the *Lp/Lp* fetus, the arrangement of the atrial chambers is the same as in the +/+ littermate. Again, the pas has fused with the superior atrioventricular endocardial cushion (arrow). c, *Pitx2c* expression in *Lp*/+ embryo at E8.5 (ventral view), showing symmetrical expression within the forebrain and asymmetrical expression in the looping heart tube, with abundant *Pitx2c* transcripts demarcating the left sinus horn, which is destined to form the coronary sinus (arrow). d, *Pitx2c* expression in a stage-matched *Lp/Lp* embryo, showing symmetrical expression in the forebrain and asymmetrical expression in the developing heart, with strong expression in the presumptive left atrial chamber (la), as in the *Lp*/+ littermate (arrow). lcv indicates left cardinal vein; ra, right atrial chamber; vv, venous valve; and sav, superior atrioventricular endocardial cushion. Bar=278 μm (a through d).

with both deficiencies in the cardiac neural crest and with abnormalities in cardiac looping.[14,20,22,23] We do not favor a neural crest abnormality as a cause of double-outlet right ventricle in the *Lp* mutant, because we observed robust cardiac neural crest migration, as shown by the expression of several well-characterized neural crest markers. In addition, comparable numbers of α-smooth muscle actin–positive neural crest cells were seen in the outflow tract cushions of all the genotypes. Normal development of other neural crest–derived structures, including the cranial ganglia, dorsal root ganglia and thymus, was also observed. These data suggest that defective neural crest cell colonization of the outflow tract is not responsible for the cardiac alignment defects seen in *Lp*. Instead, we suggest an abnormality in heart looping as the likely cause of the cardiac alignment defects seen in *Lp/Lp* fetuses.

If the processes of heart looping and remodeling are compromised, the apposition of the great vessels and ventricles can be disturbed, resulting in alignment defects, such as double-outlet right ventricle and ventricular septal defects. In the chick embryo, treatment with retinoic acid at stage 15 of development appears to induce cardiac looping abnormalities, leading to a spectrum of double-outlet right ventricle and ventricular septal defects.[20,24] The cardiac alignment defects described in these embryos closely resemble the abnormalities that we observe in *Lp/Lp* fetuses.

Cardiac Looping Abnormalities Are Likely to Be Secondary to Failure of Neural Tube Closure and/or Axial Turning Defects in *Lp*

In addition to cardiovascular defects, *Lp* homozygous embryos have a variety of other defects, including an open neural tube from the midbrain to the base of the spine, and reduction in cervical flexure, presumably as a result of alteration in mechanical forces within the open neural tube. In the chick embryo, experimental prevention of cervical flexure results in a spectrum of cardiac looping disturbances.[25] Moreover, prevention of cervical flexure can result in double-outlet right ventricle unrelated to defects in neural crest cell migration.[21] Therefore, it is possible that the looping disturbances and alignment defects in *Lp* result from a reduction in cervical flexure, which can be observed as early as E8.5, before the heart has completed looping morphogenesis.

In addition to the defects in neural tube closure, *Lp* mutants also exhibit axial rotation defects, which result in incomplete embryonic turning. These types of defects have been associated with cardiac looping abnormalities in a number of mouse mutants, including those for the *BMP2*, *no turning*, and *SIL* genes,[26–28] suggesting a close association between these two developmental processes.

Abnormal Regression/Retention of the Aortic Arch in *Lp*

The range of aortic arch malformations manifested by *Lp/Lp* fetuses can be explained on the basis of variable regression of a persistent double-sided aortic arch. The aortic arch system initially develops in a symmetrical fashion, but by E12.5 in the normal mouse embryo, the right side of the aortic arch is regressing, leaving a predominantly left-sided arch. Concomitantly, remodeling results in an asymmetrical pattern of aortic arch derivatives.[29] The mechanism behind retention and/or regression of arch elements is poorly understood. In humans, complete double-sided arch is rare and usually occurs in isolation.[30] Variations of this, such as right-sided aortic arch with aberrant origin of the subclavian artery, are much more common and are frequently found alongside other lesions, such as common arterial trunk and tetralogy of Fallot.[30] Right-sided and interrupted arch were both common findings in *Lp/Lp* fetuses, probably reflecting partial regression of a persistent double arch. Because the aortic arch phenotype in *Lp* is highly variable, it may be that the origin of the arch structures differs between embryos. For example, in some cases, the left fourth aortic arch artery may form the definitive left arch (as in the normal situation), whereas in other cases, the left third aortic arch artery might form the

definitive arch. The abnormal right side of the double arch is formed from the arterial duct (ductus arteriosus) and a retroesophageal subclavian artery. The precise origin of these vessels will be the subject of future studies.

The double-sided arch abnormality observed in *Lp* does not appear to have been previously reported in any mouse mutant. Double-sided aortic arches are found in ≈5% of rat embryos treated with the chemotherapeutic drug doxorubicin, whereas almost 25% develop a right-sided aortic arch, supporting the idea that these two abnormalities are manifestations of the same defect.[31] It is possible that aortic arch defects might have gone unnoticed in existing mouse mutants or might be associated with other, lethal anomalies that lead to death before E13.5, when abnormalities in regression/retention of the arch system become obvious. It is also possible that defects of aortic arch retention/regression are relatively specific for the *Lp* gene. The absence of mirror imagery or pulmonary and atrial appendage isomerism in *Lp/Lp* embryos and the normal expression of genes such as *Pitx2c* and *lefty 2* suggest that the *Lp* gene is unlikely to be involved in setting up the definitive left-right axis. However, *Lp* might act downstream from the genes that specify left-right symmetry; thus, the double-sided aortic arch seen in *Lp/Lp* fetuses may reflect minor abnormalities in left-right axis formation.

Acknowledgments

References

1. Edmonds LD, James LM. Temporal trends in the birth prevalence of selected congenital malformations in the Birth Defects Monitoring Program/Commission on Professional and Hospital Activities, 1979–1989. *Teratology*. 1993;48:647–649.
2. Jacobson Z, Glickstein J, Hensle T, Marion RW. Further delineation of the Opitz G/BBB syndrome: report of an infant with complex congenital heart disease and bladder exstrophy, and review of the literature. *Am J Med Genet*. 1998;78:294–299.
3. Aurora P, Wallis CE, Winter RM. The Jarcho-Levin syndrome (spondylocostal dysplasia) and complex congenital heart disease: a case report. *Clin Dysmorphol*. 1996;5:165–169.
4. Strong LC, Hollander WF. Hereditary loop-tail in the house mouse. *J Hered*. 1949;40:329–334.
5. Copp AJ, Checiu I, Henson JN. Developmental basis of severe neural tube defects in the *loop-tail* (*Lp*) mutant mouse: use of microsatellite DNA markers to identify embryonic genotype. *Dev Biol*. 1994;165:20–29.
6. Gerrelli D, Copp AJ. Failure of neural tube closure in the *loop-tail* (*Lp*) mutant mouse: analysis of the embryonic mechanism. *Dev Brain Res*. 1997;102:217–224.
7. Smith LJ, Stein KF. Axial elongation in the mouse and its retardation in homozygous looptail mice. *J Embryol Exp Morphol*. 1962;10:73–87.
8. Greene NDE, Gerrelli D, Van Straaten HWM, Copp AJ. Abnormalities of floor plate, notochord and somite differentiation in the *loop-tail* (*Lp*) mouse: a model of severe neural tube defects. *Mech Dev*. 1998;73:59–72.
9. Henderson DJ, Copp AJ. Versican expression is associated with chamber specification, septation, and valvulogenesis in the developing mouse heart. *Circ Res*. 1998;83:523–532.
10. Henderson DJ, Ybot-Gonzalez P, Copp AJ. Over-expression of the chondroitin sulphate proteoglycan *versican* is associated with defective neural crest migration in the *Pax3* mutant mouse (*splotch*). *Mech Dev*. 1997;69:39–51.
11. Henderson DJ, Ybot-Gonzalez P, Copp AJ. RhoB is expressed in migrating neural crest and endocardial cushions of the developing mouse embryo. *Mech Dev*. 2000;95:211–214.
12. Wilkinson DG. *In Situ Hybridisation: A Practical Approach*. Oxford, UK: IRL Press; 1992.
13. Franz T. Persistent truncus arteriosus in the Splotch mutant mouse. *Anat Embryol*. 1989;180:457–464.
14. Conway SJ, Henderson DJ, Anderson RH, Kirby ML, Copp AJ. Development of a lethal congenital heart defect in the *splotch* (*Pax3*) mutant mouse. *Cardiovasc Res*. 1997;36:163–173.
15. Goldmuntz E, Emanuel BS. Genetic disorders of cardiac morphogenesis: the DiGeorge and velocardiofacial syndromes. *Circ Res*. 1997;80:437–443.
16. Waller BR III, McQuinn T, Phelps AL, Markwald RR, Lo CW, Thompson RP, Wessels A. Conotruncal abnormalities in the trisomy 16 mouse: an immunohistochemical analysis with emphasis on the involvement of the neural crest. *Anat Rec*. 2000;260:279–293.
17. Bockman DE, Kirby ML. Dependence of thymus development on derivatives of the neural crest. *Science*. 1984;223:498–500.
18. Kirby ML, Waldo KL. Role of neural crest in congenital heart disease. *Circulation*. 1990;82:332–340.
19. Tomita H, Connuck DM, Leatherbury L, Kirby ML. Relation of early hemodynamic changes to final cardiac phenotype and survival after neural crest ablation in chick embryos. *Circulation*. 1991;84:1289–1295.
20. Bouman HGA, Broekhuizen MLA, Mieke A, Baasten J, Gittenberger-de Groot AC, Wenink ACG. Spectrum of looping disturbances in stage 34 chicken hearts after retinoic acid treatment. *Anat Rec*. 1995;243:101–108.
21. Kosaki K, Mendoza A, Jones KL. Cervical flexion: its contribution to normal and abnormal cardiac morphogenesis. *Teratology*. 1996;54:135–144.
22. Kirby ML, Gale TF, Stewart DE. Neural crest cells contribute to normal aorticopulmonary septation. *Science*. 1983;220:1059–1061.
23. Lin CR, Kioussi C, O'Connell S, Briata P, Szeto D, Liu F, Izpisúa-Belmonte JC, Rosenfeld MG. Pitx2 regulates lung asymmetry, cardiac positioning and pituitary and tooth morphogenesis. *Nature*. 1999;401:279–282.
24. Bouman HGA, Broekhuizen MLA, Baasten AMJ, Gittenberger-de Groot AC, Wenink ACG. Stereological study of stage 34 chicken hearts with looping disturbances after retinoic acid treatment: disturbed growth of myocardium and atrioventricular cushion tissue. *Anat Rec*. 1997;248:242–250.
25. Männer J, Seidl W, Steding G. Correlation between the embryonic head flexures and cardiac development: an experimental study in chick embryos. *Anat Embryol*. 1993;188:269–285.
26. Zhang HB, Bradley A. Mice deficient for BMP2 are nonviable and have defects in amnion chorion and cardiac development. *Development*. 1996;122:2977–2986.
27. Melloy PG, Ewart JL, Cohen MF, Desmond ME, Kuehn MR, Lo CW. *No turning*, a mouse mutation causing left-right and axial patterning defects. *Dev Biol*. 1998;193:77–89.
28. Izraeli S, Lowe LA, Bertness VL, Good DJ, Dorward DW, Kirsch IR, Kuehn MR. The *SIL* gene is required for mouse embryonic axial development and left-right specification. *Nature*. 1999;399:691–694.
29. Kaufman MH. *The Atlas of Mouse Development*. London, UK: Academic Press; 1992.
30. Tynan M, Anderson RH. Congenital heart defects. In: Julian DG, Camm AJ, Fox KM, Hall RJC, Poole Wilson PA, eds. *Diseases of the Heart*. London, UK, WB Saunders; 1996:681–755.
31. Qi BQ, Merei J, Farmer P, Hasthorpe S, Myers NA, Beasley SW, Hutson JM. Cardiovascular malformations in rat fetuses with oesophageal atresia and tracheo-oesophageal fistula induced by adriamycin. *Development*. 1997;126:1269–1280.