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


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 septation 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 mutant4 that provides a model for the human neural tube defect craniorachischisis. 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 gastrochisis, 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

<table>
<thead>
<tr>
<th>Cardiovascular Malformation</th>
<th>+/+</th>
<th>Lp/+</th>
<th>Lp/Lp</th>
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<tbody>
<tr>
<td>Double-outlet right ventricle</td>
<td>0/3</td>
<td>0/6</td>
<td>18/18</td>
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<tr>
<td>Ventricular septal defect</td>
<td>0/3</td>
<td>0/6</td>
<td>18/18</td>
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<tr>
<td>Aortopulmonary window</td>
<td>0/3</td>
<td>0/6</td>
<td>3/18</td>
</tr>
<tr>
<td>Double-sided aortic arch</td>
<td>0/3</td>
<td>0/6</td>
<td>7/19</td>
</tr>
<tr>
<td>Right-sided aortic arch</td>
<td>0/3</td>
<td>0/3</td>
<td>2/9†</td>
</tr>
<tr>
<td>Coarctation (in ink-injected fetuses*)</td>
<td>0/8</td>
<td>0/28</td>
<td>13/33</td>
</tr>
<tr>
<td>Laterality of embryos</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Embryo turned to right</td>
<td>3/3</td>
<td>3/3</td>
<td>25/25</td>
</tr>
<tr>
<td>Heart looped to right</td>
<td>3/3</td>
<td>3/3</td>
<td>25/25</td>
</tr>
</tbody>
</table>

*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. 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. The erbB3 probe was a gift from Dr C. Birchmeier (Max-Delbruck-Center for Molecular Medicine, Berlin, Germany). Pits2c and lefty-2 were obtained from Prof N. Brown (St. George’s Hospital Medical School, London, UK). The methodology for the whole-mount in situ hybridization of Wilkinson was followed, with minor modifications as described previously.

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 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 1f; 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. Lp/Lp and 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 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, 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
neural tube; as, aortic sac; and fg, foregut. Bar

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 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
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.

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...
with both deficiencies in the cardiac neural crest and with abnormalities in cardiac looping.\textsuperscript{14,20,22,23} We do not favor a neural crest abnormality as a cause of double-outlet right ventricle in the \textit{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 \textit{\alpha}-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 \textit{Lp}.

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.\textsuperscript{20,24} The cardiac alignment defects described in these embryos closely resemble the abnormalities that we observe in \textit{Lp/Lp} fetuses.

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

In addition to cardiovascular defects, \textit{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.\textsuperscript{25} Moreover, prevention of cervical flexure can result in double-outlet right ventricle unrelated to defects in neural crest cell migration.\textsuperscript{21} Therefore, it is possible that the looping disturbances and alignment defects in \textit{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, \textit{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 \textit{BMP2}, \textit{no turning}, and \textit{SIL} genes,\textsuperscript{26–28} suggesting a close association between these two developmental processes.

**Abnormal Regression/Retention of the Aortic Arch in \textit{Lp}**

The range of aortic arch malformations manifested by \textit{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{30} Right-sided and interrupted arch were both common findings in \textit{Lp/Lp} fetuses, probably reflecting partial regression of a persistent double arch. Because the aortic arch phenotype in \textit{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/retenion of the arch system become obvious. It is also possible that defects of aortic arch retention/regression are relatively specific for the 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.

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References

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