Corrigendum

Congenital heart disease reminiscent of partial trisomy 2p syndrome in mice transgenic for the transcription factor Lbh


An error in Fig. 7J of the article was not corrected before going to press. Two of the plus signs were mistakenly written as minuses. The correct figure is printed below.

The authors apologise to readers for this mistake.

![Corrected Figure](image-url)
Congenital heart disease reminiscent of partial trisomy 2p syndrome in mice transgenic for the transcription factor Lbh

Karoline J. Briegel1,*,†, H. Scott Baldwin2, Jonathan A. Epstein3 and Alexandra L. Joyner1

1Howard Hughes Medical Institute and Developmental Genetics Program, Skirball Institute of Biomolecular Medicine, New York University School of Medicine, New York, NY 10016, USA
2Departments of Pediatrics and Cell and Developmental Biology, Vanderbilt University Medical Center, Nashville, TN 37232, USA
3Cardiovascular Division, University of Pennsylvania, Philadelphia, PA 19104, USA

*Present address: Department of Biochemistry and Molecular Biology, University of Miami School of Medicine, Miami, FL 33136, USA
†Author for correspondence (e-mail: kbriegel@med.miami.edu)

Accepted 3 May 2005

Development 132, 3305-3316
Published by The Company of Biologists 2005
doi:10.1242/dev.01887

Summary

Partial trisomy 2p syndrome includes a spectrum of congenital heart disease (CHD) that is characterized by complex malformations of the outflow and inflow tracts, defects in cardiac septation, heart position, as well as abnormal ventricular development. Lbh (limb-bud and heart) is a novel, highly conserved putative transcriptional regulatory protein, which displays a unique spatiotemporal gene expression pattern during early mouse heart development. Here we show that human LBH maps to chromosome 2p23, a genomic region related to CHD in partial trisomy 2p syndrome. Remarkably, transgenic overexpression of Lbh in mice throughout the embryonic myocardium from a cardiomyocyte-specific promoter of the cardiac ankyrin repeat protein gene (Carp/Ankrd1) models CHD reported in humans with partial trisomy 2p syndrome. The malformations in Carp-Lbh transgenic mice reflect impaired pulmonary outflow tract valvulogenesis, cardiac septation, inflow tract morphogenesis, as well as abnormalities in ventricular cardiomyocyte growth. Furthermore, we demonstrate that overexpression of Lbh in cultured mammalian cells represses the synergistic activity of key cardiac transcription factors, Nkx2.5 and Tbx5, leading to reduced activation of the common target gene, Anf (Nppa). Strikingly, reduced levels of Anf expression were also observed in embryonic day 9.5 Carp-Lbh transgenic mice. Thus, repression of Nkx2.5 and Tbx5-mediated gene expression by deregulated Lbh may account in part for the cardiac anomalies observed in these mice. Our findings implicate LBH as a candidate gene for CHD associated with partial trisomy 2p syndrome and suggest an important role of Lbh in transcriptional control during normal cardiogenesis.

Key words: Lbh (Limb-bud and heart), Gene regulation, Heart development, Mouse, Congenital heart disease, Nkx2.5, Tbx5

Introduction

Congenital heart disease (CHD) is the most prominent developmental anomaly in humans with a prevalence of over 1% of live births (Hoffman and Kaplan, 2002). Yet, the molecular mechanisms leading to CHD are poorly understood. CHD is part of partial trisomy 2p syndrome, a rare autosomal disorder that is characterized by the triplication of distal regions of the short arm of chromosome 2 arising from imbalanced translocations of these chromosomal segments to various other chromosomes (Francke and Jones, 1976). Other typical phenotypes of this syndrome are mental and growth retardation, neural tube defects, characteristic craniofacial, skeletal and genital anomalies, as well as postaxial limb defects (Cassidy et al., 1977; Francke and Jones, 1976; Hahm et al., 1999; Lurie et al., 1995). Syndromic CHD involves outflow tract (OFT) defects, including pulmonary stenosis (PS) and pulmonary atresia (PA), patent ductus arteriosus (PDA), double outlet right ventricle (DORV), and the cyanotic lesion Tetralogy of Fallot (TOF) (Cassidy et al., 1977; Neu et al., 1979; Therkelsen et al., 1973). Some individuals also display ventricular and atrial septal defects (VSD and ASD), a patent foramen ovale (PFO), inflow tract (IFT) anomalies, including anomalous pulmonary return, as well as abnormally developed ventricles. In addition, dextrocardia and dextro-transposition of the great arteries (D-TGA) are observed either in isolation or in association with visceral heterotaxy (Francke and Jones, 1976; Lurie et al., 1995; Therkelsen et al., 1973). Of the approximately 60 partial trisomy 2p cases published so far, over 73% encompass duplications of chromosomal band 2p23 (Lurie et al., 1995; Taylor Clelland et al., 2000). Interestingly, CHD, as well as postaxial limb defects associated with this syndrome have been linked to this particular genomic segment (Hahm et al., 1999; Lurie et al., 1995). This suggests that one or several genes on chromosome 2p23 are responsible for patterning of the heart and the extremities.

We have previously identified a novel mouse protein, encoded by the Lbh (Limb-bud and heart) gene (Briegel and Joyner, 2001). Lbh is a member of a highly conserved family of small acidic proteins of ~12 kDa in vertebrates that do not exhibit any known structural motifs. A Xenopus orthologue of Lbh, termed Xic12, was originally cloned as a maternal RNA of unknown function that becomes activated in fertilized...
ANKR protein (stage onwards using a heart-specific promoter of the cardiac
endothelium, branchial arches, ventral tail ectoderm, urogenital
ridge, otic vesicles, oral epithelium and in neural crest-derived
sensory neurons (Briegel and Joyner, 2001). At the initial
stages of limb outgrowth, Lbh is expressed in ventral limb
ectoderm and the apical ectodermal ridge (AER). These limb
ectodermal compartments provide the cues for both ventral
limb specification and proximodistal limb outgrowth (Chen
and Johnson, 1999; Tickel, 1999). In the heart, Lbh expression
initiates in the crescent-shaped precardiac mesoderm as early
as expression of the homeodomain transcription factor Nkx2.5
(Briegel and Joyner, 2001; Lints et al., 1993). Notably, the
cardiac crescent expresses Lbh in an anterior-to-posterior
gradient with highest levels of expression in anterior pro-
cardiomyocytes (Briegel and Joyner, 2001). At the completion
of cardiac looping, Lbh expression is highest in the right
ventricle (RV), the atrio-ventricular canal (AVC) and the sinus
venosus (SV), but is excluded from atrial myocardium and
docardial structures. Once chamber formation has occurred,
the right-sided Lbh expression in ventricular myocardium is
lost and Lbh transcripts are distributed more uniformly in the
outer compact zone of RV and left ventricular (LV)
myocardium, but remain absent from atrial myocardium
(Briegel and Joyner, 2001). Interestingly, the Xenopus
orthologue, XIC12, is also specifically expressed in the
embryonic heart, suggesting a functional conservation of Lbh
proteins in vertebrate cardiogenesis (Gawantka et al., 1998).
Both Lbh and XIC12 continue to be expressed at high levels in
the adult heart (Briegel and Joyner, 2001; Paris and Philippe,
1990). Although these findings suggest important roles of Lbh
during limb and heart development, the in vivo function of Lbh
has remained unknown.

We mapped the murine Lbh locus to mouse chromosome
17E2, and the human LBH gene to human chromosome 2p23.3.
To specifically study the function of Lbh in heart development,
we engineered mice that express an Lbh transgene uniformly
throughout the developing myocardium from the 3 somite
stage onwards using a heart-specific promoter of the cardiac
ankyrin repeat protein (Carp; Ankrd1 – Mouse Genome
Informatics) (Kuo et al., 1999; Zou et al., 1997). We
demonstrate that normal anteroposterior and later
chamber-specific localization, as well as gene-dosage of Lbh in
the primitive heart tube is important for normal heart
morphogenesis because enforced expression of Lbh in mice
leads to a spectrum of cardiovascular defects. Most strikingly,
the cardiac phenotypes of Carp-Lbh transgenic mouse mimic
CHD reported in humans trisomic for chromosomal region
2p23, where LBH maps. Mice hemizygous for the Carp-Lbh
transgene develop OFT anomalies, including PS or PA due to
subvalvular obstruction of the pulmonary infundibulum with
excessive valve tissue, as well as DORV, D-TGA and TOF. In
addition, characteristic defects in IFT morphogenesis, cardiac
septation, heart position and in ventricular development were
observed. Finally, we show that Lbh expressed in tissue culture
cells inhibits Nkx2.5- and Tbx5-mediated activation of cardiac
target genes and that Anf (Nppa – Mouse Genome Informatics),
a common target gene, is downregulated in Carp-Lbh
transgenic mice. Taken together, our studies provide strong
evidence that LBH is a candidate gene for CHD associated with
partial trisomy 2p syndrome and that Lbh deregulation interferes
with normal cardiac development, in part through the
attenuation of Nkx2.5 and Tbx5 transcription factor function.

Materials and methods

Radioion hybrid mapping

Primers for mouse Lbh were STS-F (5′-GCAAGACACACTGTGAA-
GAGGCAAC-3′) and STS-B14 (5′-GTATTTCAGTGTGCAAA-
ACGG-3′), and for human Lbh hSTS-A (5′-GACATTTTACAGAA-
CAATC-3′) and hSTS-B (5′-ATAAGAGACATAGAGT-CCC-3′).
The primers were used against the mouse and human radiation hybrid
panels from Research Genetics. The data were incorporated into the
Jackson Laboratory mouse (MGM) and Stanford University human
radiation hybrid maps and compared against physical linkage and
genome sequence data of the OMIM and NCBI databases.

Generation of Carp-Lbh transgenic mice

A HindIII-BamHI fragment comprising an amino-terminally Flag-
tagged Lbh coding region from pcDNA/Flag-Lbh (Briegel and
Joyner, 2001) was blunt-end ligated into the SnaB1 site of the transgenic
vector PEVII (Kimmel et al., 2000). A 2.5 kb Carp cardiac-
specific promoter contained on a BamHI fragment (Kuo et al.,
1999; Zou et al., 1997) (generous gift from Dr Kenneth Chien), was
blunt-end ligated into the ClaI site of PEVII, creating the final
transgenic construct Carp-Lbh. The 3.5 kb transgene was released
from vector sequences by cleaving with SalI and microinjected into
the pronuclei of one-cell FVB/N embryos (Hogan et al., 1994).
Transgenic progeny were identified by PCR and confirmed by
Southern blotting analysis as previously described (Kimmel et al.,
2000).

RNA in situ hybridization

Whole-mount and section in situ hybridization was performed as
described previously (Chen et al., 2002; Schaeren-Wiemers and
probes used were to Lbh (P1) (Briegel and Joyner, 2001), lacZ (P2)
(Kimmel et al., 2000), Gata4 (Molkentin et al., 1997), Nkx2.5 (Lints
et al., 1993), Tbx5 (Bruneau et al., 1999) and Anf (Zeller et al.,
1987).

Histological analysis and immunohistochemistry

Whole mouse embryos and adult hearts were fixed for 18-24 hours
in 4% paraformaldehyde at +4°C, dehydrated in increasing
concentrations of ethanol and embedded in paraffin wax. Transverse,
frontal and coronal sections were cut at 4-6 μm and stained with
Haematoxylin and Eosin. Anti-phospho-histone H3 staining on
paraffin sections was performed as described by Shin et al. (Shin et
al., 2002), except that after immunostaining, sections were mounted
in SlowFade Antifade with DAPI (Molecular Probes). Phospho-
histone H3-positive cells were quantified by histomorphometry using
Metamorph software (Universal Imaging Corporation). This data was
statistically analyzed with a paired Student’s t-test.

Polymeric dye injections

After CO2 euthanasia of mice, blue Batson’s no. 17 casting solution
Development and disease

(Polysciences) was infused into the RV followed by injection of red Batson’s no. 17 polymeric dye solution into the LV of sick adult transgenic mice and normal wild-type mice. After dye polymerization overnight at room temperature, the tissue was digested with maceration solution according to the manufacturer’s protocol.

Transfections

NIH3T3 cells (2.5×10^5 cells/well of a 12-well plate on the day prior to transfection) were transfected using Lipofectamine 2000 reagent (Invitrogen) with 200 ng of Anf-human growth hormone (Anf-hGH) reporter (Chen et al., 2002), 200 ng each of pCGN-Nkx2.5 and Gate4 (Durocher et al., 1997) and 500 ng of pcDNA3-N-FLAG-TBX5 (Hiroi et al., 2001) expression plasmids. For synergy studies 600 ng of a pcDNA/NFLAG-"Lbh" expression plasmid (Briegel and Joyner, 2001) were co-transfected. 50 ng of pRL-CMV (Promega) were used to normalize for transfection efficiencies and pBluescript was added to transfection) were transfected using Lipofectamine 2000 reagent (Durocher et al., 1997) and 500 ng of pcDNA3-N-FLAG expression plasmids. For synergy studies 600 ng of a pcDNA/NFLAG-"Lbh" expression plasmid (Briegel and Joyner, 2001) were co-transfected. 50 ng of pRL-CMV (Promega) were used to normalize for transfection efficiencies and pBluescript was added to equalize the amount of DNA to 1.6 µg per transfection. Representative results of at least three independent experiments performed in duplicates were statistically analyzed using a paired Student’s t-test.

Results

Mammalian LBH loci map to a 2p23 syntenic group

Radiation hybrid mapping and bioinformatics (see Materials and methods) were used to determine the chromosomal positions of mammalian Lbh loci. The murine Lbh gene maps to band E2 on the distal arm of mouse chromosome 17 and is linked with a LOD score of 20.0 to marker D17Mit92 (Fig. 1). The human LBH locus maps to chromosome 2p23.3 proximal to linkage marker D2S352 within a region that is syntenic between human chromosome 2p21-23 and mouse chromosome 17 (Fig. 1). Interestingly, chromosomal band 2p23 is frequently triplicated in partial trisomy 2p syndrome and has been related to CHD associated with this syndrome (Fig. 1) (Lurie et al., 1995).

Transgenic mis-expression of Lbh throughout the developing myocardium

The cardiac expression pattern of Lbh during mouse embryogenesis and the linkage of Lbh to human chromosome 2p23 suggest that Lbh gain of function could play a role in CHD commonly associated with partial trisomy 2p syndrome. To test this hypothesis and also to examine the function of Lbh in cardiovascular development, we designed an experiment to perturb the normal Lbh gene dosage and expression pattern during heart morphogenesis in mice. Transgenic mice were generated that express an Lbh transgene uniformly throughout the developing myocardium from the 3-somite stage onwards under the control of a cardiomyocyte-specific promoter of the Cardiac ankyrin repeat protein gene (Carp) (Kuo et al., 1999; Zou et al., 1997) (Fig. 2A). Transgene expression was determined by RNA in situ analysis with a lacZ-specific antisense probe (P2) that detects a short lacZ tag contained in the transgene (Fig. 2A,D,F,H). Of the initial 10 transgenic lines, six showed robust cardiac-specific transgene expression and germline transmission, and, hence, were used for further analyses (see Table 1). In contrast to endogenous Lbh expression in ventricular myocardium of wild-type embryos with highest levels of expression in RV, AVC and SV (Fig. 2C,E,G) (Briegel and Joyner, 2001), the Carp-Lbh transgene was uniformly expressed in both ventricular and atrial cardiomyocytes in all of 38 stable transgenic embryos from the six selected transgenic lines that were analyzed on embryonic days 9.0 to 12.5 (E9.0-12.5) (Fig. 2D,F,H). Five lines expressed the Carp-Lbh transgene at comparably high levels (lines no. 3, 9, 16, 23 and 25; Table 1). Only one line (line no. 17) showed a slightly weaker, albeit equally broad transgene expression in the heart (Table 1). Transgene expression was also visible in somites (Fig. 2D,F), but alterations in somite formation were not evident, and hence not analyzed further. As expected, neither endogenous Lbh, nor the Lbh transgene were expressed in endocardial structures (Fig. 2G,H). Thus, during cardiogenesis the Carp promoter fragment drives expression of Lbh ectopically in atrial cardiomyocytes and dramatically increases the level of expression in myocardium of the OFT and LV.

Viability and phenotype penetrance of Carp-Lbh transgenic mice

The founder mice of the six independent Carp-Lbh transgenic lines were viable and indistinguishable from wild-type littermates until early adulthood. However, analysis of three transient transgenic litters at E13 revealed that two of eight transgene-positive embryos exhibited severe cardiac dysfunction, evident by pericardial edema, whereas all wild-
type littersates (n=30) had normally functioning hearts (data not shown). This suggests that approximately 25% of Carp-Lbh transgenic embryos had died of intrauterine heart failure. At 3-6 months of age, four of the six hemizygous Carp-Lbh transgenic founder mice (no. 3, 9, 16 and 23) displayed signs of illness: a hunched body posture, difficulties in breathing, lethargy and cyanosis (Fig. 2B; Table 1). Two of these sick animals (no. 3 and 9), as well as one other transgenic founder mouse (no. 25) died prematurely (Table 1). Only one transgenic founder animal (no. 17) appeared healthy and viable throughout life. Autopsy of three founder transgenic mice with signs of illness and/or premature death (no. 3, 9 and 23) revealed drastically enlarged hearts resulting from multiple structural defects, and even the one seemingly normal transgenic (no. 17) had an intra-cardiac defect, as assessed by histological examination (Table 1; Fig. 3F, Fig. 4B; data not shown).

Similar cardiovascular anomalies and a reduced viability were observed in hemizygous and homozygous Carp-Lbh transgenic F1 and F2 offspring of all founders. A total of 12 of 51 Carp-Lbh transgenic mice analyzed from different generations appeared ill and three of these died prematurely. In addition, seven of 51 transgenic mice that appeared normal died suddenly (Table 1; Fig. 3F, Fig. 4B; data not shown).

To investigate potential abnormalities in blood flow that frequently occur in common with these OCT anomalies, we injected a blue polymeric dye into the RV (venous blood), followed by injection of red polymeric dye into the LV (systemic blood) of adult wild-type (n=2) and Carp-Lbh transgenic (n=2) animals showing signs of sickness. Whereas in wild-type hearts the aorta was filled with red polymeric dye only, one of the Carp-Lbh transgenic hearts displayed
abnormal filling of the aorta with both red and blue polymeric dyes, indicative of mixing of systemic with venous blood, consistent with an overriding aorta and a PDA (Fig. 3G,H). Moreover, PS and a VSD (identified by the filling of the LV with blue dye) were apparent in this transgenic animal (Fig. 3G,H). This combination of RVOT obstruction, pulmonary vein development, and septation TOF† 2 TGA 1 VSD † TOF‡ ASD† ASD† 1 TGA, ASD †

Moreover, six of 51 postnatal and adult Carp-Lbh transgenic mice (12%) displayed an abnormal heart position in the absence of visceral heterotaxy. The transgenic hearts were shifted either to the right (dextrocardia; 4/51), to the left (levocardia; 1/51) or to the middle of the body (mesocardia; 1/51; Table 1). An adult Carp-Lbh transgenic heart with dextrocardia is shown in Fig. 4F. Whereas in wild-type mice, the heart is positioned to the left of the midline with the apex inclined towards the left, the transgenic heart was rotated to the right with dextro-positioned apex, great arteries and IFT (Fig. 4E-H). This phenotype most probably was secondary to hypoplastic growth of LV cardiomyocytes (Fig. 4I,J), rather than caused by defects in cardiac laterality.

### Table 1. Spectrum of cardiovascular phenotypes in postnatal and adult Carp-Lbh transgenic mice

<table>
<thead>
<tr>
<th>Transgenic line</th>
<th>Generation</th>
<th>Age of analysis</th>
<th>Abnormal OFT and septation</th>
<th>Abnormal IFT</th>
<th>Abnormal heart positioning</th>
<th>Cardiomyopathy</th>
<th>Hypoplastic heart</th>
<th>Transgene expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 (n=1)</td>
<td>F0</td>
<td>Adult (n=1)</td>
<td>+ PA, TOF^†</td>
<td>+</td>
<td>–</td>
<td>+ WH + RVH</td>
<td>–</td>
<td>++</td>
</tr>
<tr>
<td>9 (n=23)</td>
<td>F1</td>
<td>Adults (n=1)</td>
<td>1 PS, 2 TGA</td>
<td>+</td>
<td>–</td>
<td>4 RVH + RA</td>
<td>–</td>
<td>+++</td>
</tr>
<tr>
<td>16 (n=4)</td>
<td>F2</td>
<td>Adult (n=1)</td>
<td>0, 1 VSD^‡</td>
<td>+</td>
<td>–</td>
<td>1 BiV + RA</td>
<td>–</td>
<td>+++</td>
</tr>
<tr>
<td>17 (n=10)</td>
<td>F1*</td>
<td>P0-20, 3 litters (n=3)</td>
<td>+ PS, TOF^‡</td>
<td>+</td>
<td>–</td>
<td>+ LVH</td>
<td>–</td>
<td>+++</td>
</tr>
<tr>
<td>23 (n=5)</td>
<td>F0</td>
<td>Adult (n=1)</td>
<td>+ PS, ASD^§</td>
<td>+</td>
<td>–</td>
<td>1 RVH, 1 LVH + RA</td>
<td>–</td>
<td>+++</td>
</tr>
<tr>
<td>25 (n=8)</td>
<td>F1</td>
<td>Adult (n=1)</td>
<td>+ PS, 1 TGA, ASD^§</td>
<td>+</td>
<td>–</td>
<td>+ BiV, RA</td>
<td>1 LVH</td>
<td>+++</td>
</tr>
</tbody>
</table>

All wild-type littermates appeared normal, which was confirmed by sectioning of 10 wild-type animals at embryonic, postnatal and adult stages. *From 3 litters only 3 transgenics were obtained out of 16 offspring. Based on morphology and/or histological sections. Based on polymeric dye injections. Dextrocardia (DC), medocardia (MC), levocardia (LC). EH indicates that entire heart was hypoplastic, in association with reduced body size. **RV, right ventricular hypoplasia; LV, left ventricular hypoplasia. ††Difficulties in breathing, hunched body posture, lethargy (=physical appearance, not based on histopathology). ‡‡Heart not recovered. ‡§Refers to transgenic animals that did not exhibit cardiac defects, as assessed by postmortem histopathology. ¶¶Lethal. F0, hemizygous founder generation; F1, first hemizygous offspring; F2, second generation of mice from heterozygous intercrosses; P, postnatal day; OFT, outflow tract; IFT, inflow tract; PA, pulmonary atresia; TOF, Tetralogy of Fallot; PS, pulmonary stenosis; ASD, atrial septal defect; TGA, transposition of great arteries; WH, biventricular hypertrophy and dilated aatria; BiV, biventricular hypertrophy; RA, dilated right atrium; RVH, right ventricular hypertrophy; LVH, left ventricular hypertrophy; nd, not determined; sac, sacrificed; n, number of transgenic animals.
Abnormal ventricular development in Carp-Lbh transgenic mice

Fourteen out of 51 postnatal and adult Carp-Lbh transgenic hearts (28%) displayed various degrees of ventricular cardiomyopathy (Table 1), including RV hypertrophy (n=6), LV hypertrophy (n=3) and bilateral ventricular enlargement (n=4; Fig. 3F and Fig. 5B) with or without unilateral or bilateral atrial dilation (n=8; Fig. 4B,D and Fig. 6B). Severe ventricular hypertrophy in postnatal transgenic mice correlated with signs of illness and premature cardiac heart failure (Table 1). In most cases (n=11), ventricular hypertrophy was due to increased hypertrophic growth of cardiomyocytes (Fig. 3E,F), and arose probably because of secondary changes in hemodynamics and blood pressure as a result of coinciding OFT and IFT defects. However, three of 14 postnatal transgenic hearts displayed LV (n=2) and biventricular (n=1) enlargement in the absence of other obvious morphological defects (Table 1; Fig. 5B). Histopathologic examination of wild-type and Carp-Lbh transgenic littermates at P15 showed that ventricular hypertrophy in these transgenic mice was due to cardiomyocyte hyperplasia rather than hypertrophy of individual cardiomyocytes (Fig. 5A,B,D,E). In addition, immunohistochemistry with an antibody specific to the mitosis marker phospho-histone H3, and quantitative
histomorphometry revealed a marked increase in cardiomyocyte cell proliferation with the highest proliferation rate (approx. twofold; \( P=0.0016 \)) in LV myocardium, followed by intraventricular septum (IVS) and RV myocardium (Fig. 5G,H,J). Ventricular hyperplasia was also evident in two E13 transient transgenic embryos with pericardial effusion (Fig. 6G-J).

Furthermore, some Carp-Lbh transgenic mice displayed hypoplasia of the RV \((n=3)\), the LV \((n=1)\) or of the entire heart \((n=3)\) in the absence of other cardiac structural defects (Table 1, Fig. 5C,F). Severe cardiac hypoplasia coincided with a drastic reduction in body size (50% of the normal size) and resulted in premature death between P5 and P15. Although cardiomyocyte proliferation rates were normal in these transgenic hearts (Fig. 5D,F), the occurrence of these extreme phenotypes in a subset of Carp-Lbh transgensics suggest that some of the observed cardiomyopathies were congenital rather than acquired abnormalities in myocardial development and function. Interestingly, both ventricular hyperplasia and hypoplasia are also observed in individuals with partial 2p trisomy syndrome (Cassidy et al., 1977; Neu et al., 1979).

**Histological analysis of neonatal and embryonic Carp-Lbh transgenic hearts**

To examine disease progression and the primary morphological lesions that cause the cardiac disease phenotype in Carp-Lbh transgenic mice, we performed histopathology on wild-type and transgenic mice at different stages in heart development. To assess the effect of Lbh overexpression at birth, when the pulmonary blood circulation becomes established and vital because of closures of the intra-atrial foramen ovale and the ductus arteriosus, we analyzed the hearts of Carp-Lbh transgenic mice \((n=2)\) from two independent lines that had died shortly after birth. As shown in Fig. 6, the pulmonary valves of P0 wild-type hearts consist of three thin leaflets derived from endocardial tissue, and the RVOT beneath the valves forms a cavity (Fig. 6A,C). In the P0 Carp-Lbh transgenic mouse shown, the pulmonary valves appeared normal, however, the RVOT below the valves was filled with excessive valve tissue and myocardial cells (Fig. 6B,D), causing a subvalvular obstruction of the pulmonary infundibulum. In addition, the transgenic heart displayed a PFO with a right to left shunting most probably caused by increased pressures on the right side of the heart as a result of the pulmonic obstruction (Fig. 6F).

The second transgenic newborn that died exhibited isolated IFT anomalies (data not shown).

To investigate cardiac valve and septae formation in more detail, we analyzed transient transgenic embryos \((n=8)\), as postnatal Carp-Lbh founder transgensics showed the most severe phenotypes (Table 1), between E12-14. Fig. 6G-L shows histological sections from a wild-type and a transient transgenic littermate at E13. In addition to levocardia, ventricular hypertrophy and abnormal trabeculation, severe OFT anomalies were observed in the Carp-Lbh transgenic embryo (Fig. 6H,J,L). In wild-type embryos, the ridge-like endocardial cushions of the OFT, which give rise to the septae and the valves of the great arteries (Fishman and Chien, 1997), only line the inner conotruncal myocardium (Fig. 6I). However, in the transgenic embryo shown, the OFT ridges were not only drastically enlarged, but also extended to ectopic sites in the cavity of the RV (Fig. 6H,J). Moreover, a DORV and a VSD were apparent (Fig. 6J,L). Thus, the cardiovascular phenotypes of neonatal and embryonic Carp-Lbh transgenic mice are consistent with the spectrum of cardiac malformations observed in late postnatal and adult animals.

**Molecular analysis of embryonic Carp-Lbh transgenic hearts**

To understand the basis for the cardiac defects in Carp-Lbh transgenic hearts, we assessed cardiomyocyte proliferation with the highest proliferation rate (approx. twofold; \( P=0.0016 \)) in LV myocardium, followed by intraventricular septum (IVS) and RV myocardium (Fig. 5G,H,J). Ventricular hyperplasia was also evident in two E13 transient transgenic embryos with pericardial effusion (Fig. 6G-J).

Interestingly, both ventricular hyperplasia and hypoplasia are also observed in individuals with partial 2p trisomy syndrome (Cassidy et al., 1977; Neu et al., 1979).

**Histological analysis of neonatal and embryonic Carp-Lbh transgenic hearts**

To examine disease progression and the primary morphological lesions that cause the cardiac disease phenotype in Carp-Lbh transgenic mice, we performed histopathology on wild-type and transgenic mice at different stages in heart development. To assess the effect of Lbh overexpression at birth, when the pulmonary blood circulation becomes established and vital because of closures of the intra-atrial foramen ovale and the ductus arteriosus, we analyzed the hearts of Carp-Lbh transgenic mice \((n=2)\) from two independent lines that had died shortly after birth. As shown in Fig. 6, the pulmonary valves of P0 wild-type hearts consist of three thin leaflets derived from endocardial tissue, and the RVOT beneath the valves forms a cavity (Fig. 6A,C). In the P0 Carp-Lbh transgenic mouse shown, the pulmonary valves appeared normal, however, the RVOT below the valves was filled with excessive valve tissue and myocardial cells (Fig. 6B,D), causing a subvalvular obstruction of the pulmonary infundibulum. In addition, the transgenic heart displayed a PFO with a right to left shunting most probably caused by increased pressures on the right side of the heart as a result of the pulmonic obstruction (Fig. 6F).

The second transgenic newborn that died exhibited isolated IFT anomalies (data not shown).

To investigate cardiac valve and septae formation in more detail, we analyzed transient transgenic embryos \((n=8)\), as postnatal Carp-Lbh founder transgensics showed the most severe phenotypes (Table 1), between E12-14. Fig. 6G-L shows histological sections from a wild-type and a transient transgenic littermate at E13. In addition to levocardia, ventricular hypertrophy and abnormal trabeculation, severe OFT anomalies were observed in the Carp-Lbh transgenic embryo (Fig. 6H,J,L). In wild-type embryos, the ridge-like endocardial cushions of the OFT, which give rise to the septae and the valves of the great arteries (Fishman and Chien, 1997), only line the inner conotruncal myocardium (Fig. 6I). However, in the transgenic embryo shown, the OFT ridges were not only drastically enlarged, but also extended to ectopic sites in the cavity of the RV (Fig. 6H,J). Moreover, a DORV and a VSD were apparent (Fig. 6J,L). Thus, the cardiovascular phenotypes of neonatal and embryonic Carp-Lbh transgenic mice are consistent with the spectrum of cardiac malformations observed in late postnatal and adult animals.

**Molecular analysis of embryonic Carp-Lbh transgenic hearts**

To understand the basis for the cardiac defects in Carp-Lbh transgenic hearts, we assessed cardiomyocyte proliferation with the highest proliferation rate (approx. twofold; \( P=0.0016 \)) in LV myocardium, followed by intraventricular septum (IVS) and RV myocardium (Fig. 5G,H,J). Ventricular hyperplasia was also evident in two E13 transient transgenic embryos with pericardial effusion (Fig. 6G-J).

Interestingly, both ventricular hyperplasia and hypoplasia are also observed in individuals with partial 2p trisomy syndrome (Cassidy et al., 1977; Neu et al., 1979).
transgenic mice, we analyzed markers for cardiac cell specification and differentiation. RNA in situ hybridization analysis of E9.5 wild-type (n=2) and transient Carp-Lbh transgenic embryos (n=2) revealed normal distribution and expression levels of the cardiac transcription factors Nkx2.5, Tbx5 and Gata4 (Fig. 7A-F). Expression of dHand (Hand2 – MGI), eHand (Hand1) and Pitx2 was also normal (data not shown). However, mRNA levels of Anf, a common target of Nkx2.5, Tbx5 and Gata4 (Bruneau et al., 2001; Durocher et al., 1997; Garg et al., 2003), were downregulated in transgenic embryos (Fig. 7G,H). Expression of other atrial and ventricular markers, Irx4, MLC2a (My17), MLC2v (My12), in contrast, was not affected (data not shown). Thus, ectopic Lbh expression in the heart decreases Anf mRNA levels despite normal gene dosage of key cardiac transcription factors.

To investigate whether Lbh misexpression could have affected the transcriptional activities of the factors regulating Anf expression, we employed a transient reporter assay in heterologous NIH3T3 cells. As anticipated, Nkx2.5, Tbx5 and GATA4 individually or synergistically activated the promoter of the Anf gene in these cells (Fig. 7I,J) (Bruneau et al., 2001; Durocher et al., 1997; Garg et al., 2003; Hiroi et al., 2001). Conversely, cells transfected with vector alone, or cells transfected with an Lbh expression plasmid displayed no significant activation of an Anf-human growth hormone reporter (Anf-hGH; Fig. 7J). Strikingly, co-transfection of increasing amounts of Lbh expression plasmid with Nkx2.5, Tbx5 or Gata4 expression constructs predominantly suppressed the transcriptional activities of Nkx2.5 and Tbx5 (Fig. 7I,J). A low concentration of Lbh (0.2 µg) inhibited Nkx2.5 and Tbx5 activities up to 50% (P<0.001), whereas it had only a modest effect on the activity of Gata4 (Fig. 7I). A 50% reduction (P<0.05) of Gata4 activity was achieved only with the highest concentration of Lbh expression plasmid (1.0 µg) used (Fig. 7I). Furthermore, whereas increasing amounts of the Lbh expression plasmid did not yield a greater inhibition of Nkx2.5, higher concentrations of Lbh led to a linear reduction of Tbx5 activity up to 85% (P<0.001) (Fig. 7I). Lbh also produced a
Lbh mutant mice

strong inhibitory effect (~3.3-fold; P = 0.008) on the synergistic activation of Anf by Nkx2.5 and Tbx5 (Fig. 7J). In contrast, co-transfection of an Lbh expression construct in combination with Nkx2.5 and Gata4, or Tbx5 and Gata4 expression plasmids had no or only a minimal effect (~1.4-fold reduction) on the synergistic activities of these factors (Fig. 7J). These results suggest that the cardiac dysmorphogenesis observed in Carp-Lbh transgenic mice, as well as in individuals with partial trisomy 2p syndrome, may be due in part to attenuation of Nkx2.5 and Tbx5 protein function by deregulated Lbh.

Discussion

Ubiquitous expression of Lbh in the embryonic myocardium of transgenic mice causes a unique spectrum of cardiovascular anomalies reminiscent of CHD associated with human partial trisomy 2p syndrome: PS or PA, TOF, TGA, DORV, VSD, ASD, PDA, IFT dysmorphogenesis, heart mispositioning and ventricular abnormalities. In keeping with this finding, the human LBH gene maps to chromosomal band 2p23, which consistently is triplicated in individuals with partial trisomy 2p syndrome, as well as in individuals with partial trisomy 2p syndrome, may be due in part to attenuation of Nkx2.5 and Tbx5 protein function by deregulated Lbh.

Range of cardiovascular malformations in Carp-Lbh transgenic mice

The phenotypes of Carp-Lbh transgenic mice suggest that enforced expression of Lbh in heart muscle cells from the 3-somite stage onwards affects different steps in heart morphogenesis, including OFT valvulogenesis, IFT morphogenesis, cardiac septation, as well as cardiomyocyte growth and function, leading to secondary cardiovascular defects and dysfunction in postnatal and adult transgenic animals. The spectrum of cardiac malformations was consistent between the independent transgenic lines, but was more severe in some lines and varied among different individuals of one line (Table 1). Although this could reflect different Lbh transgene levels, we detected only slight variations in transgene levels in five out of six lines, as assessed by RNA in situ hybridization. Furthermore, homozygous animals with clearly elevated transgene expression levels did not show more severe phenotypes than their hemizygous littermates (Table 1; data not shown). This suggests that the diversity of cardiac defects in Carp-Lbh transgenic mice could have been evoked not only by a gene dosage effect, but also by the abnormal distribution of Lbh in transgenic hearts.

Lbh interferes with OFT valvulogenesis

Carp-Lbh transgenic mice displayed a spectrum of OFT defects (PS and PA with or without TOF) that is characteristic for DiGeorge Syndrome, a human neural crest (NC) ablation defect, caused by haploinsufficiency of genes located on chromosome 22p11, in particular TBX1 (Goldmuntz et al., 1998; Jerome and Papaioannou, 2001; Lindsay et al., 1999; Merscher et al., 2001). Cardiac NC colonizes the primordial endocardial cushions, which is a prerequisite to cardiac valve formation.
and septae formation (reviewed by Kirby, 1999). However, in Carp-Lbh transgenic mice these OFT anomalies appear to be of myocardial origin, because the Carp-Lbh transgene was not expressed in endocardium or cardiac NC (Fig. 2) (Kuo et al., 1999; Zou et al., 1997). Valve formation is controlled by reciprocal signaling between the myocardium and endocardial cushions in valve-forming regions (OFT, AVC), which induces epithelial-mesenchymal transformation (EMT) in the valve cushions (reviewed by Barnett and Desgrosellier, 2003). In the OFT these tissue interactions also control a process called ‘myocardialization’, in which myocardial cells invade the conotruncal mesenchymal cushions, contributing to muscular pulmonary infundibulum and outlet septum (van den Hoff et al., 1999). Overgrowth of the sub-pulmonic region of embryonic and P0 Carp-Lbh transgenic mice with both mesenchymal valve tissue and myocardial cells, leading to pulmonary obstruction and eventually to a regression of the pulmonary artery in adult transgensics, suggests that overexpression of Lbh in OFT myocardium perturbed the myocardial signaling that controls both of these processes. Notch signaling has been shown to promote EMT in OFT myocardial cushions in valve-forming regions (OFT, AVC), which induces epithelial-mesenchymal transformation (EMT) in the valve cushions (reviewed by Barnett and Desgrosellier, 2003). In the OFT these tissue interactions also control a process called ‘myocardialization’, in which myocardial cells invade the conotruncal mesenchymal cushions, contributing to muscular pulmonary infundibulum and outlet septum (van den Hoff et al., 1999).

Overgrowth of the sub-pulmonic region of embryonic and P0 Carp-Lbh transgenic mice with both mesenchymal valve tissue and myocardial cells, leading to pulmonary obstruction and eventually to a regression of the pulmonary artery in adult transgensics, suggests that overexpression of Lbh in OFT myocardium perturbed the myocardial signaling that controls both of these processes. Notch signaling has been shown to promote EMT in endocardial cushions (Noseda et al., 2004; Timmerman et al., 2004). Notably, gene mutations in Jagged 1 (JAG1), a Notch ligand, in human Alagille Syndrome (Li et al., 1997; Oda et al., 1997) or gene ablation of Hey2, encoding a hairy/Enhancer-of-split-related basic helix-loop-helix transcription factor acting downstream of Notch, in mice (Donovan et al., 2002), cause OFT and cardiac septation defects similar to those observed in Carp-Lbh transgenic mice. Another myocardial signal that regulates EMT during cardiac valve formation, as well as OFT myocardialization, is transforming growth factor β2 (Tgfβ2) (Bartram et al., 2001; Boyer et al., 1999; Camenisich et al., 2002). Tgfβ2-deficient mice, not only have hyperplastic valves but also DORV with accompanying VSD (Bartram et al., 2001), an anomaly we also observed in Carp-Lbh transgenic mice.

Mis-regulation of Lbh perturbs late sino-atral morphogenesis

Ectopic expression of Lbh in atrial cardiomyocytes most probably contributed to both abnormal pulmonary venous return and to defects in atrial septation in Carp-Lbh transgenic mice. The primordial pulmonary veins originate as an outgrowth of atrial muscle cells that anastomose with the pulmonary venous plexus and subsequently colonize the pulmonary veins in a caudocranial fashion (Larsen, 1997; Millino et al., 2000). Although the role of myocardium in pulmonary vein formation is not known, mis-expression of Lbh in atrial muscle cells that encase the pulmonary veins indicates that proper pulmonary vein muscle development is a prerequisite for the correct positioning of the pulmonary veins. Alternatively, ectopic Lbh might have altered atrial myocardial function, which could impair atrial-venous differentiation and cardiac morphology by changing the normal blood flow and hemodynamics (Huang et al., 2003; le Noble et al., 2003). Altered hemodynamic forces may also have contributed to ASD, PFA and other morphological defects in Carp-Lbh transgenic mice (Larsen, 1997; le Noble et al., 2003). Furthermore, Lbh transgene expression might have directly interfered with the molecular pathways that control atrial septation (see below).

Overexpression of Lbh impairs ventricular cardiomyocyte growth

During heart development, Lbh is predominantly expressed in proliferative ventricular myocardium (Briegel and Joyner, 2001), suggesting a role of Lbh in ventricular cardiomyocyte growth. In keeping with this idea, ventricular growth defects including hyperplasia and hypoplasia were observed in a cohort of Carp-Lbh transgenic mice. Proliferation of cardiomyocytes normally stops after birth and transits into hypertrophic cell growth, which is accompanied by increased myofibril density and cardiomyocyte cell fusion (Pasumarthi and Field, 2002). However, in hyperplastic transgenic hearts, proliferation of ventricular cardiomyocytes was prolonged into postnatal stages. In contrast, hypoplasia in transgenic mice was due to a failure of ventricular cardiomyocytes to undergo hypertrophic growth, which was evident by reduced cardiomyocyte size and fusion (Fig. 5F). As the Carp promoter is down-regulated upon birth (Kuo et al., 1999; Zou et al., 1997), these postnatal ventricular growth defects could be the result of transgenic Lbh protein being more stable than its mRNA. Furthermore, the abnormal position of some Carp-Lbh transgenic hearts appeared to be the consequence of asymmetric ventricular hypoplasia (Fig. 4J) or hyperplasia (Fig. 6H), rather than of aberrant left-right patterning, as expression of Pitx2, a POU-homeodomain transcription factor and major determinant for organ asymmetry (Capdevila et al., 2000), and early looping morphogenesis was unaltered in these mice (data not shown).

What is the molecular basis for Lbh function?

The biochemical properties of Lbh suggest a role of this protein in cardiac gene regulation (Briegel and Joyner, 2001). Consistent with this notion, we found that expression of Lbh in tissue culture cells predominantly represses the transcriptional activities of Nkx2.5 and Tbx5 (Bruneau et al., 2001; Hiroi et al., 2001), two key regulators of cardiogenesis (Biben et al., 2000; Bruneau et al., 2001; Liberatore et al., 2000; Lyons et al., 1995; Schott et al., 1998; Takeuchi et al., 2003). In accord with these data, expression of Anf, a common Nkx2.5/Tbx5 target gene (Bruneau et al., 2001), was markedly downregulated in early Carp-Lbh transgenic embryos. Since Lbh lacks a DNA binding domain and therefore could not compete for Nkx2.5/Tbx5 DNA binding sites in the promoters of cardiac genes, we favor the idea that Lbh modulates Nkx2.5 transcriptional activities by directly interacting with these factors at the protein level. In support of this idea, the cardiac disease phenotypes observed in Carp-Lbh transgenic mice, as well as in individuals with partial trisomy 2p syndrome are remarkably similar to CHD caused by haploinsufficiency of NKKZ2.5 or TBX5: ASD, TOF and ventricular abnormalities (Basson et al., 1997; Biben et al., 2000; Bruneau et al., 2001; Schott et al., 1998). In addition, Carp-Lbh transgenic mice and individuals with partial trisomy 2p syndrome have in common with NKKZ2.5 haploinsufficiency, PS, DORV and PDA (Benson et al., 1999; Schott et al., 1998), whereas anomalous pulmonary return and left ventricular hyperplasia have also been reported in TBX5-haploinsufficient individuals with Holt-Oram syndrom and in Tbx5 mouse mutants (Basson et al., 1997; Bruneau et al., 2001). However, unlike NKKZ2.5 and TBX-deficiencies (Bruneau et al., 2001; Schott et al., 1998) Carp-Lbh transgenic mice did not display...
cardiac rhythm disturbances, as assessed by electrocardiograms (data not shown), which could be due to the absence of *Carp*-promoter activity in the conduction system (Kuo et al., 1999; Zou et al., 1997). There is also a significant overlap between the cardiac phenotypes observed in *Carp*-Lbh transgenic mice and Gata4 deficiencies: ASD, TOF, PS, PDA, DORV, VSD membranous type, excessive pulmonary valve tissue, dextrocardia and ventricular hypoplasia (Garg et al., 2003; Pu et al., 2004). Although we did measure only a modest inhibitory effect of Lbh on Gata4 transcriptional activity in cell-based reporter assays, our genetic data would suggest that the Lbh transgene also interfered with Gata4-dependent pathways. Taken together, our data indicate that Lbh can regulate cardiac gene expression by modulating the combinatorial activities of key cardiac transcription factors, as well as their individual functions in cardiogenesis.

**Potential role of LBH in human congenital heart disease**

Most importantly, Lbh gain of function during heart development of transgenic mice virtually mimics CHD that has been reported in individuals trisomic for the chromosomal region 2p23, to which the human LBH maps. This provides the first evidence that CHD associated with partial trisomy 2p syndrome is due in part to increased gene dosage, and most likely also abnormal cardiac distribution of LBH in some individuals. A deregulation of LBH gene expression could result if the translocation breakpoint occurs in proximity of the LBH locus. Indeed, in over one-third of these individuals the chromosomal breakpoint maps within 2p23 (Taylor Clelland et al., 2000). Interestingly, triplication of chromosomal band 2p23 also seems to be associated with postaxial limb defects, such as polydactyly, clinodactyly, long tapering fan-like digits and bilateral simian creases (Cassidy et al., 1977; Francke and Jones, 1976; Hahm et al., 1999; Lurie et al., 1995). These anomalies might in part be due to dysfunction of the AER, but also to defective dorsoventral limb patterning. Since Lbh is expressed both in the AER and in ventral limb ectoderm during mouse limb development (Briegel and Joyner, 2001), it is tempting to speculate that increased LBH gene dosage is also involved in the partial trisomy 2p limb phenotypes. In conclusion, our findings suggest a pivotal role of Lbh in normal heart development, as well as in human CHD, as a trans-acting modulator of key cardiac transcription factors.

We are indebted to Kenneth Chien, Issei Komura, Eric Olson and Christine Seidman for providing crucial reagents. We thank Jimmy Lao for technical assistance and the Skirball transgenic facility for injection of zygotes. We also thank Deborah Yelon for critical reading of the manuscript, and Chaitanya Jain for continuous support. A.L.J. is an Investigator and K.J.B. was a Research Associate of the Howard Hughes Medical Institute. K.J.B. also received support from a Long-term Fellowship from the Human Frontiers of Science Organization.

**References**


