Abrogation of both short and long forms of latent transforming growth factor-β binding protein-1 causes defective cardiovascular development and is perinatally lethal

Masahito Horiguchi[1], **Vesna Todorovic**[1,2], **Krassimira Hadjiolova**[1], **Ralf Weiskirchen**[3] **and Daniel B. Rifkin**[1,2]

*1 - **Department of Cell Biology,** New York University Langone School of Medicine, 550 First Avenue, New York, NY 10016, USA*
*2 - **Department of Medicine,** New York University Langone School of Medicine, 550 First Avenue, New York, NY 10016, USA*
*3 - **Institute of Molecular Pathobiochemistry,** Experimental Gene Therapy and Clinical Chemistry, RWTH University Hospital Aachen, Pauwelsstr. 30, D-52074 Aachen, Germany*

***Correspondence to Daniel B. Rifkin:** Department of Cell Biology, NYU Langone School of Medicine, 550 First Avenue, NY, NY 10016, USA. Daniel.Rifkin@nyumc.org*
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Abstract

Latent transforming growth factor-β binding protein-1 (LTBP-1) is an extracellular protein that is structurally similar to fibrillin and has an important role in controlling transforming growth factor-β (TGF-β) signaling by storing the cytokine in the extracellular matrix and by being involved in the conversion of the latent growth factor to its active form. LTBP-1 is found as both short (LTBP-1S) and long (LTBP-1L) forms, which are derived through the use of separate promoters. There is controversy regarding the importance of LTBP-1L, as *Ltbp1L* knockout mice showed multiple cardiovascular defects but the complete null mice did not. Here, we describe a third line of *Ltbp1* knockout mice generated utilizing a conditional knockout strategy that ablated expression of both L and S forms of LTBP-1. These mice show severe developmental cardiovascular abnormalities and die perinatally; thus these animals display a phenotype similar to previously reported *Ltbp1L* knockout mice. We reinvestigated the other "complete" knockout line and found that these mice express a splice variant of LTBP-1L and, therefore, are not complete *Ltbp1* knockouts. Our results clarify the phenotypes of *Ltbp1* null mice and re-emphasize the importance of LTBP-1 in vivo.

Introduction

Transforming growth factor-β (TGF-β), the prototype member of the TGF-β family, exerts numerous essential functions in vivo. TGF-β is involved in physiological processes, such as embryogenesis, organ development, and the maintenance of tissue homeostasis [1]. TGF-β also participates in pathological conditions, such as inflammation, tissue fibrosis, suppression and development of tumors, and the pathology of several connective tissue diseases [2]. There are three different TGF-β isoforms (TGF-β1, 2, and 3), all of which have been studied extensively and seem to have unique functions in vivo, as there is little overlap in the phenotypes of knockout mice for individual TGF-β isoforms [3–6].

The exquisite control of the production of active TGF-β cytokine, which is essential in maintaining physiological functions in vivo, is exerted at three levels: expression, storage, and activation. Unlike other members of the TGF-β family, such as the bone morphogenetic proteins, TGF-β is secreted from cells complexed non-covalently with its propeptide, referred to as the latency associated peptide (LAP). This complex of mature cytokine and LAP is called the small latent complex (SLC), and in this complex TGF-β is blocked from interacting with its receptor [7]. SLC can be secreted by itself, but LAP is usually covalently bound to a carrier protein by disulfide bonds [8]. This large carrier protein is named the latent TGF-β binding protein (LTBP), whereas the complex of SLC and LTBP is referred to as the large

latent complex (LLC) [8–10]. LLC is incorporated into extracellular matrix (ECM) via interaction between LTBP and ECM components, such as fibrillin and fibronectin [11–13]. This allows latent TGF-β to be stored in the ECM in a ready-to-use state [14]. Mature TGF-β needs to be released from LLC and specifically from LAP via a process called activation in order to bind to its high affinity surface receptor. Several mechanisms have been proposed to activate latent TGF-β including, but not limited to, mechanical traction by integrins [15–17], degradation of LLC by proteases [18–21], and release of free cytokine by mechanical shear [22].

The formation of LLC is essential for the control of TGF-β function. This has been shown by a series of studies with knock-in mice in which the cysteine residue in LAP used for binding to LTBP was replaced by a serine residue. These mice (*Tgfb1C33S*) showed severe systemic inflammation, development of gastrointestinal tumors, and decreased TGF-β signaling [23–25]. These phenotypes are similar to those observed with *Tgfb1* null mice and indicate the importance of LLC formation for proper TGF-β1 activity.

LTBP-1 is the most studied isoform in the LTBP family, which is comprised of 4 members, LTBP-1, 2, 3, and 4. *LTBP1* contains two transcription initiation sites controlled by independent promoters, resulting in long and short isoforms (LTBP-1L and LTBP-1S, respectively) [26]. LTBP-1 has an important role in regulating TGF-β availability, as LTBP-1 and 3 readily bind to all three isoforms of latent TGF-β, whereas LTBP-4 inefficiently interacts only with latent TGF-β1 and LTBP-2 does not bind to any latent TGF-β isoform [27]. LTBP-1 is involved not only in activating latent TGF-β, but also in storing the cytokine in the ECM. The C-terminal region of LTBP-1 interacts with fibrillin microfibrils [11]. LTBP-1 also deposits in the ECM in the absence of fibrillin microfibrils via interaction with fibronectin [18,28,29]. LTBP-1 is essential for the activation of latent TGF-β1 via interaction with integrin β6 [15].

The importance of LTBP-1 in vivo has been underscored by a series of studies with *Ltbp1L* knockout mice. Targeted deletion of either exon 1 or 2 led to the same phenotype, developmental cardiovascular abnormalities, such as persistent truncus arteriosus (PTA), interrupted aortic arch (IAA), atrial septal defect (ASD), ventricular septal defect (VSD), and thickening of cardiac valves due to aberrant epithelial–mesenchymal transformation (EMT) [30,31]. *Ltbp1L* null mice showed complete penetrance of PTA and partial penetrance of IAA, which are perinatally lethal after closure of the ductus arteriosus. As the targeted exons in these mice are upstream of the first exon of LTBP-1S, it was predicted that the *Ltbp1L* null mice retained the expression of LTBP-1S, and this was verified by Northern blotting [30].

However, a contradiction that was difficult to explain occurred when the complete knockout mouse of *Ltbp1* in which exon 5, the first exon shared by LTBP-1L and -1S, was targeted in order to abolish expression of both isoforms was reported [32]. These total knockout mice were viable, were fertile, and showed only mild craniofacial abnormalities, but there were no developmental cardiovascular defects including PTA or IAA, as would have been expected with loss of *Ltbp1L*. The reason for the observed discrepancy between the expected phenotype and the observed phenotype of *Ltbp1L* knockout mice and these mice, hereafter designated as *Ltbp1* Δ*Ex5* mice, was unclear.

To resolve this discrepancy, we disrupted the long and short forms of LTBP-1 by targeting exon 8, which is common for both forms and is never spliced out, by utilizing a conditional knockout *Ltbp1* mouse generated by the International Knockout Mouse Consortium (IKMC) and the Cre–loxP system. The new *Ltbp1* complete knockout mice, hereafter designated as *Ltbp1* Δ*Ex8* mice, died perinatally, and showed PTA and IAA, similar to *Ltbp1L* knockout mice. We also reexamined *Ltbp1* Δ*Ex5* mice and found that these mice express a splice variant of LTBP-1 by splicing out exon 5. These results indicate that LTBP-1 is essential in the developing cardiovascular system as was first reported.

Results

Generation of *Ltbp1* Δ*Ex8* mice

The European Conditional Mouse Mutagenesis Program (EUCOMM) Consortium, a member of IKMC, generated a gene-targeted mouse of the *Ltbp1* allele (Fig. 1A) by knocking in an L1L2_Bact_P cassette, which is a promoter-driven selection cassette (Fig. 1B), to produce a knockout-first allele (Fig. 1C). We obtained sperm from these mice, generated animals and crossed them with mice that ubiquitously express flippase to generate a conditional *floxed* allele, in which exon 8 of *Ltbp1* is flanked by loxP sequences (Fig. 1D). These mice were crossed with mice that express Cre recombinase ubiquitously to generate the "Δ*Ex8*" allele, which lacks the 103-base pair long exon 8 (Fig. 1E). The deletion should cause a frameshift mutation and result in the degradation of protein coding transcripts by nonsense-mediated decay (NMD). Heterozygous mice were bred to each other to produce $Ltbp1^{\Delta Ex8/\Delta Ex8}$ mice.

Disruption of exon 8 abrogates expression of *Ltbp1* mRNA and protein

We first performed experiments to verify if the targeting strategy effectively abrogated the expression of both long and short forms of LTBP-1. PCR genotyping of neonatal tissue showed bands of the expected size for the correct targeting (Fig. 2A).