Disruption of LTBP-4 function reduces TGF-β activation and enhances BMP-4 signaling in the lung

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Disruption of latent TGF-β binding protein (LTBP)-4 expression in the mouse leads to abnormal lung development and colorectal cancer. Lung fibroblasts from these mice produced decreased amounts of active TGF-β, whereas secretion of latent TGF-β was significantly increased. Expression and secretion of TGF-β2 and -β3 increased considerably. These results suggested that TGF-β activation but not secretion would be severely impaired in LTBP-4 −/− fibroblasts. Microarrays revealed increased expression of bone morphogenetic protein (BMP)-4 and decreased expression of its inhibitor gremlin. This finding was accompanied by enhanced expression of BMP-4 target genes, inhibitors of differentiation 1 and 2, and increased deposition of fibronectin-rich extracellular matrix. Accordingly, increased expression of BMP-4 and decreased expression of gremlin were observed in mouse lung. Transfection of LTBP-4 rescued the −/− fibroblast phenotype, while LTBP-1 was inefficient. Treatment with active TGF-β1 rescued BMP-4 and gremlin expression to wild-type levels. Our results indicate that the lack of LTBP-4-mediated targeting and activation of TGF-β1 leads to enhanced BMP-4 signaling in mouse lung.

Introduction

The three mammalian TGF-β isoforms regulate a wide variety of cellular processes during embryonic development and in adult organisms (for reviews see Dumont and Arteaga, 2000; Massagué et al., 2000). TGF-β potently inhibit cellular proliferation and regulate cellular differentiation and adhesion and ECM production and degradation. At early stages of cancer development, TGF-βs can act as tumor suppressors, but as tumor cells develop resistance to the growth inhibitory action of TGF-β, they can enable tumor growth (for reviews see Dumont and Arteaga, 2000; Derynck et al., 2001) via modulation of immune functions, angiogenesis, and regulation of the ECM.

High molecular mass latent TGF-β complexes contain the 25-kD mature TGF-β associated noncovalently with its propeptide (latency-associated peptide) and a latent TGF-β binding protein (LTBP; for review see Saharinen et al., 1999). Recent evidence indicates that LTBPs play important roles not only in the secretion of TGF-βs (Miyazono et al., 1991) but also in the targeting of the complexes to specific extracellular sites and in the activation process (for reviews see Taipale et al., 1998; Annes et al., 2003). These complexes are activated through various mechanisms. Thrombospondin- and integrin-mediated mechanisms are probably among the most significant (Crawford et al., 1998; Munger et al., 1999). Activation by proteolytic enzymes also appears to be important, for example, during invasive processes (Mignatti and Rifkin, 1993).

LTBPs, together with fibrillins, constitute a family of extracellular glycoproteins, which are mainly composed of repeated domain structures that are important for the function of LTBPs (for reviews see Oklu and Hesketh, 2000; Koli et al., 2001a). For example, the LTBPs contain EGF-like repeats and 8-Cys repeats, which are involved in protein–protein interactions. Proline-rich regions in the molecule provide flexibility to the protein, are sensitive to proteolytic cleavage, and also contain heparin-binding sequences that may be important for cell recognition and matrix association (Annes et al., 2004).

Three mammalian fibrillins have been identified, of which fibrillins-1 and -2 are major constituents of the 10-nm microfibrils (for review see Ramirez and Pereira, 1999). Of the four LTBPs (Kanzaki et al., 1990; Moren et al., 1994; Saharinen et al., 1998; Penttinen et al., 2002), three associ-
ate with the small latent TGF-β. The third 8-Cys repeats of 
LTBP-1, -3, and -4 are responsible for the covalent association 
with latency-associated peptide (Saharinen and Keski-Oja, 2000).
Although LTBP-1 and -3 bind all three isoforms of TGF-β, 
LTBP-4 associates with TGF-β1 only (Saharinen and Keski-Oja, 2000). 
LTBP-2 does not associate with the small latent 
TGF-β, but has functions in cell adhesion and migration 
(Hyytiäinen and Keski-Oja, 2000). LTBP-2 does not associate with the small latent 
LTBP-4 associates with TGF-β

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**Results**

**Expression and secretion of TGF-β in lung fibroblasts**

To determine if the functional inactivation of LTBP-4 interferes with TGF-β secretion and/or activation, we established lung fibroblast cultures from wt and LTBP-4 −/− mice (Sterner-Kock et al., 2002). As expected, LTBP-4 mRNA was not expressed in the −/− fibroblasts (Fig. 1A). TGF-β activity was quantified from cell-conditioned medium using TGF-β responsive reporter cells that produce luciferase activity in response to TGF-β (Abe et al., 1994). Fibroblast-conditioned medium (harvested at 24 h) was incubated with the reporter cells overnight, after which the luciferase activity was measured. The amount of active TGF-β in the conditioned medium of wt cells was ~50 pg/ml/10⁶ cells, whereas the amount of active TGF-β was down-regulated ~50% in the −/− fibroblasts.
Neutralizing anti–TGF-β antibodies drastically reduced the activity of the medium. These results correlated well with previous in vivo findings, which indicated significantly less TGF-β signaling in the lungs of the −/− mice (Sterner-Kock et al., 2002) and analyzed with the reporter cell system. In contrast to the reduced levels of active TGF-β, the secretion of latent TGF-βs was prominently increased in the −/− fibroblasts (Fig. 1 B). This indicated that the reduced TGF-β activity cannot be attributed to decreased secretion. The results imply that the TGF-β activation process is significantly impaired in LTBP-4 −/− fibroblasts, probably due to perturbed targeting.

Because the −/− fibroblasts secreted increased amounts of latent TGF-β, we analyzed the expression and secretion of all three isoforms of TGF-β. Northern analyses of mRNA expression indicated that TGF-β1 levels were not significantly altered, whereas TGF-β2 and -β3 levels were highly up-regulated in −/− fibroblasts (Fig. 1 C and see Fig. 4 C). In accordance with mRNA data, their protein levels were clearly up-regulated (Fig. 1 D), whereas the total levels of TGF-β1 did not change appreciably. Although the mechanism of this induction is unclear, one possible explanation is that low levels of TGF-β1 secretion induced up-regulation of the other isoforms. Importantly, TGF-β1 is a potent repressor of TGF-β2 and -β3 expression (Bascom et al., 1989).

LTBP-4 −/− fibroblasts produce increased amounts of ECM

TGF-β1 is a potent regulator of ECM production and degradation that generally causes an accumulation of ECM. Therefore, we analyzed whether or not the lack of LTBP-4–mediated TGF-β1 targeting would reduce ECM production. Cells were metabolically labeled for 2 d, after which extracellular matrices were prepared and analyzed by SDS-PAGE. Unexpectedly, the amount of sodium deoxycholate–resistant matrix was considerably higher in −/− fibroblasts (Fig. 2 A), and the amount of the 250-kD fibronectin polypeptide was particularly increased. Immunofluorescence analyses of 5-d-old fibroblast cultures indicated that in wt cells, fibronectin was localized into fibrillar structures in the matrices, whereas in −/− cells fibronectin was more abundant and formed a uniform sheetlike structure around the cells (Fig. 2 B). Because the altered ECM structure may change the sodium deoxycholate solubility of the ECM,
associated fibronectin, the massive increase seen in Fig. 2 A is plausibly due to both increased protein deposition and decreased solubility. Neither fibronectin mRNA levels in vitro (Fig. 2 D) or in vivo (not depicted) nor secreted protein levels in −/− fibroblasts (not depicted) were up-regulated, suggesting increased deposition and/or decreased turnover. The cell surface expression levels of the fibronectin receptor α5β1 integrin were not changed in −/− fibroblasts (unpublished data). Accordingly, immunohistochemical analysis of lung tissues indicated that fibronectin levels were highly up-regulated in the −/− mice (Fig. 2 C). LTBP-4 binds to fibrillin-1 (Isogai et al., 2003) and loss of fibrillin fibers in mice leads to changes in the levels of active TGF-β (Neptune et al., 2003). Therefore, fibrillin-1 distribution was evaluated, but no significant changes were observed (Fig. 2 C).

We examined whether or not the expression of certain ECM regulatory proteins other than TGF-β would be altered in −/− fibroblasts. These proteins included connective tissue growth factor (CTGF), plasminogen activator inhibitor-1 (PAI-1), and matrix metalloproteinase (MMP)-2. CTGF belongs to the CCN (CTGF, cyr61, nov) family of immediate early genes (Leask and Abraham, 2003) and its expression is induced in several fibrotic diseases. Our Northern hybridization analyses indicated that CTGF mRNA levels were low in wnt fibroblasts treated with TGF-β/H9252 (Fig. 2 D) and protein levels (not depicted) were observed (Fig. 2 C). ECM regulatory proteins other than TGF-β were observed (Fig. 2 C). MMP-2 is also regulated by TGF-β. MMP-2 levels were down-regulated below detection limit in −/− fibroblasts.

Inhibition of TGF-β signaling does not reverse the −/− fibroblast phenotype

Although the amount of active TGF-β in the conditioned medium was decreased, the −/− fibroblasts exhibited a phenotype similar to TGF-β–treated wt cells. TGF-β induces the production of ECM and enhances the expression of PAI-1 and CTGF in wt cells (unpublished data). The enhanced secretion of latent forms of TGF-β led us to speculate whether or not TGF-β might be activated by a distinct mechanism. For example, direct activation of TGF-β and subsequent binding to cell surface receptors may occur without release into the medium. To address this possibility, TGF-β signaling was inhibited by stable expression of a dominant-negative TGF-β type II receptor (ΔRII) in −/− fibroblasts (see Materials and methods). This form of the receptor lacks the kinase domain–containing cytoplasmic tail, and is therefore unable to transduce TGF-β signals (Wieser et al., 1993). To verify that the transgene prevented TGF-β signaling, the cells were transiently transfected with a TGF-β responsive promoter ((CAGA)12-luc; Dennler et al., 1998), followed by overnight treatment with TGF-β1. In −/− fibroblasts, TGF-β1 (100 pg/ml) induced the activity of (CAGA)12-luc promoter by ∼30-fold, whereas in cells expressing ΔRII the response was almost completely lost (Fig. 3 A). Analyses of ECM production revealed that the inhibition of TGF-β signaling in −/− fibroblasts did not lead to reversal of the phenotype (Fig. 3 B and see Fig. 7), which suggested that enhanced TGF-β activity is not involved in the induction of this phenotype.

The −/− phenotype is LTBP-4 dependent

The phenotype observed in −/− fibroblasts results from multiple alterations in their gene expression pattern (Fig. 1, Fig. 2, and Table I). To determine whether or not these changes are truly LTBP-4 dependent, we introduced LTBP-4 expression into the −/− fibroblasts by stable transfection of hLTBP-4S/pEF-IRES expression vector (see Materials and methods). Immunoblotting analyses of LTBP-4 from concentrated cell-conditioned medium indicated that the transgene was expressed and the protein secreted by the cells (Fig. 4 A, top). Analyses of ECM production indicated that the LTBP-4–expressing −/− cells accumulated ECM components at levels almost equivalent to wt (Fig. 4 B). As a result of this LTBP-4 expression, the mRNA levels of CTGF, PAI-1, TGF-β2, and TGF-β3 were reduced to wt levels or even lower (Fig. 4 C). These results suggest that the accumulation of ECM and the specific changes in gene expression levels could be reversed by restoring LTBP-4 expression.

Next, we tested if stable expression of LTBP-1 could substitute for LTBP-4 in the reversal of the phenotype in −/− fibroblasts. Detectable levels of LTBP-1 were expressed in −/−...
fibroblasts, and highly enhanced levels were obtained by transgene expression (Fig. 4 A, bottom). Analyses of ECM production indicated that the overexpression of LTBP-1 did not decrease ECM production and thus was unable to compensate for the loss of LTBP-4 in −/− fibroblasts (Fig. 4 B). This finding suggests an isoform-specific function for LTBP-4 in lung fibroblasts.

Gene expression profiling of fibroblasts
To identify candidate genes involved in the development of the −/− phenotype, we analyzed differentially expressed genes by oligonucleotide microarrays for over 12,000 mouse genes comparing RNA from −/− and wt lung fibroblasts (see Materials and methods). A total of 119 genes or ESTs were either up- or down-regulated greater than threefold in −/− lung fibroblasts (see Online supplemental material, available at http://www.jcb.org/cgi/content/full/jcb.200403067/DC1). The expression of LTBP-4, which was lost, served as an internal control for the analyses. LTBP-1 and -3 were expressed at comparable levels in wt and −/− fibroblasts, whereas LTBP-2 expression levels were slightly enhanced in −/− fibroblasts (unpublished data). Special attention was paid to genes regulated greater than fivefold, genes known to be involved in the TGF-β response pathway, genes involved in the regulation of the ECM, and genes of growth factors or their receptors (Table I). The expression of FGF-7 was found to be down-regulated. Although FGF-7 plays a role in lung development, knockout animals have no obvious defects in the lung (Guo et al., 1996). In addition, several ECM components or proteases were either up- or down-regulated in LTBP-4 −/− cells, probably contributing to the enhanced matrix phenotype. One of the most prominent changes was down-regulation of the expression of thrombospondin-2, which is a matricellular protein involved in the regulation of cell–matrix interactions (for review see Bornstein, 2001).

A prominent observation in the −/− fibroblasts was a fivefold increase in the expression of BMP-4, in parallel with almost complete loss of expression of the BMP inhibitor gremlin. Northern analyses confirmed these observations (Fig. 5 A). Immunoblotting of cell-conditioned medium revealed increased levels of BMP-4 protein in the medium of −/− fibroblasts (Fig. 5 B). In addition, the BMP-4 target gene inhibitor of differentiation (Id)2 was induced in the gene array. Northern analyses of Id2 as well as Id1 indicated 5–10-fold increases in expression levels (Fig. 5 A). The expression levels of the BMP-4 receptors BMPR-IA (ALK-3), BMPR-IB (ALK-6), and BMPR-II were comparable in wt and −/− fibroblasts (gene array analyses; unpublished data). These results suggest that BMP-4 signaling is considerably increased in LTBP-4 −/− fibroblasts.

Table I. A panel of selected genes expressed differentially in LTBP-4 −/− and control wt fibroblasts

| Gene                  | Accession | Fold change |
|-----------------------|-----------|-------------|
| Genes up-regulated in −/− fibroblasts |           |             |
| Id2                   | AF077861  | 8           |
| BMP-4                 | L47480    | 5           |
| IGFBP-6               | X81584    | 6           |
| Genes down-regulated in −/− fibroblasts |           |             |
| p21                   | U09507    | >10         |
| c-Fos                 | V00727    | 6           |
| Mdm2                  | AI853375  | 6           |
| IL-6                  | X54542    | 5           |
| Laminin-2 a2 chain    | U12147    | 6           |
| PDGF-Rβ               | X04367    | 5           |
| IGF-II                | X71922    | >10         |
| Thrombospondin-2      | I07803    | >10         |
| Gremlin               | AF045801  | >10         |
| ADAM-19 (meltrin β)   | AA726223  | >10         |
| FGF-7                 | Z22703    | 5           |
| CCN-3 (Nov)           | Y09257    | >10         |

The GenBank accession numbers and change in expression levels (fold change) as coupled by Genespring software array are indicated. 
*BMP pathway genes.
fibroblasts efficiently inhibited the induction of (BRE)2-luc expression levels of both CTGF and TGF-
Cumulation by gremlin expression caused a significant decrease in matrix accumulation by
especially increased, from undetectable to high levels, which probably controls BMP-4 signaling levels. As expected, ECM production was significantly decreased in −/− fibroblasts after restoration of TGF-β1 activity (Fig. 9 B).

Discussion
In our previous paper, we observed that LTBP-4 −/− mice expressing only trace amounts of LTBP-4 protein develop severe pulmonary aplasia/emphysema (Sterner-Kock et al., 2002). The condition was already present at birth and deteriorated over time. Histologically, the alveolar spaces were enlarged, inflated, and significantly reduced in number. Immunohistochemical analyses revealed less active TGF-β1 in the lung tissues of −/− mice, leading to reduced levels of Smad2 phosphorylation. As Smad2 is a direct target of TGF-β receptor activation, this finding indicates that a lack of LTBP-4 severely affects the TGF-β signaling pathway. To understand the ob-
served changes in the −/− phenotype, we have isolated lung fibroblasts from wt and LTBP-4 −/− mice. We found that the −/− fibroblasts produced increased amounts of fibronectin-rich ECM, and the expression of numerous genes involved in the regulation of matrix turnover was altered. The correlation of the in vitro findings in cultured fibroblasts with the situation in mouse lungs in vivo was evaluated by immunohistochemical analyses of lung tissues. A clear increase in the expression of fibronectin was observed in lung tissues from −/− mice, which corresponds with our observations with cultured cells. The excessive accumulation of fibronectin and the gene expression profile of the −/− fibroblasts were very similar in TGF-β–treated wt cells. However, neutralization of TGF-β signaling did not rescue the phenotype and ruled out the involvement of enhanced levels of TGF-β in the induction of this phenotype. In accordance with our previous in vivo findings (Sterner-Kock et al., 2002), we observed that the amount of active TGF-β was significantly decreased in the −/− fibroblasts. Interestingly, the −/− fibroblasts produced increased amounts of latent forms of TGF-β. The expression and secretion of TGF-β2 and −β3 increased considerably in −/− fibroblasts, but the activation of TGF-β complexes was compromised. TGF-β1 down-regulates the expression of TGF-β2 and −β3 (Bascom et al., 1989), and the lack of LTBP-4–mediated activation of TGF-β1 might contribute to the observed increase in

Figure 6. Decreased levels of gremlin and increased levels of BMP-4 and phosphorylated Smad1 in −/− lung tissue. Lung sections from wt (+/+) and LTBP-4 −/− mice were stained for BMP-4, gremlin, phosphorylated Smad1, and total Smad1. Positive staining is reddish-brown.

Figure 7. Inhibition of BMP-4 signaling pathway partially rescues the LTBP-4 hypomorphic (−/−) fibroblast phenotype. (A) −/− lung fibroblasts expressing mouse gremlin (+gremlin) were transiently transfected with (BRE)2-luc promoter, treated with indicated concentrations of BMP-4, and analyzed for luciferase activity as in Fig. 3 (see Materials and methods). The results are expressed as relative luciferase activities (untreated −/− fibroblast samples equal one). (B) Lung fibroblasts were cultured in the presence of labeled amino acids for 2 d followed by isolation of the ECM. Polypeptides of the ECM preparations were separated by SDS-PAGE under reducing conditions and visualized by fluorography. The migration of the molecular mass markers (kD) is indicated on the left. (C) Total RNA was isolated from cultured lung fibroblasts, and mRNA expression levels of CTGF, PAI-1, TGF-β2, and TGF-β3 were analyzed by Northern blotting. mRNA expression of a constant gene, GADPH, was used to control loading.
expression of LTBP-1 in fibroblasts. Because both LTBP-1 and -3 are expressed in the ECM. Polypeptides of the ECM preparations were separated by SDS-PAGE under reducing conditions and visualized by fluorography. The migration of the molecular mass markers (kD) is indicated on the left. (B) Total RNA was isolated from cultured lung fibroblasts, and mRNA expression levels of CTGF, PAI-1, TGF-β2 and TGF-β3 were analyzed by Northern blotting. mRNA expression of a constant gene, GADPH, was used to control loading.

the levels of the other isoforms. Interestingly, BMP-4 regulated TGF-β3 levels in wt fibroblasts, so increased secretion of BMP-4 may also regulate TGF-β3 mRNA expression in −/− fibroblasts. Because both LTBP-1 and -3 are expressed in the mouse lung (Yin et al., 1995; Noguera et al., 2003), they may compensate for the loss of LTBP-4 in the secretion of the small latent complexes. However, the pericellular locations and activation mechanisms of various complexes are likely to be different. This finding is supported by the observation that overexpression of LTBP-1 in −/− fibroblasts could not substitute for the loss of LTBP-4 in the reversal of the phenotype. Similarly, isoform-specific function of LTBP-1 in TGF-β1 activation cannot be substituted by LTBP-3 in αvβ6 integrin–mediated activation model (Annes et al., 2004).

The microarray analyses revealed that BMP-4 expression was induced in −/− fibroblasts in parallel with the loss of expression of its inhibitor gremlin. Activation of the BMP pathway was also reflected by the up-regulation of BMP-4 target genes Id1 and -2. In addition, CTGF, which was highly up-regulated in −/− fibroblasts, is a direct target of both BMP-4 and TGF-β1 (Abreu et al., 2002). Inhibition of the BMP-4 pathway partially rescued the −/− phenotype, further indicating the importance of this signaling cascade for the development of the LTBP-4 −/− phenotype. The observations were further supported by in vivo data because immunohistochemical staining of −/− mouse lung tissue revealed increased BMP-4 levels in parallel with decreased gremlin levels and increased phosphorylation of the BMP-specific Smad protein.

Several genes important for the development of the lung were dysregulated in LTBP-4 −/− fibroblasts. FGF-7, BMP-4, and TGF-β1–3 have specific expression patterns in the developing lung (for review see Warburton et al., 2000). FGF-7 promotes epithelial proliferation and functions as a differentiation factor for the developing lung. However, FGF-7 null animals do not show any detectable lung phenotype (Guo et al., 1996), which may be due to overlapping functions of lung morphogens. BMP-4 is an important lung morphogen and its overexpression under the surfactant protein promoter in the lung could severely perturb lung development (Bellusci et al., 1996). The correct targeting and expression levels of these factors are crucial for lung morphogenesis. The up-regulation of TGF-β activity in fibrillin-1–deficient mice led to emphysema, which was rescued by inhibition of TGF-β1 and -β2 activities with neutralizing antibodies (Neptune et al., 2003). Interestingly, the down-regulation of TGF-β activation in LTBP-4 −/− and β6 integrin null mice could also lead to emphysema–like conditions in the lung (Sterner-Kock et al., 2002; Morris et al., 2003). Although the mechanisms differ, the correct balance of growth factor activation appears crucial for the development of the lung.

TGF-β regulates the expression of gremlin in mesangial cells (McMahon et al., 2000). Gremlin expression levels are drastically down-regulated in −/− fibroblasts, which probably allows BMP-4 signaling and accumulation of the fibronectin-rich ECM. Restoring wt levels of TGF-β activity to −/− fibroblasts reverts gremlin expression and reduces ECM accumulation, suggesting that TGF-β is a strong regulator of gremlin expression and BMP-4 signaling also in lung fibroblasts. LTBP-4 affects the targeting and activation of TGF-β1 in an isoform-specific manner. Although the accumulation of matrix com-

![Figure 8](image_url) Figure 8. BMP-4 induces matrix production in wt fibroblasts. [A] wt fibroblasts (+/+) were cultured in the presence of the indicated concentrations of BMP-4 and labeled amino acids for 2 d, followed by isolation of the ECM. Polypeptides of the ECM preparations were separated by SDS-PAGE under reducing conditions and visualized by fluorography. The migration of the molecular mass markers (kD) is indicated on the left. [B] Total RNA was isolated from cultured lung fibroblasts, and mRNA expression levels of CTGF, PAI-1, TGF-β2 and TGF-β3 were analyzed by Northern blotting. mRNA expression of a constant gene, GADPH, was used to control loading.

![Figure 9](image_url) Figure 9. TGF-β1 stimulation restores BMP-4 and gremlin expression to wt levels. Lung −/− fibroblasts were cultured in the presence of 0.5 ng/ml TGF-β1 for 2 wk (+TGF-β1). [A] Total RNA was isolated from cultured lung fibroblasts, and mRNA expression levels of gremlin and BMP-4 were analyzed by Northern blotting. mRNA expression of a constant gene, GADPH, was used to control loading. (B) Lung fibroblasts were cultured in the presence of labeled amino acids for 2 d followed by isolation of the ECM. Polypeptides of the ECM preparations were separated by SDS-PAGE under reducing conditions and visualized by fluorography. The migration of the molecular mass markers (kD) is indicated on the left.
ponents is reduced in TGF-β1−/− mouse embryo fibroblasts, mRNA levels remain unchanged for TGF-β2 and -β3 (Sudarshan et al., 1998). These differences may reflect the differentiation status of the cells. A contribution of LTBP functions that are independent of TGF-β in the induction of the −/− phenotype cannot be ruled out. Our findings suggest that TGF-β1 targeting as well as LTBP-4–dependent modulation of growth factor pathways are important for the development of the lung. Because BMP-4 is essential for pulmonary morphogenesis, and its transgenic overexpression produces abnormal lungs (Beluscι et al., 1996), it is likely that the pulmonary aplasia/embryosoma in LTBP-4−/− mice results at least in part from the switch from TGF-β to BMP-4 signaling.

Materials and methods

Antibodies and growth factors

Rabbit antibodies against human TGF-β1 and human LTBP-4 have been described previously (Taipale et al., 1992; Saharinen et al., 1998). TGF-β2, TGF-β3, fibrillin-1 (H-109), Smad1 (A-4), and BMP-4 (H22.3) antibodies were obtained from Santa Cruz Biotechnology, Inc. BMP-4 antibody used in immunohistochemistry was obtained from R&D Systems (AF355). Antibodies used for immunoblotting and immunohistochemistry were purchased from Sigma-Aldrich (FN-15) and Santa Cruz Biotechnology, Inc. (N-20), respectively. P-Smad1 antibody (Persson et al., 1998) was provided by P. ten Dijke (The Netherlands Cancer Institute, Amsterdam, Netherlands). Rabbit antibodies against human LTBP-1 (Ab39) were provided by C.-H. Heldin (Ludwig Institute for Cancer Research, Uppsala, Sweden). Recombinant BMP-4 was obtained from R&D Systems, and TGF-β1 was obtained from Oncogene Research Products.

cDNA constructs

Full-length human LTBP-4S (Saharinen et al., 1998) was cloned into pEF-IRESP vector (Hobb’s et al., 1998) as an XhoI–EcoRI fragment generating a construct hLTBP-4S/pEF-IREP. The dominant-negative TGF-β1 receptor (ΔR1) cDNA insert was removed from kdTβR1 (construct was provided by M.G. Brattain, Roswell Park Cancer Institute, Buffalo, NY; Ko et al., 1998) as an EcoRI–XbaI fragment and cloned into pEF-IRESP generating a ΔR1/pEF-IREP construct. mGremlin/pEF-IREP was constructed by RT-PCR amplification of the coding sequence of mouse gremlin followed by cloning into pEF-IREP as an XhoI–EcoRI fragment. The primer sequences used for the amplification were 5′-CCCGTCCGAGATAGCTCCGCGGA-3′ and 5′-GGAATTCCTGACGGCTGATGTCG-3′. Construct pLTBP-1 has been described previously (Saharinen and Keski-Oja, 2000).

Cell culture and transfections

Lung fibroblast cultures from adult wt and LTBP-4−/− mice were established according to standard protocols. In brief, the lung tissues were finely chopped, and primary explant cultures were allowed to attach and produce outgrowth for 10 d. This was followed by trypsinization of the cells, removal of the primary explants, and culturing the cells as adherent monolayer cultures until confluency. During the following 10–12 wk in culture the cells to be transiently transfected were plated in 6-well plates. The transfection was performed the next day using 3 μg of plasmid DNA and 2 μl of FuGENE 6 transfection reagent (Roche) according to the manufacturer’s instructions. After transfection, the cells were washed and incubated overnight with serum containing growth medium. The antibiotic selection was started a day after transfection with 2 μg/ml of puromycin (Sigma-Aldrich). A gradual increase of puromycin concentration was performed if higher expression levels were needed.

Cells to be transiently transfected were plated in 6-well plates. The transfection was performed the next day using 3 μg of plasmid DNA and 2 μl of FuGENE 6 transfection reagent (Roche). After 24 h, the cells were changed to serum-free medium, and TGF-β1 or BMP-4 were added at the indicated concentrations. Subsequently, 48 h after transfection, the cells were lysed and subjected to luciferase activity measurements by a Dual Luciferase Kit (Promega) and Dignee DCR-1 luminometer (MGM Instruments).

TGF-β activity assay

Mink lung epithelial cells stably transfected with a fragment of PAI-1 promoter fused to luciferase gene (TMLC) were provided by D.B. Rifkin (New York University School of Medicine, New York, NY). These cells produce luciferase in response to TGF-β. TGF-β standards and medium samples to be assayed for TGF-β activity were analyzed as described previously (Abe et al., 1994). The assays were performed at least three times. The results have been presented as relative value of TGF-β activity (wt medium set as one). The specificity was controlled by adding neutralizing pan-specific TGF-β antibodies where indicated (AB-100-NA; R&D Systems).

Isolation of extracellular matrices

Cells were cultured in the presence of 35S-labeled amino acids (EasyTag Express−[35]S; NEN Life Sciences Products) for 2 d. Extracellular matrices were prepared as described previously (Hedman et al., 1997; Taipale et al., 1992). In brief, cell cultures were washed once with PBS, and then treated three times with 0.5% sodium deoxycholate in 10 mM Tris-HCl buffer, pH 8.0, at 0°C for 10 min. The plates were then washed again with PBS, allowed briefly to dry, and the ECM samples were collected by extraction with Laemmli sample buffer and heat treated at 95°C for 5 min. Aliquots of matrix samples were separated by SDS-PAGE and the radiolabeled proteins were visualized by fluorography.

SDS-PAGE and immunoblotting

SDS-PAGE was performed using 7.5% or 4–15% gradient Tris-HCl polyacrylamide gels (Bio-Rad Laboratories). Conditioned cell culture medium, equalized according to cell number, was concentrated 10-fold using Microcon YM-10 or YM-30 centrifugal filters (Millipore) where indicated. Electrophoretically separated proteins were transferred to nitrocellulose membranes using semidry blotting system (Bio-Rad Laboratories). Immunodetection was performed as described previously (Penttinen et al., 2002).

Immunofluorescence analysis and microscopy

Cells were grown on glass coverslips for 5 d. Coverslips were washed three times with PBS and the cells fixed in ice-cold methanol at −20°C for 15 min. After washing three times with PBS, the cells were incubated in Dulbecco’s PBS containing 3% BSA to prevent nonspecific binding of the antibodies. The cells were incubated with the primary antibody in Dulbecco’s PBS for 1 h. The binding of the antibodies was detected using FITC-conjugated secondary antibodies (Jackson Immunoresearch Laboratories). The coverslips were finally washed in water, mounted on glass slides using Vectashield anti-fading reagent (Vector Laboratories), and examined under the imaging microscope (model Axioplan 2; Carl Zeiss Mikromaging, Inc.) using a 40× objective. Images were acquired with a camera (model AxioCamHRc; Carl Zeiss Mikromaging, Inc.) and software (AxioVision 3.1; Carl Zeiss Mikromaging, Inc.).

Immunohistochemistry

Paraffin sections of mouse tissues harvested at the age of 6 wk were prepared and stained using standard histology procedures. For immunostainings (immunoperoxidase), the deparaffinized and rehydrated tissue slices were first treated for 30 min with 3% H2O2 to inactivate the endogenous peroxidases. After rinsing in ddH2O and soaking in PBS for 5–10 min, the slices were treated with 10% (wt/vol) BSA in PBS to saturate nonspecific protein binding sites. The slides were exposed to the specific antibodies at 4°C overnight. After removing excess antibody, the slices were treated with appropriate biotin-labeled secondary antibodies (Dianova) at 37°C for 40 min, and finally with HRP-labeled streptavidin (Zymed Laboratories) at 37°C for 20 min. After washing, the slices were incubated in diaminobenzidine (Sigma-Aldrich) at RT for 5 min. The slides were finally examined under a conventional microscope (Carl Zeiss Mikromaging, Inc.) after removing the excess substrate in ddH2O.

RNA isolation and RT-PCR

Total cellular RNA was isolated using RNeasy Mini kit (QiAGEN) according to manufacturer’s instructions. RNA concentration and purities were determined spectrophotometrmetrically (Ultrospec 3000; Amersham Biosciences) as well as by agarose gel electrophoresis followed by ethidium bromide staining.

Reverse transcription was performed with Random primers (Life Technologies) and Superscript II reverse transcriptase (Life Technologies)
using 2.5 μg of total RNA according to manufacturer’s instructions. The cDNAs were amplified using AmpliTaq Gold [PerkinElmer] in a 30-cycle PCR reaction as described previously [Koli et al., 2001b]. The primer sequences used for the amplification of the LTBP-4 fragment were 5′-CCCGTCGCTTATAACATG-3′ and 5′-AGGAAACCCTCCGGAC-3′. The amplified products were analyzed by agarose gel electrophoresis.

Northern hybridization analyses
10 μg of total cellular RNA was fractionated on 1.2% agarose gels containing formaldehyde and transferred to Hybond-N nylon membranes (Amersham Biosciences) by capillary transfer. Prehybridization and hybridization were performed at 68°C in ExpressHyb hybridization solution (CLONTECH Laboratories, Inc.). cDNA probes for genes of interest and a control gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were labeled with [α-32P]dCTP (&gt;3,000 Ci/mmol; Amersham Biosciences) using a Ready-To-Go DNA Labeling Kit [Amersham Biosciences]. Radioactivity levels were quantified with a bio-imaging analyzer [model BAS-2500; Fuji] where indicated, and the results expressed as mRNA expression levels relative to the GAPDH levels.

Oligonucleotide microarray analyses
Total RNA was extracted using TRIzol (Invitrogen) according to the manufacturer’s instructions. To assure that the gene expression measured by microarray assay was not affected by degradation of the RNA extracted from the lung fibroblast cells, we used the Bioanalyzer-system (Agilent Technologies, Inc.). cDNA probes for genes of interest and a control gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were labeled with [α-32P]dCTP (&gt;3,000 Ci/mmol; Amersham Biosciences) using a Ready-To-Go DNA Labeling Kit [Amersham Biosciences]. Radioactivity levels were quantified with a bio-imaging analyzer [model BAS-2500; Fuji] where indicated, and the results expressed as mRNA expression levels relative to the GAPDH levels.

Online supplemental material
The complete data from the gene array analyses (see Gene array analyses) is available at http://www.jcb.org/cgi/content/full/jcb.200303067/DC1.

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