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# Elevated formation of pyridinoline cross-links by profibrotic cytokines is associated with enhanced lysyl hydroxylase 2b levels

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**Abstract**

The hallmark of fibrosis is the excessive accumulation of collagen. The deposited collagen contains increased pyridinoline cross-link levels due to an overhydroxylation of lysine residues within the collagen telopeptides. Lysyl hydroxylase 2b (LH2b) is the only lysyl hydroxylase consistently up-regulated in several forms of fibrosis, suggesting that an enhanced LH2b level is responsible for the overhydroxylation of collagen telopeptides. The present paper reports the effect of profibrotic cytokines on the expression of collagen, lysyl hydroxylases and lysyl oxidase in normal human skin fibroblasts, as well as the effect on pyridinoline formation in the deposited matrix. All three isoforms of TGF-β induce a substantial increase in LH2b mRNA levels, also when expressed relatively to the mRNA levels of collagen type I α2 (COL1A2). The TGF-β isoforms also clearly influence the collagen cross-linking pathway, since higher levels of pyridinoline cross-links were measured. Similar stimulatory effects on LH2b/COL1A2 mRNA expression and pyridinoline formation were observed for IL-4, activin A, and TNF-α. An exception was BMP-2, which has no effect on LH2b/COL1A2 mRNA levels nor on pyridinoline formation. Our data show for the first time that two processes, i.e., up-regulation of LH2b mRNA levels and increased formation of pyridinoline cross-links, previously recognized to be inherent to fibrotic processes, are induced by various profibrotic cytokines.


*Keywords:* Fibrosis; Lysyl hydroxylase 2b; Pyridinoline; Cytokine; TGF-β; Collagen

## 1. Introduction

Fibrosis is a complex process finally resulting in the excessive deposition of extracellular matrix, which is mainly composed of collagen. The collagen deposited in the fibrotic lesions contains highly increased pyridinoline cross-link levels [1–6]. Pyridinolines are trifunctional cross-links, derived from the hydroxyallysine cross-link route. In this pathway, the hydroxylated lysine residue within the telopeptide is converted to a hydroxyallysine ($Hyl^{ald}$), a reaction catalyzed by lysyl oxidase (LOX). Subsequently, the $Hyl^{ald}$ reacts with a lysine (Lys) or hydroxylysine (Hyl) residue within the triple helix to form di- and tri-functional cross-links [7–10].

The conversion of Lys into a Hyl in the collagen molecule is catalyzed by lysyl hydroxylases (LH; EC1.14.11.4). Three different lysyl hydroxylases have been described: LH1, LH2 and LH3 [11–15]. Only for LH2 splice variants have been described (LH2a and LH2b) [16,17]. Mutations in the gene encoding LH1 (Ehlers–Danlos syndrome type VI) [18] and LH2 (Bruck syndrome) [19] revealed that LH1 predominantly catalyzes the hydroxylation of lysine residues within the triple helix, whereas LH2 predominantly catalyzes the formation of Hyl in the

*Abbreviations:* B2M, β2-microglobulin; BMP-2, bone morphogenetic protein-2; COL1A2, collagen type I α2; ET-1, endothelin-1; $Hyl^{ald}$, hydroxyallysine; Hyl, hydroxylysine; HP, hydroxylysylpyridinoline; IL-4, interleukin 4; LH, lysyl hydroxylase; LP, lysylpyridinoline; LOX, lysyl oxidase; Lys, lysine; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α

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telopeptides. Furthermore, compelling evidence that LH2 has telopeptide lysyl hydroxylase activity has been shown along various research lines [20–22]. The lysine substrate specificity (telopeptides and/or triple helix) of LH3 is unknown. Interestingly, LH3 has been found to be a multifunctional enzyme as it is able to catalyze the lysyl hydroxylation reaction as well as the sugar transfer reactions, enabling consecutive steps in the formation of glucosylgalactosylhydroxylysyl residues [23,24].

The increased amount of pyridinoline cross-links in fibrosis is the result of an overhydroxylation of the lysine residues within the telopeptides. We have recently found that LH2b is the only lysyl hydroxylase that is consistently up-regulated in several forms of fibrosis [19,25]. This suggests that increased LH2b expression is responsible for the overhydroxylation and the concomitant formation of pyridinoline cross-links in several forms of fibrosis.

The fibrotic process involves an inflammatory response, and cytokines and growth factors constitute a major class of mediators in this process. Transforming growth factor-β (TGF-β) has been considered to be one of the key mediators in fibrosis. Increased amounts of TGF-β have consistently been found in fibrotic lesions [26–28]. Administration of exogenous TGF-β to laboratory animals leads to the development of fibrosis [29,30], whereas administration of anti-TGF-β inhibits experimentally induced fibrosis [31–34]. TGF-β exists in three isoforms, namely TGF-β1, TGF-β2 and TGF-β3 [35]. Although the biological activities of the three isoforms are highly similar in vitro, their difference in function in vivo is shown by the unique isoform-specific phenotypes displayed by the different TGF-β knock-out mice [36–38].

Fibroblasts stimulated with TGF-β show increased expression levels of various extracellular matrix proteins, including collagen. Furthermore, they secrete reduced amounts of several matrix metalloproteinases, whereas the production of tissue inhibitors of metalloproteinases is increased (reviewed in Ref. [26]). These changes are characteristic for fibrotic cells. Another general process in fibrosis is the recently observed increased expression of LH2b and the formation of pyridinoline cross-links [25]. This prompted us to investigate whether TGF-β is also able to induce LH2b expression and pyridinoline cross-link formation. Besides TGF-β, several other cytokines, including interleukin 4 (IL-4), bone morphogenetic protein-2 (BMP-2), activin A, and tumor necrosis factor-α (TNF-α), can be indicated as profibrotic [39,40]. For example, overexpression of activin A in the skin of transgenic mice induces fibrosis [41], BMP-2 induces scar formation [42], and anti-IL-4 or anti-TNF-α treatment prevents collagen deposition in animal models of fibrosis [43,44]. So far, the effect of these cytokines on pyridinoline cross-link levels is unknown. Therefore, we examined whether these cytokines affect LH2b expression and pyridinoline cross-link formation as well.

We found that LH2b mRNA levels in human primary fibroblasts are induced by all three TGF-β isoforms. Furthermore, the TGF-β isoforms have considerable effects on the cross-linking as the stimulated cells deposit collagen with significantly increased pyridinoline levels. These stimulatory effects on LH2b mRNA levels and pyridinoline cross-linking of the collagen were also found for the cytokines IL-4, activin A, and TNF-α. The observed effects of these profibrotic cytokines are in perfect agreement with our previous studies in which we showed that increased LH2b mRNA levels in fibrotic cells are associated with elevated pyridinoline cross-linking in the deposited matrices [19,25] and indicate that these processes play an important role in fibrosis.

## 2. Materials and methods

### 2.1. Cytokine stimulation experiments

Primary cultured human fibroblasts, obtained from the split skin of seven healthy individuals, were established by routine methods. Cells were grown in 10-$cm^2$ wells in Dulbecco's modified Eagle's medium (DMEM; BioWhittaker) supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin and 100 U/ml streptomycin at 37 °C in 5% $CO_2$. Fibroblasts that reached subconfluence were used for stimulation experiments. Before stimulation, cells were washed in PBS and starved for 3 h in DMEM supplemented with 0.1% bovine serum albumin (BSA), 100 U/ml penicillin and 100 U/ml streptomycin. Dose- and time-dependent effects of TGF-β1, -β2 or -β3 on mRNA expression levels were studied by culturing fibroblasts of one donor in the presence or absence of different concentrations of recombinant human TGF-β1, -β2 or -β3 (Sigma) for 24 h and by culturing cells of one donor in the presence or absence of 1 ng/ml TGF-β1, -β2 or -β3 for 6, 12, 24, 48 or 72 h. Furthermore, fibroblasts of seven donors were cultured in DMEM supplemented with 0.1% BSA, 100 U/ml penicillin and 100 U/ml streptomycin in the presence or absence of 1 ng/ml TGF-β1, -β2 or -β3 for 24 h. Fibroblasts of four donors were stimulated with or without 10 ng/ml human recombinant IL-4 (R&D Systems), 10 ng/ml human recombinant BMP-2 (Sigma), 5 ng/ml human recombinant activin A (Sigma), or 10 ng/ml human recombinant TNF-α (R&D Systems) in DMEM supplemented with 0.1% BSA, 100 U/ml penicillin and 100 U/ml streptomycin for 24 h. The above-mentioned cultures were used for total RNA isolation.

For extracellular matrix analysis, fibroblasts were cultured for 4 weeks in DMEM supplemented with 5% FCS, 100 U/ml penicillin, 100 U/ml streptomycin and 50 μg/ml ascorbic acid in the presence or absence of 1 ng/ml TGF-β1, -β2 or -β3 ($n$=7) or in the presence or absence of 10 ng/ml IL-4, 10 ng/ml BMP-2, 5 ng/ml activin A, or 10 ng/ml TNF-α. Medium was replenished every 3 days. After 4 weeks of culture, the deposited matrix was washed in PBS; the cells present in the matrix were killed by two

freeze-thaw cycles. Finally, the remaining matrix was incubated in PBS at 37 °C for another week. We have found that at 4 weeks the level of pyridinoline cross-link was 0.0229±0.0005 mol/mol collagen and this increased to 0.0333±0.0006 mol/mol collagen after the described incubation in PBS. In vitro maturation into tri-functional cross-links has been observed by others as well [45]. Thus, during the latter incubation the di-functional collagen cross-links further mature into tri-functional pyridinoline cross-links; consequently, the level of pyridinoline cross-links per collagen molecule reflects more closely the in vivo situation, because maturation of collagen cross-links is a time-dependent process which is facilitated in vivo by the long half-life of collagen.

### 2.2. Real-time PCR analysis

Total RNA was isolated from cytokine-stimulated fibroblast cultures using the RNeasy kit (Qiagen). Subsequently, the RNA was reverse transcribed into cDNA and subjected to real-time PCR amplification. Real-time PCR amplification of cDNA sequences was performed for LH1, LH2b, LH3, collagen type I α2 (COL1A2) and LOX. To standardize for differences in the total amount of cDNA a real-time PCR was performed of β-2-microglobulin (B2M), a constitutively expressed gene and which expression was not affected by the different stimulations. The expression of the target genes was calculated relatively to B2M expression. Each cDNA was amplified using specific primers and specific molecular beacons as described earlier [19]. Data were analyzed using sequence detector V1.7 software. LH2a expression was measured using a PCR amplifying both LH2a and LH2b [19].

### 2.3. Cross-link analysis

Measurements were performed on the extracellular matrix obtained from long-term (4 weeks) fibroblast cultures. Hydroxyproline (Hyp) and Hyl levels were determined in acid hydrolysates of matrix samples after derivatization with 9-fluorenylmethyl chloroformate by reverse-phase high-performance liquid chromatography [46]. Hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP) cross-links were measured in the same acid hydrolysates as described previously [47,48]. Pyridinoline and Hyl levels were expressed as total amount of residues per collagen molecule, assuming 300 Hyp residues per triple helix.

### 2.4. Statistical analysis

Statistical analysis was performed using SPSS 11.0 software. Differences between stimulated and unstimulated cells were analyzed by one-way analysis of variance (ANOVA) followed by LSD or Scheffé post-hoc tests. A $P$ value of $\leq 0.05$ was considered to indicate statistically significant differences.

## 3. Results

### 3.1. TGF-β stimulation of skin fibroblasts

We have recently found that LH2b is increased in cells present in several fibrotic tissues [19,25]. Since TGF-β is an important factor for the induction of fibrosis, we examined

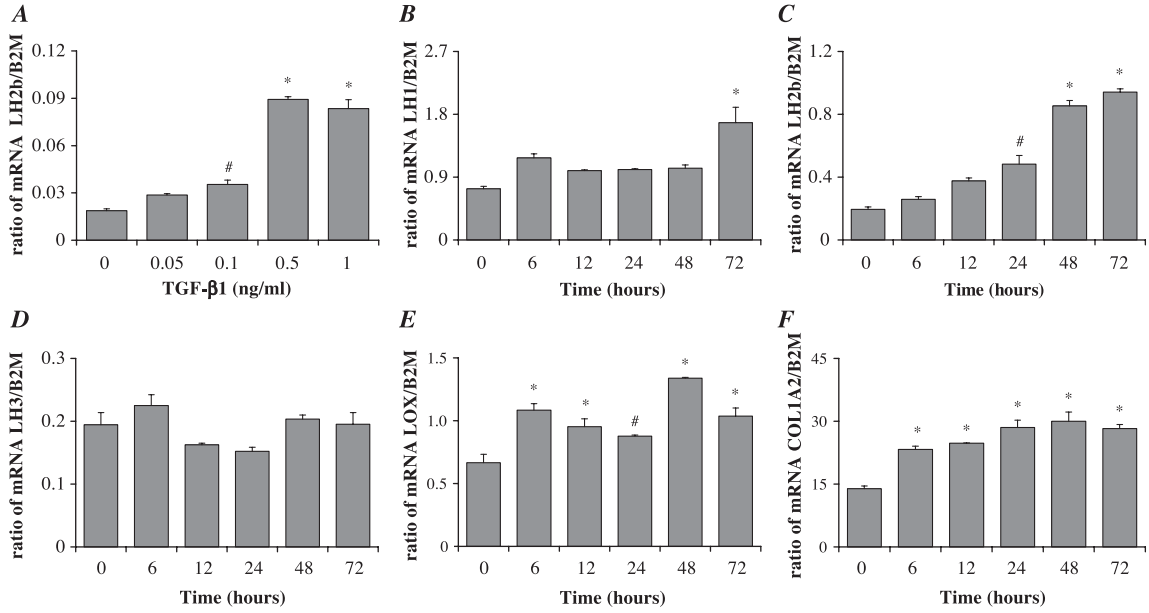


Fig. 1. Concentration- and time-dependent increase in LH2b expression induced by TGF-β1. Concentration-dependent up-regulation of LH2b mRNA expression in TGF-β1 stimulated skin fibroblasts is shown in panel A (after 24 h). The other panels demonstrate the time-dependent mRNA expression relative to B2M of LH1 (B), LH2b (C), LH3 (D), LOX (E) and COL1A2 (F) in TGF-β1 stimulated cells (1 ng/ml). * indicates $P \leq 0.01$ and # indicates $P \leq 0.05$ for TGF-β1 stimulated cells versus unstimulated cells. Data are presented as mean±S.E.

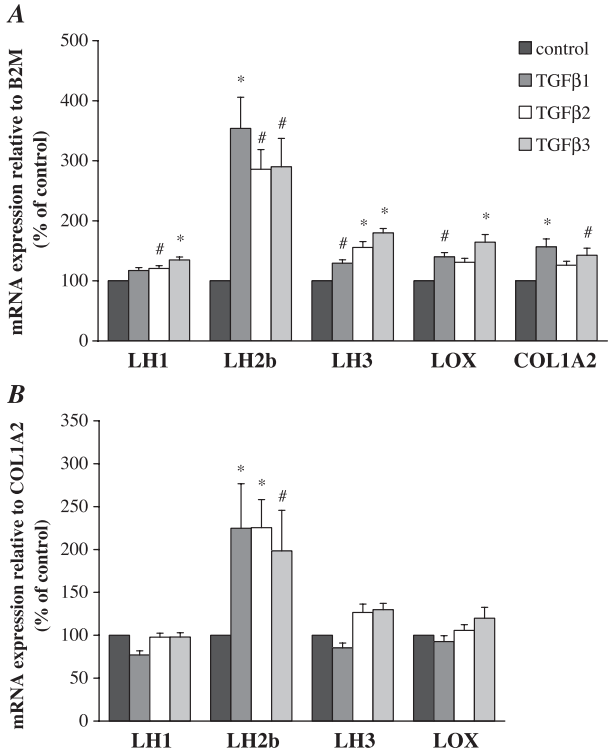


Fig. 2. Increased LH2b expression in TGF-β1, -β2, and -β3 stimulated fibroblasts. Expression levels of LH1, LH2b, LH3, LOX and COL1A2 relative to B2M expression in TGF-β1, -β2 and -β3 stimulated skin fibroblasts ($n$=7; 1 ng/ml; 24 h) are shown in panel A. Expression levels of LH1, LH2b, LH3 and LOX relative to COL1A2 expression are depicted in panel B. * indicates $P \leq 0.01$ and # indicates $P \leq 0.05$ for TGF-β stimulated cells versus unstimulated cells. Data are presented as mean±S.E.

the effect of TGF-β stimulation on LH2b mRNA expression in skin fibroblasts. To explore the concentration-dependent effect of TGF-β, fibroblasts of one donor were stimulated for 24 h with different concentrations TGF-β1, -β2, or -β3, rising from 0.05 to 1 ng/ml. Analysis of LH2b mRNA expression demonstrated that TGF-β1 indeed increased LH2b mRNA expression and that the optimum concentration for TGF-β1-induced LH2b up-regulation is 1 ng/ml (Fig. 1A). Similar LH2b expression patterns were found for TGF-β2 or -β3 stimulation (data not shown).

To examine the temporal TGF-β-induced mRNA expression patterns, fibroblasts of one donor were cultured for 6, 12, 24, 48 and 72 h in the presence or absence of 1 ng/ml TGF-β1, -β2, or -β3. Real-time PCR analysis revealed that LH2b mRNA expression did not show a significant increase until 24 h after TGF-β stimulation and expression was further increased after this time point, reaching high levels (fivefold induction) both at 48 and 72 h (Fig. 1C). A significant increase in LH1 mRNA expression could only be detected at 72 h (Fig. 1B). LH3 mRNA expression was not affected at all by TGF-β1 stimulation (Fig. 1D). Induction of LOX and COL1A2 mRNA expression by TGF-β1 was much faster than LH1 and LH2b, since both genes were approximately 1.5-fold up-regulated directly after 6 h and did not further increase at later time-points (Fig. 1E and F, respectively). Equal temporal expression patterns of the mentioned genes were found in fibroblasts after stimulation with TGF-β2 or -β3 (data not shown).

To exclude whether the induction of LH2b by TGF-β in skin fibroblasts is a donor-specific effect, fibroblasts of seven donors were cultured in the presence or absence of 1 ng/ml TGF-β1, -β2, or -β3 for 24 h. Real-time PCR analysis revealed highly increased LH2b mRNA expression in the fibroblasts of all donors stimulated with either TGF-β1, -β2 or -β3 (up to 3.5-fold compared to controls; Fig. 2A). The stimulatory effect on LH2b mRNA expression was not significantly different between the three TGF-β isoforms. In previous experiments, we have found that LH2a is undetectable in normal skin fibroblast [19]. However, a PCR amplifying LH2a and LH2b revealed that the three TGF-β isoforms slightly induce LH2a expression, although the expression was still very low (data not shown). Analysis of LH1 mRNA expression (Fig. 2A) in fibroblasts stimulated with either TGF-β2 or -β3 showed only a 1.3-fold increase compared to controls; TGF-β1 stimulation did not result in a significant increase of LH1. Interestingly, in contrast to the donor of the previous experiment, increased LH3 mRNA expression after TGF-β stimulation was found in the other six donors (Fig. 2A). LOX and COL1A2 mRNA expression was also slightly elevated in TGF-β-stimulated

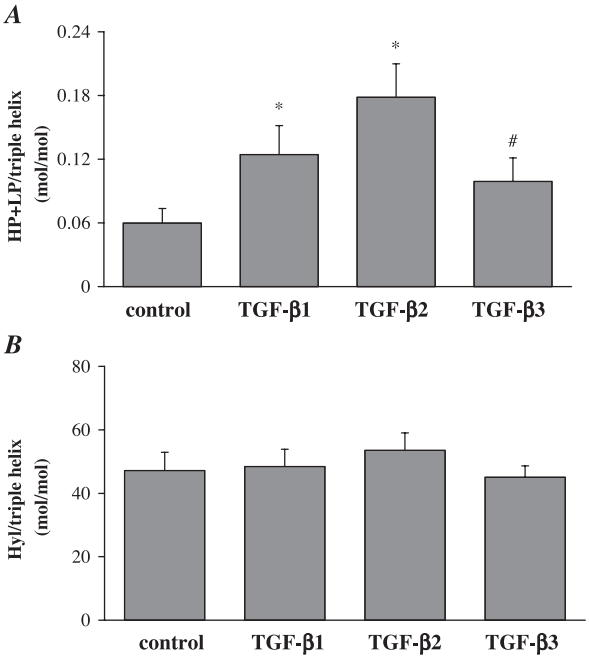


Fig. 3. TGF-β1, -β2, and -β3 induce Lys overhydroxylation of the telopeptides, but not of the triple helix. Pyridinoline cross-link (HP+LP) levels in the collagen matrix deposited by TGF-β1, -β2, -β3 stimulated fibroblasts ($n$=7; 1 ng/ml) are depicted in panel A. Hyl levels within the collagen triple helix are shown in panel B. * indicates $P \leq 0.01$ and # indicates $P \leq 0.05$ for TGF-β stimulated cells versus unstimulated cells. Data are presented as mean±S.E.

fibroblasts (up to 1.5-fold compared to controls; Fig. 2A). No large differences in LH1, LH3, COL1A2 and LOX mRNA expression were found between the three TGF-β isoforms. When mRNA expression levels were calculated relative to COL1A2, only LH2b was more than twofold increased in fibroblasts stimulated with TGF-β (Fig. 2B), whereas no significant increase was found in LH1, LH3 and LOX expression levels (Fig. 2B).

To examine whether TGF-β stimulation also results in the induction of pyridinoline cross-links formation, the extracellular matrix deposited by fibroblasts stimulated with TGF-β1, -β2 or -β3 was analyzed. Indeed, an increase in pyridinoline cross-links levels was found in the collagen deposited by TGF-β stimulated cells (Fig. 3A). The total amount of Hyl residues within the collagen triple helix was unaltered in the matrix deposited by TGF-β1, -β2, and -β3 stimulated cells as compared to unstimulated cells (Fig. 3B).

### *3.2. IL-4, BMP-2, activin A, and TNF-α stimulation of skin fibroblasts*

In order to examine whether other profibrotic cytokines are able to induce LH2b mRNA expression, fibroblasts were cultured in the presence of IL-4, BMP-2, activin A, or TNF-α for 24 h and compared to their unstimulated counterparts. Real-time PCR analysis revealed that all tested cytokines increased LH2b mRNA expression (Fig. 4A). LH2b expression relative to COL1A2 expression showed a 2.5-fold increase for IL-4 and activin A, when compared to controls (Fig. 4B). This increase was due to the changes in LH2b mRNA levels and not due to changes in COL1A2 expression, which was hardly altered (Fig. 4A). TNF-α induced a fourfold increase in LH2b expression relative to COL1A2 (Fig. 4B) as a result of increased LH2b expression and decreased COL1A2 expression (Fig. 4A). Fibroblasts stimulated with BMP-2 did not increase LH2b expression relative to COL1A2.

LH1 expression (Fig. 4A) was only induced in activin A stimulated cells when compared to unstimulated cells. However, LH1 expression relative to COL1A2 was not changed, due to a slight increase in COL1A2 expression (Fig. 4B). All tested cytokines did not affect LH3 expression (Fig. 4A) nor LH3 expression relative to COL1A2 (Fig. 4B). LOX expression and LOX expression relative to COL1A2 was only increased in fibroblasts stimulated with IL-4 (Fig. 4A and B, respectively), whereas activin A and TNF-α did not show any effect. BMP-2 stimulated cells showed a twofold increase in LOX expression, however, LOX expression relative to COL1A2 was not significantly increased when compared to unstimulated cells (Fig. 4A and B, respectively).

The effect of IL-4, BMP-2, activin A, and TNF-α on the pyridinoline cross-links levels in the deposited matrix is shown in Fig. 5A. All tested cytokines, with the exception of BMP-2, showed an increase in pyridinoline cross-link levels. Hyl levels within the collagen triple helix were

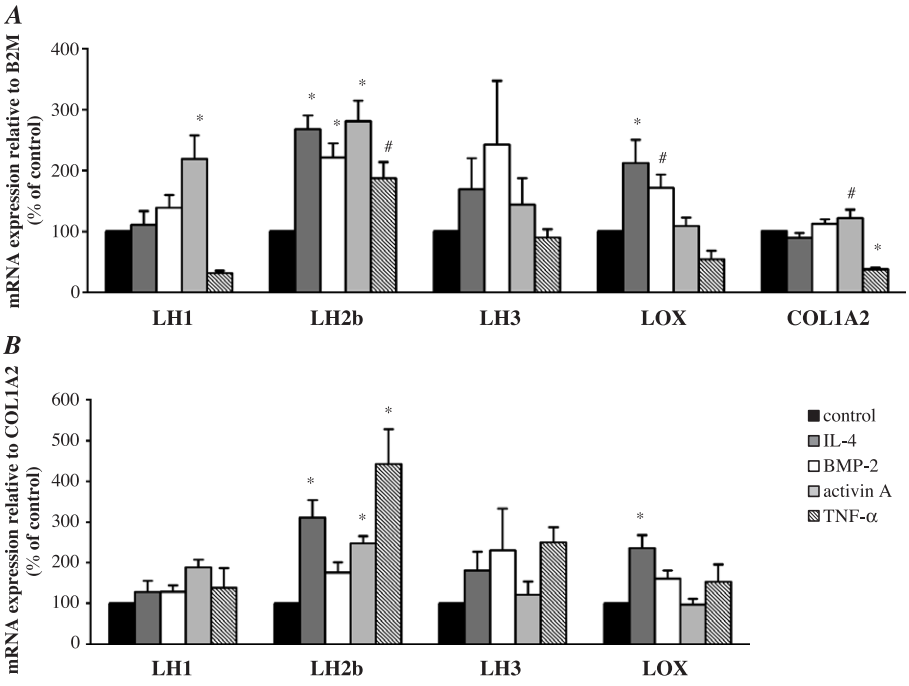


Fig. 4. Profibrotic cytokines elevate LH2b expression. Expression levels of LH1, LH2b, LH3, LOX and COL1A2 relative to B2M expression in IL-4 (10 ng/ml), BMP-2 (10 ng/ml), activin A (5 ng/ml), and TNF-α (10 ng/ml) stimulated skin fibroblasts ($n$=4) are shown in panel A (after 24 h). Expression levels of LH1, LH2b, LH3 and LOX relative to COL1A2 expression are depicted in panel B. * indicates $P \leq 0.01$ and # indicates $P \leq 0.05$ for stimulated cells versus unstimulated cells. Data are presented as mean±S.E.

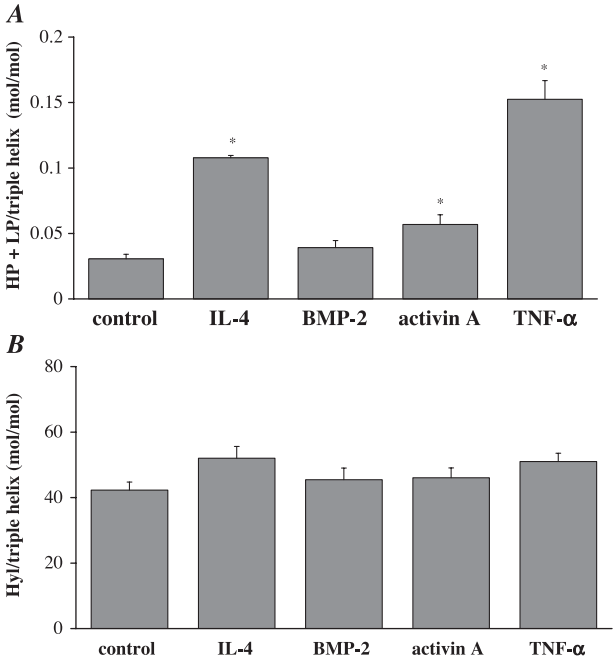


Fig. 5. Profibrotic cytokines induce pyridinoline cross-link formation but do not affect triple helical Hyl levels. Pyridinoline cross-link (HP+LP) levels in the collagen matrix deposited by IL-4 (10 ng/ml), BMP-2 (10 ng/ml), activin A (5 ng/ml) and TNF-α (10 ng/ml) stimulated fibroblasts ($n$=4) are depicted in panel A. Hyl levels within the collagen triple helix are shown in panel B. * indicates $P \leq 0.01$ and # indicates $P \leq 0.05$ for stimulated cells versus unstimulated cells. Data are presented as mean±S.E.

unchanged in all stimulated cells when compared to unstimulated cells (Fig. 5B).

## 4. Discussion

Fibrosis is characterized by the accumulation of collagen, containing highly increased pyridinoline cross-link levels [1–6]. We have recently found that in several forms of fibrosis an increased LH2b mRNA expression level is associated with increased formation of pyridinoline cross-links, which suggests that LH2b is responsible for the overhydroxylation of telopeptide lysine residues and the concomitant increase in pyridinoline cross-links in fibrotic lesions [19,25]. The stimulatory effect of the profibrotic cytokines TGF-β1, -β2, and -β3 on LH2b gene expression, as shown in this study, underlines the significant role of LH2b up-regulation in fibrotic processes. In contrast to LH1 and LH3, LH2b mRNA expression is far more induced by TGF-β than the mRNA expression of COL1A2, thus changing the proportion between LH2b and COL1A2 expression. Analysis of the matrix deposited by fibroblasts stimulated with TGF-β clearly showed increased pyridinoline cross-linking, but no increase in overall hydroxylation of the lysine residues in the collagen molecule. This means that only the lysine residues within the telopeptides are overhydroxylated and not in the triple helical part. Since LH2b is the only lysyl hydroxylase which is induced to a greater extent than collagen expression by stimulation with TGF-β, it is plausible that telopeptide lysyl hydroxylases activity of LH2b is responsible for the increased formation of pyridinoline cross-links in the matrix deposited by TGF-β stimulated cells. This is further endorsed by the fact that the TGF-β isoforms did not induce the expression of LOX relative to COL1A2 expression. The increased formation of pyridinolines is therefore most likely not due to an increased aldehyde formation (a step necessary for cross-link formation and catalyzed by LOX).

Not only TGF-β isoforms, but also other profibrotic cytokines, including IL-4, activin A and TNF-α, appeared to be capable of inducing LH2b expression, whereas the expression of the lysyl hydroxylases LH1 and LH3 relative to COL1A2 was unaffected by these cytokines. As for TGF-β isoforms, above-mentioned cytokines also stimulated the formation of pyridinoline cross-links in the deposited matrix. An exception appeared to be BMP-2. This cytokine is clearly not involved in the regulation of LH2b and we can perfectly match that with our observation that the amount of pyridinoline cross-links in the matrix deposited by BMP-2-stimulated cells was not increased as well. Again the obtained results for the profibrotic cytokines activin A and TNF-α point out that it is very likely that LH2b is responsible for the overhydroxylation of the lysine residues in the telopeptides, leading to increased formation of pyridinoline cross-links. In the case of IL-4, the induced formation of pyridinoline cross-links might also be due to increased LOX expression or to a combination of induced LH2b and LOX expression. But clearly this was not the case for activin A and TNF-α, since no differences in LOX expression relative to COL1A2 were observed in stimulated cells.

TGF-β time curves of LH1, LH2b, LH3, COL1A2 and LOX mRNA expression revealed differences in the induction rate of the TGF-β induced mRNA expression: COL1A2 and LOX expression is up-regulated to the maximum already after 6 h, whereas LH2b and LH1 expression reaches high levels at 48 and 72 h, respectively. The slower induction rate of LH1 and LH2b suggests that the effect of TGF-β on LH1 and LH2b gene expression is probably not accomplished directly via the intracellular pathways downstream the TGF-β receptor. It is most likely a secondary effect. It could be that the increased gene expression of LH1 and LH2b is accomplished by profibrotic factors that are rapidly up-regulated by TGF-β, such as connective tissue growth factor [49] or endothelin-1 (ET-1) [50,51]. Evidence for such involvement of ET-1 has been obtained by microarray analysis studies of lung fibroblasts, showing a 2.5-fold increase in LH2 expression within 4 h after ET-1 stimulation [52].

Interestingly, two research groups showed that a hypoxic environment, which favors the formation of collagen deposits, induces increased LH2 expression levels [53,54]. A hypoxic environment has a significant pathophysiological impact on pulmonary and cardiac fibrosis. Our findings

together with the above-mentioned observations reveal that a broad range of profibrotic cytokines/conditions induces LH2b expression, highlighting that up-regulation of LH2b is an important general phenomenon in fibrotic processes. In addition, our study showed for the first time that in association with increased LH2b expression levels, profibrotic cytokines are capable to induce pyridinoline cross-link formation. These results provide strong evidence that LH2b is responsible for the overhydroxylation of telopeptide lysine and the concomitant pyridinoline formation in fibrotic lesions.

The occurrence of hydroxyallysine-derived cross-links (such as pyridinolines) in the matrix deposited in fibrotic lesions is an important criterion in assessing the irreversibility of fibrosis [4,5,55–57]. Specific inhibition of LH2b to decrease the formation of pyridinoline cross-links is expected to result in a more reversible deposition of collagen. Specific compounds inhibiting LH2b enzyme activity or compounds that reduce LH2b gene expression, such as antisense molecules [21], hold promise for future therapies to prevent irreversible fibrosis. The differences in induction rate of the different extracellular matrix-related genes by TGF-β [this paper, 58], together with the fact that from the measured collagen-modifying enzymes TGF-β only induces the expression of LH2b relative to COL1A2, suggest that different intracellular pathways are involved in the TGF-β-induced gene regulation of LH1, LH2b, LH3, COL1A2 and LOX. Unraveling the processes involved in the up-regulation of LH2b mRNA expression may provide tools to find new targets enabling interference specifically with LH2b expression in order to attenuate fibrosis.

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## References

[1] D.A. Brenner, T. Waterboer, S.K. Choi, J.N. Lindquist, B. Stefanovic, E. Burchardt, M. Yamauchi, A. Gillan, R.A. Rippe, New aspects of hepatic fibrosis, J. Hepatol. 32 (2000) 32–38.

[2] J. Brinckmann, C.M. Neess, Y. Gaber, H. Sobhi, H. Notbohm, N. Hunzelmann, P.P. Fietzek, P.K. Muller, J. Risteli, R. Gebker, K. Scharffetter-Kochanek, Different pattern of collagen cross-links in two sclerotic skin diseases: lipodermatosclerosis and circumscribed scleroderma, J. Invest. Dermatol. 117 (2001) 269–273.

[3] T. Moriguchi, D. Fujimoto, Cross-link of collagen in hypertrophic scar, J. Invest. Dermatol. 72 (1979) 143–145.

[4] S. Ricard-Blum, S. Bresson-Hadni, D.A. Vuitton, G. Ville, J.A. Grimaud, Hydroxypyridinium collagen cross-links in human liver fibrosis: study of alveolar echinococcosis, Hepatology 15 (1992) 599–602.

[5] S. Ricard-Blum, P. Esterre, J.A. Grimaud, Collagen cross-linking by pyridinoline occurs in non-reversible skin fibrosis, Cell. Mol. Biol. (Noisy-le-grand) 39 (1993) 723–727.

[6] K. Uzawa, M.K. Marshall, E.P. Katz, H. Tanzawa, H.N. Yeowell, M. Yamauchi, Altered posttranslational modifications of collagen in keloid, Biochem. Biophys. Res. Commun. 249 (1998) 652–655.

[7] D.R. Eyre, M.A. Paz, P.M. Gallop, Cross-linking in collagen and elastin, Annu. Rev. Biochem. 53 (1984) 717–748.

[8] D. Herbage, M. Le Lous, L. Cohen-Solal, S. Bazin, Study of Collagen Cross-links, Front. Matrix Biol. 10 (1985) 59–91.

[9] K. Reiser, R.J. McCormick, R.B. Rucker, Enzymatic and nonenzymatic cross-linking of collagen and elastin, FASEB J. 6 (1992) 2439–2449.

[10] S.P. Robins, Analysis of the cross-linking components in collagen and elastin, Methods Biochem. Anal. 28 (1982) 329–379.

[11] T. Hautala, M.G. Byers, R.L. Eddy, T.B. Shows, K.I. Kivirikko, R. Myllyla, Cloning of human lysyl hydroxylase: complete cDNA-derived amino acid sequence and assignment of the gene (PLOD) to chromosome 1p36.3–p36.2, Genomics 13 (1992) 62–69.

[12] K. Passoja, K. Rautavuoma, L. Ala-Kokko, T. Kosonen, K.I. Kivirikko, Cloning and characterization of a third human lysyl hydroxylase isoform, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 10482–10486.

[13] M. Risteli, O. Niemitalo, H. Lankinen, A.H. Juffer, R. Myllyla, Characterization of collagenous peptides bound to lysyl hydroxylase isoforms, J. Biol. Chem. 279 (2004) 37535–37543.

[14] M. Valtavaara, H. Papponen, A.M. Pirttila, K. Hiltunen, H. Helander, R. Myllyla, Cloning and characterization of a novel human lysyl hydroxylase isoform highly expressed in pancreas and muscle, J. Biol. Chem. 272 (1997) 6831–6834.

[15] M. Valtavaara, C. Szpirer, J. Szpirer, R. Myllyla, Primary structure, tissue distribution, and chromosomal localization of a novel isoform of lysyl hydroxylase (lysyl hydroxylase 3), J. Biol. Chem. 273 (1998) 12881–12886.

[16] H. Ruotsalainen, S. Vanhatupa, M. Tampio, L. Sipila, M. Valtavaara, R. Myllyla, Complete genomic structure of mouse lysyl hydroxylase 2 and lysyl hydroxylase 3/collagen glucosyltransferase, Matrix Biol. 20 (2001) 137–146.

[17] H.N. Yeowell, L.C. Walker, Tissue specificity of a new splice form of the human lysyl hydroxylase 2 gene, Matrix Biol. 18 (1999) 179–187.

[18] B. Steinmann, D.R. Eyre, P. Shao, Urinary pyridinoline cross-links in Ehlers–Danlos syndrome type VI, Am. J. Hum. Genet. 57 (1995) 1505–1508.

[19] A.J. van der Slot, A.M. Zuurmond, A.F. Bardoel, C. Wijmenga, H.E. Pruijs, D.O. Sillence, J. Brinckmann, D.J. Abraham, C.M. Black, N. Verzijl, J. DeGroot, R. Hanemaaijer, J.M. TeKoppele, T.W. Huizinga, R.A. Bank, Identification of PLOD2 as telopeptide lysyl hydroxylase, an important enzyme in fibrosis, J. Biol. Chem. 278 (2003) 40967–40972.

[20] D.K. Mercer, P.F. Nicol, C. Kimbembe, S.P. Robins, Identification, expression, and tissue distribution of the three rat lysyl hydroxylase isoforms, Biochem. Biophys. Res. Commun. 307 (2003) 803–809.

[21] S. Pornprasertsuk, W.R. Duarte, Y. Mochida, M. Yamauchi, Lysyl hydroxylase-2b directs collagen cross-linking pathways in MC3T3-E1 cells, J. Bone Miner. Res. 19 (2004) 1349–1355.

[22] K. Uzawa, W.J. Grzesik, T. Nishiura, S.A. Kuznetsov, P.G. Robey, D.A. Brenner, M. Yamauchi, Differential expression of human lysyl hydroxylase genes, lysine hydroxylation, and cross-linking of type I collagen during osteoblastic differentiation in vitro, J. Bone Miner. Res. 14 (1999) 1272–1280.

[23] J. Heikkinen, M. Risteli, C. Wang, J. Latvala, M. Rossi, M. Valtavaara, R. Myllyla, Lysyl hydroxylase 3 is a multifunctional protein possessing collagen glucosyltransferase activity, J. Biol. Chem. 275 (2000) 36158–36163.

[24] C. Wang, H. Luosujarvi, J. Heikkinen, M. Risteli, L. Uitto, R. Myllyla, The third activity for lysyl hydroxylase 3: galactosylation of

hydroxylysyl residues in collagens in vitro, Matrix Biol. 21 (2002) 559–566.
[25] A.J. van der Slot, A.M. Zuurmond, A.J. van den Bogaerdt, M.M. Ulrich, E. Middelkoop, W. Boers, H.K. Ronday, J. DeGroot, T.W. Huizinga, R.A. Bank, Increased formation of pyridinoline cross-links due to higher telopeptide lysyl hydroxylase levels is a general fibrotic phenomenon, Matrix Biol. 23 (2004) 251–257.
[26] M.H. Branton, J.B. Kopp, TGF-beta and fibrosis, Microbes Infect. 1 (1999) 1349–1365.
[27] T.J. Broekelmann, A.H. Limper, T.V. Colby, J.A. McDonald, Transforming growth factor beta 1 is present at sites of extracellular matrix gene expression in human pulmonary fibrosis, Proc. Natl. Acad. Sci. U. S. A. 88 (1991) 6642–6646.
[28] C.P. Denton, D.J. Abraham, Transforming growth factor-beta and connective tissue growth factor: key cytokines in scleroderma pathogenesis, Curr. Opin. Rheumatol. 13 (2001) 505–511.
[29] P.J. Sime, Z. Xing, F.L. Graham, K.G. Csaky, J. Gauldie, Adenovector-mediated gene transfer of active transforming growth factor-beta1 induces prolonged severe fibrosis in rat lung, J. Clin. Invest. 100 (1997) 768–776.
[30] S. Kanzler, A.W. Lohse, A. Keil, J. Henninger, H.P. Dienes, P. Schirmacher, S. Rose-John, K.H. zum Buschenfelde, M. Lessing, TGF-beta1 in liver fibrosis: an inducible transgenic mouse model to study liver fibrogenesis, Am. J. Physiol. 276 (1999) G1059–G1068.
[31] J.S. Huang, Y.H. Wang, T.Y. Ling, S.S. Chuang, F.E. Johnson, S.S. Huang, Synthetic TGF-beta antagonist accelerates wound healing and reduces scarring, FASEB J. 16 (2002) 1269–1270.
[32] Z. Qi, N. Atsuchi, A. Ooshima, A. Takeshita, H. Ueno, Blockade of type beta transforming growth factor signaling prevents liver fibrosis and dysfunction in the rat, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 2345–2349.
[33] Q. Wang, Y. Wang, D.M. Hyde, P.J. Gotwals, V.E. Koteliansky, S.T. Ryan, S.N. Giri, Reduction of bleomycin induced lung fibrosis by transforming growth factor beta soluble receptor in hamsters, Thorax 54 (1999) 805–812.
[34] J. George, D. Roulot, V.E. Koteliansky, D.M. Bissell, In vivo inhibition of rat stellate cell activation by soluble transforming growth factor beta type II receptor: a potential new therapy for hepatic fibrosis, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 12719–12724.
[35] E. Piek, C.H. Heldin, P. Ten Dijke, Specificity, diversity, and regulation in TGF-beta superfamily signaling, FASEB J. 13 (1999) 2105–2124.
[36] L.P. Sanford, I. Ormsby, A.C. Gittenberger-de Groot, H. Sariola, R. Friedman, G.P. Boivin, E.L. Cardell, T. Doetschman, TGFbeta2 knockout mice have multiple developmental defects that are non-overlapping with other TGFbeta knockout phenotypes, Development 124 (1997) 2659–2670.
[37] V. Kaartinen, J.W. Voncken, C. Shuler, D. Warburton, D. Bu, N. Heisterkamp, J. Groffen, Abnormal lung development and cleft palate in mice lacking TGF-beta 3 indicates defects of epithelial–mesenchymal interaction, Nat. Genet. 11 (1995) 415–421.
[38] J.J. Letterio, A.G. Geiser, A.B. Kulkarni, N.S. Roche, M.B. Sporn, A.B. Roberts, Maternal rescue of transforming growth factor-beta 1 null mice, Science 264 (1994) 1936–1938.
[39] S.P. Atamas, Complex cytokine regulation of tissue fibrosis, Life Sci. 72 (2002) 631–643.
[40] M. Gharaee-Kermani, S.H. Phan, Role of cytokines and cytokine therapy in wound healing and fibrotic diseases, Curr. Pharm. Des. 7 (2001) 1083–1103.
[41] B. Munz, H. Smola, F. Engelhardt, K. Bleuel, M. Brauchle, I. Lein, L.W. Evans, D. Huylebroeck, R. Balling, S. Werner, Overexpression of activin A in the skin of transgenic mice reveals new activities of activin in epidermal morphogenesis, dermal fibrosis and wound repair, EMBO J. 18 (1999) 5205–5215.
[42] E.J. Stelnicki, M.T. Longaker, D. Holmes, K. Vanderwall, M.R. Harrison, C. Largman, W.Y. Hoffman, Bone morphogenetic protein-2 induces scar formation and skin maturation in the second trimester fetus, Plast. Reconstr. Surg. 101 (1998) 12–19.
[43] K. Zhang, M. Gharaee-Kermani, B. McGarry, D. Remick, S.H. Phan, TNF-alpha-mediated lung cytokine networking and eosinophil recruitment in pulmonary fibrosis, J. Immunol. 158 (1997) 954–959.
[44] C. Ong, C. Wong, C.R. Roberts, H.S. Teh, F.R. Jirik, Anti-IL-4 treatment prevents dermal collagen deposition in the tight-skin mouse model of scleroderma, Eur. J. Immunol. 28 (1998) 2619–2629.
[45] D.R. Eyre, Cross-link maturation in bone collagen, in: A. Veis (Ed.), The Chemistry and Biology of Mineralized Connective Tissues, Elsevier Science, New York, 1981, pp. 51–59.
[46] R.A. Bank, E.J. Jansen, B. Beekman, J.M. TeKoppele, Amino acid analysis by reverse-phase high-performance liquid chromatography: improved derivatization and detection conditions with 9-fluorenylmethyl chloroformate, Anal. Biochem. 240 (1996) 167–176.
[47] R.A. Bank, B. Beekman, N. Verzijl, J.A. De Roos, A.N. Sakkee, J.M. TeKoppele, Sensitive fluorimetric quantitation of pyridinium and pentosidine cross-links in biological samples in a single high-performance liquid chromatographic run, J. Chromatogr., B, Biomed. Sci. Appl. 703 (1997) 37–44.
[48] S.P. Robins, A. Duncan, N. Wilson, B.J. Evans, Standardization of pyridinium cross-links, pyridinoline and deoxypyridinoline, for use as biochemical markers of collagen degradation, Clin. Chem. 42 (1996) 1621–1626.
[49] A. Leask, A. Holmes, D.J. Abraham, Connective tissue growth factor: a new and important player in the pathogenesis of fibrosis, Curr. Rheumatol. Rep. 4 (2002) 136–142.
[50] F. Rodriguez-Pascual, M. Redondo-Horcajo, S. Lamas, Functional cooperation between Smad proteins and activator protein-1 regulates transforming growth factor-beta-mediated induction of endothelin-1 expression, Circ. Res. 92 (2003) 1288–1295.
[51] P. Teder, P.W. Noble, A cytokine reborn? Endothelin-1 in pulmonary inflammation and fibrosis, Am. J. Respir. Cell Mol. Biol. 23 (2000) 7–10.
[52] X. Shi-Wen, Y. Chen, C.P. Denton, M. Eastwood, E.A. Renzoni, G. Bou-Gharios, J.D. Pearson, M. Dashwood, R.M. du Bois, C.M. Black, A. Leask, D.J. Abraham, Endothelin-1 promotes myofibroblast induction through the ETA receptor via a rac/phosphoinositide 3-kinase/Akt-dependent pathway and is essential for the enhanced contractile phenotype of fibrotic fibroblasts, Mol. Biol. Cell 15 (2004) 2707–2719.
[53] K.H. Hofbauer, B. Gess, C. Lohaus, H.E. Meyer, D. Katschinski, A. Kurtz, Oxygen tension regulates the expression of a group of procollagen hydroxylases, Eur. J. Biochem. 270 (2003) 4515–4522.
[54] S.B. Scheurer, J.N. Raybak, C. Rosli, D. Neri, G. Elia, Modulation of gene expression by hypoxia in human umbilical cord vein endothelial cells: a transcriptomic and proteomic study, Proteomics 4 (2004) 1737–1760.
[55] A.J. Bailey, S. Bazin, T.J. Sims, M. Le Lous, C. Nicoletis, A. Delaunay, Characterization of the collagen of human hypertrophic and normal scars, Biochim. Biophys. Acta 405 (1975) 412–421.
[56] A.J. Bailey, N.D. Light, Intermolecular cross-linking in fibrotic collagen, Ciba Found. Symp. 114 (1985) 80–96.
[57] J.A. Last, T.E. King, A.G. Nerlich, K.M. Reiser, Collagen cross-linking in adult patients with acute and chronic fibrotic lung disease. Molecular markers for fibrotic collagen, Am. Rev. Respir. Dis. 141 (1990) 307–313.
[58] R.C. Chambers, P. Leoni, N. Kaminski, G.J. Laurent, R.A. Heller, Global expression profiling of fibroblast responses to transforming growth factor-beta1 reveals the induction of inhibitor of differentiation-1 and provides evidence of smooth muscle cell phenotypic switching, Am. J. Pathol. 162 (2003) 533–546.