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Inhibitory role of Id1 on TGF-β-induced collagen expression in human dermal fibroblasts

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ABSTRACT

Inhibitor of DNA binding 1 (Id1) is a basic helix-loop-helix (bHLH) protein that has a variety of functional roles in cellular events including differentiation, cell cycle and cancer development. In addition, it has been demonstrated that Id1 is related with TGF-β and Smad signaling in various biological conditions. In this study, we investigated the effect of Id1 on TGF-β-induced collagen expression in human dermal fibroblasts. When Id1-b isoform was overexpressed, TGF-β-induced collagen expression was markedly inhibited. Consistent with this result, Id1-b significantly inhibited TGF-β-induced collagen gel contraction. In addition, Id1-b inhibited TGF-β-induced phosphorylation of Smad2 and Smad3. Finally, immunohistochemistry showed that Id1 expression was decreased in fibrotic skin diseases while TGF-β signaling was increased. Together, these results suggest that Id1 is an inhibitory regulator on TGF-β-induced collagen expression in dermal fibroblasts.

1. Introduction

Transforming growth factor-β (TGF-β) is a multifunctional cytokine that regulates a variety of biological activities, including cell proliferation, differentiation, apoptosis, angiogenesis and wound healing [1]. In skin, TGF-β stimulates the growth and migration of fibroblasts, and also positively regulates the expression and deposition of extracellular matrix (ECM) components such as type I collagen and fibronectin [2]. In pathological condition, enhanced TGF-β signaling can induce excessive ECM accumulation, leading to the development of skin diseases such as hypertrophic scar and keloid [3–5].

The inhibitor of DNA binding (Id) proteins are basic helix-loop-helix (bHLH) proteins, which belong to a structurally-related family that are comprised of four members (Id1, Id2, Id3 and Id4). They all share common HLH-dimerization domain but lack a basic DNA binding domain. Id proteins can bind to other bHLH transcription factors to make heterodimeric complexes. However, these heterodimeric complexes are unable to bind to target DNA, thus Id proteins inhibit the action of bHLH transcription factors on gene expression [6,7]. Interestingly, it has been demonstrated that Id proteins also interact with non-HLH proteins. For instance, Id2 binds to retinoblastoma (Rb), thereby reversing the Rb-mediated cell cycle inhibition [8,9]. In other example, Id1 interacts physically with caveolin-1, a cell membrane protein, such an interaction plays a key role in the epithelial–mesenchymal transition and increased cell migration of prostate cancer cells [10].

Many evidences indicate that Id proteins are related with TGF-β and Smad signaling in various biological conditions. For example, TGF-β-activated Smad3 directly induces ATF3 expression, and then ATF3 and Smad3 form a complex that directly mediates Id1 repression in epithelial cells [11]. Inhibition of TGF-β results in upregulation of Id1 expression, which is necessary for the increased yield of functional endothelial cells capable of in vivo neo-angiogenesis [12]. Conversely, inactivation of Id1 increases the cellular susceptibility to TGF-β1-induced growth arrest in prostate epithelial cells [13]. Overexpression of Id2 attenuates TGF-β-induced apoptosis in gut epithelial cells [14]. These results suggest that TGF-β and Id proteins can counteract each other to control homeostasis in a context-dependent manner.

Although there are many evidences supporting the link between TGF-β and Id proteins, the functional relationship between these molecules in collagen production of skin dermal fibroblasts is not well elucidated. In this study, we demonstrate that Id1 has an inhibitory role on TGF-β-induced collagen expression in human dermal fibroblasts.

2. Materials and methods

2.1. Skin specimens

All human skin samples were obtained under the written informed consent of donors, in accordance with the ethical

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committee approval process of the Institutional Review Board of Chungnam National University School of Medicine.

2.2. Reagents and antibodies

The following primary antibodies were used in this study: collagen type 1 (α1), collagen type 1 (α2), Id1, GFP (Santa Cruz Biotechnology, Santa Cruz, CA), Smad2, phospho-Smad2, Smad3, phospho-Smad3, TGF-β (Cell Signaling Technology, Beverly, MA), actin (Sigma–Aldrich, St. Louis, MO). Recombinant human TGF-β1 was purchased from R&D System (Minneapolis, MN).

2.3. Cell culture

Dermal fibroblasts were primary cultured from human foreskin tissues as previously reported [15]. Briefly, skin tissues were briefly sterilized in 70% ethanol, minced, and then treated with dispase for overnight at 4 °C. The dermis was separated from epidermis and placed in culture dish for explant culture. Cells were grown in DMEM supplemented with 10% FBS (Life Technologies Corporation, Grand Island, NY). Cells passaged between 4 and 10 were used in this study.

2.4. Western blot

Cells were lysed in Proprep solution (Intron, Daejeon, Korea). Total protein was measured using a BCA Protein Assay Reagent (Pierce Biotechnology, Rockford, IL). Samples were run on SDS–polyacrylamide gels, transferred onto nitrocellulose membranes and incubated with appropriate antibodies. Blots were then incubated with peroxidase-conjugated secondary antibodies, visualized by enhanced chemiluminescence (Intron).

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNAs were isolated from human dermal fibroblasts using Easy-blue RNA extraction kit (Intron). Two μg of total RNAs were reverse transcribed with moloney-murine leukaemia virus (M-MLV) reverse transcriptase (ELPIS Biotech, Daejeon, Korea). Aliquots of RT mixture were subjected to PCR cycles with appropriate primer sets. The sequences for primers were as follows: collagen type 1 (α1), 5′-GGCTTAAAGGGACACAA and 5′-TTCTTGGC TGGGATGTTTTC; collagen type 1 (α2), 5′-GAGAGCATGACCGATGG ATT and 5′-TTTTGAGGGGGTTCAGTTTG; Id1-a, 5′-CGTAGATCTATG AAAGTCGCCAGTGG and 5′- TATCTCGAGTCAGCGACACAAGATGC; Id1-b, 5′-CGTAGATCTATGAAAGTCGCCAGTGG and 5′-TATCTCGAG CTAGTGGTCGGATCTGG; cyclophilin, 5′-CTCCTTTGAGCTGTTTGCAG and 5′-CACCACATGCTTGCCATCCA.

2.6. Adenovirus creation

The amplified full-length cDNA for Id1-b was subcloned into the pENT/CMV-GFP vector that had attL sites for site specific recombination with a Gateway destination vector. Replication-incompetent adenoviruses were created using the Virapower adenovirus expression system (Invitrogen, Carlsbad, CA). The adenovirus was purified with cesium chloride.

2.7. Collagen gel contraction assay

Gel contractions assay was performed using a Cell contraction assay kit (Cell Biolabs Inc., San Diego, CA), according to the manufacturer's protocol. Briefly, 5×10^5 cells were mixed with collagen gel and polymerized in Transwell permeable supports (3.0 μm polycarbonate membrane) (Corning Incorporated, Corning, NY). After collagen polymerizaiton, 5 ml of medium was added atop each collagen gel lattice.

2.8. Immunohistochemistry

Paraffin sections were dewaxed, rehydrated, then washed three times with phosphate-buffered saline (PBS). After treatment with proteinase K (1 mg/ml) for 5 min at 37 °C, sections were treated with H_2O_2 for 10 min at room temperature, blocked in 0.1% Tween-20, 1% bovine serum albumin (BSA) in PBS for 30 min, and followed by reaction with appropriate primary antibodies. Sections were incubated sequentially with peroxidase-conjugated secondary antibodies and visualized with Chemmate envision detection kit (Dako, Carpinteria, CA).

3. Results

It has been well established that TGF-β induces collagen expression via the Smad signaling pathway in human dermal fibroblasts [16]. As an initial step, we confirmed whether TGF-β induces expression of type I collagen in our system. Consistent with previous data, TGF-β treatment of cultured human dermal fibroblasts resulted in marked increase of collagen type 1 (α1) and collagen type 1 (α2) at both translational and transcriptional levels in a time-dependent manner (Fig. 1A and B). We next investigated the effect of TGF-β on Id1 gene expression. As the isoforms of Id1 transcripts can be generated by alternative splicing, we examined what isoforms are affected by TGF-β. As a result, both isoforms (Id1-a and Id1-b) were downregulated by TGF-β, with more profound effect on Id1-b isoform (Fig. 1C). These results suggest

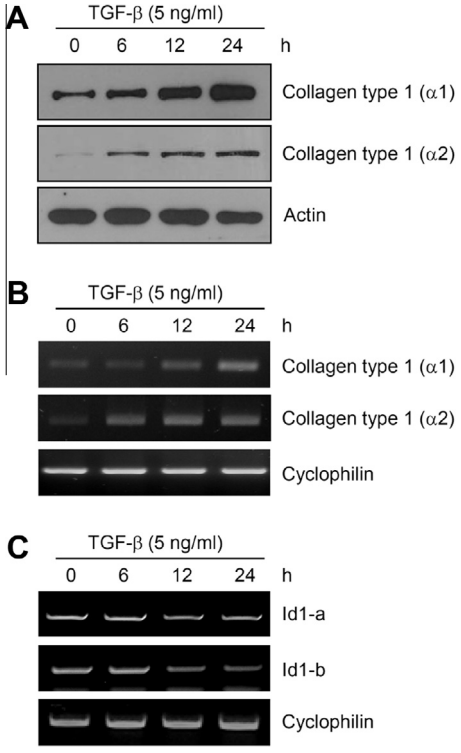

Fig. 1. Effects of TGF-β on expression of collagen and Id1. (A) Dermal fibroblasts were treated with TGF-β for the indicated time points. Expression of type I collagen (α1 and α2) was detected using Western blot analysis. Expression of type I collagen was increased in a time-dependent manner. (B) The mRNA level for type I collagen was determined by RT-PCR. (C). Effects of TGF-β on expression Id1 was determined by RT-PCR. Both isofoms of Id1 (Id1-a and Id1-b) were decreased by TGF-β in a time-dependent manner.

a possible link between Id1 expression and TGF-β-induced collagen synthesis.

To address the question whether Id1 affects TGF-β-induced collagen expression, we exogenously overexpressed green fluorescent protein-tagged Id1-b isoform (GFP-Id1-b) using a recombinant adenovirus. Western blot analysis showed that overexpression of Id1-b significantly inhibited the TGF-β-induced type I collagen expression (Fig. 2A). To further examine the effect of Id1-b, we next performed collagen gel contraction assay, a well-established experimental model for wound contraction [17]. After TGF-β treatment, the 3-D gel containing fibroblasts overexpressing GFP (control group) was markedly contracted in a temporal manner, while the gel contraction was significantly inhibited by Id1-b overexpression in fibroblasts (Fig. 2B, Suppl. Fig. 1). These results suggest that Id1 has an inhibitory role on TGF-β-induced collagen expression in human dermal fibroblasts.

As Smad2 and Smad3 are the major players in TGF-β signaling, we investigated whether Id1-b affects the activation of Smad proteins in terms of phosphorylation. Treatment of fibroblasts with TGF-β induced phosphorylation of Smad2 and Smad3 in control group (GFP overexpressing fibroblasts). In contrast, overexpression of Id1-b blocked significantly the TGF-β-induced phosphorylation of Smad2 and Smad3 (Fig. 3). These results suggest that inhibitory role of Id1 is likely via the modulation of intracellular signaling pathway.

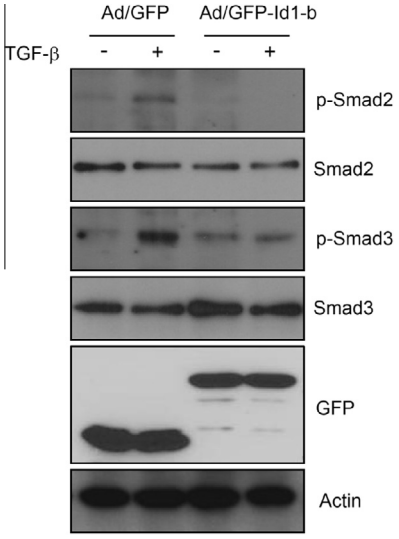

Fig. 3. Effect of Id1-b on TGF-β-induced phosphorylation of Smad 2 and Smad 3. Dermal fibroblasts were transduced with 10 multiplicity of infection (MOI) of adenovirus expressing GFP-tagged Id1-b (Ad/GFP-Id1-b) or control adenovirus (Ad/GFP) for 6 h. Cells were replenished with fresh medium, and incubated for a further 2 d. Then, cells were treated with TGF-β (5 ng/ml) for 30 min. Phosphorylation of Smad2 and 3 was determined by Western blot. TGF-β-induced phosphorylation of Smad2 and 3 was significantly inhibited by overexpression of GFP-Id1-b.

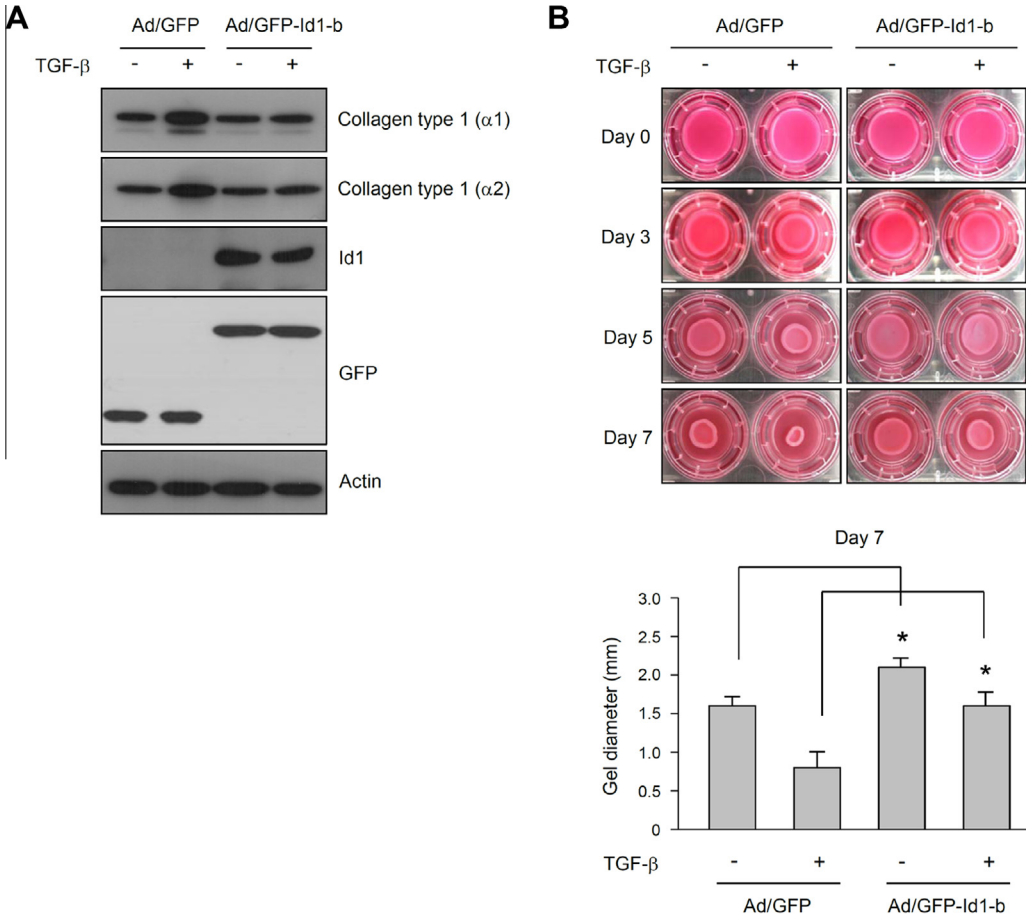

Fig. 2. Effect of Id1-b on TGF-β-induced collagen expression. (A) Dermal fibroblasts were transduced with 10 multiplicity of infection (MOI) of adenovirus expressing GFP-tagged Id1-b (Ad/GFP-Id1-b) or control adenovirus (Ad/GFP) for 6 h. Cells were replenished with fresh medium, and incubated for a further 2 d. Then, cells were treated with TGF-β (5 ng/ml) for 24 h. Protein level for type I collagen was determined by Western blot. TGF-β-induced collagen expression was significantly inhibited in GFP-Id1-b overexpressed group compared to GFP overexpressed control group. (B) Collagen gel contraction assay. Collagen gel containing fibroblasts were prepared, then adenovirus was added to the gel and incubated for 6 h. After replenishing with fresh medium, TGF-β was treated and incubated for the indicated time points. In GFP-Id1-b overexpressed group, TGF-β-driven gel contraction was significantly inhibited compared to control group. Lower graph shows the results measured from three independent experiments (±SD). $^{*}P < 0.05$.

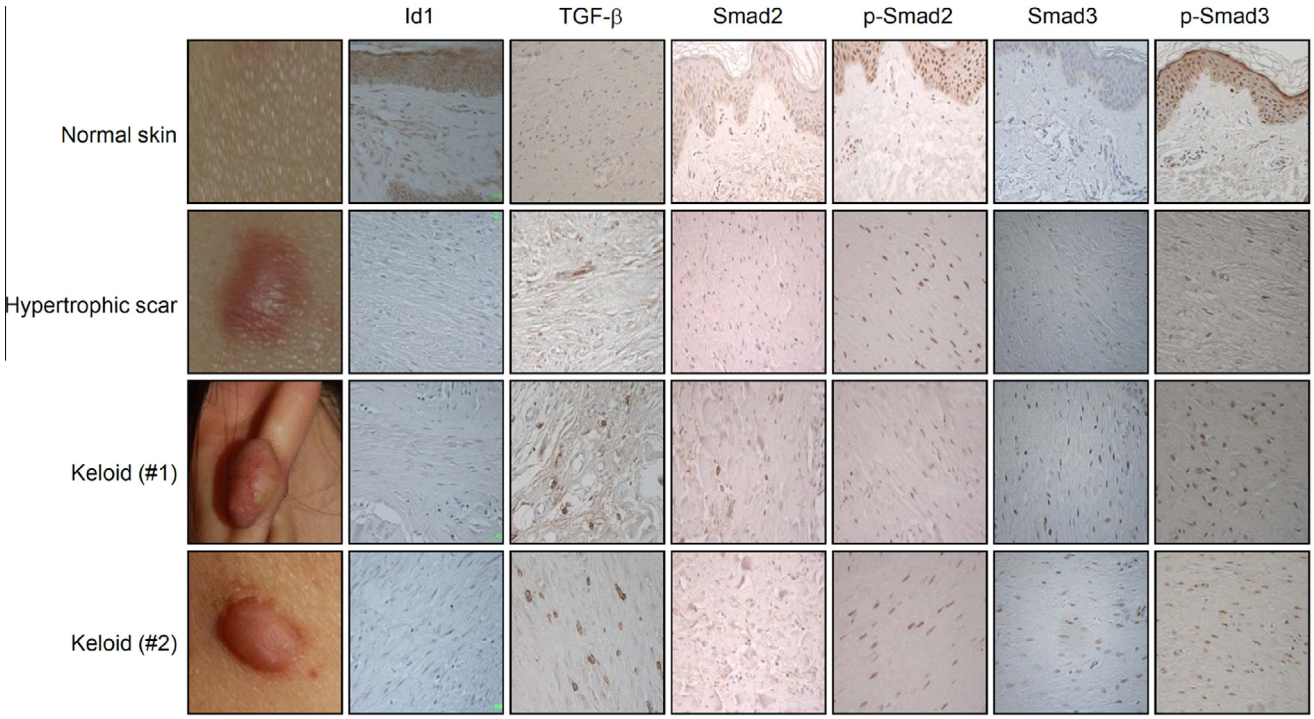

Fig. 4. Expression of Id1 and TGF-β signaling molecules in fibrotic skin diseases. Paraffin sections were stained with indicated antibodies. Id1 was barely detected in fibrotic skin diseases such as hypertrophic scar and keloid, while TGF-β signaling molecules were increased in these skin diseases compared to normal skin.

Based on the fact that Id1 has an inhibitory role in TGF-β-induced collagen expression, we speculated that Id1 is implicated in some fibrotic skin diseases. To address this question, we performed immunohistochemistry analysis against fibrotic skin diseases such as hypertrophic scar and keloid. Positive staining for Id1 was readily observed in normal dermis, while it was barely detected in hypertrophic scar and keloid. Conversely, TGF-β and its downstream signaling players such as Smad2 and Smad3 were detected in these fibrotic skin diseases more intensively than normal skin dermis (Fig. 4, Suppl. Fig. 2). These results support the idea that Id1 exerts its action as an inhibitory regulator on TGF-β-induced collagen expression in skin dermal fibroblasts.

4. Discussion

The results presented here highlight the role of Id1 on TGF-β-induced collagen expression in human dermal fibroblasts. Specifically, we demonstrate that TGF-β decreases Id1 expression; overexpression of Id1-b inhibits the TGF-β-induced type I collagen expression; overexpression of Id1-b inhibits the TGF-β-induced phosphorylation of Smad2 and Smad3; and finally, Id1 expression is decreased in fibrotic skin diseases.

Id proteins have been known to exert their action as the dominant negative regulators on bHLH transcription factors, owing to their lack of DNA binding domain. In many cell types, Id proteins inhibit the expression of differentiation-related genes and enhance cell proliferation [18]. In addition to their nuclear roles, it has been also demonstrated that Id proteins can bind to cytoplasmic and membrane proteins, exerting their specified roles in a context-dependent manner. For example, Id1 binds to the estrogen receptor β1 (ERβ1) in the cytoplasm and participates in ERβ1-mediated growth inhibition of breast cancer cells [19]. In other example, Id1 binds to the membrane protein caveolin-1, and this interaction promotes Akt activation in prostate cancer cells [10].

In our study, overexpression of Id1-b significantly inhibited the TGF-β-induced type I collagen expression, concurrently with marked inhibition of phosphorylation of Smad2 and 3. These results suggest that inhibitory role of Id1 is likely via the inhibition of intracellular signaling, not by the direct nuclear effect of Id1. This speculation is partly supported by the fact that exogenously expressed GFP-Id1-b was localized in cytoplasm predominantly rather than nucleus, in both the absence and/or presence of TGF-β (Suppl. Fig. 3). The precise mechanism underlying inhibition of Smad2 and 3 activation by Id1 remains to be determined. Interestingly, as mentioned before, previous study indicates that caveolin-1 is a binding partner for Id1 [10]. And it has been demonstrated that caveolin-1 attenuates TGF-β1 signaling by directly interacting with the TGF-β receptor. That is, caveolin-1 interacts with TGF-β type I receptor (TGFβRI) and its downstream effector Smad2, leading to the sequestration of TGFβRI and prevention of Smad phosphoryltion [20]. Additionally, caveolin-1-dependet internalization of TGF-β1 and TGFβRI has also been demonstrated in alveolar epithelial cells [21]. Thus, one possible hypothesis underlying the link between Id1 and TGF-β signaling is that Id1 binds to caveolin1, which facilitates the internalization of TGFβRI and inhibition of Smad activation. Elucidation of crosstalk between molecular players in this setting will be an interesting further study.

In our immunohistochemistry analysis, TGF-β and its downstream Smad signaling was activated in fibrotic skin diseases such as hypertrophic scar and keloid, well consistent with previous reports [22,23]. Interestingly, in many cases of fibrotic skin diseases (4 out of 5 cases), Id1 expression was hardly detected. The inverse correlation between Id1 and TGF-β signaling, if not all, suggests that Id1 is an actual player balancing the TGF-β signaling in skin fibroblasts (Suppl. Fig. 2). The direct causative relationship between Id1 and fibrotic skin diseases should be investigated further.

In summary, we demonstrate that Id1 inhibits the TGF-β-induced type I collagen expression via inhibition of Smad signaling. Our findings provide new insights into the link between Id1 and TGF-β-induced collagen expression, and may help to develop new target for fibrotic skin diseases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.01.010.

References

[1] S. Dennler, M.J. Goumans, P. ten Dijke, Transforming growth factor β signal transduction, J. Leukoc. Biol. 71 (2002) 731–740.
[2] F. Verrecchia, A. Mauviel, Transforming growth factor-β signaling through the Smad pathway: role in extracellular matrix gene expression and regulation, J. Invest. Dermatol. 118 (2002) 211–215.
[3] P. Schmid, P. Itin, G. Cherry, C. Bi, D.A. Cox, Enhanced expression of transforming growth factor-β type I and type II receptors in wound granulation tissue and hypertrophic scar, Am. J. Pathol. 152 (1998) 485–493.
[4] A. Leask, D.J. Abraham, TGF-β signaling and the fibrotic response, FASEB J. 18 (2004) 816–827.
[5] D. Wolfram, A. Tzankov, P. Pülzl, H. Piza-Katzer, Hypertrophic scars and keloids-a review of their pathophysiology, risk factors, and therapeutic management, Dermatol. Surg. 35 (2009) 171–181.
[6] J.D. Norton, ID helix-loop-helix proteins in cell growth, differentiation and tumorigenesis, J. Cell Sci. 113 (2000) 3897–3905.
[7] J. Perk, A. Iavarone, R. Benezra, Id family of helix-loop-helix proteins in cancer, Nat. Rev. Cancer 5 (2005) 603–614.
[8] A. Iavarone, P. Garg, A. Lasorella, J. Hsu, M.A. Israel, The helix-loop-helix protein Id-2 enhances cell proliferation and binds to the retinoblastoma protein, Genes Dev. 8 (1994) 1270–1284.
[9] A. Lasorella, M. Noseda, M. Beyna, Y. Yokota, A. Iavarone, Id2 is a retinoblastoma protein target and mediates signalling by Myc oncoproteins, Nature 407 (2000) 592–598.
[10] X. Zhang, M.T. Ling, Q. Wang, C.K. Lau, S.C. Leung, T.K. Lee, A.L. Cheung, Y.C. Wong, X. Wang, Identification of a novel inhibitor of differentiation-1 (ID-1) binding partner, caveolin-1, and its role in epithelial-mesenchymal transition and resistance to apoptosis in prostate cancer cells, J. Biol. Chem. 282 (2007) 33284–33294.
[11] Y. Kang, C.R. Chen, J. Massagué, A self-enabling TGFβ response coupled to stress signaling: Smad engages stress response factor ATF3 for Id1 repression in epithelial cells, Mol. Cell 11 (2003) 915–926.
[12] D. James, H.S. Nam, M. Seandel, D. Nolan, T. Janovitz, M. Tomishima, L. Studer, G. Lee, D. Lyden, R. Benezra, N. Zaninovic, Z. Rosenwaks, S.Y. Rabbany, S. Rafii, Expansion and maintenance of human embryonic stem cell-derived endothelial cells by TGFβ inhibition is Id1 dependent, Nat. Biotechnol. 28 (2010) 161–166.
[13] K. Di, M.T. Ling, S.W. Tsao, Y.C. Wong, X. Wang, Id-1 modulates senescence and TGF-β1 sensitivity in prostate epithelial cells, Biol. Cell 98 (2006) 523–533.
[14] Y. Cao, X. Liu, W. Zhang, X. Deng, H. Zhang, Y. Liu, L. Chen, E.A. Thompson, C.M. Townsend Jr, T.C. Ko, TGF-β repression of Id2 induces apoptosis in gut epithelial cells, Oncogene 28 (2009) 1089–1098.
[15] J.S. Lee, D.H. Kim, D.K. Choi, C.D. Kim, G.B. Ahn, T.Y. Yoon, J.H. Lee, J.Y. Lee, Comparison of gene expression profiles between keratinocytes, melanocytes and fibroblasts, Ann. Dermatol. 25 (2013) 36–45.
[16] F. Verrecchia, M.L. Chu, A. Mauviel, Identification of novel TGF-β/Smad gene targets in dermal fibroblasts using a combined cDNA microarray/promoter transactivation approach, J. Biol. Chem. 276 (2001) 17058–17062.
[17] P. Ngo, P. Ramalingam, J.A. Phillips, G.T. Furuta, Collagen gel contraction assay, Methods Mol. Biol. 341 (2006) 103–109.
[18] X.H. Sun, N.G. Copeland, N.A. Jenkins, D. Baltimore, Id proteins Id1 and Id2 selectively inhibit DNA binding by one class of helix-loop-helix proteins, Mol. Cell. Biol. 11 (1991) 5603–5611.
[19] L. Chen, J. Qiu, C. Yang, X. Yang, X. Chen, J. Jiang, X. Luo, Identification of a novel estrogen receptor β1 binding partner, inhibitor of differentiation-1, and role of ERβ1 in human breast cancer cells, Cancer Lett. 278 (2009) 210–219.
[20] B. Razani, X.L. Zhang, M. Bitzer, G. von Gersdorff, E.P. Böttinger, M.P. Lisanti, Caveolin-1 regulates transforming growth factor (TGF)-β/SMAD signaling through an interaction with the TGF-β type I receptor, J. Biol. Chem. 276 (2001) 6727–6738.
[21] Y. Shi, B.R. Gochuico, G. Yu, X. Tang, J.C. Osorio, I.E. Fernandez, C.F. Risquez, A.S. Patel, Y. Shi, M.G. Wathelet, A.J. Goodwin, J.A. Haspel, S.W. Ryter, E.M. Billings, N. Kaminski, D. Morse, I.O. Rosas, Syndecan-2 exerts antifibrotic effects by promoting caveolin-1-mediated transforming growth factor-β receptor I internalization and inhibiting transforming growth factor-β1 signaling, Am. J. Respir. Crit. Care Med. 188 (2013) 831–841.
[22] G.S. Chin, W. Liu, Z. Peled, T.Y. Lee, D.S. Steinbrech, M. Hsu, M.T. Longaker, Differential expression of transforming growth factor-β receptors I and II and activation of Smad3 in keloid fibroblasts, Plast. Reconstr. Surg. 108 (2001) 423–429.
[23] A.B. Campaner, L.M. Ferreira, A. Gragnani, J.M. Bruder, J.L. Cusick, J.R. Morgan, Upregulation of TGF-β1 expression may be necessary but is not sufficient for excessive scarring, J. Invest. Dermatol. 126 (2006) 1168–1176.