The integrin $\alpha_V\beta_6$ binds and activates latent TGFβ3

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Abstract Transforming growth factors-β (TGFβ1, 2 and 3) are secreted in a complex with their propeptides (latency-associated peptide 1 (LAP1), 2 and 3). TGFβ signaling requires the dissociation of LAP and TGFβ, a process termed latent TGFβ activation. This process is a critical but incompletely understood step in the regulation of TGFβ function. In particular, the extent to which activation mechanisms differ among the three TGFβ isoforms is relatively unexplored. We show here that $\alpha_V\beta_6$ binds and activates latent TGFβ3.

Key words: Transforming growth factor-β; Integrin; Activation; Ligand; Latency-associated peptide; Isoform

1. Introduction

Transforming growth factor-βs (TGFβ) are pleiotropic growth factors that regulate cell differentiation, cell division, immune function and extracellular matrix production [1]. The three mammalian TGFβ isoforms (TGFβ1, 2 and 3) are secreted as homodimeric proteins derived from the carboxy-termini of pro-TGFβ dimers [2]. The remnant amino-terminal dimer is named the latency-associated peptide (LAP) because it remains non-covalently associated with TGFβ and prevents TGFβ from binding TGFβ receptors [3]. Activation of latent TGFβ requires the dissociation of LAP and TGFβ. The latent TGFβ complex also includes a third protein, latent TGFβ-binding protein, which is linked by disulfide bonds to LAP.

There are several candidate mechanisms for converting latent TGFβ to the active form [4], but our understanding of this process is rudimentary. Defining the mechanisms by which the various TGFβ isoforms are activated would enhance our understanding of their specific functions, but so far most work has focused on TGFβ1. The integrin $\alpha_V\beta_6$ is a recently identified activator of latent TGFβ1 [5]. $\alpha_V\beta_6$ binds the RGD sequence in LAP1 and activates TGFβ1, apparently by causing a conformational change in LAP1 [5]. $\alpha_V\beta_6$ is expressed exclusively in epithelial cells [6]. Mice lacking the β_6 gene have inflammation in the lung and skin, but do not develop lung fibrosis after exposure to bleomycin [5,7]. In bleomycin-treated mice, the majority of TGFβ-responsive genes upregulated in control mice are not increased in β_6-null animals [8], suggesting that the protection of β_6-null mice is due to decreased TGFβ activity.

Interestingly, LAP3 and LAP1 contain RGD sequences at comparable locations, suggesting that TGFβ3 activity is also modulated by RGD-binding integrin(s). However, the sequence amino-terminal to the RGD differs between LAP3 and LAP1, indicating that LAP1 and LAP3 might be ligands for different integrins. Furthermore, the phenotype of β_6 integrin null mice does not suggest a deficit of TGFβ3 activity, as there is no phenotypic overlap between $\beta_6^{-/-}$ and TGFβ3$^{-/-}$ mice. (In contrast, $\beta_6^{-/-}$ and TGFβ1$^{-/-}$ mice both develop inflammation.) Nevertheless, we tested whether LAP3 is a ligand for $\alpha_V\beta_6$, and whether cells expressing $\alpha_V\beta_6$ can activate latent TGFβ3 since this knowledge is important for understanding the biologic functions of both TGFβ3 and the β_6 integrin.

2. Materials and methods

2.1. Cell culture, antibodies and reagents

HT-1080 cells were from the American Type Tissue Culture Collection (Manassas, VA, USA), 293T cells were from David Ron (NYU, New York, NY, USA), Vector-transfected and β_6-integrin-transfected 293 cells and SW480 cells were from Dean Sheppard (UCSF, San Francisco, CA, USA) [9]. Vector-transfected and β_6-integrin-transfected HT-1080 cells were generated as described in Weinacker et al. [9]. TGFβ1$^{-/-}$ liver fibroblast cells from Anita Roberts (NIH, Bethesda, MD, USA) were stably transfected with either pCDNA3.1/Hygro(-) (TGFβ1$^{-/-}$ cells) (Invitrogen; Carlsbad, CA, USA) or pCDNA3.1/Hygro(-) containing the β_6 cDNA (TGFβ1$^{-/-}$/β_6 cells), and cloned by limiting dilution. Transfected mink lung epithelial cells (TMLC), which produce luciferase in response to TGFβ, were as described [10]. Mouse anti-$\alpha_V\beta_6$ Mab 10D5 [11] was a gift of Dean Sheppard. MAb 1D11 against active TGFβ (all isoforms), anti-TGFβ1 (AF-101-NA), anti-TGFβ2 (AB-112-NA) and anti-TGFβ3 (AB-244-NA) were from R&D Systems (Minneapolis, MN, USA). GM6001 was from Calbiochem. Other reagents were from Roche Diagnostics Corporation (Indianapolis, IN, USA).

2.2. TGFβ bioassays

To measure activation of endogenous TGFβ3, we plated TGFβ1$^{-/-}$/β_6 cells (8×10^4) or TGFβ1$^{-/-}$ cells in 35-mm wells in Dulbecco's modified Eagle's medium (DMEM)/0.1% bovine serum albumin (BSA). After 16 h, the cells were trypsinized and replated (1.5×10^4 per well) in 50 μl of DMEM/0.1% BSA in 96-well plates. TMLC (2.5×10^4), suspended in DMEM/0.1% BSA, were added to the test cells in an equal volume. When appropriate, anti-TGFβ1 (1 μg/ml), anti-TGFβ2 (10 μg/ml), anti-TGFβ3 (25 μg/ml), 10D5 (20 μg/ml), 1D11 (25 μg/ml) or LAP (100 ng/ml) was added. Cell lysates and total TGFβ in conditioned media were assayed as described [10,12]. All experiments were done in duplicate and repeated three times with similar results. The data presented are the mean and the standard error of the mean of a single experiment.

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Abbreviations: TGFβ, transforming growth factor-β; LAP, latency-associated peptide

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To measure activation of TGFβ after transfection of TGFβ1$^{-/-}$ and TGFβ1$^{-/-}$/β$_6$ cells with various TGFβ cDNAs, we plated cells at 8×10^4 cells per 35-mm well in DMEM/fetal calf serum (FCS). After 16 h, cells were transfected with 400 ng per well using Lipofectamine Plus (Life Technologies; Grand Island, NY, USA) per the manufacturer's protocol. After 16 h the cells were collected in 3 ml of DMEM/10% FCS and replated in 96-well and 24-well plates (50 or 500 μl per well, respectively). 96-well plates were used to measure TGFβ activation, and 24-well plates were used to measure total (latent plus active) TGFβ secretion, as follows. After 4 h, the media were replaced with equal volumes of DMEM/0.1% BSA. TMLC (2×10^4 cells/well) were added to the 96-well plates (final volume, 100 μl per well). Additional reagents were added as appropriate. Conditioned media were generated in the 24-well plate mono-cultures. After 16–24 h, TGFβ activation was assessed by measuring luciferase activity in the cell lysates from co-culture wells. Also after 16–24 h, total secreted TGFβ was measured by activating latent TGFβ in media from mono-culture wells (80°C for 10 min). These samples were incubated with TMLC overnight and luciferase activity measured [10]. Experiments were repeated four times with similar results. Error bars show the standard deviation of a single experiment.

2.3. Constructs

Mouse TGFβ1 was obtained from G.J. Thorbecke (NYU, New York, NY, USA). Human TGFβ3 and human TGFβ2 pRK5 expression vectors, from R. Derynck (UCSF, San Francisco, CA, USA), were transferred into pCDNA3.1/Zeo(+) (Invitrogen; Carlsbad, CA, USA). The cDNA sequences encoding LAP2 and LAP3 (*without* the 3′-TGFβ sequence) were amplified by polymerase chain reaction using the above cDNAs as templates, cloned into the pCDNA-Fc vector (gift of Carl Blobel, Memorial Sloan-Kettering Cancer Center, New York, NY, USA) and used for protein production and purification. A Factor Xa protease site was inserted between the last amino acid of LAP2 or LAP3 and the first amino acid of the Fc. To maximize LAP expression, cysteine 27 was mutated to serine in the LAP3 sequence and cysteine 24 was mutated to serine in the LAP2 sequence (GeneEditor, Promega; Madison, WI, USA). Mutant versions of mTGFβ1 (D246E), hTGFβ3 (D263E), and hLAP3 C27S (D263E), in which the RGD amino acid motif is changed to RGE, were made in the same way. All cDNAs were sequenced prior to use.

2.4. Production and purification of LAPs

Recombinant LAP1 was produced using a baculovirus system [13]. A related procedure was used to purify LAP2, LAP3 and LAP3-RGE, as follows. 293T cells were transfected with the appropriate LAP-Fc construct using Lipofectamine Plus. Conditioned media were collected 72 h post-transfection. LAP-Fc fusion proteins were removed from the media with protein-A agarose. LAPs were released by digestion with Factor Xa, which was removed with soybean trypsin inhibitor beads (Sigma). We confirmed protein purity by silver stain. Adhesion assays were carried out as described [13]. Results are expressed as absorbance or as a percentage where absorbance of serum-coated and BSA-coated wells is 100 and 0%, respectively.

3. Results

3.1. TGFβ3 LAP is a ligand for the integrin $\alpha_V\beta_6$

We recently showed that the propeptide of TGFβ1 (LAP1) is a ligand for the integrins $\alpha_V\beta_1$, $\alpha_V\beta_5$, and $\alpha_V\beta_6$ [13]. To determine whether LAP3 is also a ligand for $\alpha_V\beta_6$, we tested the ability of $\alpha_V\beta_6$-expressing cells to adhere to recombinant LAP1, LAP3 and LAP3-RGE. Four different cell types stably transfected with either vector alone or an expression vector containing the β$_6$ integrin cDNA were allowed to adhere to protein-coated wells (Fig. 1). In β$_6$-expressing cells, the β$_6$ subunit pairs with endogenous α_V subunit to produce the $\alpha_V\beta_6$ integrin. $\alpha_V\beta_6$-expressing cells adhered equally well to LAP1 and LAP3 (Fig. 1). Mock-transfected cells did not adhere to either LAP. Adhesion of $\alpha_V\beta_6$-expressing cells to LAP3 requires the RGD sequence as cells did not attach to LAP3 lacking the RGD sequence (LAP3-RGE) (Fig. 1).

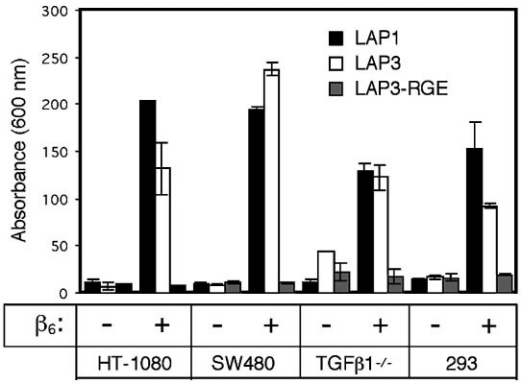

Fig. 1. Cells transfected with the β$_6$ integrin adhere to LAP1 and LAP3 in an RGD-dependent fashion. Wells were coated with LAP1, LAP3 or LAP3-RGE. HT-1080, SW480, TGFβ1$^{-/-}$ and 293 cells, either stably transfected with a β$_6$ integrin cDNA or a control vector, were allowed to adhere to the coated wells.

3.2. LAP1 and LAP3 support adhesion with similar efficacy

The integrin $\alpha_V\beta_6$ binds LAP1 present in the latent TGFβ complex and generates active TGFβ1 [5], while another LAP1-binding integrin, $\alpha_V\beta_1$, does not activate TGFβ1 [13]. In adhesion assays, cells expressing $\alpha_V\beta_6$ can attach to LAP1 at coating concentrations that are 10-fold lower than the those needed for attachment of $\alpha_V\beta_1$-expressing cells [5,13]. We compared the abilities of LAP1, LAP2 and LAP3 to promote $\alpha_V\beta_6$-mediated adhesion at various coating concentrations. As shown in Fig. 2A, SW480/β$_6$ cells adhered to LAP1 and LAP3 with similar dose-response. More than 50% of the cells adhered to coating concentrations of less than 1 μg/ml of protein, whereas in our prior work with $\alpha_V\beta_1$, no adhesion occurred at 1 μg/ml [13]. The specificity of the adhesion shown in Fig. 2A is demonstrated by the lack of adhesion to LAP3-RGE or LAP2. Also, the anti-$\alpha_V\beta_6$ antibody 10D5 blocked cell adhesion to LAP3 (Fig. 2B).

3.3. TGFβ1 knockout cells expressing $\alpha_V\beta_6$ activate endogenous TGFβ3

We previously found that the most sensitive way to detect $\alpha_V\beta_6$-mediated activation of TGFβ is to co-culture test cells with TGFβ-responsive reporter cells. To determine if $\alpha_V\beta_6$ activates latent TGFβ3, we sought a cell system in which TGFβ3 but not TGFβ1 is expressed to eliminate a back-

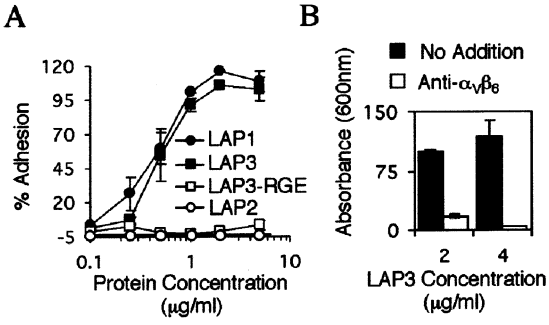

Fig. 2. LAP1 and LAP3 support $\alpha_V\beta_6$-dependent adhesion with similar efficacy. A: SW480 cells, stably transfected with a β$_6$ integrin cDNA, were allowed to adhere to wells coated with different concentrations of LAP1, LAP3, LAP3-RGE or LAP2. B: SW480/β$_6$ cells, in the absence or presence of an $\alpha_V\beta_6$-blocking antibody, were allowed to adhere to wells coated with LAP3.

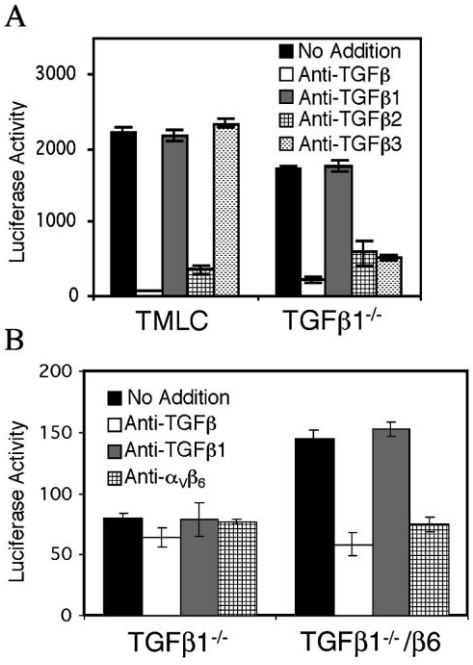

Fig. 3. TGFβ1$^{-/-}$ cells that express the integrin $\alpha_V\beta_6$ activate a latent TGFβ isoform other than TGFβ1. A: Serum-free media were conditioned by TGFβ reporter cells (TMLC) or TGFβ1$^{-/-}$ cells. Media were collected, heated to 80°C for 10 min to activate latent TGFβ, and incubated overnight with TGFβ reporter cells (TMLC). Antibodies were added to inhibit the activity of specific TGFβ isoforms. Luciferase activity indicates the amount of active TGFβ in the sample. B: TGFβ1$^{-/-}$ cells or TGFβ1$^{-/-}$/β_6 cells were co-cultured for 16–20 h with reporter cells in the presence of various antibodies. Luciferase activity indicates the amount of TGFβ activated in the culture.

ground signal of TGFβ1 activation. We selected a TGFβ1-null fibroblast line that secretes latent TGFβ2 and TGFβ3 (Fig. 3A, right) and stably transfected them with empty expression vector (TGFβ1$^{-/-}$ cells) or vector encoding β_6 integrin (TGFβ1$^{-/-}$/β_6 cells). The TMLC reporter cells [10] secrete only the TGFβ2 isoform (Fig. 3A, left). Analysis of medium from co-cultures of TGFβ1$^{-/-}$ cells and TMLC revealed only TGFβ2 and TGFβ3 (not shown), indicating that co-culture conditions do not induce TGFβ1 expression by TMLC.

Co-culture experiments indicated that TGFβ1$^{-/-}$ cells expressing $\alpha_V\beta_6$ activate endogenous latent TGFβ3. TMLC cultured with TGFβ1$^{-/-}$/β_6 cells had increased luciferase levels (indicating the presence of active TGFβ) compared to TMLC cultured with TGFβ1$^{-/-}$ cells (Fig. 3B). This increase did not occur when either an $\alpha_V\beta_6$-specific blocking antibody or a monoclonal antibody that inhibits all three TGFβs was added. The addition of isoform-specific antibody against TGFβ1 to the co-culture did not affect the luciferase activity induced by TGFβ1$^{-/-}$/β_6 cells (Fig. 3B), although this antibody blocks activation of TGFβ1 by other β_6-expressing cells [5]. These results suggest that the TGFβ1$^{-/-}$/β_6 fibroblasts activate a TGFβ isoform other than TGFβ1. Thus, these experiments strongly imply, but do not directly demonstrate, that $\alpha_V\beta_6$ activates latent TGFβ3.

3.4. The integrin $\alpha_V\beta_6$ activates transfected latent TGFβ3

To demonstrate conclusively that TGFβ3 is activated by $\alpha_V\beta_6$, we modified our assay in order to test specific TGFβ isoforms and mutants thereof. We transfected TGFβ1$^{-/-}$ cells or TGFβ1$^{-/-}$/β_6 cells with an expression vector (empty or containing TGFβ1, TGFβ3, TGFβ1-RGE, TGFβ3-RGE, or TGFβ2 cDNAs) and cultured these cells with TGFβ reporter cells (Fig. 4A). When $\alpha_V\beta_6$-expressing cells were transfected with TGFβ1 or TGFβ3 cDNA, active TGFβ was generated as indicated by an increase in luciferase activity. No active TGFβ was detected when non-$\alpha_V\beta_6$-expressing cells were similarly transfected. No increase of luciferase activity above the β_6-dependent activation of endogenous TGFβ was seen when the vector control, TGFβ1-RGE, TGFβ3-RGE or the TGFβ2 cDNAs were used for transfection (Fig. 4A). Therefore, TGFβ1 and TGFβ3 activation by $\alpha_V\beta_6$ require the RGD sequence in LAP. Addition of protease inhibitors (aprotinin, leupeptin, and the matrix metalloproteinase (MMP) inhibitor GM6001) to the cultures did not decrease $\alpha_V\beta_6$-mediated activation (data not shown); these results agree with previous studies of $\alpha_V\beta_6$-mediated activation of TGFβ1 [5].

To confirm that transfection efficiencies were similar, we measured total TGFβ levels in conditioned media from the

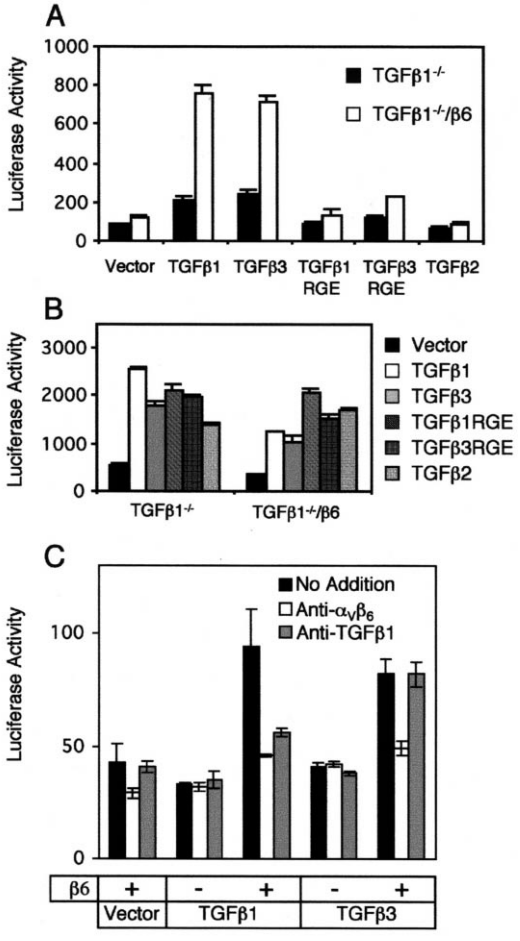

Fig. 4. Cells expressing the integrin $\alpha_V\beta_6$ activate latent TGFβ3 in an RGD-dependent manner. A: TGFβ1$^{-/-}$ cells or TGFβ1$^{-/-}$/β_6 cells were transfected with an expression vector (no insert, TGFβ, TGFβ3, TGFβ1-RGE, TGFβ3-RGE or TGFβ2). Transfected cells were co-cultured with TGFβ reporter cells (see Section 2). B: The relative amounts of total TGFβ (active and latent) secreted by the transfected cells were determined as in Fig. 3A. C: TGFβ1$^{-/-}$ cells or TGFβ1$^{-/-}$/β_6 cells were transfected with an expression vector (no insert, TGFβ1 or TGFβ3), then co-cultured with TGFβ reporter cells. Anti-$\alpha_V\beta_6$ or anti-TGFβ1 were added as indicated.

various TGFβ-transfected cells (Fig. 4B). TGFβ activity produced by TGFβ1$^{-/-}$/β_6 cells transfected with TGFβ1 or TGFβ3 cDNA is prevented by the addition of an antibody against $\alpha_V\beta_6$ (Fig. 4C). Also, a TGFβ1-specific antibody prevented the induction of luciferase activity observed when TGFβ1$^{-/-}$/β_6 cells are transfected with TGFβ1 but not when transfected with TGFβ3 (Fig. 4C).

4. Discussion

Activation of latent TGFβ is a key regulatory step in TGFβ action. The extent to which activation mechanisms vary among the three TGFβ isoforms is not known, but significant differences appear to be possible because of sequence differences among the LAPs. For instance, LAP1 and LAP3, but not LAP2, contain the integrin recognition sequence RGD, suggesting that TGFβ1 and TGFβ3 may be regulated by RGD-binding integrins. TGFβ1 can be activated by $\alpha_V\beta_6$ [5] and $\alpha_V\beta_8$ (S. Nishimura, personal communication). In contrast, cells expressing $\alpha_V\beta_1$ and $\alpha_5\beta_1$ can bind LAP1 but do not activate TGFβ, and cells transfected to express $\alpha_V\beta_3$, $\alpha_{IIb}\beta_3$, or $\alpha_8\beta_1$ do not activate endogenous TGFβ (unpublished data). To investigate the effect of integrins on TGFβ3 function, we tested whether LAP3 is a ligand for $\alpha_V\beta_6$ and whether TGFβ3 is activated by $\alpha_V\beta_6$. We identified LAP3 as a ligand for the integrin $\alpha_V\beta_6$ and demonstrated that $\alpha_V\beta_6$ can activate latent TGFβ3 but not TGFβ2.

LAP1 is clearly a physiologic ligand for $\alpha_V\beta_6$ and appears to promote adhesion in vitro more effectively than do other $\alpha_V\beta_6$ ligands [14]. Our result that LAP1 and LAP3 are equally effective ligands for $\alpha V\beta_6$ suggests that LAP3 is also a physiologic ligand.

Little is known about TGFβ3 activation. MMP-2, -3 and -9 can activate all three isoforms of TGFβ [15], and to our knowledge this is the only prior report of TGFβ3 activation. Thrombospondin-1 (TSP1) activates TGFβ1 and TGFβ2, and is predicted to activate TGFβ3, but experiments with TGFβ3 have not been published [16]. Plasmin is perhaps the most studied TGFβ activator, but no published evidence addresses the ability of plasmin to activate TGFβ2 or TGFβ3.

TGFβ1-null mice die of inflammation beginning around 3 weeks of age [17]. The only reported defects in TGFβ3-null mice are cleft palate and abnormal pulmonary development [18]. Presumably, TGFβ3 activity during palatal fusion and lung morphogenesis requires a TGFβ3 activator. Because the phenotypes of mice deficient for β_6 integrin, TSP1, plasminogen, or MMP-2, -3, -9 do not show these defects, none of them is likely to be a unique TGFβ3 activator in palatal fusion or lung morphogenesis. Thus, these processes involve redundant latent TGFβ3 activators or an undiscovered unique activator.

Undoubtedly, TGFβ3 plays roles not revealed by TGFβ3-null mice. One such role might be to regulate inflammation, as TGFβ1 does. Because TGFβ3-null animals die perinatally, it is not known if these mice would develop postnatal inflammation. β_6-null mice have lung and skin inflammation, and are resistant to bleomycin-induced pulmonary fibrosis [7]. Based upon the significant expression of TGFβ3 in the skin and lung and the nearly identical in vitro functions of TGFβ1 and TGFβ3, it is possible that the phenotype of $\beta_6^{-/-}$ animals is due to a combined lack of TGFβ1 and TGFβ3 activities. It will be interesting to examine other epithelial processes where TGFβ3 is involved. For instance, TGFβ3 influences spermatogenesis in the epididymis [19] and mammary gland involution in the breast [20]. In both organs, TGFβ3 and the β_6-integrin are expressed in the same cell types [6].

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