Adenoviral gene transfer of BMP-7, Id2, or Id3 suppresses injury-induced epithelial-to-mesenchymal transition of lens epithilium in mice

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Saika, Shizuya, Kazuo Ikeda, Osamu Yamanaka, Kathleen C. Flanders, Yoshitaka Ohnishi, Yuji Nakajima, Yasuteru Muragaki, and Akira Ooshima. Adenoviral gene transfer of BMP-7, Id2, or Id3 suppresses injury-induced epithelial-mesenchymal transition of lens epithilium in mice. Am J Physiol Cell Physiol 290: C282–C289, 2006. First published August 24, 2005; doi:10.1152/ajpcell.00306.2005.—We have examined the effect of adenovirus-mediated expression of bone morphogenetic protein-7 (BMP-7) and inhibitors of differentiation 2 and 3 (Id2 and Id3) on injury-induced epithelial-to-mesenchymal transition (EMT) of lens epithilium in mice. Id2 and Id3 are known to be upregulated by BMP-7 and to antagonize Smad2/3 signaling. The Cre-LoxP system adenoviral gene transfer was used. Three microliters of adenoviral solution (2 × 10^10 PFU/μl) were injected into the right lens of adult male C57BL/6 mice (n = 144) at the time of capsular incision using a hypodermic needle under both general and topical anesthesia. A mixture of Cre-adenovirus (Cre-Ad) and vector encoding mBMP-7, mId2, or mId3 was administered in a test group. Control lenses were treated with Cre-Ad alone. After healing intervals of 5 or 10 days, the animals were killed and then we performed histological processes or RNA extraction from the lens. RT-PCR, real-time RT-PCR, and immunohistochemistry showed expression of each introduced gene in the lens. Exogenous BMP-7 upregulated expression of Id2 and Id3 in injured lenses, and gene introduction of Id2 or Id3 also upregulated BMP-7 expression. Gene transfer of BMP-7, Id2, or Id3 delayed injury-induced EMT of the lens epithelial cells as evaluated by histology and expression patterns of α-smooth muscle actin and collagens in association with reduction of Smad2 COOH-terminal phosphorylation. Gene transfer of BMP-7, Id2, or Id3 delayed injury-induced EMT of lens epithelial cells and subsequent sealing of the capsular break with fibrous tissue in mice.}

Materials and Methods

All experimental procedures were approved by both the DNA Recombination Experiment Committee and the Animal Care and Use Committee of Wakayama Medical University, Wakayama, Japan, and were conducted in accordance with the Association for Research in Vision and Ophthalmic and Vision Research Statement for the Use of Animals in Ophthalmic and Vision Research.

Adenovirus vector construction and virus purification. We used the Adenovirus Cre/LoxP-Regulated Expression Vector Set (no. 6151; TaKaRa, Tokyo, Japan) to generate recombinant adenovirus. Cosmid pAxCALNLmBMP-7, pAxCALNLId2, or pAxCALNLmId3 was constructed by inserting mouse cDNA of BMP-7, Id2, or Id3 into the SmaI cloning site of pAxCALNLw (37). Using the COS-TPC method (21), we generated the recombinant adenovirus of each gene by

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transfected human embryonic kidney (HEK)-293 cells with the pAxCALNLmBMP-7, pAxCALNLmId2, or pAxCALNLmId3. AxCALNCre was generated by transfecting HEK-293 cells with AxCALNLCreDNA-TPC as described in the manufacturer’s protocol. The titer of recombinant adenoviruses was measured using the 50% tissue culture infectious dose method (14). Each adenovector was used at the concentration of 2 × 10⁷ plaque-forming units (PFU)/μl. When these two viral vectors coinfected cells, Cre recombinase expressed under the CAG promoter (cytomegalovirus enhancer, chicken β-actin promoter, plus a part of 3′-untranslated region of rabbit β-globin) activates the stuffer poly(A)⁺ RNA through the Cre-LoxP system.

Experimental protocol. Evaluation of the efficacy of adenosiviral gene transfer into the mouse lens epithelium in vivo by coinfection of viruses carrying Cre recombinase under the CAG promoter and LNL-green fluorescent protein (GFP) or by infection of virus carrying GFP alone in an injured lens was reported previously (22). In these previous studies, we reported that Cre-LoxP system of adenosiviral gene introduction by TaKaRa was satisfactorily efficient in gene introduction to the mouse lens epithelium in vivo.

To induce transcription of BMP-7, Id2, or Id3, LNL-BMP-7, LNL-Id2, or LNL-Id3 and CAG-Cre vectors (total of 3 μl) were coinfected directly into the affected lens at the time of the formation of injury. Eight eyes of eight animals (2 Cre-Ad-treated, 2 BMP-7-Ad-treated, 2 BMP-7-Ad, or Id2-Ad, or Id3-Ad-treated, and 2 Id3-Ad-treated) were processed for cryosectioning at day 5. In brief, each enucleated eye was embedded in optimal cutting temperature compound, cut as a 7-μm section, and fixed in cold acetone.

**RNA extraction.** The mouse lens (n = 48) was injured and was treated with adenoviral vectors described above for extraction of RNA from the lens. The animals were killed, and their eyes were enucleated at days 5 and 10. Each group (BMP-7-Ad-, Id2-Ad-, or Id3-Ad-treated group) included six lenses at each time point. The crystalline lens was removed carefully without breaking the capsule in each eye under a binocular microscope and was processed for RNA extraction using the Sigma RNA extraction kit (GenElute Mammalian Total RNA Mini-Prep; Sigma-Aldrich, St. Louis, MO).

**RT-PCR.** To detect expression of the BMP-7 gene introduced by adenosiviral vector, we performed RT-PCR as previously reported (28).

**Real-time RT-PCR.** To detect expression of Id2 and Id3 genes either endogenously expressed or introduced by adenosiviral vector, we performed real-time RT-PCR. Extracted RNA was processed for semiquantitative real-time RT-PCR for mRNA of mouse Id2 or Id3 using the One-Step Cyber Green RT-PCR Master Mix Reagents Kit (TaKaRa) and Prism 7700 (Applied Biosystems, Foster City, CA). Primers and oligonucleotide probes were designed according to the cDNA sequences in the GenBank database using Primer Express software (Applied Biosystems) and are listed in Table 1. The RT-PCR reaction mixture contained 10 μl of 2× One Step SYBR RT-PCR buffer, 0.4 μl of TaKaRa Ex Taq Hot Start (5 U/μl), 0.2 μl of Moloney murine leukemia virus RNase (200 U/μl), 0.4 μl of RNase inhibitor (40 U/μl), 0.4 μl of forward primer (10 μM), 0.4 μl of reverse primer (10 μM), 0.4 μl of ROX reference dye (50×; Invitrogen, Carlsbad, CA), 100 ng of RNA, and distilled water to a final volume of 20 μl. The following RT-PCR conditions were used: 1 cycle of 42°C for 15 min, followed by 1 cycle of 95°C for 2 min, 40 cycles of 95°C for 15 s, 60°C for 1 min, and for the dissociation curve analysis, 1 cycle of 95°C for 15 s, 1 cycle of 60°C for 1 min, and 1 cycle of 95°C for 15 s.

**Immunohistochemistry.** Deparaffinized sections (5 μm thick) were allowed to react with primary antibodies appropriately diluted with PBS after being blocked with 5% dried milk and 5% serum as previously reported (33). After being washed in PBS, the specimens were treated with FITC-conjugated secondary antibodies. Antibodies against phosphorylated (Ser465/467) Smad2 (1:50 dilution; Chemicon International, Temecula, CA), α-smooth muscle actin (α-SMA, 1:200 dilution; NeoMarker, Fremont, CA), and collagen type VI (1:200 dilution; Southern Biotechnology, Birmingham, AL) were used as previously reported (16, 40). BrdU immunostaining was performed as previously described (16, 40). In brief, deparaffinized sections were treated with 2 N HCl for 1 h at 37°C and allowed to react with anti-BrdU antibody (1:11 dilution; Roche Diagnostics, Mannheim, Germany) and then with peroxidase-conjugated secondary antibody after being washed in PBS. After the color reaction with diaminobenzidine and methyl green counterstaining, the number of labeled cells in healing epithelium was determined. Six specimens were used to obtain the number of labeled cells in each group at days 5 and 10. As for the semiquanification of phosphorylased Smad2 in lens epithelium, five specimens were examined for each experimental condition and the number of cells with positive immunoreactivity with each antibody was determined in the specific field around the capsular break site (200 × 150 μm). Histology was observed by staining tissues with hematoxylin and eosin (HE).

**Fixed cryosections** were allowed to react with antibodies against BMP-7, Id2, or Id3 (each at 1:100 dilution in PBS; Santa Cruz Biotechnology, Santa Cruz, CA), phospho-Smad2/1/5/8 (each at 1:100 dilution in PBS; Cell Signaling, Beverly, MA) overnight at 4°C and then processed for FITC-conjugated secondary antibody as previously reported (32, 36). Negative control staining was performed by omission of each primary antibody.

**RESULTS**

Expression of BMP-7, Id2, or Id3 mRNA and protein in injured lens treated with adenosiviral gene transfer. RT-PCR detected endogenous BMP-7 gene expression in injured lens specimens infected with control Cre-Ad at day 5 but not at day 10 postinjury. The expression level was much higher in a BMP-7-Ad-treated lens than in a lens treated with Cre-Ad (Fig. 1A). Introduction of Id2 or Id3 genes also upregulated BMP-7 expression at days 5 and 10.

Figure 1, B and C, shows the relative expression level of mRNA of Id2 or Id3, respectively, in an injured lens at day 5.**
or day 10. Endogenous Id2 mRNA was upregulated 47-fold in a BMP-7-Ad-treated lens at day 5. The Id2 mRNA level in a lens treated with Id2-Ad at day 5 or day 10 was much higher compared with not only a control injured lens but also a lens treated with BMP-7-Ad, indicating that exogenous adenovirus-derived Id2 was expressed. Similar results are shown for Id3 (Fig. 1C).

Immunohistochemistry showed enhanced protein expression of BMP-7 (Fig. 2, A and D), Id2 (Fig. 2 B and E), or Id3 (Fig. 2, C and F) in specimens treated with the corresponding adenoviral vector compared with controls at day 5. Similar results were observed at day 10 (data not shown). Each gene product was highly expressed after exogenous introduction of each gene. Although BMP-7 gene introduction upregulated endogenous Id2 and Id3 mRNA, immunohistochemistry did not detect Id2 and Id3 proteins (data not shown).

Expression of phosphorylated Smads. BMP family members utilize Smad1/5/8 as their signaling transmitters. To examine whether the exogenously introduced BMP-7 activates Smad1/5/8 signal, we immunostained cryosections with anti-phospho-Smad1/5/8 antibody. At both day 5 (Fig. 3a) and day 10 (data not shown), phospho-Smad1/5/8 was not detected in control specimens (Fig. 3aA), whereas marked expression of phospho-Smad1/5/8 was observed in specimens treated with BMP-7 gene introduction (Fig. 3aB). Epithelial cells in specimens treated with Id2 (Fig. 3aC) or Id3 (Fig. 3aD) gene introduction were labeled faintly with anti-phospho-Smad1/5/8 antibody.

We then immunostained paraffin sections of tissue specimens with anti-COOH-terminal phospho-Smad2 (Ser465/467) antibody to determine the status of TGF-β/Smad signaling. The nuclei of lens epithelial cells adjacent to the capsular injury, which had undergone EMT, were well labeled with anti-phospho-Smad2 antibody at day 5 (Fig. 3aE). At day 10, expression of phospho-Smad2 was still detected, mainly in the cytoplasm but almost not at all in the nuclei (Fig. 3aI). On the other hand, the majority of lens cells in an eye treated with...
either BMP-7-Ad (Fig. 3, aF and aJ), Id2-Ad (Fig. 3, aG and aK), or Id3-Ad (Fig. 3, aH and aL) were negative for phospho-Smad2 at day 5. At day 10, phospho-Smad2-positive cells were observed in these three specimens, although the incidence of positive cells seemed less with BMP-7 gene introduction. Figure 3b shows the number of cells with nuclei stained for phospho-Smad2 adjacent to the capsular break. These findings indicate that gene introduction of BMP-7, Id2, or Id3 delayed TGF-β/Smad activation but that the mechanism of action by BMP-7 and Id might differ. Negative control staining did not yield any specific staining (data not shown).

Histology of injured lens epithelial cells and expression of EMT markers. HE staining showed that the capsular break was sealed by a plaque of elongated, fibroblast-like lens epithelial cells as early as day 5 (Fig. 4, A and A’) and remained at day 10 (Fig. 4, E and E’) in Cre-Ad-treated eyes, whereas the capsular opening was not closed at day 5 in an injured lens that had been treated with adenoviral gene transfer of BMP-7 (Fig. 4b), Id2 (Fig. 4c) or Id3 (Fig. 4d). Rather, these groups showed round or ovoid epithelium-like cells surrounding the break (Fig. 4, B’–E’). At day 10, lens cells adjacent to the capsular break exhibited an elongated fibroblast-like morphology, regardless of treatment (Fig. 4, E–H, E–H’). These histological findings suggest that gene transfer introduction of BMP-7, Id2, or Id3 might perturb injury-induced EMT of lens epithelial cells.

To test this hypothesis, we performed immunohistochemistry for the EMT markers α-SMA (Fig. 5) and collagen type VI (Fig. 6). At day 5, marked immunofluorescence for α-SMA was observed in multilayered cells in the control Cre-Ad-treated lens (Fig. 5a), with faint α-SMA staining detected in cells of a lens treated with BMP-7-Ad (Fig. 5b), Id2-Ad (Fig. 5c), or Id3-Ad (Fig. 5d). At day 10, faint immunoreactivity was detected in lens epithelial cells in the multilayer around the capsular break in BMP-7-Ad-treated group (Fig. 5f), compared with strong immunofluorescence observed in control Cre group cells (Fig. 5e). The cells accumulated around the capsular break in Id2-Ad-treated lenses (Fig. 4g) or Id3-Ad-treated lenses (Fig. 5h) also were labeled markedly with anti-α-SMA at day 10. The monolayer cells far outside the capsule aggregates that had been formed adjacent to the capsular break were not labeled with anti-α-SMA antibody as previously reported (16) (data not shown).

We next examined the expression of collagen type VI, a component of fibrotic tissue, at day 5 (Fig. 6, aα–aD) and day 10 (Fig. 6, aE–aH) postinjury. As expected, the results indicated that fibroblast-like lens epithelial cells were labeled with anti-collagen type VI in the control group (Fig. 5a) but not in the BMP-7-Ad (Fig. 6aB), Id2-Ad (Fig. 6aC), and Id3-Ad groups (Fig. 6aD). At day 10, the cell multilayer formed beneath the broken capsule was labeled with anti-collagen VI in each treatment group (Fig. 6, aE–aH). Real-time RT-PCR for collagen Iα2 mRNA was performed to examine further the effects of introduced genes on fibrous reactions by the lens epithelium EMT (Fig. 6b). At day 5, transfer of cDNA of BMP-7, Id2, or Id3 all suppressed collagen Iα2 mRNA expression by lens cells, whereas such inhibitory effects were observed only with BMP-7 gene introduction but not with Id2 or Id3 gene transfer. These findings indicate that gene introduction of BMP-7, Id2, or Id3 suppresses injury-induced lens cell EMT at day 5 but not at day 10. Negative control staining did not yield any specific staining (data not shown).

Epithelial cell proliferation. Previous reports showed that adenoviral gene transfer of Id2 or Id3 partially counteracted the proliferation-suppressing effect of TGF-β in vitro in epithelial cell lines but did not completely abolish it (32–34). We...
therefore examined the effect of gene transfer of BMP-7, Id2, or Id3 on cell proliferation of epithelial cells in an injured lens using BrdU labeling. The results showed that adenoviral gene introduction of BMP-7, Id2, or Id3 did not affect the incidence of BrdU-labeled cells in injured lenses (data not shown).

DISCUSSION

The present study has shown that adenoviral gene transfer of mBMP-7 cDNA delays injury-induced EMT in lens epithelial cells in vivo as demonstrated by its suppression of expression of α-SMA, a marker of lens cell EMT. Although BMP-7 gene transfer is known to suppress EMT in cultured cell types, similar inhibitory effects on EMT in vivo in the same cell lineages examined were not reported previously. Expression of an exogenous gene that was transferred by adenoviral vector into the lens epithelium lasted longer than it did in the corneal epithelium than we previously reported (29), although the exact cause of this phenomenon remains to be uncovered. One possible explanation is that lens epithelial cells might survive for a longer period with less turnover than corneal epithelium upon injury. Introduction of this gene suppressed upregulation of collagen types I and VI. Low expression levels of endogenous BMP-7 mRNA and protein were induced in the injured lens at day 5 but were not detected at day 10. However, such injury-induced endogenous BMP-7 is considered not to be sufficient to perturb EMT.
Aqueous humor contains abundant TGF-β2, and our previous study showed that an injured lens epithelium upregulates TGF-β1. Breaking the lens capsule allowed influx of the aqueous humor into the lens. Thus phosphorylation and nuclear translocation of Smad2 upon lens capsular injury might be promoted by both TGF-β1 and TGF-β2 in an injured lens. It is widely thought that an activated Smad2/3 signal is required for lens epithelium EMT. Nevertheless, the whole mechanism of suppression of EMT by BMP-7 is not clear. Exogenous BMP-7 enhanced the expression of collagen I and TGF-β1 and TGF-β2 in an injured lens. It is expected that BMP-7’s inhibitory effects against EMT lasted until day 10, HE histological staining showed relatively elongated fibroblastic morphology in the cells, even with BMP-7 gene transfer, suggesting that the cells that lacked α-SMA expression had begun to undergo EMT. Our previous study showed that Smad7 gene introduction kept the cell shape of an epithelial, nonelongated type with suppression of Smad activation even at day 10 (30). On the other hand, the present study has shown that the number of nuclear COOH-terminal phospho-Smad2-positive cells was increased at day 10 compared with day 5 in the BMP-7 gene transfer group. Thus the effects of BMP-7 were less potent than those of Smad7. The explanation of the difference in the duration of effects of Smad7 (signaling mediator) and BMP-7 (extracellular ligand) is that Smad7 and Smad3 are intracellular proteins; thus their continued overexpression exerts persistent effects on signaling pathways, whereas BMP-7 function is cell surface receptor mediated. Even though the expression of BMP-7 persisted up to day 10, the receptor and/or signaling molecules might be downregulated at day 10; thus the overexpression was no longer effective in preventing EMT. Although the expression pattern of α-SMA coincided with upregulation of COOH-terminal phospho-Smad2, whether expression of EMT markers is regulated by Smad2 signal or other TGF-β-related signals remains to be discovered. In the present study, we did not examine the role of phosphorylation of middle linker regions of Smad2/3, which is induced by various growth factors via MAPK or COOH-JNK in the process of EMT.

TGF-β/Smad3 signaling is required for EMT in several cell types, both in vivo and in vitro, although various signals might be activated in parallel with the Smad3 signal during EMT (2, 32). It has been reported that BMP-derived Smads (Smad1/5/8) directly block Smad2/3 on the E-cadherin gene promoter (47–49), but BMP also upregulates molecules such as Id2 and Id3, which are capable of blocking Smad2/3 signaling indirectly (16, 18). The knockdown of Id by siRNA in cultured cells allowed BMP-7 to induce EMT, suggesting the critical role of Id induction in blocking TGF-β/Smad signaling (16, 18). As expected in our present study, we detected upregulation of mRNA Id2 and Id3 in injured lenses treated with the BMP-7 gene. Expression of Id2 and Id3 proteins in BMP-7-Ad-treated specimens, which was observed using real-time RT-PCR, was not detected using immunohistochemistry, presumably because the protein expression level of these components was below the level required for immunohistochemical detection. This is the
reason why exogenous Id mRNA expression was more marked compared with exogenous BMP-7 mRNA expression, although the stability of mRNA might differ among each gene. We next examined the effects of gene transfer of mId2 or mId3 on injury-induced EMT of lens epithelium and showed that adenviral introduction of these genes also suppressed injury-induced lens cell EMT at day 5. The expression level of exogenous Id3 was higher at day 10 than at day 5, which was different from exogenous Id2 gene expression that had decreased at day 10 compared with day 5, although the exact reason for this difference remains unknown. Anti-TGF-β/Smad signal and anti-EMT effects at day 10 were more marked with BMP-7 cDNA compared with cDNA of Id2 or Id3, although each of the introduced genes, BMP-7, Id2, and Id3, was expressed even at this time point. Smad2 was readily phosphorylated even with overexpression of Id2 or Id3 at day 10 but was only weakly activated with BMP-7 gene introduction. The exact reason for this phenomenon is unknown. The cells might develop an alternative intracellular signaling machinery, i.e., upregulation of molecules that act against anti-Smad effects by Id2/Id3, to escape the inhibitory effects of Id2 or Id3 against Smad signaling. The present study also has shown that gene introduction of Id2 or Id3 resulted in weak downregulation of BMP-7 mRNA in an injured lens, although the exact mechanism of gene expression regulation remains to be clarified.

Our previous work showed that loss of Smad3 results in the abrogation of upregulation of TGF-β1 and ECM components in lens epithelial cells, and this phenomenon was mimicked by gene transfer of Smad7 into the lens (30, 32). Suppression of EMT by gene transfer of BMP-7, Id2, or Id3 suggests that they may act through Smad signaling pathways. However, our unpublished data show that severe corneal exposure to alkali is associated with EMT and cataract development in lens epithelium and that this type of EMT is observed even in Smad3-null mice, although the extent of EMT is much less. Similarly, overexpression of active TGF-β1 in lens epithelium using adenoviral gene introduction or transgenic technology using the crystalline gene promoter induced EMT in lens epithelium that was greatly reduced but still present in the absence of Smad3 (2, 11). These findings suggest that overexpression of active TGF-β in the lens epithelium may induce EMT through signaling molecules other than Smads. Interestingly, loss of Smad3 did not affect the expression of α-SMA in cultured ocular fibroblasts (Saika S and Yamanaka O, unpublished data), indicating that the transcriptional mechanism of upregulation of this molecule during EMT and during fibroblast-to-myofibroblast conversion may differ. This hypothesis is consistent with a report that expression of α-SMA in fibroblasts depended on Smad2 signaling (8, 7, 22).

The TGF-β-Smad pathway is also potentially involved in the regulation (mainly the suppression) of cell proliferation in many organs and tissues (6, 17, 20, 40). We previously showed that Smad7 overexpression enhanced the injury-induced proliferation of lens epithelial cells, although this finding was not observed after lens injury in Smad3-null mice (2, 32). Similarly to the results with the Smad3-null mice, BMP-7, Id2, or Id3 overexpression did not affect lens epithelial cell proliferation. The suppression of Smad signaling by BMP-7, Id2, or Id3 might be incomplete or proliferation may be controlled by other signals such as Smad2 or various MAP kinases.

In conclusion, lens capsule contraction by fibrosis is clinically unfavorable, and blocking Smad signaling by gene transfer of BMP-7, Id2, or Id3 may be a strategy to prevent such a clinical problem. Whether combinations of genes, i.e., Smad7, BMP-7, Id2, or Id3, might increase the suppressive effect on lens cell EMT compared with treatment with each factor alone remains to be determined.


