

Single small-interfering RNA expression vector for silencing multiple transforming growth factor- β pathway components

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ABSTRACT

Although RNA interference (RNAi) is a popular technique, no method for simultaneous silencing of multiple targets by small-hairpin RNA (shRNA)-expressing RNAi vectors has yet been established. Although gene silencing can be achieved by synthetic small-interfering RNA (siRNA) duplexes, the approach is transient and largely dependent on the transfection efficiency of the host cell. We offer a solution: a simple, restriction enzyme-generated stable RNAi technique that can efficiently silence multiple targets with a single RNAi vector and a single selection marker. In this study, we succeeded in simultaneous stable knockdown of transforming growth factor β (TGF- β) pathway-related Smads—Smad2, Smad3 and Smad4—at the cellular level. We observed distinct phenotypic changes in TGF- β -dependent cellular functions such as invasion, wound healing and apoptosis. This method is best suited for an analysis of complex signal transduction pathways in which silencing of a single gene cannot account for the whole process.

INTRODUCTION

Transforming growth factor β (TGF- β) is a multipotent cytokine that delivers mainly cytostatic signals (1). TGF- β signals via the receptor complex of TGFBR2 and TGFBR1, and Smad transcription factors mediate the growth inhibitory

effect of TGF- β in many cell types. The TGF- β cytostatic response is of interest because its loss contributes to tumor development. Various genetic and epigenetic alterations of the components of the TGF- β –Smad pathway have been identified in several human cancers (2–4).

Although synthetic small-interfering RNA (siRNA) duplexes can be used for loss of function analysis of the pathway, the establishment of stable knockdown cell lines whose targets are silenced by the integration of a siRNA expression unit by plasmid vectors have various advantages for such purposes. First, the knockdown efficiency of synthetic siRNA duplexes is largely dependent on the transfection efficiency of the host cell line. We optimize the efficacy when we transfect plasmids expressing siRNA; however, we do not need to introduce a plasmid into the majority of cells, as with synthetic siRNA duplexes. Second, the transient nature of siRNA duplexes makes it difficult to silence some targets with long half-lives. Third, most transfection methods are cytotoxic, which makes it difficult to observe important phenotypic changes like cell death, apoptosis and cell growth by transient transfection assay (5). The establishment of stable knockdown cell lines whose targets are silenced permanently by plasmid vectors could overcome these problems.

Although tandem-type U6 promoter-driven siRNA vectors expressing each strand of siRNA separately are sufficient for silencing endogenous gene expression (6), we used hairpin-type siRNA expression vectors because they proved to be better suppressors (7). Although we previously reported the functional analysis of Smad4 using hairpin-type single stable RNA interference (RNAi) to analyze a complex signal transduction pathway (8,9), it is sometimes desirable to knock down several genes simultaneously. To meet this challenge,

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we positioned tandem U6-driven short-hairpin RNAs targeting different genes. The particular properties of this system allow the efficient, stable and simultaneous knockdown of multiple genes.

MATERIALS AND METHODS

siRNA design and construction

Four different sequences targeting the Smad2, Smad3 or TGFBR2 genes were selected using the original algorithm (7). To improve the silencing activity and to overcome technical obstacles described in the text, multiple C to T or A to G mutations were introduced within the sense strand of the hairpin loop. To construct hairpin-type single RNAi vectors, 5 µl (100 mM) of the synthesized (Qiagen, Hilden, Germany) sense and antisense oligonucleotides (Table 1) were combined with 1 µl of 1 M NaCl and annealed by incubation at 95°C for 2 min, followed by rapid cooling to 72°C, and ramp cooling to 4°C over a period of 2 h. We diluted the annealed oligonucleotides 200-fold with TE buffer, and used 1 µl for ligation with plasmid DNA, which was prepared as follows: 3–5 µg of pcPUR+U6i cassette plasmid was digested with BspMI in a reaction volume of 100 µl. The reaction mixture was electrophoresed, gel pieces containing the DNA fragments were excised and the DNA was purified using a MinElute Gel purification kit (Qiagen). After ligation with DNA Ligation Kit Ver.2.1 (Takara, Tokyo, Japan), we transformed *Escherichia coli* host cells with the ligation products. A Smad2 and -3 double-knockdown construct was generated as follows: pcPUR+U6-Smad2i was digested with BamHI and ScaI, and pcPUR+U6-Smad3i was digested with ScaI and BglII (Step 1 in Figure 1C). The fragments containing the U6 promoter and hairpin loop units were purified (Step 2 in Figure 1C) and ligated to construct the double-knockdown vector (Step 3

in Figure 1C). Sites produced by BglII and BamHI are cohesive, but cannot be cut after ligation; consequently, the same procedure can be repeated to construct a vector with multiple siRNAs. The ScaI site is in the ampicillin-resistance gene, which reduces the number of background bacterial colonies. The same method was applied to other double- or triple-knockdown constructs (Figure 1D). The pcPUR+U6i cassette was provided by iGENE Therapeutics (Tsukuba, Japan); pcPUR+U6iGFP and pcPUR+U6-Smad4i were described previously (6,9); pCMV5-TGFβ RII/HA was provided by J.L. Wrana; pcDEF3-Flag(N)-Smad2 and pcDEF3-Flag(N)-Smad3 were gifts from K. Miyazono; (CAGA)₉-luc (10) was a gift from S.E. Kern; and p3TP-Lux was a gift from J. Massague. pRL-SV40 (Promega, Madison, WI), which contains the simian virus 40 promoter upstream of the coding sequence of *Renilla* luciferase, was used as the internal control in the luciferase assay.

Cell culture and transfection

The HeLa cell line was purchased from the American Type Culture Collection (Rockville, MD) and was maintained in DMEM containing 10% fetal bovine serum (FBS) (Sigma, St Louis, MO). The human keratinocyte cell line HaCaT was generously provided by N.E. Fusenig (DKFZ, Heidelberg, Germany) and was maintained in DMEM containing 10% FBS. Transfections were performed using Effectene (Qiagen), according to the manufacturer's instructions. To establish cell lines stably expressing siRNAs, we transfected siRNA plasmids into HaCaT cells, and cultured cells in the presence of 1 µg/ml of puromycin. Colonies resistant to puromycin appeared within 2 weeks, after which the cells were expanded for another 3 weeks to make the original stock cells. The expression of endogenous Smads or siRNA in the original stock and cells after 20 passages was examined using western or northern blotting, respectively. All biological assays

Table 1. siRNAs targeting TGFBR2, Smad2 and Smad3

Target	Site	Sequence
TGFBR2	site 1	sense 5'-caccatgagaaatgagratcaccgtgtgctgtccggtgatgctgcagttgctcattttt-3' antisense 5'-gcataaaaaatgagcaactgcagcatcaccggacagcacacggtgatactacaattactcat-3'
	site 2	sense 5'-caccagcraagtgaagtagaacagtgtgctgtccctgttctcctcagcttggccttttt-3' antisense 5'-gcataaaaaaggccaagctgaagcagaacaggacagcacactgttctacttcaacttagctc-3'
	site 3	sense 5'-caccacggttctctaaaractaccagtgtgctgtccctgtagtgttttagggagccgttttt-3' antisense 5'-gcataaaaaaggcctcctaactaccaggacagcacactggtagtatttagaaccgt-3'
	site 4	sense 5'-caccgaaggatgtcttcttagaratcgtgtgctgtccgatgctgagaagatgctcttttt-3' antisense 5'-gcataaaaaaggacatctctcagacatcggacagcacagatataagaagacatcctt-3'
Smad2	site 1	sense 5'-caccggattgagcttattgaaatggtgtgctgtccattcagatgaagttcaatccttttt-3' antisense 5'-gcataaaaaaggattgaactctatctgaatgggacagcacaccattcaataaagctcaatcc-3'
	site 2	sense 5'-caccggcttgaatctttagatratcgtgtgctgtccgatgactgtgaagatcaggccttttt-3' antisense 5'-gcataaaaaaggcctgatcttcacagtcacggacagcacagataactataaagatcaagcc-3'
	site 3	sense 5'-caccggttacttctcraagttaaacgtgtgctgtccgttaacattggagagtaaaccttttt-3' antisense 5'-gcataaaaaagggttactctcaatgttaacggacagcacagtttaactagaagaataaac-3'
	site 4	sense 5'-caccgccactcttggatatataggtgtgctgtccctgatataatccagagaggtgcttttt-3' antisense 5'-gcataaaaaaggcactcctgatataatcaggacagcacactaatatcaagaagtgcc-3'
Smad3	site 1	sense 5'-caccggccagactgrragacracctgtgctgtccggtggctgtgcagctgtgcttttt-3' antisense 5'-gcataaaaaaggcagacactgcacagccaccggacagcacacggtatgctatacaagtggcc-3'
	site 2	sense 5'-caccggattgagcttctgaatctgaatggtgtgctgtccattcagtgagcagcctcaatccttttt-3' antisense 5'-gcataaaaaaggattgagctgcacctgaatgggacagcacaccattcagatacaactcaatcc-3'
	site 3	sense 5'-caccgagttgtctttaatgaaggtgtgctgtccctcattgaagggcgaactcttttt-3' antisense 5'-gcataaaaaaggattcgcctcaatgaagggacagcacacccttattaaagacaaactc-3'
	site 4	sense 5'-caccgggtgtgtcttcaatgtraacgtgtgctgtccgttgcattggagagcagcccttttt-3' antisense 5'-gcataaaaaaggctgctcctcaatgtaacggacagcacagtttaactgagaacaacc-3'

siRNA target sequences are not underlined; mutated sequences are in italics; the loop sequence is underlined; the transcription stop codon is double underlined; and the BspMI site is indicated by a dotted underline.

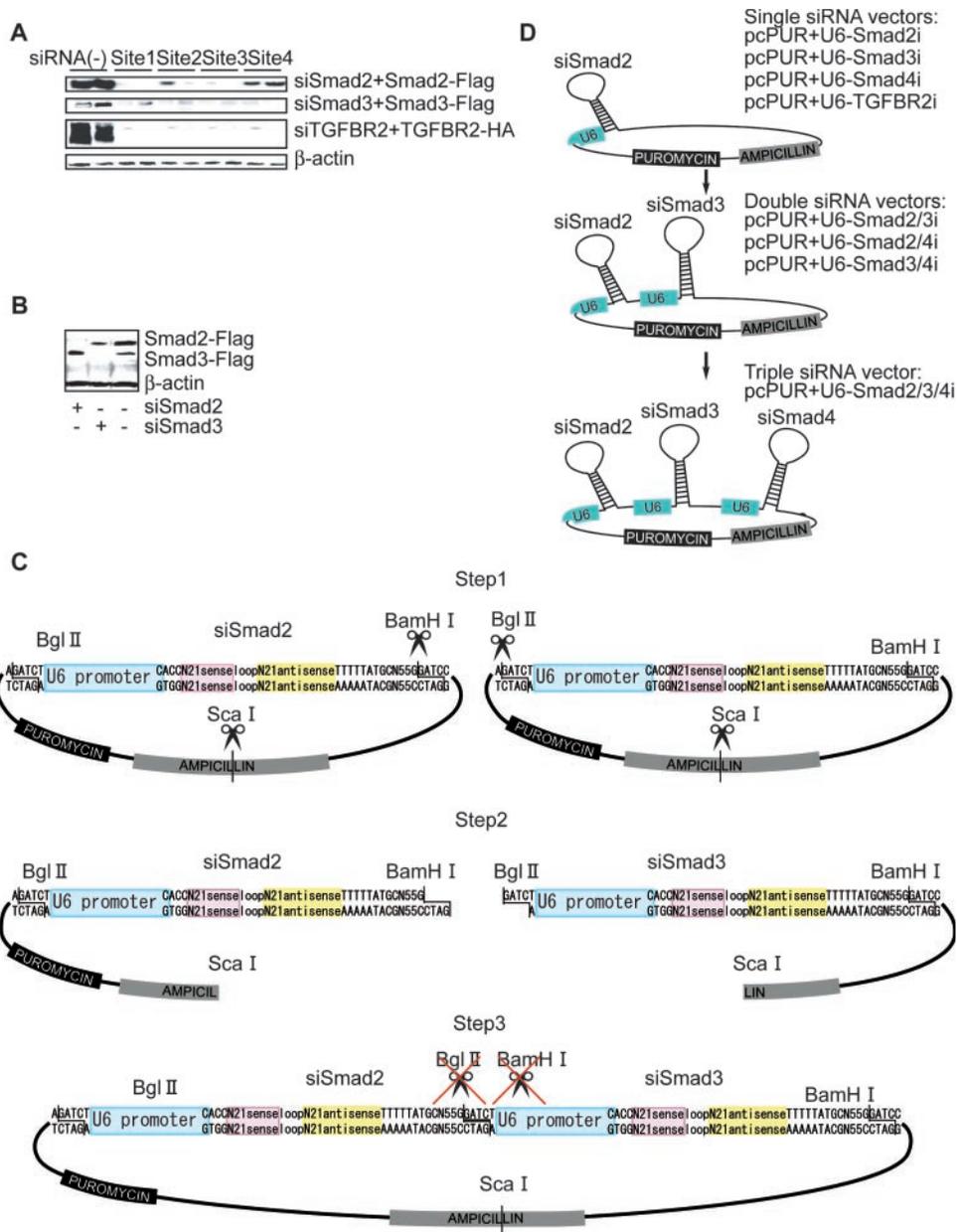


Figure 1. RNAi vector construction. (A) Four target sites for each of the Smad2, Smad3 or TGFBR2 genes were designed and subcloned into pcPUR+U6-i cassettes, which were transiently cotransfected with the Smad2-, Smad3- or TGFBR2-expressing vectors into HeLa cells, to screen the silencing efficacy by western blot analysis. The same samples were loaded in duplicate. (B) Specific knockdown of Smad2 or -3. HaCaT cells were transiently transfected with pcDEF3-Flag(N)-Smad2, pcDEF3-Flag(N)-Smad3 and pcPUR+U6-Smad2i (left lane), pcPUR+U6-Smad3i (middle lane) or pcPUR+U6-GFPi (right lane). After lysing the cells, we performed western blot analysis. (C) Restriction enzyme-based generation of multi-target siRNA vectors. The pcPUR+U6-i cassette has restriction enzyme sites for BglII upstream of the U6 promoter, BamHI downstream of the RNAi cloning site and ScaI in the ampicillin-resistance gene; all of these sites are unique in this plasmid. To make a knockdown vector that targets both Smad2 and -3, pcPUR+U6-Smad2i was digested with BamHI and ScaI and pcPUR+U6-Smad3i was digested with BglII and ScaI. The fragments containing expression cassettes were ligated. (D) Schematic representation of single-, double- and triple-siRNA vectors generated in this study. pcPUR+U6-Smad4i was described previously (8,9).

performed in this study used cells within five passages from the original cell stock.

Western blot

Western blotting was performed as described previously (11). Equal amounts of protein were electrophoresed, and the proteins were detected using the anti-Smad2/3 or anti-Smad4 antibody (BD Transduction Laboratories, San Diego, CA) at a dilution of 1:250, the anti-β-actin or anti-Flag antibody

(Sigma) at a dilution of 1:2500, or the anti-HA antibody (Roche Molecular Biochemicals, Indianapolis, IN) or anti-PAI-1 antibody (BD Transduction Laboratories) at a dilution of 1:1000.

Northern analysis

Total RNA was extracted from the stable knockdown cells using Isogen Reagent (Wako, Tokyo, Japan). The total RNA (5 μg) was size-fractionated on an 18% (wt/vol)

polyacrylamide-urea gel and transferred to Hybond N+ membrane (Amersham, Little Chalfont, UK). The resulting membrane was dried at room temperature and fixed with ultraviolet light. The membrane was prehybridized in 30% formamide, 10% dextran sulfate, 5× SSC, 0.5% SDS, 1× Denhardt's solution and 0.2 mg/ml salmon sperm DNA (Sigma Aldrich, St Louis, MO). Hybridizations were performed at 36°C for 3 h with radiolabeled synthetic oligonucleotide probes that were complementary to the sequences of siRNAs against Smad2, -3 or -4. A probe complementary to human tRNA valine was used as a loading control. The sequences of the probes were as follows:

tRNA valine; 5'-GAACGTGATAACCACTACTACG-GAAACCCCTATAGTGAGTCGATTAGGCGGGAACCGCCTAATACGACTCACTATAGG-3',
Smad2 siRNA; 5'-GGATTGAACTTCATCTGAA-3',
Smad3 siRNA; 5'-GGATTGAGCTGCACCTGAA-3', and
Smad4 siRNA; 5'-GTACTTCATACCATGCCGA-3'.

The synthetic probes were ³²P-labeled (Amersham, Little Chalfont, UK) using T4 polynucleotide kinase (Takara Shuzo, Kyoto, Japan). The membrane was washed with 2× SSC twice at 36°C and analyzed using a Fujix Bio-Image Analyzer BAS1000 (Fuji Photo Film, Tokyo, Japan).

Luciferase assay

The luciferase assay was performed as described previously (11,12). Cells were transfected with (CAGA)₉-luc or p3TP-Lux and pRL-SV40. After 24 h, the cells were incubated with or without 2.5 ng/ml of TGF-β1 (R&D Systems, Minneapolis, MN) for an additional 24 h, and the dual luciferase assay was performed. The firefly luciferase activity of (CAGA)₉-luc or p3TP-Lux was normalized to the *Renilla* luciferase activity of pRL-SV40. The level of luciferase in cells in the absence of TGF-β1 was assigned a value of 1.0, and the relative activities were calculated. The experiments were performed three times in duplicate.

Quantitative RT-PCR

Quantitative RT-PCR analysis was performed using the ABI 7000 real-time PCR system (Applied Biosystems, Foster City, CA), as described previously (9). The ratio of the mRNA level of each gene to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was calculated, and the value of 1.0 was assigned to the parental cells. Each experiment was repeated twice in triplicate. The following primers were used:

BTBD1, forward (5'-CAAGGCGTCGCTGAAGGA-3'), reverse (5'-CCAGTACGAAGCGCACAT-3');
prominin 2, forward (5'-TGCCCTCTGTGGACCATGT-3'), reverse (5'-GGCGTTGAAGGTGCTGTTCT-3');
OAS1, forward (5'-AGGTGGTAAAGGGTGGCTCC-3'), reverse (5'-ACAACCAGGTCAGCGTCAGAT-3');
GAPDH, forward (5'-CCACCCATGGCAAATTC-3'), reverse (5'-TGGGATTTCCATTGATGACAAG-3'); and
M13 (for sequence confirmation), reverse (5'-CAGGAAA-CAGCTATGAC-3').

Invasion assay

Invasion was measured using BD BioCoat Matrigel Invasion Chambers (BD Biosciences, San Jose, CA) with 8 mm

diameter, matrigel-coated polycarbonate membrane (8 μm pore size). A total of 10⁵ knockdown or control HaCaT cells were seeded into the upper chamber of the well. After 24 h, the cells were incubated in the presence or absence of 5 ng/ml of TGF-β1 for an additional 24 h. Then, the cells on the upper surface of the filter were wiped off using a cotton swab, and the cells that had invaded the underside of the filter were fixed, stained with DiffQuick (Kokusai Shiyaku, Kobe, Japan), and counted under bright-field microscopy at ×200 magnification in five random fields of view. The experiments were repeated three times.

Wound closure assay

The wound closure assay was performed as described previously (9). Confluent cell monolayers were wounded by scraping using a pipette tip of 1.2 mm width, and incubated with or without 5 ng/ml of TGF-β1 for 24 h. Cell migration was carefully observed at various times. The experiments were repeated three times.

Apoptosis

A total of 2 × 10⁶ cells in a 10-cm dish were incubated with or without 5 ng/ml of TGF-β1 for 24 h, and apoptosis was analyzed using the ApopTag Fluorescein *in situ* apoptosis detection kit S7111 (Serologicals Corporation, Norcross, GA). A Becton-Dickinson flow cytometer equipped with CellQuest software was used. The experiments were repeated three times in duplicate.

RESULTS

Development of double or triple siRNA expression vectors

We designed four target sites of 21 nt for each of the Smad2, Smad3 and TGFBR2 (Table 1) genes using software based on various algorithms for effective siRNA design, as we reported previously (7,13). To improve silencing activity and to overcome technical obstacles, such as the frequent mutations that occur in siRNA constructs or difficulties in sequencing because of tight palindromic sequences, we inserted more than two C to T or A to G point mutations within the sense strand of the hairpin (7,14,15). We subcloned chemically synthesized oligonucleotides into the pcPUR+U6-i cassette (6) to construct siRNA vectors, and we transiently transfected these siRNA vectors together with Flag-Smad2, Flag-Smad3 or TGFBR2-HA expression vectors into HeLa cells to test the silencing efficacy of the siRNA. This method of screening the optimal sequence for silencing is useful because plasmids expressing epitope-tagged cDNA and siRNA both tend to enter the same cells, and western blotting using the antibody against the epitope tag, not the protein itself, reduces the background signal of endogenous proteins from non-transfected cells. Two of four sites in Smad2, three of four in Smad3 and all sites in TGFBR2 were effective in suppressing their targets down to 0–10% (Figure 1A). For further knockdown constructs, we selected site 1 for Smad2, site 2 for Smad3 and site 1 for TGFBR2, which proved to be most effective. The constructs containing these sites were named pcPUR+U6-Smad2i, pcPUR+U6-Smad3i and pcPUR+U6-TGFBR2i (Figure 1A) to knock down the Smad2, Smad3

and TGFBR2 proteins, respectively. Using western blot analysis, we confirmed that the knockdown of Smad2 and Smad3, which are highly homologous proteins, does not exert a mutual effect (Figure 1B). We succeeded in obtaining single knockdown vectors against Smad2, Smad3 and TGFBR2.

Next, we attempted to make knockdown vectors against multiple genes by combining these constructs. The pcPUR+U6 vector has restriction enzyme sites of BglIII upstream of the U6 promoter, a BamHI site downstream of the RNAi sequence and a ScaI site in the ampicillin gene; all of these sites are unique in this plasmid. To make a knockdown vector that targets Smad2 and Smad3 simultaneously, we digested pcPUR+U6-Smad2i with BamHI and ScaI and digested pcPUR+U6-Smad3i with BglIII and ScaI (Step 1 in Figure 1C), and fragments containing expression cassettes were ligated to make a single vector that knocked down both Smad2 and Smad3 (Steps 2 and 3 in Figure 1C). The constructs against Smad2 and -3, Smad2 and -4, and Smad3 and -4 were named pcPUR+U6-Smad2/3i, pcPUR+U6-Smad2/4i and pcPUR+U6-Smad3/4i, respectively (Figure 1D). Knockdown vector pcPUR+U6-Smad2/3/4i, targeting all pathway-related Smads (Smad2, Smad3 and Smad4), was generated by the same procedure (Figure 1D). The advantage of this system is that sites produced by BglIII and BamHI are cohesive but cannot be cut after ligation, and so the same procedure is repeatable to make target vectors against multiple genes. Also, the unique ScaI site in the ampicillin-resistance gene contributes to the reduction of the number of background bacterial colonies.

Validating the Smads knockdown

To test the efficacy of these vectors, we used a human keratinocyte cell line, HaCaT, which has a functional TGF- β pathway, for further analysis. This cell line is suitable for the analysis of TGF- β signaling (16). HaCaT was transfected with these vectors and selected with 1 μ g/ml of puromycin to obtain stable knockdown cell lines S2KD, S3KD, S4KD, S23KD, S24KD, S34KD, S234KD and iGFP, respectively. We used stable cell pools, which are mixtures of the puromycin-resistant polyclonal cells, not independent cell clones, to exclude any cellular changes owing to genomic integration of the plasmid. As shown in Figure 2A, most of the stable transformants (lanes 2–8) exhibited a drastic reduction of endogenous Smad2, Smad3 and Smad4 protein expression. Although S234KD cells harbor three different siRNAs, the method resulted in an excellent knockdown of their intended targets. Northern analysis showed that the same amount of siRNA against Smad2, Smad3 or Smad4 was stably expressed in all single, double or triple knockdown cells (Figure 2B). The expression of endogenous Smads or siRNAs in the original cell stock and cells after 20 passages from the original stock was tested using western or northern blotting, respectively, and no significant changes in the Smad protein levels or siRNA expression were observed after 20 passages (data not shown). Because one of the potential problems of using polyclonal cell pools is a change in the clonality of cells after multiple passages, we performed biological assays using cells within five passages of the original stock vials as described in Materials and Methods. We were unable to obtain a stable TGFBR2 knockdown cell line because massive cell death occurred after the transfection, suggesting that TGFBR2 is important

for cell survival in this cell line. The silencing efficiency did not depend on the location along the vector or the number of siRNAs, up to three (data not shown).

Some 11–15 contiguous nucleotides of sequence identity with siRNA were reportedly sufficient to direct the silencing of non-targeted messenger RNA (mRNA) transcripts (17). Although we carefully selected the target sequence by using original algorithms that omit any sequences that are highly homologous to other genes (7,13), we could not exclude the possibility of knocking down non-specific genes. To examine off-target gene silencing in our cells, we used the BLAST database (<http://www.ncbi.nlm.nih.gov/BLAST>) to search the human genome for any complementary sequences with at least 11 contiguous nucleotides matching the RNAi sites we used. For TGFBR2 site 1, no gene appeared to have sequence similarity. For Smad2 site 1, a BTB/POZ domain-containing one protein (BTBD1) had 17 contiguous nucleotide matches, and some other hits had less similarity. A pentaspan membrane glycoprotein (prominin 2) had 15 contiguous nucleotides similar to Smad3 site 2. We examined their mRNA expressions in knockdown cells by using quantitative RT-PCR. As their expressions were not reduced in S2KD or S3KD cells compared with parental HaCaT cells (Figure 2C and D) or the iGFP control cell line (data not shown), we conclude that there was no off-target gene silencing in established cell lines. Another report demonstrated that the interferon response was induced by U6 promoter/lentivirus shRNA constructs in mammalian cells (18). The α/β interferons induce a double-stranded RNA (dsRNA)-binding enzyme, OAS1, which is activated in a dsRNA-dependent manner. Although our constructs were not derived from lentivirus, we checked the OAS1 induction to rule out any non-specific RNA degradation. Theoretically, triple-knockdown cells that might produce more siRNAs are likely to induce more OAS1, but there was no statistical difference of OAS-1 mRNA among knockdown cell lines and the parental HaCaT cell line (Figure 2E) or iGFP control cell line (data not shown). The pcPUR+U6 constructs appear to be highly specific against target genes and effective for gene silencing, and thus we proceeded to functional analysis of the TGF- β pathway in these cells.

Luciferase assays using the Smad-dependent reporter (CAGA)₉-luc showed that the canonical TGF- β -Smad-dependent signaling was markedly inhibited in all cell lines except S2KD (Figure 2F). The preserved induction of luciferase in S2KD cells by TGF- β (Figure 2F) is in agreement with the original report (10) in which Smad3 and Smad4, but not Smad2, bound to this Smad-binding element. The induction of the luciferase activity of S2KD cells by TGF- β was downregulated to 20% of that of control cells when p3TP-Lux (three TRE elements from the human collagenase gene linked to the -740/-636 region of the PAI-1 promoter, which is selectively induced by TGF- β) was used as the reporter plasmid (data not shown). We investigated the induction of PAI-1, a well-known TGF- β -responsive gene, in knockdown cell lines. The results revealed that TGF- β -mediated PAI-1 induction was impaired in all knockdown cell lines, except for the S2KD cell line (Figure 2G). TGF- β -mediated PAI-1 induction is largely dependent on the CAGA Smad binding element in its promoter region, and the results of the PAI-1 western blot were consistent with the reporter assay shown in Figure 2F.

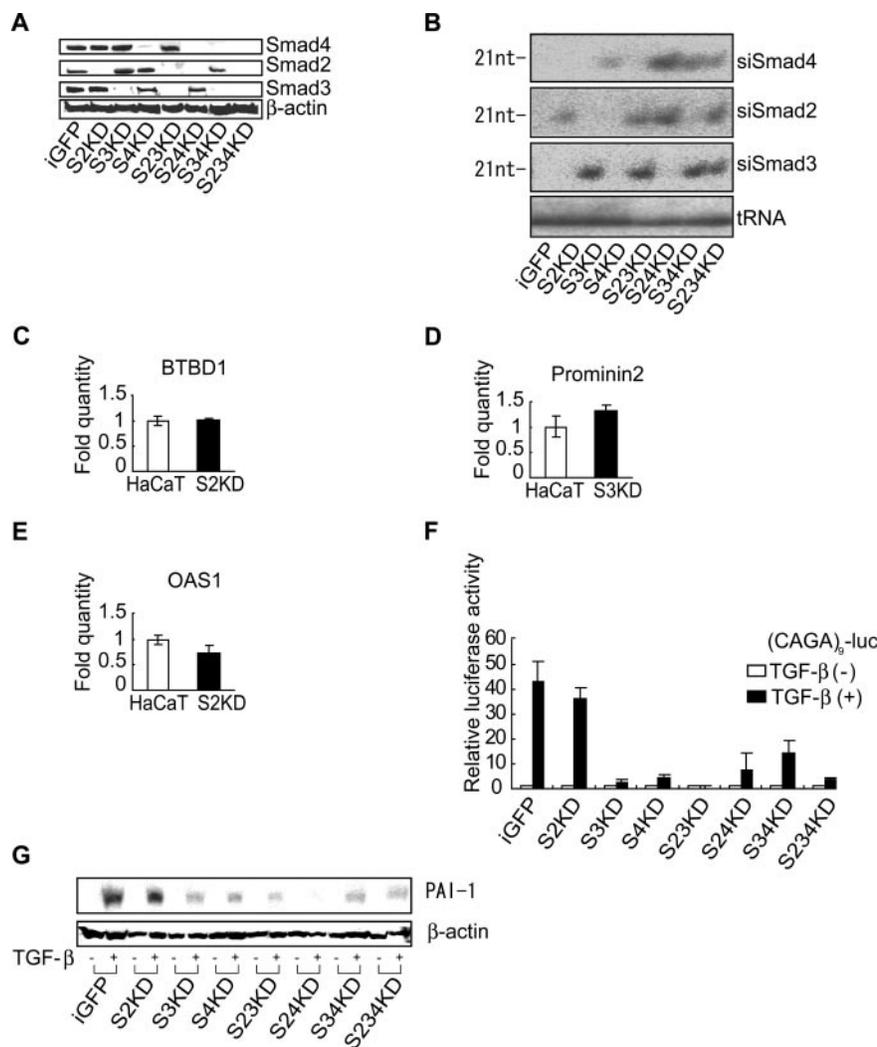


Figure 2. Efficiency and specificity of stable knockdown cells. (A) Endogenous Smad knockdown. HaCaT cells were transfected with pcPUR+U6-GFPi, pcPUR+U6-Smad2i, pcPUR+U6-Smad3i, pcPUR+U6-Smad4i, pcPUR+U6-Smad2/3i, pcPUR+U6-Smad2/4i, pcPUR+U6-Smad3/4i or pcPUR+U6-Smad2/3/4i and incubated with 1 μ g/ml of puromycin to select stably transfected cells. The total cell lysates were subjected to western blot analysis. (B) The expression of siRNAs (21 nt) against Smad2, -3 or -4 in cells was detected using northern blot analysis. tRNA valine was used as a control. (C and D) Knockdown vectors do not trigger non-specific target gene silencing or (E) interferon induction. Relative expressions of (C) BTBD1, (D) prominin2 or (E) OAS1 in the indicated cell lines were assessed by quantitative RT-PCR. The gene expression level in the parental HaCaT cells was assigned a value of 1.0. (F) Inhibition of TGF- β -Smad signaling in knockdown cells. The knockdown and control cells were transfected with (CAGA)₆-luc and pRL-SV40. After 24 h, the cells were incubated with or without 2.5 ng/ml of TGF- β 1 for an additional 24 h, and the dual luciferase assay was performed. The firefly luciferase activity of (CAGA)₆-luc was normalized to the *Renilla* luciferase activity of pRL-SV40. The level of luciferase in the cells in the absence of TGF- β 1 was assigned a value of 1.0 (open bars), and the relative activities were calculated. The experiments were performed three times in duplicate, and the mean and standard error are shown. (G) Induction of PAI-1 by TGF- β was examined by western blot. Cells were incubated with or without 5 ng/ml TGF- β for 10 h, and total cell lysates were prepared.

Phenotypes of Smads knockdown cell lines

Past knowledge of the functional differences of Smad proteins has been accumulated by using Smad dominant-negative constructs (19), antisense RNA (20) and siRNA duplexes (21). These studies focused mainly on molecular targets or changes in signal transduction rather than phenotypic changes because the transient nature of these previous methods limited such observations. In contrast, stable RNAi constructs allowed us to concentrate more on changes in TGF- β -dependent phenotypes such as invasion, wound closure and apoptosis.

Using matrigel invasion chambers, we analyzed the TGF- β -dependent invasion *in vitro*. Although there are reports that the loss of Smad4 in mice leads to increased metastasis and

tumor invasion (2,22), our results show that the invasive activity of S4KD cells without TGF- β is lower than that of any other cells, and that TGF- β strongly stimulates invasion (41.3-fold increase; Figure 3A and B). S2KD and S3KD cells, in contrast, have invasive character at the base level, but respond to TGF- β poorly (8.3- and 3.5-fold increases, respectively). The TGF- β -dependent invasion of S23KD, S24KD and S34KD cells had no statistically significant differences compared with that of control iGFP cells (4.3-, 4.3-, 3.8- and 3.8-fold increases, respectively). The fold increase in the TGF- β -induced invasion in S4KD cells was more than 10 times that of control cells with intact Smad4 (Figure 3A and B). Invasion induced by TGF- β was statistically and significantly

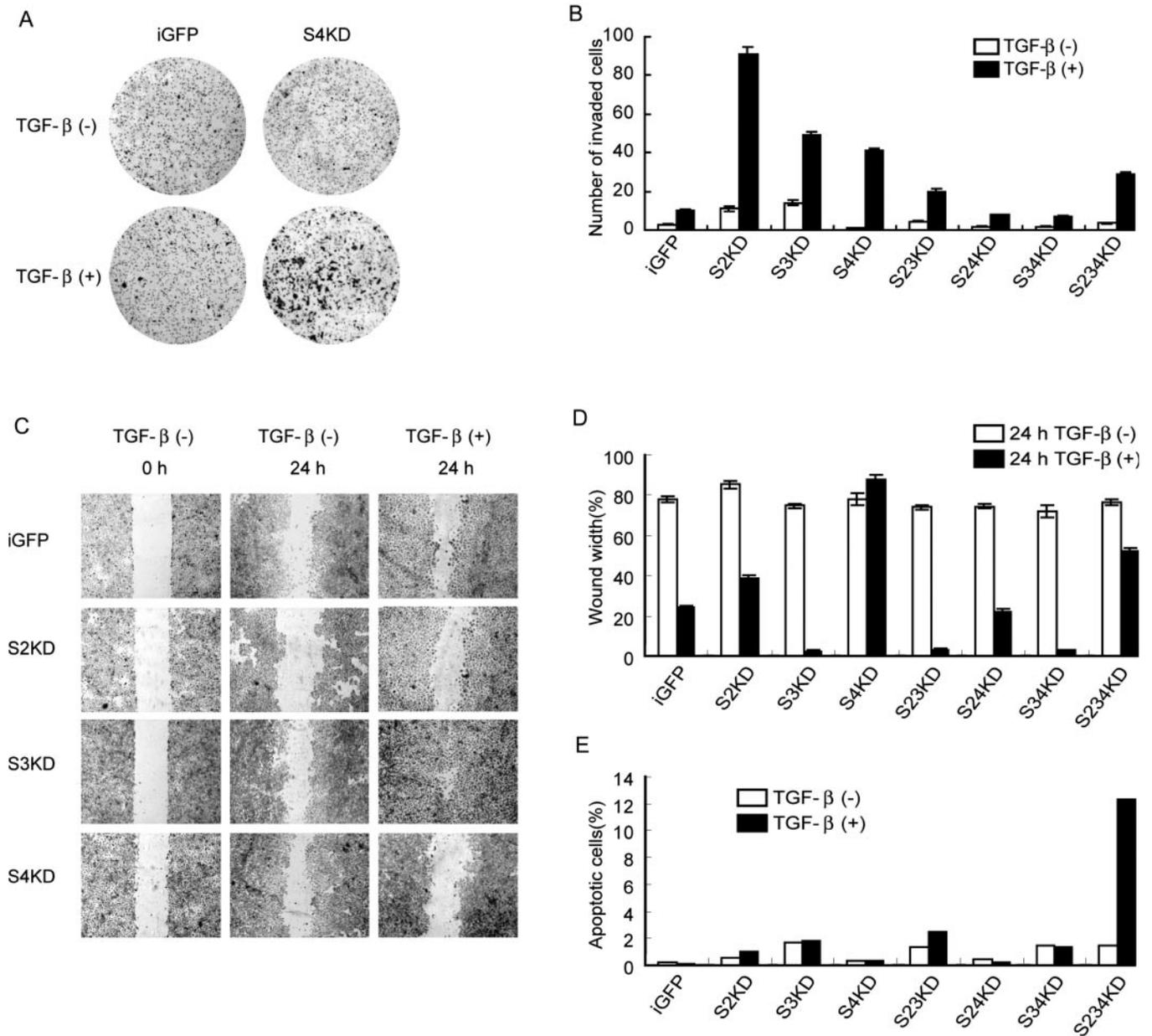


Figure 3. Analysis of TGF-β-dependent cellular phenotypes of stable knockdown cell lines. (A) Representative images of the invasion assay and (B) the number of invaded cells. The numbers of cells that invaded through the matrigel in the absence (open bars) and presence (closed bars) of TGF-β were counted in five random fields of view. The experiments were repeated three times, and the mean and standard errors are shown. (C) Representative images of the migration assay. Wound closure was assessed after 24 h with or without 5 ng/ml of TGF-β₁. (D) Percentage of distance between wound edges in the absence (open bars) or presence (closed bars) of TGF-β₁ in the indicated cell lines. The experiments were performed three times, and the mean and standard errors are shown. (E) Percentages of apoptotic cells in the absence (open bars) or presence (closed bars) of 5 ng/ml of TGF-β₁ for 24 h are shown. Three independent experiments were performed with similar results, and the representative results are shown.

reduced in the triple-knockdown cell line (8.3-fold increase) compared with that of Smad4 knockdown cells, suggesting that Smad2 and Smad3 are important for TGF-β-mediated cell invasion in cells lacking the function of Smad4.

As TGF-β is known to promote wound closure by activating cell migration, we tested the ability of TGF-β to induce wound closure in established knockdown cell lines. Confluent cell monolayers were wounded with a tip of 1.2-mm width, and wound closure was observed after 24 h of TGF-β administration. In our earlier report, Smad4 knockdown

blocked wound closure induced by TGF-β in Panc-1 cells (9); we observed similar results in HaCaT cells. As shown in Figure 3C and D, the loss of Smad3 alone or together with Smad2 or Smad4 accelerated wound closure, a finding that is consistent with the reports in Smad3-knockout mice (23). In contrast, S2KD and S234KD cells had attenuated wound closure compared with that of control cells (Figure 3C and D). Although all Smads are downstream mediators of the same TGF-β pathway, each Smad seems to contribute differently to cell migration.

The effect of TGF- β on cellular apoptosis varies greatly, depending on cell type (2). In HaCaT cells, TGF- β enhances survival by preventing apoptosis via the Akt-dependent regulation of FKHL1 (24). We tested whether this ability of TGF- β was affected by disruption of its downstream regulators (Figure 3E). The percentages of apoptotic cells for different cell types in the absence/presence of TGF- β were as follows: iGFP, 0.21/0.15; S2KD, 0.61/1.01; S3KD, 1.64/1.78; S4KD, 0.29/0.31; S23KD, 1.41/2.50; S24KD, 1.47/2.5; S34KD, 1.48/1.31; and S234KD, 1.42/12.32. To our surprise, TGF- β still prevented apoptosis in single- or double-knockdown cells. When we knocked down all Smads (Smad2, Smad3 and Smad4), TGF- β lost its apoptosis-preventing function and gained an apoptosis-inducing function. As the underlying mechanism is unknown, our data suggest that in this cell line, the Smad-independent TGF- β pathway transmits apoptotic signals in the absence of all Smads.

DISCUSSION

RNAi is arguably one of the great discoveries of humankind (25–27). Despite substantial improvements in making effective siRNA duplexes (14,15), many biologists still have difficulty performing efficient RNAi owing to low transfection efficiency and the frequent cytotoxicity associated with the transfection method (5). The development of vector-expressed siRNA represents revolutionary progress (6,28). Although some robust techniques for generating large-scale RNAi libraries have been introduced (7,29), they are costly, time-consuming and optimization is needed for the stable knockdown of multiple genes to similar levels in the same cells. Besides large-scale RNAi library screening, researchers in a particular field also need a more pinpointable, multiple stable knockdown method for a particular analysis, without using different selection antibiotics, and we believe that our newly established method fulfills these requirements. A recent study stated that small hairpin RNA Dicer substrates were more effective in promoting RNAi than siRNA duplexes (30). The results could position the shRNA expressing vectors as a dominant tool for RNAi analysis in the future.

The method described here is a rapid, simple and effective way to produce stable RNAi cell lines. In this report, we demonstrate the efficiency of this method for silencing at least three targets side by side. Although they are different techniques, multiple knockout mouse models and multiple stable RNAi share a common feature of stably suppressing gene expression. As the former has contributed enormously to the life sciences, we expect as much from the latter. The RNAi machinery is a multiple-turn enzyme complex, so once activated, a single RISC complex could potentially cleave many copies of RNA targets (31), and this feature could allow effective silencing of multiple targets simultaneously. If an effective shRNA fails to work because of the low concentration of shRNAs produced, it may be possible to achieve efficient knockdown by doubling, quadrupling or further increasing the copy number of RNAi cassettes by combining the same plasmid. This advantage of this system is very important because it increases the number of shRNAs without significantly changing the overall plasmid length. Hypothetically, a single siRNA expression plasmid could harbor >30 shRNA cassettes, if the upper length for successful plasmid DNA

transfection is 20 kb. Further experimentation is necessary to determine the maximum number of targets that can be silenced effectively.

In transient transfection experiments, a major drawback of RNAi could result from the elevated level of siRNA transcription, which could saturate the siRNA machinery and contribute to unwanted effects. The idea that large amounts of siRNA inside a transfected cell could improve the efficiency of gene silencing remains to be verified. Although we followed the stability of siRNA until 20 passages of cell culture in various cell lines and observed no apparent loss of siRNA in any cell line, these stable cell lines should be carefully followed over the long term to check the biological consequences of these vectors.

In this study, we found phenotypic changes in cells lacking Smads. As shown here, there are discrepancies in the results of biological assays, and the changes were complex, which suggests that the knockdown of Smads affects the regulation of other signaling pathways. Previous papers, including ours (8,9), suggest the existence of Smad-independent pathways, such as the MAPK, RhoA, PP2A and TAK1/MEKK pathways. To dissect the biological response to TGF- β at the molecular level, future investigation is needed.

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