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Induction of cancer cell-specific death via MMP2 promoter-dependent Bax expression

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Controlled gene expression in specific cells is a valuable tool for gene therapy. We attempted to determine whether the lentivirus-mediated Tet-On inducible system could be applied to cancer gene therapy. In order to select the genes that induce cancer cell death, we compared the ability of the known pro-apoptotreic genes, Bax and tBid, and a cell cycle inhibitor, p21cip1/waf1, and determined that Bax was the most effective. For the cancer cell-specific expression of rtTA2^{S}-M2, we tested the matrix metalloproteinase-2 (MMP-2) promoter and determined that it is highly expressed in cancer cell lines, including SNU475 cells. The co-transduction of two lentiviruses that contain sequences for TRE-Bax and rtTA2^{S}-M2, the expression of which is controlled by the MMP-2 promoter, resulted in the specific cell death of SNU475, whereas other cells with low MMP-2 expression did not evidence significant cell death. Our data indicate that the lentivirus-mediated Tet-On system using the cancer-specific promoter is applicable for cancer gene therapy. [BMB reports 2009; 42(4): 217-222]

INTRODUCTION

The regulated expression of certain genes in specific cells or tissues at desirable time points constitutes a valuable tool not only for the study of gene functions, but also for gene therapy. If pro-apoptotic genes can be specifically expressed in cancer cells and can kill the cells, this system would be an extremely useful tool for curing cancers. However, the specific delivery of pro-apoptotic genes or drugs to cancer cells has proven a major obstacle in the realization of this idea (1, 2). Many researchers have focused on finding ways to deliver genes specifically into cancer cells, and several successful cases have been previously reported (3, 4). Another approach is the specific expression of pro-apoptotic genes in cancer cells after the delivery of the genes to both normal and cancer cells, although a serious problem in this approach is the potential cytotoxic effect on normal cells. In an effort to overcome this problem, pro-apoptotic genes were expressed in a cancer cell-specific manner using gene promoters that are expressed abundantly in cancer cells (5, 6).

For the inducible expression of target genes in eukaryotic cells, the Tet-On system based on the *E. coli* tetracycline resistance operon has been used extensively (7, 8). The binding of tetracycline or its derivative Doxycycline to the reverse-tetracycline-transactivator (rtTA), a chimeric transcription activator protein, results in conformational changes of the rtTA, and allows the rtTA to bind to the tetracycline response element (TRE) sequences and activate the gene downstream of TRE.

Pro-apoptotic or cell cycle inhibitor genes are good candidates for the induction of specific cancer cell death in cancer gene therapies. For example, bcl-2, Bax, tBid, and p21cip1/waf1 genes have been investigated for their possible use in cancer gene therapies (9-13). As the ectopic expression of these genes is cytotoxic to both cancer cells and normal cells, the expression of these genes via the Tet-On inducible system has been utilized to induce cancer cell-specific death (10).

The efficient and safe delivery of target genes is a crucial issue in gene therapy. As the human immunodeficiency virus type 1 (HIV-1)-derived lentiviral vectors can integrate into the host genome and be transduced into both dividing and non-dividing cells *in vitro* and *in vivo*, these vectors are considered to be efficient tools for gene therapy applications (1).

In this study, we combined the recently available techniques and attempted to determine whether the lentivirus-mediated Tet-On inducible system could be applicable to cancer gene therapy applications. Via the transduction of two independent lentiviruses, one harboring TRE-Bax and the other harboring sequences for rtTA2^{S}M2 expression controlled by the Matrix metalloproteinase-2 (MMP-2) promoter, we demonstrated that cancer cell-specific death occurred in an inducible fashion. Our data strongly indicate that the lentivirus-mediated Tet-On system using cancer-specific promoters is a valuable tool for cancer gene therapy.

RESULTS

Selection of Bax and MMP-2 promoter for the induction of cancer-cell specific death

To determine whether cancer cell-specific death could be in-

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duced, we adopted the lentivirus-mediated Tet-On inducible system (Fig. 1A). We hypothesized that cancer cell-specific deaths could be induced via the inducible expression of pro-apoptotic genes under the control of a promoter that is abundantly expressed in cancer cells.

Two pro-apoptotic genes (tBid and Bax) and p21, which negatively regulates cell cycle progression, were selected for our experiments. As tBid, Bax, and p21 were good candidates for gene therapy to induce cancer cell-specific death, we cloned them in an inducible vector and compared their ability to induce cell death in order to select the gene that most efficiently causes cancer cell death when its expression is transiently induced. The results of Western blot analysis demonstrated that the cloned genes in the pBI-EGFP cloning vector were expressed in an inducible manner upon treatment with Doxycycline (Fig. 1B). In order to determine which genes most effectively induced cell death, pCMV-rtTA2^S-M2 was transfected transiently into HEK293T cells with TRE-plasmid harboring EGFP, p21, EGFP/tBid or EGFP/Bax, respectively, and the number of viable cells was counted via trypan blue staining (Fig. 2). The inducible expression of p21 did not significantly induce the cell death as compared to EGFP expression alone, whereas the inducible expression of EGFP/tBid slightly induced cell death. However, most interestingly, the inducible expression of EGFP/Bax upon 10 h of Doxycycline treatment significantly induced cell death and reduced the number of viable cells. Consistent with the known function of Bax, the TUNEL staining data indicated that inducible Bax expression induces apoptotic cell death (Suppl. Fig. 1). As our data demonstrated that inducible Bax expression was most efficient in inducing cell death, we utilized Bax to induce cancer cell-specific death in our subsequent studies.

To study the inducible expression of rtTA2^S-M2 in cancer cells, the Matrix metalloproteinase-2 (MMP-2) promoter was selected. MMPs are endopeptidases that degrade extracellular matrix (ECM) components (14). MMPs are known to be responsible for the detachment of tumor cells from the tumor cell mass and the invasion of the surrounding extracellular matrix, and MMP-2 expression is highly increased in a variety of cancer cell types (15-17). We assessed MMP-2 expression in several selected cancer cell lines. We had initially planned to use a promoter that was expressed in all of the cancer cell

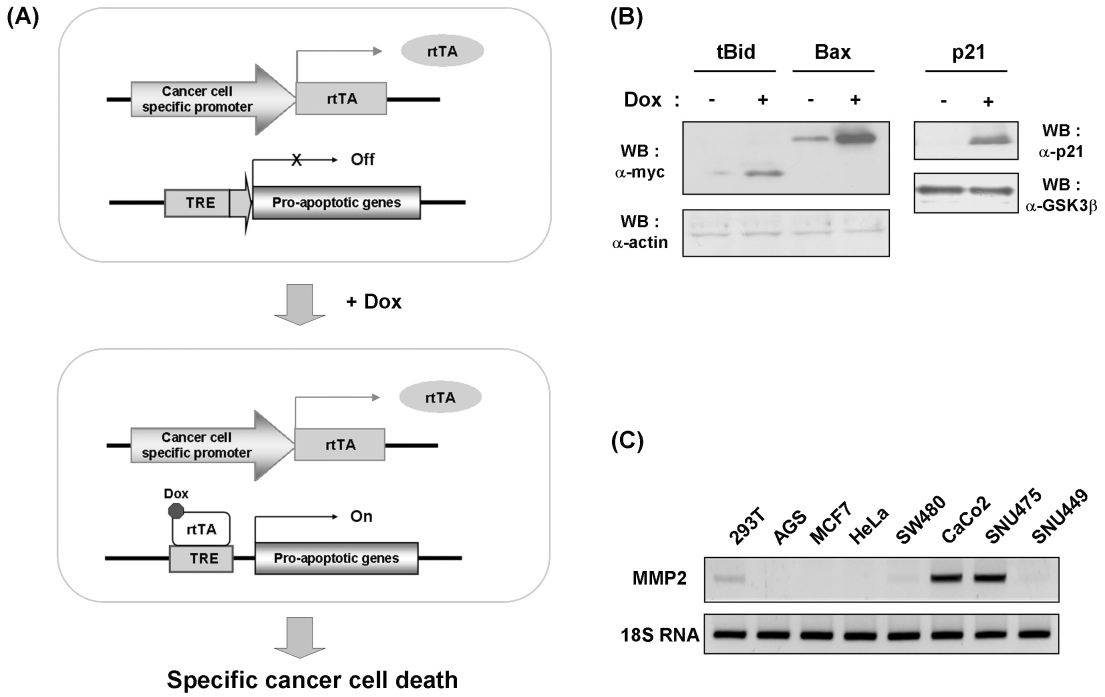

Fig. 1. (A) Schematic diagram for the induction of specific cancer cell death via the lentivirus-mediated Tet-On inducible system. Two lentiviruses, one containing rtTA2^S-M2 under a cancer-specific promoter and the other encoding for a pro-apoptotic gene controlled by the tetracycline-responsive element (TRE), are co-transduced into both normal and cancer cells. As cancer cells express more rtTA2^S-M2 than normal cells via the promoter that controls the specific expression of genes in cancer cells, Doxycycline treatment will result in the specific expression of pro-apoptotic genes in cancer cells, which will subsequently result in inducible cancer cell-specific death. (B) Inducible expression of proapoptotic genes. CMV-rtTA2^S-M2 plasmid was co-transfected with pBI-EGFP/tBid, pBI-EGFP/Bax, or pBI-p21, respectively, into HEK293T cells. Cells were incubated with or without 1 μg/ml of doxycycline in culture media for 24 h, followed by Western blotting. Actin or GSK3β expression was assessed to ensure even loading. (C) RT-PCR analysis reveals strong MMP-2 expression in the CaCo2 and SNU475 cell lines. 18S rRNA was analyzed to ensure even loading. PCR products were obtained after 35 cycles for MMP-2 and 18 cycles for 18S rRNA, respectively.

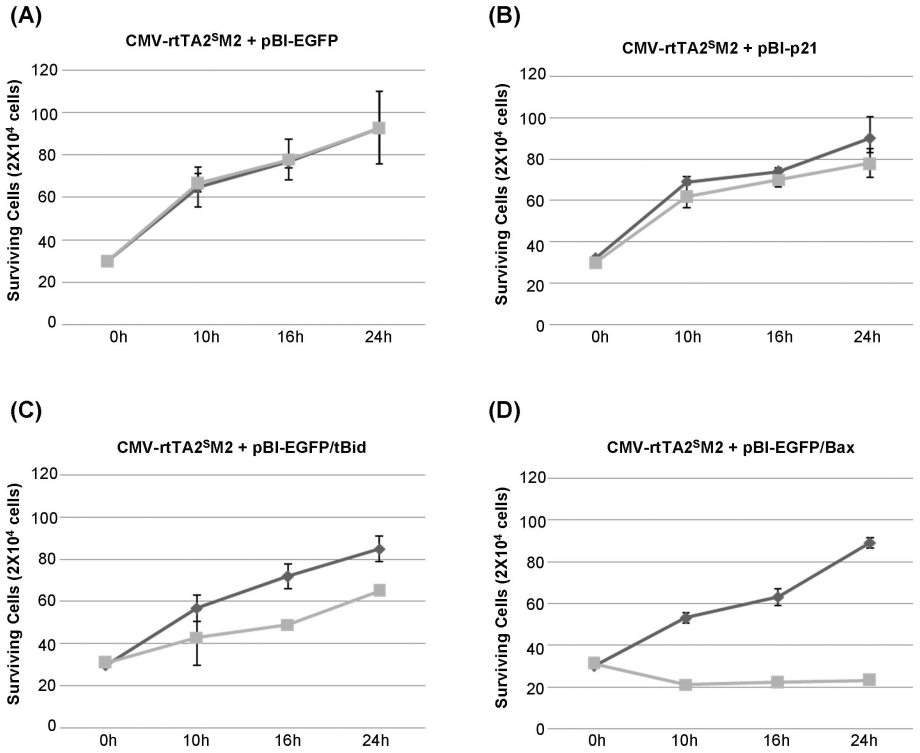

Fig. 2. Differential induction of cell death. CMV-rtTA2^S-M2 plasmid was co-transfected with pBI-EGFP (A), pBI-p21 (B), pBI-EGFP/tBid (C), or pBI-EGFP/Bax (D), respectively, into HEK293T cells. Cells were incubated in the absence or presence of doxycycline (1 μg/ml) as indicated, and the number of viable cells was counted using trypan blue. The graphs shown are representative of at least three independent experiments conducted in triplicate. The error bars represent standard deviations.

lines, but the results of our RT-PCR analysis demonstrated that MMP-2 was expressed abundantly in some, but not all, of the cancer cell lines (Fig. 1C). We do not know why these cell lines evidenced differential MMP-2 expression, but it remains possible that SNU475 and CaCo2 cells evidence higher levels of metastatic ability than other cell lines. MMP-2 expression has been shown to be higher in malignant tumors than in benign tumors (15, 16). However, we believed that the MMP-2 promoter could be utilized to assess the therapeutic use of the lentivirus-mediated Tet-On inducible system. We cloned the putative promoter of mouse MMP-2 via genomic PCR (approximately 2 kb of the putative MMP-2 promoter) and inserted it into the 5′-end of rtTA2^S-M2.

The death of SNU475 cells are specifically induced via lentivirus-mediated inducible expression of Bax

Many different methods for gene delivery in gene therapy have been developed–each system has its own advantages and disadvantages [References in (2)]. In this study, we attempted to use lentiviral vectors for efficient gene delivery. Fig. 3A shows the schematic diagram of the lentiviral vectors we employed in our experiments. First, we attempted to determine whether the lentivirus-mediated inducible expression system was working properly. Lenti-TRE-EGFP or Lenti-TRE-Bax was transduced into the HeLa cell line that had been stably transfected with CMV-rtTA2^S-M2. EGFP expression in Lenti-TRE-EGFP was verified via fluorescence microscopy (Fig. 3B). The results of our Western blot analysis demonstrated that the addition of Doxycycline to the HeLa cell line clearly induced Bax expression (Fig. 3C). We then attempted to determine whether the expression of target genes could be induced by the co-transduction of two different types of lentiviruses expressing rtTA2^S-M2 and TRE-EGFP. The inducible expression of EGFP upon Doxycycline treatment in HEK293T cells co-transduced with Lenti-TRE-EGFP and Lenti-MMP2-rtTA2^S-M2 indicates that the lentivirus-mediated Tet-On inducible system worked as expected (Fig. 3D).

Since MMP-2 expression levels are high in SNU475 cell lines (Fig. 1C), we hypothesized that the level of MMP-2 promoter-driven rtTA2^S-M2 would also be high in SNU475 cells and that the induced Bax expression from TRE-Bax would induce SNU475 cell death but might not significantly affect other cell lines. To evaluate that notion, SNU475 cells, Huh7 cells, which evidenced negligible MMP-2 expression (Suppl. Fig. 2), or normal human fibroblast (NHF) cells were co-transduced with Lenti-MMP2-rtTA2^S-M2 and Lenti-TRE-Bax, and the effect on cell death upon Doxycycline treatment was then assessed. Similar transduction efficiencies in these cell lines were verified via comparison of EGFP-positive cells following the transduction of Lenti-CMV-EGFP (data not shown). Whereas doxycycline treatment reduced the number of viable SNU475 cells by approximately 50% as compared to the untreated SNU475 cells, it did not induce death in the Huh7 and NHF cells (Fig. 4). These results indicate that the lentivirus-mediated Tet-On inducible system using the MMP-2 promoter can induce the spe-

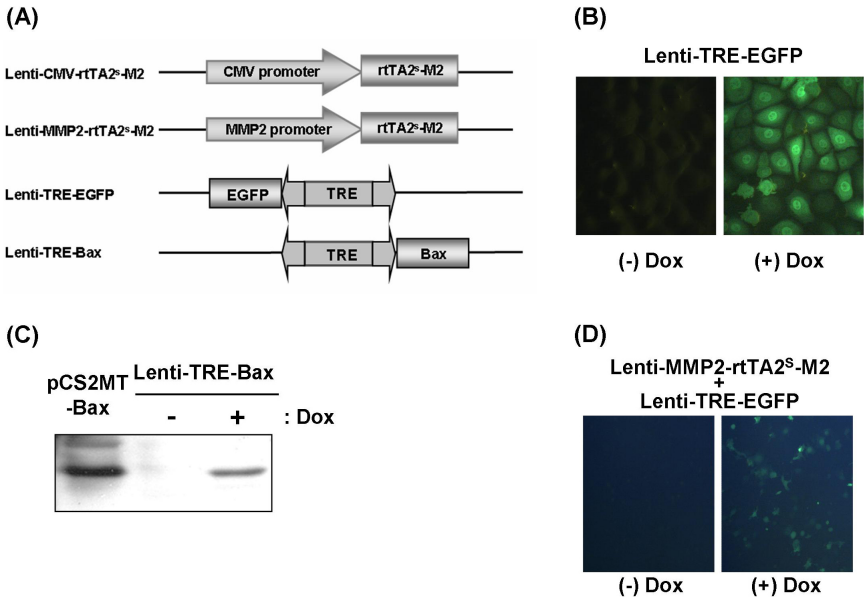

Fig. 3. Inducible expression of target genes using lentivirus-mediated Tet-On inducible system. (A) Schematic diagram of lentivirus vectors used in this work. (B) HeLa cells that stably express rtTA2^S-M2 were transduced with Lenti-TRE-EGFP and the cells were incubated for 24 h with or without 1 μg/ml of Doxycycline in culture media. EGFP expression was observed using fluorescence microscopy. (C) HEK293T cells were transfected with pCS2-MT-Bax plasmid (lane 1) and HeLa cells that stably express rtTA2^S-M2 were transduced with Lenti-TRE-Bax (lanes 2 and 3). Cells were incubated for 24 h without (lane 2) or with (lane 3) 1 μg/ml of doxycycline in culture media followed by Western blotting using anti-myc antibody. (D) Lenti-MMP2-rtTA2^S-M2 and Lenti-TRE-EGFP were co-transduced into HEK293T cells and the cells were incubated for 24 h without or with 1 μg/ml of doxycycline in culture media. Inducible EGFP expression was assessed using fluorescence microscopy.

cific death of cancer cells that express MMP-2 abundantly.

DISCUSSION

The lentivirus-mediated inducible expression of Bax under the control of the MMP-2 promoter specifically induced SNU475 cell death. Our data proved the possibility that the lentivirus-mediated Tet-On inducible system might have a viable therapeutic application. Our data strongly indicate that if certain gene promoters that are more abundantly expressed in cancer cells than in normal cells are used to express rtTA2^S-M2, the lentivirus-mediated Tet-On inducible system can be utilized for therapeutic purposes, to cure cancers via the induction of cancer cell-specific death. In addition, this approach may prove applicable to other therapeutic purposes, by using different promoters and TRE-linked target genes *in vitro*, and even *in vivo*.

Target gene expression in gene therapy must be tightly controlled in order to reduce side effects. We utillzed rtTA2^S-M2, an improved version of rtTA that has very low leakiness and high inducibility, combined with lentiviral vector. As is shown in Fig. 2, the identical growth patterns of CMV-rtTA2^S-M2- and pBI-EGFP-Bax-transfected cells and control cells in the absence of Doxycycline indicates that the leaky expression of Bax is quite low. In addition, the clearly observed induction of cell death upon only 10 h of doxycycline treatment suggests that the expression of the target gene using this system is highly inducible, and is sufficient to induce cell death. More importantly, leakiness and inducible expression appear to be more tightly controlled when the Tet-On system is delivered via lentiviral vector [Fig. 3 and (18)]. The lack of effect on cell death in Huh7 and NHF cells, which evidence very low MMP-2 expression levels even in the presence of doxycycline,

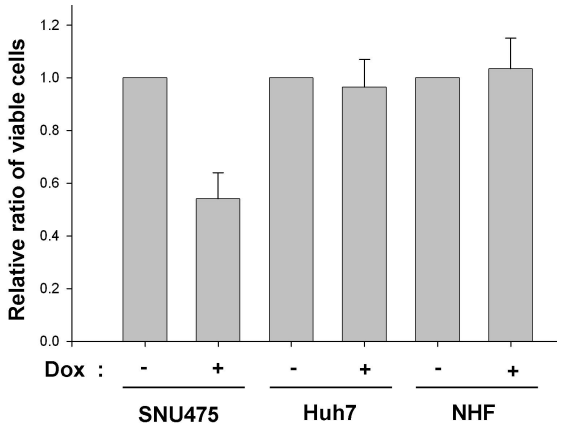

Fig. 4. Co-transduction of Lenti-MMP2-rtTA2^S-M2 and Lenti-TRE-Bax resulted in specific cell death of SNU475 cells. Lenti-MMP2-rtTA2^S-M2 and Lenti-TRE-Bax were co-transduced into the cell lines as indicated. Cells were incubated for 36 h without or with doxycycline (500 ng/ml) and the viable cells were counted using trypan blue. Since the growth rate of each cell line was different, the ratio of surviving cells incubated without and with doxycycline in the same cell line is shown in the bar graphs. The graphs shown are representative of at least three independent experiments in triplicate. The error bars represent standard deviations.

strongly indicates that the lentivirus-mediated Tet-On inducible system can be utilized for therapeutic purposes *in vivo*.

MATERIALS AND METHODS

Plasmids

Human Bax cDNA and human tBid cDNA were generated via

RT-PCR using *Pfu* DNA polymerase (Stratagene). These cDNAs were cloned into pCS2-MT vector in order to add the myc-epitope tag, and were then designated pCS2-MT-Bax and pCS2-MT-tBid, respectively. p21 cDNA was obtained from Dr. Eun Sung Hwang (University of Seoul, Korea). The myc epitope-tagged Bax and tBid inserts were cloned into pBI-EGFP vector (Clontech) and designated pBI-EGFP/Bax and pBI-EGFP/tBid, respectively. The EGFP coding sequence in the pBI-EGFP vector was replaced with p21 cDNA and designated pBI-p21. A lentiviral backbone vector, pCS-CG, which was generously provided by Dr. Inder Verma (19), was modified to enhance the cloning efficiency by reducing the size and enhancing the copy number of the vector. The *Sal*I-*Apa*I fragment (4,046 bp) of the pCS-CG plasmid was replaced with the *Sal*I-*Apa*I fragment (2,737 bp) of the pCS2-MT plasmid (20). This modified vector was designated pCS2-CG. In order to generate Lenti-TRE-EGFP, the *Hind*III-*Nhe*I fragment of pBI-EGFP harboring the TRE-EGFP sequences was cloned into the pCS2-CS vector following the removal of the sequences for the CMV promoter and GFP using *Eco*RI-*Xho*I (all sticky ends were filled-in with DNA polymerase I, Klenow fragment). Similarly, the *Spe*I-*Xho*I fragment of pBI-EGFP/Bax harboring the TRE-Bax sequences was cloned into the pCS2-CS vector following the removal of the *Eco*RI-*Xho*I fragment, and the resultant plasmid was named Lenti-TRE-Bax. The MMP2 promoter was obtained via PCR from genomic DNA isolated from L929 mouse fibroblast cells. The primers utilized were as follows: 5′-TTACTAGTGTACATGAAGTGTTCTTCAC-3′ and 5′-TTGAATTCCGTTGCGCTCCCGGGCTCCG-3′; the fragment was verified via sequencing. This promoter was exchanged with the CMV promoter from the CMV-rtTA2^{S}M2 vector (21) and the resultant construct was designated MMP2-rtTA2^{S}M2. MMP2- and the CMV-rtTA2^{S}M2 inserts were digested with *Xho*I/*Pvu*II and cloned into pCS2-CG following the removal of the *Eco*RI/*Xho*I fragment containing the CMV promoter and the GFP coding sequences. These vectors were named Lenti-MMP2-rtTA2^{S}M2 and Lenti-CMV-rtTA2^{S}M2, respectively.

Western blot analysis

Cells were lysed in lysis buffer [20 mM Hepes (pH 7.4), 0.5% Nonidet P-40, 250 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM PMSF, 2.5 μg/ml leupeptin, 100 μM sodium orthovanadate, 50 mM sodium fluoride]. The lysates were centrifuged for 10 min at 12,000 g at 4°C, and the supernatant was utilized for Western blot analysis. Total proteins were separated via SDS-PAGE and then transferred onto pre-wetted polyvinylidene fluoride (PVDF) membranes (MILLIPORE). The proteins were detected with primary antibodies and horseradish peroxidase-conjugated secondary antibodies using enhanced chemiluminescence Plus (Roche Diagnostic). We utilized anti-c-myc monoclonal antibody (9E10, Oncogene Research Products), anti-p21-HRP-conjugated antibody (c-19, Santa Cruz Biotechnology), anti-GSK3β monoclonal antibody (Transduction Laboratories), and anti-actin monoclonal antibody (Sigma).

RT-PCR

Total RNA was extracted from HEK293T, AGS, MCF7, HeLa, SW480, CaCo2, HepG2, Huh7, SNU449, and SNU 475 cell lines with TRIzol (Invitrogen), in accordance with the manufacturer's instructions. The following primers were utilized: endogenous human MMP2, 5′-GGAGGCGCTAATGGCCCG-3′ and 5′-GTATGTGATCTGGTTCTTG-3′; 18S rRNA, 5′-CGGCT ACCACATCCAAGGAA-3′ and 5′-GCTGGAATTACCGCGGCT -3′; GAPDH, 5′-AAGAAGGTGGTGAAGCAG-3′ and 5′-TCAT ACCAGGAAATGAGC-3′.

Cell cultures and generation of stable cell lines

HEK293T, AGS, MCF7, HeLa, SW480, CaCo2, HepG2, Huh7, SNU449, and SNU 475 cell lines were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Cambrex). In order to generate HeLa cells stably transfected with TRE-EGFP, the cells were transfected with pCDNA3, which includes a neomycin resistance gene as a selection marker, and a 3-fold dose of pBI-EGFP. Stable transfectants were selected with media containing 800 μg/ml of G418 (Life Technologies Gibco BRL). In order to generate HeLa cells that constitutively express rtTA2^S-M2, the cells were transfected with CMV-rtTA2^S-M2-neo (21), selected under media containing 800 μg/ml of G418, then maintained in DMEM supplemented with 10% FBS.

Viable cell counting

The number of viable cells was determined using trypan blue. Cells suspended in DMEM media were diluted at a 1:1 ratio with 0.4% trypan blue, and the unstained cells were counted.

TUNEL staining

Apoptotic cell death was examined using an ApopTag Red In Situ Apoptosis Detection Kit (CHEMICON) according to the manufacturer's protocol. The HEK293T cells were transiently transfected with plasmids using calcium phosphate methods. TUNEL assays were conducted after the cells were incubated for 16 h with doxycycline (500 ng/ml) in culture media.

Preparation of lentivirus and transduction

VSV-G-pseudotyped replication-deficient lentivirus vectors were generated via the co-transfection of three plasmids: the vector harboring the gene to be expressed (7 μg), the VSV-G-expressing construct pMD.G (7 μg), and the packaging construct pCMVΔR 8.2 (7 μg), from which all accessory protein genes are deleted. These constructs were transiently co-transfected into HEK293T cells via the calcium phosphate method (22, 23). Plasmids pMD.G and pCMVΔR 8.2 were generously provided by Dr. Inder Verma. After 20∼24 h, 70% of the media was replaced with fresh culture media. Viral supernatants were collected at 54∼60 h after transfection, followed by sterilization with a 0.45 μm cellulose acetate filter. In order to obtain a higher viral titer, the viral supernatant was

concentrated via 90 min of ultracentrifugation at 50,000 g at 4°C and the pellets were resuspended in PBS at 1/100 of the original volume (24). Lentiviral vectors were transduced into target cells with 4∼8 μg/ml of polybrene (Sigma) and incubated overnight. The culture medium was then replaced with fresh media and the cells were examined after 24∼36 h.

Acknowledgements
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