Development of a Cancer-Targeted Tissue-Specific Promoter System

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ABSTRACT

Present cancer gene therapy using proapoptotic genes has had limited success because the therapy is prone to cause side effects as a result of the lack of tissue and cancer specificity. To target cancer cells without damaging normal cells, we have designed a novel dual promoter system in which a tissue-specific transcription system under the control of a cancer-specific promoter drives expression of a therapeutic gene. The applicability of this system was demonstrated by adapting it to target lung cancer. We termed this lung cancer system TTS (TTFI gene under the control of human telomerase reverse transcriptase promoter and human surfactant protein A1 promoter). The TTS system showed much higher promoter activity in lung cancer cells compared with other kinds of cancer and normal lung cells, including stem cells. Moreover, insertion of negative glucocorticoid responsive elements in the system allows it to be drug controllable. The approaches that we have used could be adapted to target other types of cancer. We report a novel cancer-targeted tissue-specific dual promoter system designed for gene therapy.

INTRODUCTION

Gene therapies such as replacement of the tumor suppressor gene p53 (1) or the transfer of the proapoptotic suicide gene HSVtk (2) into cancer cells have been an attractive strategy for the management of tumors. In terms of the development of a cancer-specific promoter system, there have been three major systems for gene therapies. First, the cytomegalovirus (CMV) promoter along with a combination of the tumor suppressor gene p53 has been used to manage different types of cancers. Although the CMV promoter is a constitutive promoter, this has not been a problem because p53 causes apoptosis only in cancer cells and not in normal cells. This treatment has been used successfully (1), but it has a drawback because it does not function in p53-resistant cancer cells (3). Additionally, a proapoptotic suicide gene therapy would have to be developed to cause cell death in the p53-resistant cancer cells. The problem in using a proapoptotic gene is that it causes apoptosis nonspecifically in all of the cells, including normal cells. To resolve the problem of nonspecificity of targeting cancer cells, Gu et al. (4, 5) developed another system using the human telomerase reverse transcriptase (hTERT) promoter instead of the CMV promoter. The hTERT promoter, a determinant for the activity of telomerase (6, 7), is strongly active in normal prostate cells (21). The use of a tissue-specific promoter for targeting cancer cells with a proapoptotic suicide gene under the control of hTERT-responsive elements that provide a means of controlling the system in vivo. We show that the TTS system is tissue and cancer specific, and is controllable by dexamethasone. Present gene therapy treatments have had limited success because they are inefficient and prone to cause side effects as a result of the lack of tissue and cancer specificity. We report a novel tissue- and cancer-specific promoter system that can be adapted to target different types of cancers and tissues.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. The human large cell lung carcinoma cells H1299 were the generous gift of Dr. Adi F. Gazdar (Hamon Center for Therapeutic Oncology Research, University of Texas Southwestern Medical Center, Dallas, TX) and were grown as monolayer in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS). The human adenocarcinoma cells A549 and H441, and the human breast cancer cells MCF7 were obtained from American Type Culture Collection (Manassas, VA), and grown in Ham’s F12 (A549 cells) and RPMI 1640 (H441 and MCF7 cells) supplemented with 10% heat-inactivated FBS. The transformed human embryonic kidney cells HEK293T were maintained in high glucose DMEM supplemented with 10% heat-inactivated FBS. The human hepatoblastoma cells HepG2 were maintained in Eagle’s MEM supplemented with 1 mM sodium pyruvate, 0.1% nonessential amino acids, and 10% heat-inactivated FBS. The normal human lung fibroblast cells NHLF and the normal human small airway epithelial cells SAEC (Clonetics, San Diego, CA) were grown in culture medium supplied by the manufacturer. The mouse embryonic feeder cells were obtained from Stem Cell Technologies (Vancouver, British Columbia, Canada) and grown in high glucose DMEM supplemented with 10% heat-inactivated FBS and 100 μM 2-mercaptoethanol. The mouse embryonic stem cells ES D3 were from the American Type Culture Collection and grown in KNOCKOUT DMEM with 15% KNOCKOUT SR (Invitrogen, Carlsbad, CA). 2 mM l-glutamine, 100 μM MEM nonessential amino acid solution, 100 μM 2-mercaptoethanol, and 1000 units/ml leukemia inhibitory factor. All of the cell lines were cultured in 5% CO2 at 37°C.

Plasmids. The hTERT promoter region ~378/+79 was kindly provided by Dr. Bingliang Fang (Department of Thoracic and Cardiovascular Surgery, University of Texas M.D. Anderson Cancer Center, Houston, TX) and subcloned into the pGL3-Basic luciferase reporter construct (pGL3.hTERT/Luc; Promega, Madison, WI; Fig. 1A; Ref. 23). Dr. Parviz Minoo (Department of Pediatrics, Women’s and Children’s Hospital, University of Southern California School of Medicine, Los Angeles, CA) kindly provided the plasmid that
The hSPA1 promoter was obtained from genomic DNA of H441 cells. The luciferase reporter constructs (Fig. 3C) were generated by subcloning multiple parts of the hSPA1 promoter region: −253/+59, −180/+59, −169/+59, −157/+59, and −83/+59 into pGL3-Basic luciferase reporter construct. The hSPA1 TATA box (TATAAA) free region −157/~36 was generated from pGL3.hSPA1−253 (Fig. 3C) and subcloned into pGL3.hSPA1−157 (pGL3.hSPA1−157 2x). The plasmids pGL3.hSPA1−157 3x to 5x were constructed in the same manner (Fig. 4A).

Bicistronic plasmid phTERTTTF1:hSPA1−157 5x/Luc (Fig. 1C, top) was constructed by excising hTERT/TTF1 and SV40 late polyadenylic acid signal fragment from pGL3.hTERTTTF1 and ligating it into pGL3.hSPA1−157 5x. All of the recombinant plasmids were sequenced to ensure insert identity and proper insert orientation.

Transient Transfection Reporter Assays. All of the transfections were carried out in six-well plates. Cells were seeded 1 day before transfection at the following densities: 0.3 × 10^5/well for H1299 cells; 0.4 × 10^5/well for A549, H441, Hep3B, and MCF7 cells; and 0.50 × 10^5/well for NHLF and SAEC cells. Transfections were carried out with Lipofectin (Invitrogen) in accordance with the manufacturer’s protocol as indicated. Transfected NHLF and SAEC cells were harvested 40 h after lipofection. All of the other transfected cells were harvested at 24 h. Results of one representative experiment are presented as fold induction of relative light units normalized to β-galactosidase activity relative to that observed for the control vectors. Each experiment was repeated at least three times. Error bars indicate the SD from the average of the triplicate samples in one experiment.

Immunoblot and Gel Retardation Assays. Crude nuclear extracts were prepared from cells overexpressing human TTF1 as described previously (22, 24). Equal amounts of protein were transferred electrophoretically to a Hybond polyvinylidene difluoride transfer membrane (Amersham, Arlington Heights, IL) that was incubated with primary and secondary antibodies. The Supersignal West Pico chemiluminescence protocol (Pierce, Rockford, IL) was used to detect secondary antibody binding. Electromobility shift assays were performed as described previously using the putative TTF1 binding sites of the hSPA1 promoter (22, 24). The following oligonucleotides were used as probes: Probe a, hSPA1 −187/~158: 5’TTCTGTGCTCCCC TCAAGGGCTCTTT-GAGA; Probe b, hSPA1 −157/−128: 5’TCTCCACGCTGATGCTCTTG-GGGGAAAACA; and Probe c, hSPA1 −96/~67: 5’CTGAGGCACAGGTGGTGTT-GTGTTAGGGCTCTC.”
Fig. 3. Characterization of human surfactant protein A1 (SPA1) promoter. A, sequence comparison of thyroid transcription factor 1 (TTF1) binding elements (TBEs) between baboon SPA1, 2 (hSPA1, 2), and human SPA1 (hSPA1). Sequences of hSPA1 were reported previously (50). hSPA1, 2 and cyclic AMP response element for SPA promoter (CRESPA) and TBEs were identified by Li et al. (35). The hSPA1 promoter region sequences −208/−203 (CTGGAG), −175/−169 (CTCCCAAG), −164/−161 (TTTGG), −155/−149 (CCTTGG), and −83/−78 (TTTGG), corresponding to baboon TBEs, are shown as potential TBEs. B, the sequence of the proximal promoter of hSPA1. Potential TBEs, CRESPA element, negative glucocorticoid responsive elements, and probes A–C are underlined by single solid, double solid, broken solid, and single thin lines, respectively. TATA box, GT box consensus motif, and the region of the hSPA1 promoter that mediates repression of transcription by dexamethasone are also indicated. C, schematic representation of hSPA1 promoter deletion reporter constructs. The hSPA1 promoter fragments containing potential TBEs were inserted into the luciferase (Luc) reporter vector pGL3.Basic. The name of each reporter construct was assigned according to the 5′-end of the nucleotide number of the inserted promoter sequences. D, transient transfection reporter assays in Hep3B and A549 cells with the indicated hSPA1 luciferase reporter constructs (2 μg), either pGL3.hTERT or pGL3.hTERT/TTF1 (2 μg), plus pCMV β-galactosidase (2 μg). Results are presented as fold induction of relative light units normalized to β-galactosidase activity relative to that observed for control constructs. E, gel shift assay to identify TTF1 binding sites in the proximal promoter of hSPA1. Nuclear extracts from 293T cells transfected with either pGL3.hTERT or pGL3.hTERT/TTF1 were incubated with 32P-labeled probes ± antibody to TTF1 shown above each lane. Probes are shown in A and B and in “Materials and Methods.” The top arrow designates supershifted bands. Antibody to TTF1 changed the mobility of TTF1 protein and hSPA1 probe complex in Lanes 16 and 17, but the antibody did not detect a complex in Lane 18, indicating that probe c does not have a TBE.

units (pfu)/cell, and all of the other human cells were infected at an MOI of 40 pfu/cell. Mouse embryonic stem cells were infected at an MOI of 100 pfu/cell.

Flow Cytometric Analysis for GFP. Quantitation of GFP expression was performed by FACScan (Becton Dickinson, Mountain View, CA). Thirty-six h after infection, cells were harvested and washed once with PBS. Cells were resuspended in 400 μl PBS for FACS analysis. Cell doublets and cell debris were gated out, and the analysis was done using CellQuest version 3.3 software (Becton Dickinson).

Dexamethasone Inhibition of hSPA1 Promoter Activity. A549 cells were treated with dexamethasone (0–500 nm) for 24 h after lipofection with bicistronic plasmid pHtERT/TTF1-hSPA1−157 5xLuc (2 μg) plus pCMV β-galactosidase (2 μg), or 36 h after Ad-TTS/GFP infection at an MOI of 40 pfu/cell. Results are presented as percentage of relative light units normalized to β-galactosidase activity relative to that observed for dexamethasone, or as percentage of GFP-positive cells by flow cytometric analysis.

RESULTS

Design of Cancer-Targeted Tissue-Specific Promoter System. To develop a transfection system that might be targeted only to cancer cells in normal tissues, we designed a novel tissue- and cancerspecific promoter system (Fig. 5). This system consists of two parts. First, a tissue-specific transcription factor is induced in cancer cells by using a cancer-specific promoter. Second, the induced tissue-specific transcription factor transactivates target gene expression with the tissue-specific promoter, which corresponds to the DNA binding site of the tissue-specific transcription factor, presumably along with other endogenous tissue-specific cofactors (26, 27). To test the effectiveness and applicability of this system, we decided to develop a lung cancer-specific promoter system.

Application of Cancer-Targeted Tissue-Specific Promoter System for Lung Cancer. To develop a lung cancer-specific gene therapy system using our dual promoter system, we used the hTERT promoter as a cancer-specific promoter (Figs. 2 and 5). To provide tissue specificity, we next looked for a lung-specific transcription system (Figs. 3 and 5). The TTF1 and human surfactant protein A (hSPA) have been shown to be tissue-specific proteins that are expressed in normal lung respiratory epithelium (28). TTF1 is an 83,000 nuclear transcriptional protein and is a member of the Nkx homeodomain gene family. TTF1 has been shown to bind and transactivate some lung-specific promoters, including that of hSPA (29, 30). hSPA is an abundant, M, 38,000 nuclear transcriptional protein and is a member of the Nkx homeodomain gene family. TTF1 has been shown to bind and transactivate some lung-specific promoters, including that of hSPA (29, 30). hSPA is an abundant, M, 38,000–36,000 glycoprotein secreted by respiratory epithelial cells in the lung. It is involved in surfactant physiology, structure, and metabolism (31, 32), and is produced in the lung in at least two cell types: alveolar type II cells and nonciliated bronchiolar cells known as Clara cells (33). In humans and baboons,
two highly similar genes, \( \text{SPA1} \) and \( \text{SPA2} \), encode \( \text{SPA} \), whereas in rabbits, rats, and mice, a single-copy gene encodes \( \text{SPA} \) (34). Li et al. (35) identified TTF1 binding elements (TBEs) in the baboon \( \text{SPA} \). The TBEs were found to reside within a 255-bp region flanking the 5′-end of the \( \text{SPA1} \) and \( \text{SPA2} \) genes (Fig. 3A; Ref. 36). A feature of the hSPA1 promoter that additionally makes it potentially useful in gene therapy is that it has a region that enables glucocorticoid-mediated inhibition of transcription enabling the in vivo control of gene therapy by glucocorticoid drugs. Hoover et al. (36) identified the region of the hSPA1 promoter between +14 and +59 that is repressed by glucocorticoid, and this region included two negative glucocorticoid responsive elements (Fig. 3B). In H441 cells (human lung adenocarcinoma cancer cells), dexamethasone (a glucocorticoid) decreases total hSPA mRNA levels in a dose- and time-dependent manner (37, 38). Therefore, the use of negative glucocorticoid responsive elements in a transactivation system for gene therapy can enable the therapy to be drug controlled. The hSPA1 promoter has been used to target TTF1-positive H441 cells, but the hSPA1 promoter is not active in A549 cells (human lung adenocarcinoma) that do not express TTF1 (Fig. 2; Ref. 39). To explore the usefulness of TTF1 and the hSPA1 promoter for use in gene therapy for lung cancers, we put the hSPA1 promoter under the control of TTF1 driven by the hTERT promoter, which is active in cancer cells.

Use of Cancer-Specific hTERT Promoter to Drive Lung-Specific Transcription Factor TTF1 Expression. First, we measured the ability of hTERT to drive the expression of TTF1 in cells that otherwise do not produce TTF1. A construct containing only hTERT as a control (pGL3.hTERT) and a construct containing hTERT with TTF1 (pGL3.hTERT/TTF1) were tested for their ability to promote TTF1 expression in cells that normally do not express TTF1, including transformed embryonic kidney cells (293T), lung cancer cells (A549), and non-lung cancer cells (Hep3B; Fig. 2). Immunoblot analysis of TTF1 expression showed that TTF1 protein could only be detected in cells that contained the plasmid with the hTERT/TTF1 combination and in the H441 cells that normally produce TTF1 (Fig. 2). This result demonstrates the ability of the hTERT promoter to drive expression of TTF1 in cells that normally do not express TTF1. The ability to drive expression of TTF1 in cancer cells coupled with the tissue specificity conferred by the use of the hSPA1 promoter confers lung- and cancer-specific expression.

Identification of the Region in the hSPA1 Promoter That Confers Tissue-Specific Transactivation When Under the Control of TTF1 Driven by the hTERT Promoter. To identify hSPA1 TBEs that are homologous to the baboon TBEs, we analyzed the hSPA1 sequence and found the following to be potential TTF1 binding sites based on sequence similarity to those found in the baboon: −208/−203 (CTGGAG), −175/−169 (CTCAAG), −164/−161 (TTTG), −155/−149 (CTCAG), and −83/−78 (TGTGG; Fig. 3, A and B). To identify a region of the hSPA1 promoter that might be useful for lung cancer-specific transactivation by TTF1, we made deletion constructs of the hSPA1 promoter (Fig. 3C) and compared their effectiveness in promoting transcription mediated by a construct (pGL3.hTERT/TTF1) that expresses TTF1 driven by the hTERT promoter. These constructs were used in transient transfection reporter assays in A549 cancer cells (human lung adenocarcinoma) and Hep3B cells (human hepatoblastoma; Fig. 3D), and we measured their ability to promote the expression of the reporter gene luciferase. The ideal construct would have minimal activity in non-lung cancer Hep3B cells but good induction in lung cancer A549 cells. In the control, which lacked pGL3.hTERT/TTF1, all of the hSPA1 deletion constructs had little transcriptional activity (0.58–1.32-fold; Fig. 3D, left). In the presence of pGL3.hTERT/TTF1, the proximal 253-bp fragment of the hSPA1 promoter exhibited the highest activity (3.30–8.36-fold). The 83-bp fragment conferred no activity in Hep3B and A549 cells above control levels. Promoter deletions of −180, −169, and −157 showed stepwise decreases in transcriptional activity in Hep3B cells (1.62–1.17-fold) and A549 cells (4.30–2.68-fold; Fig. 3D, right). The −157 construct showed the most promise as a lung cancer-cell-specific promoter because it had good transcriptional activity in A549 lung cancer cells with little activity in Hep3B cells.

To determine the region in which TTF1 binds to the hSPA1 promoter, we used a gel shift assay (Fig. 3E). Radiolabeled oligonucleotide probes were constructed that spanned the following regions of the hSPA1 promoter: probe a = −187 to −158; probe b = −157 to −128; and probe c = −96 to −67 (Fig. 3, A and B). There was binding of all of the probes to the nuclear extracts from 293T cells
whether they contained TTF1 (Fig. 3E, Lanes 4–9 and 13–18). This indicates that there may be many factors in the nuclear extracts that are capable of binding to the hSPA1 promoter. However, in the presence of TTF1 antiserum, only probes a and b formed one supershifted band (Fig. 3E, Lanes 16 and 17, top arrow). The supershifted band represents a complex of hSPA1 promoter fragment and TTF1 antibody bound to TTF1. There was no complex formed with the portion of the hSPA1 promoter represented by probe c (Fig. 3E, Lane 18), and there were no complexes formed when TTF1 was not present. T 

Taken together with the transient transfection reporter assays, these results suggest that the 157-bp fragment upstream of transcription initiation sites of the hSPA1 promoter is the region that best confers lung cancer-specific transactivation under the control of TTF1 driven by the hTERT promoter.

Analysis of the Effect of Tandem hSPA1 Promoters. To see if we could increase the level of transcriptional activity, we tested the effect of adding additional tandem copies of the hSPA1 – 157/-36 promoter region (without a TATA box) to a construct (pGL3.hSPA1 – 157 1x; Fig. 4A) that was under the control of TTF1 driven by the hTERT promoter (pGL3.hTERT/TTF1; Fig. 4B). In A549 cells, transcriptional activity increased 11.23-fold with the addition of one extra hSPA1 cassette – 157/-36 (pGL3.hSPA1 – 157 1x versus pGL3.hSPA1 – 157 2x; Fig. 4B). Each additional hSPA1 cassette resulted in increased transcriptional activity. Moreover, with five tandem copies (pGL3.hSPA1 – 157 5x), the activity increased 26.17-fold (Fig. 4B). These results demonstrated that pGL3.hTERT/TTF1 and pGL3.hSPA1 – 157 5x generated the highest lung cancer-specific transactivation, and we call it the TTS system (TTF1 gene under the control of hTERT promoter and hSPA1 promoter system).

Comparison between the hTERT Promoter and TTS Systems. To compare the TTS system (Fig. 1B) with the hTERT promoter-mediated gene expression system (Fig. 1A), we did transient transfection reporter assays in different kinds of cells. As shown in Fig. 1A, hTERT promoter activity was higher in human cancer cells (H441, A549, H1299, MCF7, and Hep3B; 4.61–13.16-fold) than in normal human lung cells (NHLF and SAEC; 1.26–2.14-fold). These results are consistent with previous studies (4, 5, 7, 40). Conversely, the TTS system generated high transcriptional activity (15.04–53.11-fold) only in lung cancer cells (H441, A549, and H1299) compared with normal lung cells and other cancer cells (NHLF, SAEC, MCF7, and Hep3B; Fig. 1C). These data suggest that the combination of pGL3.hTERT/TTF1 and pGL3.hSPA1 – 157 5x can control hTERT activity and generate high lung cancer-specific promoter activity.

A single bicistronic construct or a viral vector for gene expression is desirable and convenient because clinical use of two vectors could raise several concerns, such as cotransfection or coinfection efficiency, and safety issues (5, 20). Thus, we combined the hTERT, TTF1, and hSPA1 sequences, and created the TTS bicistronic plasmid pH TERT/TTF1-hSPA1 – 157 5x (Fig. 1C, top). To evaluate the efficiency of the combined system, we added the reporter gene luciferase to the TTS construct and tested promoter activity in different types of human cells. Fig. 1C (bottom) shows that the bicistronic TTS plasmid had increased promoter activity compared with the two separate constructs (pGL3.hTERT/TTF1 and pGL3.hSPA1 – 157 5x) in A549 (32.01-fold versus 21.03-fold) and H1299 cells (49.40-fold versus 15.04-fold), and maintained lung cancer specificity in all of the lung cancer cells. For additional testing of the TTS system, we created an adenovirus TTS construct expressing GFP as a reporter (Ad-TTS/ GFP; Fig. 6A). Flow cytometric analysis showed that Ad-TTS/GFP induced GFP production in the lung cancer cells A549, H441, and H1299, whereas it showed little expression in normal lung SAEC cells or in other non-lung cancer cells such as MCF7 and Hep3B cells (Fig. 6B).

No Promoter Activity Is Detectable in Stem Cells by the TTS System. To evaluate the effects of the TTS system in stem cells or progenitor cells, we examined GFP expression induced by Ad-TTS/ GFP in mouse embryonic stem cells (Fig. 6C). When a construct driven by the CMV promoter was tested, the population of cells showing fluorescence (caused by the expression of GFP) reached 45.93% in the embryonic stem cells. When a construct driven by hTERT was tested, 38.71% of the cell population was fluorescent because embryonic stem cells have high telomerase activity (12, 16). Conversely, Ad-TTS/GFP did not show any significant fluorescent population (4.57%). These data suggest that the TTS system does not target normal cells, including stem cells.

Glucocorticoid Suppression of TTS System. An important feature of the TTS system is that it contains the two negative glucocorticoid responsive elements of the hSPA1 promoter (Fig. 1B, Fig. 3B, and Fig. 6A). Therefore, we examined the effect of dexamethasone (a glucocorticoid) on the TTS system.Transient transfection reporter
inhibition of green fluorescent protein (GFP) expression mediated by Ad-TTS/GFP (Fig. 7). Dexamethasone dose-dependent reduction of GFP production (Fig. 7A). Flow cytometric analysis with Ad-TTS/GFP-infected A549 cells also showed dexamethasone dose-dependent repression in A549 cells down to 31.45% (Fig. 7A).

A. A549/Bicistronic vector: hTERT/TFI · hSPA1-157 5x/Luc. B. A549 lung cancer cells

Fig. 7. TTS system controlled by glucocorticoid. A. transient transfection reporter assays in A549 cells with the bicistronic construct pH31/TTFI·hSPA1–157 5x/5Luc (Fig. 1C, top) with dexamethasone (Dex). Transfected A549 cells were treated with Dex as the indicated concentration. Results are presented as percentage of relative light units normalized to β-galactosidase activity relative to that observed for no treatment. B. Dex inhibition of green fluorescent protein (GFP) expression mediated by Ad-TTS/GFP (Fig. 7B) in A549 cells. Results are presented as percentage of GFP-positive cells by flow cytometric analysis.

assays demonstrated that dexamethasone dose-dependently repressed the promoter activity of TTS. The TTS plasmid containing the luciferase reporter gene (pH31/TTFI·hSPA1–157 5x/Luc) was repressed in A549 cells down to 31.45% (Fig. 7A). Flow cytometric analysis with Ad-TTS/GFP-infected A549 cells also showed dexamethasone dose-dependent reduction of GFP production (Fig. 7B).

**DISCUSSION**

Cancer gene therapies using proapoptotic genes have been tested extensively (43–45). One limitation of these therapies is the toxicity to normal cells. Targeted expression of therapeutic genes is desired to prevent this toxicity. The hTERT promoter has been expected to be useful for targeted transgene expression (4, 5, 7, 40), but its mediated gene expression system has the potential to affect negatively some normal cells or tissues that have high telomerase activity, such as stem cells (Fig. 6C; Refs. 12–17). To improve the efficacy and safety of lung cancer gene therapy, we designed the TTS system, a cancer-targeted tissue-specific promoter system combining the TFI gene driven by the hTERT promoter with five tandem copies of a portion of the hSPA1 promoter generating a novel lung cancer-specific promoter system that does not target normal cells, including stem cells.

In the process of making a cancer-targeted lung-specific promoter, we identified the hSPA1 promoter region –157/-159 containing one TBE that generated high lung cancer-specific transcription under the control of TFI driven by the hTERT promoter (Fig. 3D). In the presence of TFI driven by the hTERT promoter and pGL3.hSPA1–157 5x that has five additional TBEs, there was no promoter activity in non-lung cancer Hep3B cells (human hepatoblastoma), which have high telomerase activity and no endogenous TFI (Fig. 2, Fig. 3D, and Fig. 4B). TFI may not be the only factor recruited on the hSPA1 promoter based on the amount of binding of the hSPA1 promoter fragment to unknown nuclear factors as seen in the gel shift assay (Fig. 3E). Therefore, it is possible that the lung cancer specificity of the TTS system also may be based on the existence of additional unknown lung-specific accessory factors that interact with TFI on the hSPA1 promoter (26, 27, 42).

Potential toxicity to stem cells has been recognized as one of the major concerns regarding the use of the hTERT promoter to drive proapoptotic or cytotoxic gene expression. Hematopoietic stem cells, lymphohematopoietic cells, and germ cells are known to have high telomerase activity (12, 15, 16). Recent studies show that stem cells located in the bottom third of intestinal crypts, stem cell layers of the skin, and epithelial liver stem cells also have telomerase activity (12, 13, 16). Moreover, regenerative and precancerous lesions in cirrhosis and chronic hepatitis have been reported to have high telomerase activity (14, 17). Thus, the hTERT promoter-mediated gene expression system may affect negatively these cells and tissues.

Although relatively high doses of recombinant adenovirus vectors are required to infect hematopoietic stem cells or embryonic stem cells (43–45), it has been reported that successful IFN-α gene transfection into human CD34+ hematopoietic stem cells was achieved by infection with Ad-CMV/IFN-α at an MOI of 120–240 pfu/cell (43, 44). In our study, the fluorescent population of embryonic stem cells reached 45.93% 36 h after infection with Ad-CMV/GFP at an MOI of 100 pfu/cell (Fig. 6C) and 59.44% at an MOI of 500 pfu/cell (data not shown). In the case of Ad-hTERT/GFP, 38.71% population of the cells was GFP positive at an MOI of 100 pfu/cell, whereas with Ad-TTS/GFP, it induced only 4.57%. These data suggested that hTERT promoter-mediated gene expression also could target the stem cells and other tissues with high telomerase activity such as cirrhosis. We proved that the TTS system targeted only lung cancer cells and not stem cells compared with hTERT-mediated gene expression system. Thus, the TTS system is expected to be much safer than hTERT, SV40, or CMV promoter-mediated target gene expression systems because of potentially fewer side effects.

For practical gene delivery, we chose the adenovirus-mediated delivery system with the TTS system (Fig. 6) because the adenovirus system is used most extensively for lung cancer gene therapy (1). Moreover, when used systemically, this system could be used not only for apoptosis-based therapy but also for the diagnostic purpose to detect metastasis in other organs by tracing GFP signals (18, 46). As an additional potential improvement, the system can be used with retroviruses, liposomes, or new delivery systems such as bacteria, adenoassociated and herpes simplex viruses, lentiviruses, cationic polymer-DNA complexes, and electroporation, which would bring more cancer specificity (47).

Safety for patients is the major consideration in clinical gene therapy, and every possible effort must be attempted to minimize the risks (48). For low-risk cancer gene therapy, drug-controllable transactivation system is clinically important (49). In this study, we show that dexamethasone suppressed 70% of the promoter activity of the TTS system and can be used potentially to down-regulate the TTS system in case complications do arise. It may be possible to insert more negative glucocorticoid responsive elements into the system to induce additional suppression of the activity. The combination of dexamethasone control and lung cancer-specific gene expression...
should make the TTS system ideal for the gene therapy management of lung cancer. In addition, the approaches that we have used could be adapted to target other types of cancer cells and tissues.

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