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## Repression of Human Telomerase Reverse Transcriptase Using Artificial Zinc Finger Transcription Factors

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## Signaling and Regulation

Repression of Human Telomerase Reverse Transcriptase  
Using Artificial Zinc Finger Transcription FactorsJoon Hyung Sohn<sup>1,4</sup>, Byung-II Yeh<sup>1,2,3</sup>, Jong-Wan Choi<sup>1,2</sup>, Joonho Yoon<sup>1</sup>, Jun Namkung<sup>1</sup>, Kwang-Kyun Park<sup>4</sup>, and Hyun-Won Kim<sup>1,2</sup>

## Abstract

Telomerase activation is a key step in the development of human cancers. Expression of the catalytic subunit, human telomerase reverse transcriptase (hTERT), represents the limiting factor for telomerase activity. In this study, we have used artificial zinc finger protein (ZFP) transcription factors (TF) to repress the expression of hTERT in human cancer cell lines at the transcriptional level. We have constructed four-fingered ZFPs derived from the human genome which binds 12-bp recognition sequences within the promoter of the *hTERT* gene and fused them with a KRAB repressor domain to create a potent transcriptional repressor. Luciferase activity was decreased by >80% in all of the transcriptional repressors with luciferase reporter assay. When they were transfected into the telomerase-positive HEK293 cell line, a decrease of mRNA level and telomerase activity together with shortening of telomere length was observed. Actual growth of HEK293 cells was also inhibited by transfection of artificial ZFP-TFs. The repression was maintained for 100 days of culture. The repression of telomerase expression by artificial ZFP-TFs targeting the promoter region of the *hTERT* presents a new promising strategy for inhibiting the growth of human cancer cells. *Mol Cancer Res*; 8(2); 246–53. ©2010 AACR.

## Introduction

A key property of malignant tumors is their immortality or limitless replicative potential (1). It is believed that the maintenance of telomeres is essential for the immortality of cancer cells. Telomeres are maintained by a specialized reverse transcriptase, the ribonucleoprotein telomerase, which is composed of a ubiquitously expressed RNA subunit, human telomerase RNA component (hTERC; ref. 2), and a protein catalytic subunit, human telomerase reverse transcriptase (hTERT), the expression of which is highly regulated (3, 4). The expression of hTERT represents the limiting factor for telomerase activity. Therefore, the regulation of hTERT at the transcriptional level could be an effective method for cancer regulation.

Promoter characterizations of the *hTERT* gene have been previously described (5–7). It has been suggested that the *hTERT* core promoter, encompassing the proximal 283 bp region upstream of the initiation ATG codon, is essential for transcriptional activation (Fig. 1A; ref. 8). A

large number of transcription factors act on the promoter of *hTERT* such as SP1 (6), c-MYC (9), the estrogen receptor (10), E2F-1 (11), WT-1 (12), and MZF-2 (13). These have been reviewed in recent investigations (14, 15).

The Cys2-His2 class of zinc finger protein (ZFP) is the most common DNA-binding motif in the human genome and provides an ideal scaffold for designing artificial transcription factors (TF) with novel DNA sequence specificities. Due to their modular nature, zinc fingers could be used like molecular building blocks to create multi-finger domains recognizing extended DNA sequences (16).

Various artificially engineered ZFPs with DNA-binding specificities were created by using phage display (17, 18). These ZFPs could be used as modular building blocks in the construction of sequence-specific DNA-binding proteins. Fusion of these ZFPs with transcriptional repression domains or activation domains could generate potent transcriptional repressors or transcriptional activators controlling endogenous human genes as well as plant and viral genes (19–24).

ZFPs could also be isolated by screening, in yeast, plasmid libraries that encode zinc fingers derived from human genomes (25). Unlike ZFPs using phage display in which mutations were incorporated at key positions in a given zinc finger framework, ZFPs with sequences in the human genome could be used intact as wild-type, and were revealed to be more natural and efficient for regulating human endogenous gene expression than those selected *in vitro* using phage display.

Telomerases are activated in a variety of tumors, and only few tumors maintain telomeres by an alternative mechanism which relies on recombination (26–28). In

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contrast to normal somatic cells, telomerase is highly reactivated in cancer cells. Thus, telomerase could be an important target in developing new anticancer agents. Many recent strategies have been targeting telomerase inhibition (15, 29-31).

In this study, we have artificially constructed four-fingered ZFPs using the domains derived from the human genome, which bind 12 bp recognition sequences within the core promoter of the endogenous *hTERT* gene (Fig. 1A). We have created a potent transcriptional repressor domain by fusing these ZFPs with a KRAB repressor domain (Fig. 1B). Our results show that the artificial ZFP-TFs technology could provide a new efficient method of hTERT inhibition.

## Materials and Methods

### Plasmid Construction

The cloning of ZFP-TFs has been previously described (32, 33). The amino acid sequences of six ZFPs are: NH<sub>3</sub><sup>+</sup>-PGEKPYKCGCQCGKSFYSQVSHLTRHQKIHTGEKPYKPCDCGKSFSSQSSSLIRHQRTHTGKPYK-CMECGKAFNRRSHLTRHQRIHTGKPYECEKC-GKAFNQSSNLTRHKKSHTEK-CO<sub>2</sub> [F-197R], NH<sub>3</sub><sup>+</sup>-PGEKPYVCDVEGCTWKFARSDDELNRHKKRHTGKPYKMECGKAFNRRSHLTRHQRIHTGKPYKCKQCGKAFGCPNLRRHGRHTGTEKPFQCKTCQRKFSRSDHLKTHTRHTGK-CO<sub>2</sub> [F-153R], NH<sub>3</sub><sup>+</sup>-PGEKPYKMECGKAFNRRSHLTRHQRIHTGKPYECNYCGKTFVSSTLIRHQRIHTGKPYKMECGKAFNRRSHLTRHQRIHTGKPYKCGCQCGKSFYSQVSHLTRHQKIHTGK-CO<sub>2</sub> [F-122R], NH<sub>3</sub><sup>+</sup>-PGEKPFQCKTCQRKFSRSDHLKTHTRHTGKPYKMECGKAFNRRSHLTRHQRIHTGKPYECNYCGKTFVSSTLIRHQRIHTGKPYKMECGKAFNRRSHLTRHQRIHTGK-CO<sub>2</sub> [F-105R], NH<sub>3</sub><sup>+</sup>-PGEKPYKMECGKAFNRRSHLTRHQRIHTGKPYICRKCGRGFSRKSNIIRHQRTHTGKPYKMECGKAFNRRSHLTRHQRIHTGKPYECEKC-GKAFNQSSNLTRHKKSHTEK-CO<sub>2</sub> [F-97R], NH<sub>3</sub><sup>+</sup>-PGEKPYKMECGKAFNRRSHLTRHQRIHTGKPYVCDVEGCTWKFARSDDELNRHKKRHTGKPYKMECGKAFNRRSHLTRHQRIHTGK-CO<sub>2</sub> [F-5R]. Each plasmid encoded the following fusion protein: hemagglutinin tag-nuclear localization signal-ZFP-KRAB (Fig. 1B). The KRAB repression domain used in these studies contained 63 amino acids. They constitute the minimal segment which retains transcriptional repressor activity (32).

DNA segments that encode ZFPs were isolated by digestion with *EcoRI* and *NotI*, and were inserted into pGEX-4T2 (Amersham Pharmacia) for protein expression. These constructs encoded ZFP-glutathione-S-transferase fusion protein (Fig. 1C). The DNA fragment of the *hTERT* core promoter, which is located 334 bp upstream of the ATG initiation codon, was inserted into the *KpnI* and *NcoI* restriction site of pGL3-basic (Promega) promoterless and

enhancerless firefly luciferase reporter vector. Then, inserted DNA was amplified by nested PCR using GC-rich PCR system (Roche) from HeLa Genomic DNA. The primer sequences used for the first PCR were 5'-CAA TGC GTC CTC GGG TTC GTC C-3' (forward) and 5'-TCT CCG CAT GTC GCT GGT TCC C-3' (reverse), and for the second PCR were 5'-GGG GTA CCC CGG AGC AGC TGC GCT GT-3' (forward) and 5'-CAT GCC ATG GCG CGG GGG TGG CCG-3' (reverse). The construct was named pGL3-hTERT. Each construct was purified by Miniprep Kits (Qiagen) and confirmed by sequencing (Genotech).

### Cell Culture and Transfection of HEK293 Cells

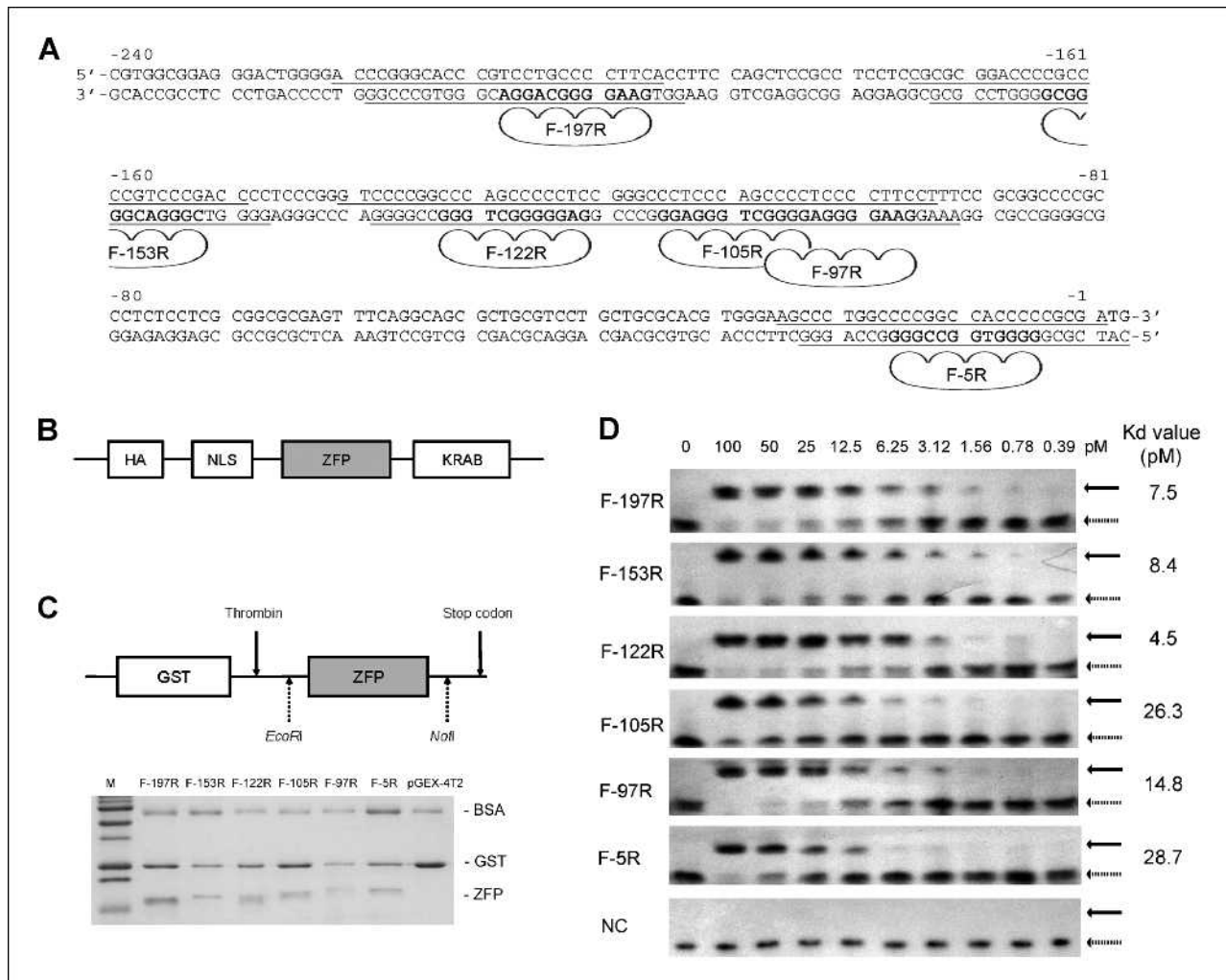
HEK293 cells (American Type Culture Collection) were maintained in DMEM supplemented with 100 units/mL of penicillin, 100 mg/mL of streptomycin, and 10% fetal bovine serum (all from Life Technologies) at 37°C in a 5% CO<sub>2</sub> humidified incubator. For transient transfections, HEK293 cells (2 × 10<sup>4</sup> cells/well) were precultured for 24 h in 96-well culture plates (Becton Dickinson) that contained 100 μL of the medium described above without antibiotics. For some experiments, 10<sup>5</sup> cells per well were cultivated in 24-well culture plates. Unless otherwise indicated, the HEK293 cells were transfected with a nucleic acid mixture at a total amount of 320 ng (for 96-well culture plates) or 1 μg (for 24-well culture plates). The nucleic acid mixtures contain effector plasmids encoding ZFPs (Fig. 1B), reporter plasmids encoding firefly luciferase (Fig. 2A), and internal control *Renilla* luciferase (pRL-SV40; Promega). Transfection was done with the LipofectAMINE 2000 reagent (Invitrogen) according to the instructions of the manufacturer.

### Electrophoretic Mobility Shift Assay

ZFPs in pGEX-4T2 were then expressed in *Escherichia coli* strain BL21 (DE3) as fusion proteins linked to glutathione-S-transferase. The fusion proteins were purified using glutathione affinity chromatography (Amersham Pharmacia) and the connection between the glutathione-S-transferase moiety and the ZFPs was digested with thrombin. Probe DNAs were synthesized, annealed, and labeled with <sup>32</sup>P using T4 polynucleotide kinase. Next, electrophoretic mobility shift assays were carried out as described previously (25, 32).

### Luciferase Assay

After incubation at 37°C for 48 h after transfection, cultured cells were washed twice with PBS and mixed with passive lysis buffer (30 μL; Promega). Firefly and *Renilla* luciferase activities in the cell lysates were measured in dual wavelength in a MicroLumatPlus LB96 luminometer (Berthold) after sequential addition of the LAR II reagent (40 μL), stop buffer (40 μL), and the Glo reagent to the lysates (8 μL). The Dual-luciferase reporter assay kit was purchased from Promega. To compare the results, the mean values of relative luciferase activity were used. The levels of the different constructs were



**FIGURE 1.** Artificial ZFPs that bind a 12-bp recognition sequence within the core promoter of the *hTERT* gene and the electrophoretic mobility shift assay. A, schematic representation of the sequences of the *hTERT* core promoter and the ZFP-TF. The sequences of the four zinc finger domain-binding sites are shown in boldface. The probe DNA sequences for electrophoretic mobility shift assay are underlined. B, expression vector constructs for ZFP-KRAB artificial transcriptional repressors. Zinc finger domains were designed and assembled on a pcDNA3 vector that included the hemagglutinin-epitope, nuclear localization signal, and the KRAB repression domain. The resulting zinc finger expression vectors pF-197RK, pF-153RK, pF-122RK, pF-105RK, pF-97RK, and pF-5RK consist of four zinc finger domains. The amino acid sequence of the each ZFP DNA binding domain is described in Materials and Methods. C, pGEX-4T2 protein expression vector construct for ZFPs. Fusion proteins were purified using glutathione-S-transferase affinity columns and then digested with thrombin. D, to determine dissociation constants, 2-fold serial dilutions of the ZFPs were incubated with labeled probe DNA (100 pmol/L) at room temperature for 1 h, and then the reaction mixtures were subjected to gel electrophoresis. Autoradiography signals were quantitated by NIH ImageJ software. Arrows, positions of the ZFP DNA-bound shift bands. Dotted line arrows, positions of the free-probe bands. Negative control (NC) means that ZFP-TF expression vector which does not target the *hTERT* core promoter.

compared with the level of the pGL3-*hTERT* and to the level of the pGL3-basic vector.

#### Quantification of *hTERT* mRNA

For the reverse transcription-PCR analysis,  $2 \times 10^5$  HEK293 cells per well were precultured at 37°C in a humid atmosphere containing 5% CO<sub>2</sub> for 24 h in 24-well plates containing culture medium (0.5 mL; supplemented with 10% fetal bovine serum without antibiotics). Transfection was done with the LipofectAMINE 2000 reagent (Invitrogen) according to the instructions of the manufac-

turer. The cells were harvested for reverse transcription-PCR analysis and telomere repeat amplification protocol (TRAP) assay. RNA was extracted with the RNeasy mini kit (Qiagen) according to the instructions of the manufacturer. The reverse transcription reactions were done with 100 ng of total RNA using Quatitect reverse transcription kit (Qiagen). To analyze mRNA quantities, 1 μL of the cDNAs generated from the reverse transcription reactions were amplified using *hTERT*-specific primers [5'-GCG GAA GAC AGT GGT GAA CT-3' (forward) and 5'-AGC TGG AGT AGT CGC TCT GC-3' (reverse)].



The specific *hTERT* mRNA amounts were normalized to the glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) mRNA quantities calculated by specific amplification using primers [5'-CAA TGA CCC CTT CAT TGA CC-3' (forward) and 5'-GAT CTC GCT CCT GGA AGA TG-3' (reverse)]. Amplification of *hTERT*-specific and *GAPDH*-specific cDNAs was monitored and analyzed with QuantiTect SYBR Green PCR kit (Qiagen) and Rotorgene 3000 Real-time Cyler (Corbett Research). Calculations of relative expression levels were done using the  $2^{-\Delta\Delta C_t}$  method (34), by normalizing to *GAPDH* expression levels.

### Telomerase Activity Assay

Telomerase activity in cell lysates was measured by TRAP assay (26) using TRAP<sub>EZE</sub> telomerase detection kit (Chemicon) according to the instructions of the manufacturer. PCR products were separated by electrophoresis on 12.5% nondenaturing polyacrylamide gels, which were stained with SYBR Green (Molecular Probes). TSR8 and CHAPS lysis buffer (provided in the TRAP<sub>EZE</sub> kit) were used as positive and negative controls, respectively. Telomerase activity was semiquantified by normalizing the band intensities of the characteristic 6 bp telomerase-specific ladder to those of the 36 bp internal standard using NIH ImageJ software.

### Cell Proliferation Assay

Colorimetric cellular proliferation assay was done using Cell Counting Kit-8 (Dojindo) according to the instructions of the manufacturer. Absorbance at 450 nm was measured using VersaMax microplate reader (Molecular Devices). To compare the results, the mean values of relative proliferation rates were used. The levels of the different constructs were compared with the level of the pcDNA3-null vector and HEK293 cell.

### Telomere Length

Telomere restriction fragment length was determined using the Telo-TAGGG-Telomere Length Kit (Roche), following the instructions of the manufacturer. Genomic DNA was isolated from the pellets of cells grown in culture for 100 d using QIAamp DNA Mini Kit (Qiagen) and separated on a 0.8% agarose gel in 1× TAE buffer.

## Results

### Target DNA Binding Specificity and Affinity of the Artificial ZFP-TFs

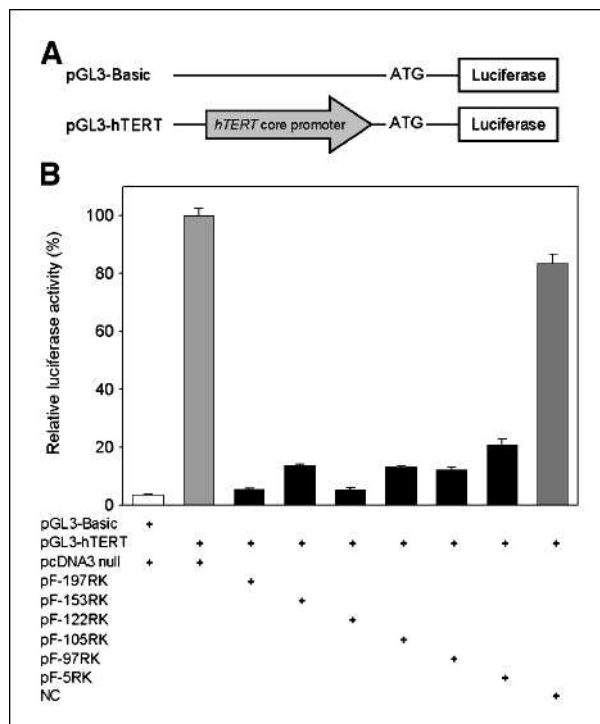
Using zinc finger domains with sequences from the human genome, six four-fingered ZFPs targeting 12-bp sequences (Table 1) could be generated at the -197, -153, -122, -105, -97, and -5 upstream regions of the ATG initiation codon in the core promoter of *hTERT* (Fig. 1A). When each ZFPs was incubated with labeled probe containing target sequences, only the protein-binding probes showed shifted bands, confirming the specificities of each artificial ZFP (Fig. 1B-D). Disso-

ciation constant ( $K_d$ ) values of F-197R, F-153R, F-122R, F-105R, F-97R, and F-5R were 7.5, 8.4, 4.5, 26.3, 14.8, and 28.7 pmol/L, respectively, indicating much higher affinity among each value than in the case of the widely used three-fingered ZFPs the  $K_d$  values of which were 0.01 to 10 nmol/L (25).

Our results suggested that the four-fingered artificial ZFP-TFs, which recognize 12-bp DNA sequences, could be a novel tool for the regulation of the target gene at the transcriptional level.

### Suppression of Reporter Gene Expression by Artificial ZFP-TFs

To examine the efficiency of the artificial ZFP transcriptional repressor in regulating genes, we used the firefly luciferase reporter gene under the *hTERT* core promoter. We developed a new reporter plasmid that contains a well-characterized *hTERT* core promoter (Fig. 2A). HEK293 cells were cotransfected with pGL3-hTERT (luciferase reporter plasmid) containing the appropriate ZFP binding



**FIGURE 2.** Effect of artificial ZFP-TFs on luciferase production driven by the *hTERT* core promoter. A, schematic representation of the reporter plasmid. Luciferase reporter pGL3-basic is a promoterless and enhancerless vector. For luciferase activity assay control, the pGL3-hTERT luciferase reporter vector was generated by the insertion of the *hTERT* core promoter, 334 bp upstream of the initiation ATG codon. For cloning of the *hTERT* core promoter, hot-start, touchdown, GC-rich, and nested PCRs were done using HeLa Genomic DNA. B, reporter vectors and ZFP-TFs expression vectors were cotransfected in HEK293 cells. After incubation for 48 h, luciferase activity assays were done. Negative control (NC) was done by ZFP-TF expression vector which does not target the *hTERT* core promoter. Graphs indicate the mean values and SEs of three independent experiments.

**Table 1. Binding sites and identity of ZFPs used in hTERT repression**

ZFP	Binding positions*	F1	F2	F3	F4	Target DNA sequence (from 5' to 3')
F-197RK	-197R <sup>†</sup>	QSHR2 <sup>‡</sup>	QSSR1	RSHR	QSNR3	GAA GGG GCA GGA
F-153RK	-153R	RDER1	RSHR	CSNR1	RDHT	CGG GAC GGG GCG
F-122RK	-122R	RSHR	VSTR	RSHR	RSNR	GAG GGG GCT GGG
F-105RK	-105R	RDHT	RSHR	VSTR	RSHR	GGG GCT GGG AGG
F-97RK	-97R	RSHR	RSNR	RSHR	QSNR3	GAA GGG GAG GGG
F-5RK	-5R	RSHR	DSCR	RDER1	RSHR	GGG GTG GCC GGG
NC	-	RSHR	RSHR	RSHR	RSHR	GGG GGG GGG GGG

\*Numbers in the binding position indicate a 3' end of the binding site relative to the ATG start codon.

<sup>†</sup>R, reverse strand.

<sup>‡</sup>Each zinc finger was named using the single abbreviation of the four amino acid residues at positions -1, 2, 3, and 6 in the  $\alpha$ -helix of the zinc finger.

sites, together with pcDNA3 (expression plasmid) which expresses ZFPs fused with KRAB transcriptional repression domain. The high luciferase activity of pGL3-hTERT remarkably decreased when appropriate ZFPs which could bind to *hTERT* core promoter were expressed. F-197RK, F-153RK, F-122RK, F-105RK, F-97RK, and F-5RK showed 94.5%, 86.2%, 94.7%, 86.8%, 87.8%, and 79.2% repression, respectively, of the *hTERT* core promoter activated transcription of the luciferase gene (Fig. 2B).

These data indicate that all of the ZFP-TFs designed in this experiment specifically bound within the promoter and actually repressed the expression of target gene at the cellular level.

#### Artificial ZFP-TFs Suppressed Endogenous *hTERT* Gene Expression, Reduction of Telomerase Activity, and Inhibition of Cell Proliferation

To achieve the transcriptional repression of endogenous *hTERT* gene expression, six ZFPs with specificities to the core promoter of *hTERT* were fused to the nuclear localization signal and the KRAB transcriptional repression domain (Fig. 1B). HEK293 cells were transfected using pF-197RK, pF-153RK, pF-122RK, pF-105RK, pF-97RK, pF-5RK, and pcDNA3-null vector (control), respectively. Transfected cells were maintained in medium with 500  $\mu$ g/mL of G-418 antibiotics. Selected cells were harvested at 2, 4, 8, and 12 days after transfection. Endogenous *hTERT* mRNA quantities measured by real-time reverse transcription-PCR were reduced as the time of culture increased (Fig. 3A).  $2^{-\Delta\Delta C_t}$  values of the pF-197RK, pF-153RK, pF-122RK, pF-105RK, pF-97RK, and pF-5RK were 0.285, 0.205, 0.219, 0.331, 0.243, and 0.219, respectively, when measured at 12 days passage, indicating stable repression of endogenous *hTERT* gene by artificial ZFPs.

Telomerase activity of HEK293 cells was also reduced by ZFP-TFs, as evaluated by the TRAP assay (Fig. 3B and C). Control HEK293 cells with or without pcDNA3-null vec-

tor showed the same high levels of telomerase activity, whereas pF-197RK, pF-153RK, pF-122RK, pF-105RK, pF-97RK, and pF-5RK all showed significantly decreased levels of telomerase activity.

Induction of six ZFP-TFs expression caused a time-dependent decrease of the cell proliferation rate (Fig. 3D). Relative cell proliferations of the pF-197RK, pF-153RK, pF-122RK, pF-105RK, pF-97RK, and pF-5RK were 53.3%, 43.4%, 48.1%, 38.6%, 60.3%, and 44.5%, respectively, at 12 days passage.

#### Stable Repression of Endogenous *hTERT*

pF-197RK, pF-153RK, pF-122RK, pF-105RK, pF-97RK, pF-5RK, and pcDNA3-null vector-transfected HEK293 cells could be cultured for 100 days in medium containing 100  $\mu$ g/mL of G-418-selective antibiotics. The inhibition of *hTERT* mRNA (Fig. 4A) and telomerase activity (Fig. 4B) persisted for up to 100 days in the culture, indicating stable repression of endogenous *hTERT* by ZFP-TFs.

#### Artificial ZFP-TFs Shorten Telomere Length

Analysis of terminal restriction fragments by Southern blotting showed that the telomere lengths observed in control pcDNA3-null vector were 4.8 kb, similar to that of parental HEK293 cells. In contrast, telomere lengths in pF-197RK, pF-153RK, pF-122RK, pF-105RK, pF-97RK, and pF-5RK were all shortened, with lengths of 3.5, 3.1, 3.9, 3.0, 3.1, and 3.2 kb, respectively, measured at 100 days after transfection (Fig. 4C). These observations indicate that stable suppression of *hTERT* by artificial ZFP-TFs functionally inhibits telomerase activity and shortens telomere length in human cancer cells.

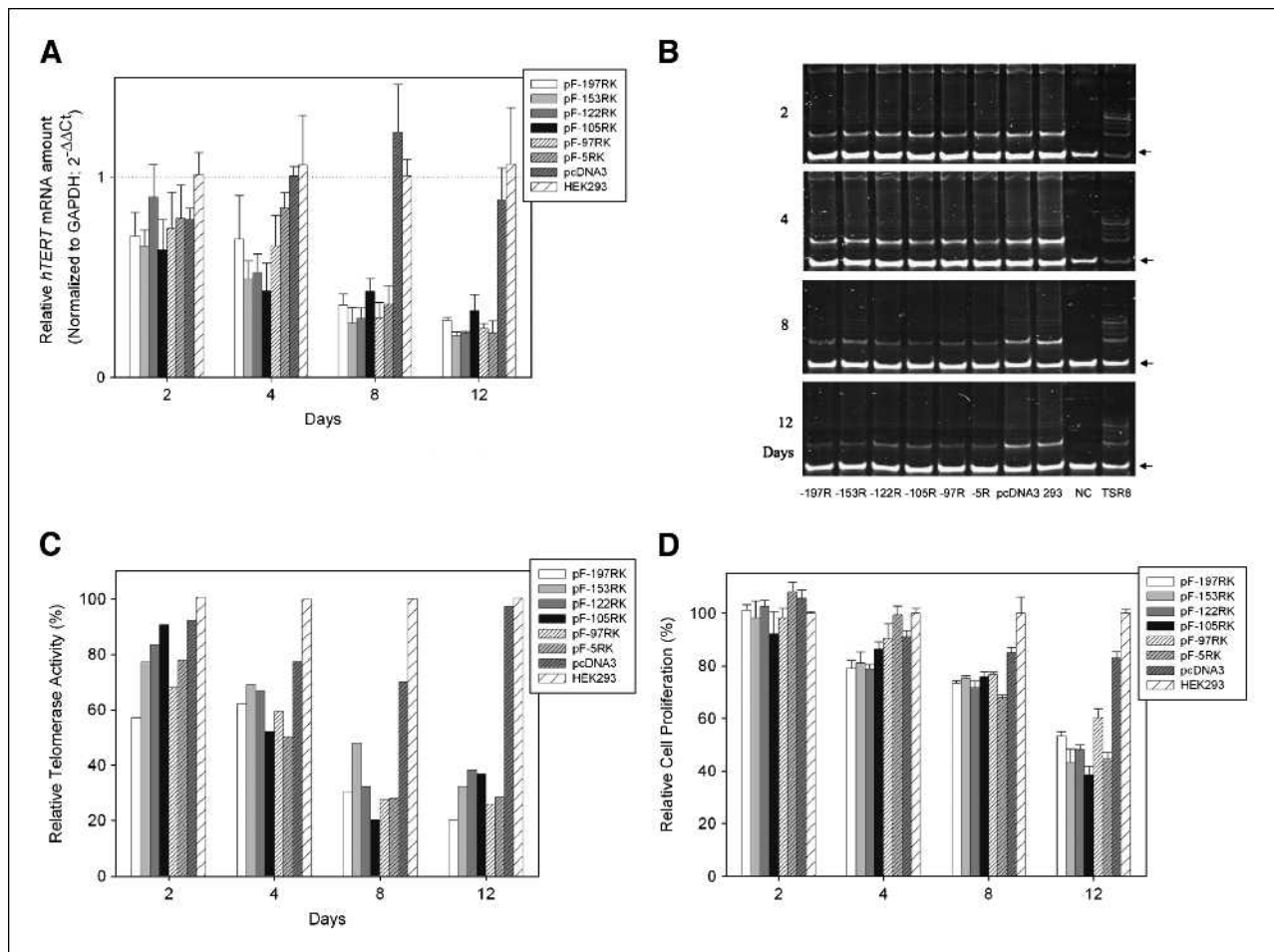
#### Discussion

We have designed four-fingered ZFP-TFs targeting the *hTERT* promoter region using zinc finger domains naturally

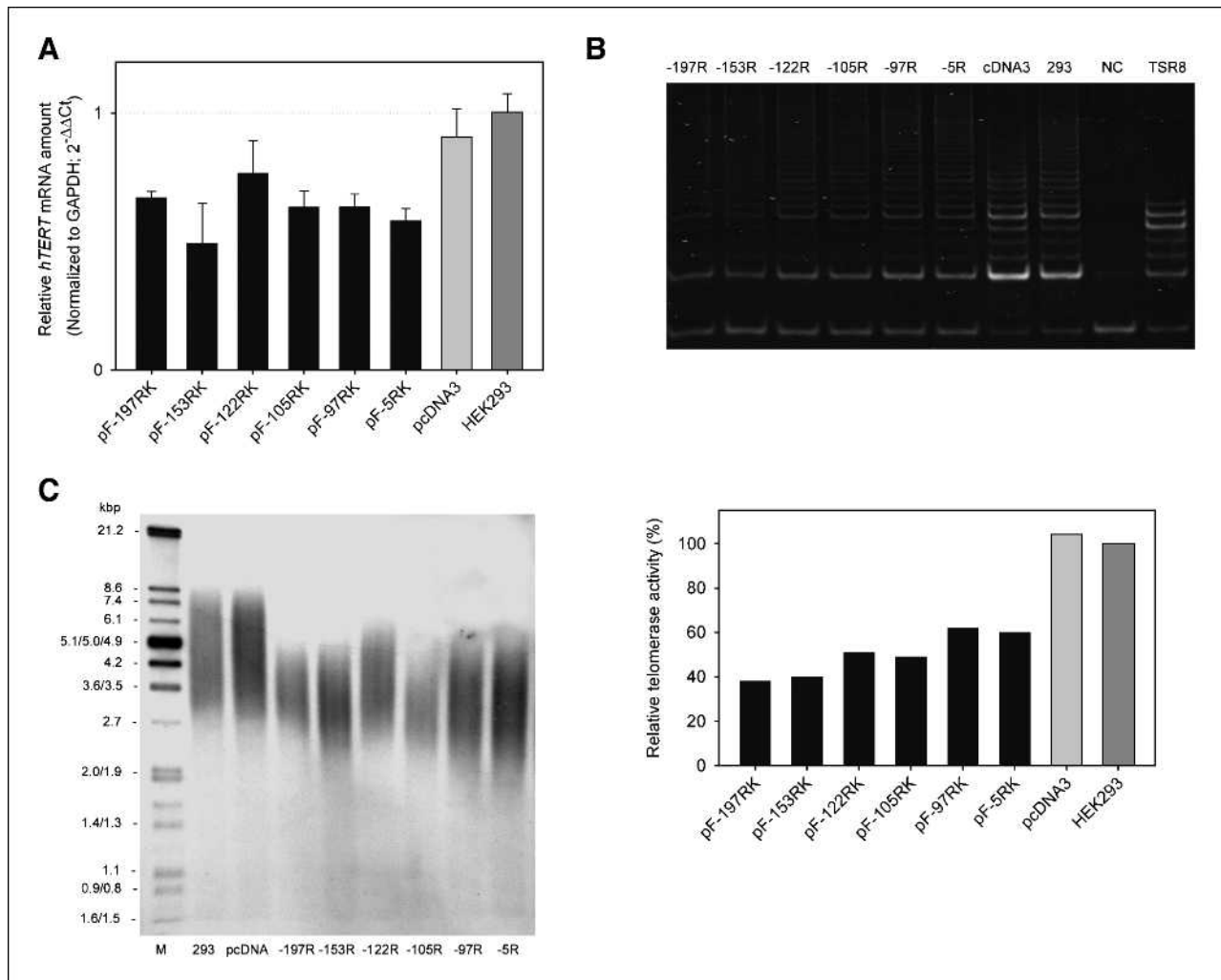
existing in the human genome. Although the probability of four-fingered ZFPs recognizing 12 bp DNA sequences ( $4^{12} = 2 \times 10^7$ ) is much lower than the whole human genome ( $3 \times 10^9$ ), considering the portion of the promoter region of unique gene in the whole genome, it is believed that four-fingered ZFPs are very safe and would not affect other genes. With high affinity and high specificity of four-fingered ZFPs as well as high efficiency of ZFPs isolated from human genome (25), the four-fingered ZFPs we have designed would be very ideal for gene controls.

Our results showed that artificial four-fingered ZFP-TFs targeting the promoter region of the *hTERT* could

reduce the expression of telomerase at the transcriptional level with actual telomere shortening, and actual proliferation of human cancer cells was inhibited, although the inhibition level was not strong enough to kill cancer cells. Several other genetic approaches have also been described to block telomerase expression or biogenesis by ribozyme, antisense, and small interfering RNA agents whose levels of telomerase inhibition, including the present results, were mostly similar but much lower than those of direct inhibitors such as pharmacologic drugs and chemical agents (15, 29-31). From the 4th day, *hTERT* expression was suppressed by artificial ZFP-TFs, whereas actual cell



**FIGURE 3.** Repression of endogenous *hTERT* using artificial ZFP-TFs. A, to analyze relative quantitative gene expression at the *hTERT* mRNA level using real-time PCR with the  $2^{-\Delta\Delta C_t}$  method, results with the reference gene (*GAPDH*) were compared with those of the target gene (*hTERT*). After RNA extraction, reverse transcription was done. The resulting cDNA was subjected to real-time PCR using gene-specific primers (sequences are shown in Materials and Methods). Gene expression profiles of pF-197RK, pF-153RK, pF-122RK, pF-105RK, pF-97RK, pF-5RK, pcDNA3-null vector, and HEK293 (control) were analyzed by real-time PCR using the  $2^{-\Delta\Delta C_t}$  method. Samples were collected at 2, 4, 8, or 12 d after transfection. A HEK293 sample was selected as the calibrator. For the calibrator,  $\Delta\Delta C_t$  equals zero and  $2^0$  equals one, so that the fold change in gene expression in HEK293 equals one, by definition. For other samples, evaluation of  $2^{-\Delta\Delta C_t}$  represents the fold change in gene expression relative to HEK293. B, telomerase activity after transfection with artificial ZFP-TFs. HEK293 cell lysates incubated for 2 to 12 d were tested for telomerase activity via the TRAP assay. PCR products were separated by gel electrophoresis, and gels were stained with SYBR Green. Arrows, positions of the 36 bp internal control bands. Negative control (NC) corresponds to use of CHAPS lysis buffer. C, to determine relative telomerase activities, signal densities of the bands were quantitated by NIH ImageJ software, and then each unit of total product generated (TPG) was calculated according to the instructions of the manufacturer. Telomerase activity values represent percentage values of TPG relative to HEK293 cells. D, cell proliferation assay. Each of six ZFP-TFs inhibits the growth of HEK293 cells.



**FIGURE 4.** Stable repression of endogenous *hTERT*. Samples were collected at 100 d after transfection. **A**, quantitative gene expression profiles of six ZFP-TFs, pcDNA3-null vector, and HEK293 (control) were analyzed by real-time PCR. **B**, TRAP assay. Relative signal densities of the bands are summarized (bottom). **C**, Southern blot assay to measure the telomere length of HEK293 cells, control clones (HEK293 and pcDNA3-null vector), and artificial ZFP-TF expressing clones (PF-197RK, PF-153RK, PF-122RK, PF-105RK, PF-97RK, and PF-5RK).

proliferation was beginning to be inhibited as late as almost the 12th day of culture. This might indicate the possibility of another direct controlling pathway to inhibit cell growth other than telomere shortening-related cellular inhibition.

Many approaches using designed ZFPs with activator domains were used to activate dormant genes (16, 24). Recent activation of dormant tumor suppressor gene showed the inhibition of proliferation and invasion of tumor cell line (35, 36). We expect that the attachment of an activator domain to our ZFP-TF targeting the *hTERT* promoter region could also lead to interesting results, as telomerase activity and telomere lengths are closely related to cell aging. There are many trials using gene insertion (37, 38). Telomerase activation using ZFPs should be unique in that a dormant gene is activated.

Although there are many trials for inhibition of telomerase (15, 29-31), telomerase gene repression using gene-regulating proteins such as ZFP-TFs could be advantageous, as this could suggest the new possibility of protein drugs regulating gene expression. Each isolated human ZFP tagged with protein transduction domains (39, 40) could be directly used for repression or activation of telomerase activity. Many combinations of ZFP-TFs could even be possible for maximum efficiency. Further studies are expected in this field.

Telomerase and telomeres are attractive targets for anticancer therapy. As tumors have relatively short telomeres, human cancers can be more susceptible to cancer drug when telomerase is inhibited. The repression of telomerase expression by artificial ZFP-TFs presents a new promising strategy for inhibiting the growth of human cancer cells.



## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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