Citation: Molecular Therapy–Nucleic Acids (2013) 2, e87; doi:10.1038/mtna.2013.12

www.nature.com/mtna

Design and Development of Artificial Zinc Finger Transcription Factors and Zinc Finger Nucleases to the hTERT Locus

Kimberly A Wilson[1,2], Morgan L Chateau[3] and Matthew H Porteus[1,2,4]

The ability to direct human telomerase reverse transcriptase (hTERT) expression through either genetic control or tunable regulatory factors would advance not only our understanding of the transcriptional regulation of this gene, but also potentially produce new strategies for addressing telomerase-associated disease. In this work, we describe the engineering of artificial zinc finger transcription factors (ZFTFs) and ZF nucleases (ZFNs) to target sequences within the hTERT promoter and exon-1. We were able to identify several active ZFTFs that demonstrate a broadly tunable response when screened by a cell-based transcriptional reporter assay. Using the same DNA-binding domains, we generated ZFNs that were screened in combinatorial pairs in cell-based extrachromosomal single-strand annealing (SSA) assays and in gene-targeting assays using stably integrated constructs. Selected ZFN pairs were tested for the ability to induce sequence changes in a Cel1 assay and we observed frequencies of genomic modification up to 18.7% at the endogenous hTERT locus. These screening strategies have pinpointed several ZFN pairs that may be useful in gene editing of the hTERT locus. Our work provides a foundation for using engineered ZF proteins (ZFPs) for modulation of the hTERT locus.

Molecular Therapy–Nucleic Acids (2013) **2**, e87; doi:10.1038/mtna.2013.12; published online 23 April 2013
Subject Category: Gene Insertion, Deletion, and Modification

Introduction

Telomeres are specialized chromosomal end caps comprised of 5′-TTAGGG-3′ hexanucleotide repeats which protect human chromosomes from genomic instability and replicative attrition which is a consequence of their linear structure and the inability of DNA polymerases to completely replicate chromosomal ends.[1] Telomerase extends and maintains telomere length, which allows a cell to keep proliferative capacity. Alternatively, the absence of active telomerase results in telomere shortening with every cell cycle.[2] This becomes an important mechanism for cellular aging, lifespan, proliferative potential, and senescence. Telomere shortening is also an important tumor suppressive mechanism.[3] The human telomerase holoenzyme is a complex of two components: hTR, a single-stranded RNA template, and hTERT, a reverse transcriptase. As the limiting component, human telomerase reverse transcriptase (hTERT) expression is constitutively repressed in normal somatic cell types.[4] The expression of this gene can be upregulated to affect changes in proliferation potential, but this alone is insufficient to initiate oncogenesis.[4–6]

The promoter region controlling hTERT expression is densely composed of many regulatory elements. Many of these can be found within the first 300 basepairs (bp), which is considered to be the core promoter.[7–9] Studies have suggested that transcriptional regulation of hTERT is done in a cell context-dependent manner and the transcription factors controlling the promoter at different sites can vary between cell lines.[7] Haploinsufficiencies and mutations in hTERT have also been implicated in a number of inheritable genetic diseases that result from increasingly shortened telomere lengths. These diseases (such as idiopathic pulmonary fibrosis, aplastic anemia, and dyskeratosis congenita) exhibit attrition of fast-growing tissues, stem cell depletion, and present at earlier ages in each successive generation (anticipation).[10–12] The ability to modify the promoter of hTERT, therefore, would have a wide range of research and potentially therapeutic applications. In addition, systematic site-specific mutational analysis of the endogenous hTERT locus or the use of a tunable regulatory factor at the hTERT promoter across many human cell lines would yield useful insights about the regulation of this crucial gene.

Artificial zinc finger transcription factors (ZFTFs) and ZF nucleases (ZFNs) are methods to modulate the activity of specific genes. Both chimeric proteins are based on the well-characterized ZF DNA-binding protein domains (ZFPs). The polydactyl ZFP is custom engineered for a chosen target site and are commonly generated through one of four publicly available methods: (i) combining individual ZFs in a modular fashion, (ii) using randomly recombined libraries of ZFs in the bacterial-2-hybrid (B2H) selection-based OPEN platform, (iii) context-dependent assembly, or (iv) a hybrid methodology that incorporates elements of both.[13–17] A novel, chimeric protein with site-specific activity is then made by attaching another functional effector domain to the ZFP.

ZFPs, when linked to a transcriptional effector domain, become ZFTFs that will recruit the cellular machinery necessary to activate or repress transcription and are typically

[1]Department of Biochemistry, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas, USA; [2]Department of Pediatrics, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas, USA; [3]Department of Microbiology and Immunology, University of North Carolina, Chapel Hill, North Carolina, USA; [4]Department of Pediatrics, Stanford University, Stanford, California, USA. Correspondence: Matthew H Porteus, Lokey Stem Cell Research, Building G3040, 265 Campus Drive, Stanford Medical School, Stanford, California 94305, USA. E-mail: mporteus@stanford.edu
Keywords: artificial transcription factors; gene editing; telomerase; TERT; zinc finger nuclease
Received 24 March 2012; accepted 5 February 2013; advance online publication 23 April 2013. doi:10.1038/mtna.2013.12

developed to sequences found in the promoter regions of the target gene. Due to the ability of a ZFTF to site specifically regulate transcription in cell culture and *in vivo*, these artificial proteins are useful tools for drug development.[18,19] Recently, Sohn *et al.* (2010) developed a set of four-fingered ZFTFs that target sequences within the hTERT core promoter and demonstrated transcriptional repression of hTERT in HEK293 cells by linking the KRAB repressor domain to ZFPs made by modular assembly.[20] Several ZFTFs have also been successfully made to other target genes such as ErbB-2, VEGF, and utrophin, all of which have therapeutic potential.[21–23]

ZFNs are made by joining a ZFP to the nuclease domain of the FokI endonuclease and are developed in pairs to "full target sites" of the general structure: 5′-(ZFN target site 1)-spacer-(ZFN target site 2)-3′.[24,25] The binding of the two ZFNs at the cognate target half-sites allows the nuclease domain to dimerize in the spacer region and create a DNA double-stranded break (DSB).[26] If the DSB is repaired by homologous recombination using an exogenously supplied donor DNA fragment, then the ZFN-targeted locus can be edited to introduce any number of modifications from single point mutations to the installment of large transgenes. Repair of the DSB through error-prone pathways (e.g., non-homologous end joining) can also result in changes to the nucleotide content of the target site.[27,28] ZFNs have been used to perform genomic editing in human cells and are currently undergoing rapid development for gene therapy and clinical trials using ZFNs directed against the CCR5 gene that have already been initiated.[29–31]

In this study, we report on the progress towards engineering new ZFTFs for transcriptional activation and ZFNs that target the hTERT locus. We have identified five ZFN full sites (10 target half-sites total) within the hTERT promoter and exon-1. Multiple three-fingered ZFPs were generated for each target half-site using the OPEN platform strictly for eight target half-sites and a hybrid methodology that incorporated module fingers at the Finger 1 or Finger 2 positions in the OPEN protocols for the other two target half-sites. The resulting ZFPs were converted into ZFTFs or ZFNs by linking these DNA-binding domains to either a VP16 transactivator domain or a nuclease, respectively. ZFTFs were screened for the ability to upregulate transcriptional activity through cotransfection with an episomal hTERT promoter-driven green fluorescent protein (GFP) reporter construct in HEK293 cells. We found that the ZFTFs could not only induce GFP expression in a coarsely tunable manner, but could also be used in combination. ZFN versions of many of the same ZFPs used as ZFTFs were screened and assayed using a extrachromsomal single-strand annealing (SSA) assays, a chromosomally integrated GFP gene-targeting reporter assay, and a

Table 1 hTERT target sites and sequences

Full site name	Half-site name	Sequence	Start[a]	End
hTERT5		5′-AGCCACTACcgcgaGGTGCTGCC-3′	46	68
	hTERT5L	5′-GTAGTGGCT-3′	46	54
	hTERT5R	5′-GGTGCTGCC-3′	60	68
hTERT5C		5′-CACTACCGCgaggtGCTGCCGCT-3′	49	71
	hTERT5CL	5′-GCGGTAGTG-3′	49	57
	hTERT5CR	5′-GCTGCCGCT-3′	63	71
hTERT5S		5′-CCCGCCCTCtcctcGCGGCGCGA-3′	−85	−63
	hTERT5SL	5′-GAGGGCGGG-3′	−85	−77
	hTERT5SR	5′-GCGGCGCGA-3′	−71	−63
hTERT6		5′-CCCGGCCACccccgcGATGCCGCG-3′	−16	8
	hTERT6L	5′-GTGGCCGGG-3′	−16	−8
	hTERT6R	5′-GATGCCGCG-3′	−1	8
hTERT6B		5′-GCCAGCGGCcaaaggGTCGCCGCA-3′	−507	−484
	hTERT6BL	5′-GCCGCTGGC-3′	−507	−499
	hTERT6BR	5′-GTCGCCGCA-3′	−492	−484

hTERT, human telomerase reverse transcriptase.
[a]Position is given relative to the translational start codon.

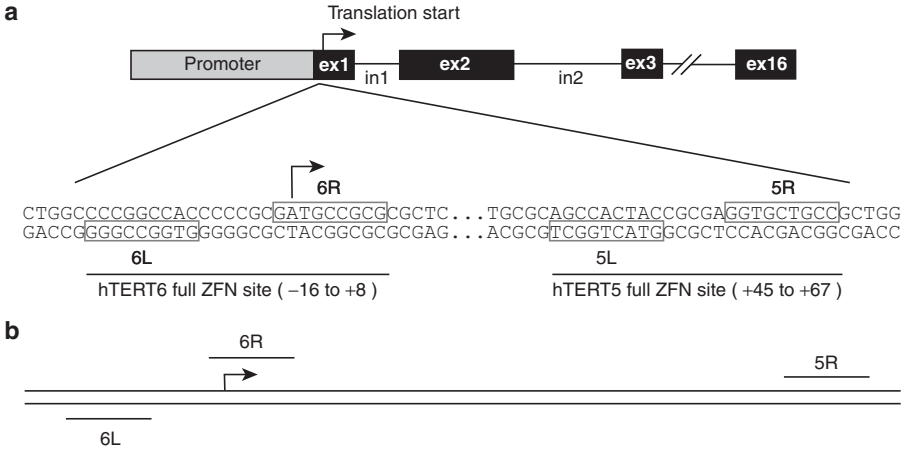

Figure 1 Approximate location and sequence of the human telomerase reverse transcriptase 5 (hTERT5) and hTERT6 sites in the hTERT locus. (**a**) The hTERT5 and hTERT6 zinc finger nuclease (ZFN) full target sites were identified in the hTERT promoter and exon-1. Genomic sequences are provided in the traditional 5′-3′ orientation of the top strand and 3′-5′ of the bottom strand with position is given relative to the translational start codon. (**b**) Schematic of hTERT promoter locus showing relative position of hTERT6L, hTERT6R, and hTERT5R zinc finger transcription factor-binding sites at scale. The arrow represents the site of the initiation ATG.

Table 2 Zinc finger DNA-binding domains generated in this study for the hTERT5 and hTERT6 target sites

hTERT5L	**GTA**	**GTG**	**GCT**		
Method	**Finger 3[a]**	**Finger 2**	**Finger 1**	**ZFTF**	**ZFN**
OPEN	QSPSLNR	RREVLMN	NAHQLKR	—	pKW741
OPEN	QSTSLQR	RHEVLVN	GRQALDR	—	pKW744[b]
OPEN	QKVSLKR	RKQVLTN	QRQALDR	—	pKW745
OPEN	QIVSLRR	RIEILRN	NGQGLRR	—	pKW746
hTERT5R	**GGT**	**GCT**	**GCC**		
Method	**Finger 3**	**Finger 2**	**Finger 1**	**ZFTF**	**ZFN**
OPEN	QNHGLLR	LSQTLKR	DRRTLDR	pKW639	pKW663
OPEN	LMHQLNR	LSQTLKR	DGGTLHR	pKW640	pKW664[b]
OPEN	QNHGLLR	LSQTLKR	DSSTLAR	—	pKW665
OPEN	LMHGLVR	LSQTLKR	DSSTLAR	pKW641[c]	—
OPEN	HRHGLGR	LSQTLKR	DSTTLRR	pKW642	pKW666
OPEN	LMHGLVR	LSQTLKR	DRRTLDR	pKW643	—
OPEN	VMHHLAR	LSQTLKR	DRRTLDR	pKW644	—
OPEN	LMHGLVR	LSQTLKR	ERRGLDR	pKW645	—
OPEN	LMHGLVR	LSQTLKR	DGRGLER	pKW646	—
hTERT6L	**GTG**	**GCC**	**GGG**		
Method	**Finger 3**	**Finger 2**	**Finger 1**	**ZFTF**	**ZFN**
Modular	RSDALTR	DRSDLTR	RSDHLSR	pKW308	—
OPEN	RADALTR	DSSVLRR	RREKLRI	pKW616	—
OPEN	RADSLTR	DRSVLVR	RQDHLAR	—	pKW632
OPEN	RRDALLR	DTSVLNR	KQDHLNR	pKW618	pKW633
OPEN	RRDLATR	DRSVLAR	KKDHLDR	pKW619	pKW634
OPEN	RADSLTR	DVSVLKR	ARDKLVR	pKW620[c]	pKW635[b]
OPEN	RVDALTR	DVSVLKR	KQDHLAR	pKW621	pKW637
OPEN	RRDALTR	DTSVLTR	RREQLGR	pKW622	pKW638
OPEN	RGDSLTR	DTSVLNR	RKEKLRI	pKW623	—
hTERT6R	**GAT**	**GCC**	**GCG**		
Method	**Finger 3**	**Finger 2**	**Finger 1**	**ZFTF**	**ZFN**
Modular	QSSNLAR	DRSDLTR	RSDDLTR	pKW309	—
OPEN	VRSNLGR	DRSVLRR	RQHSLRR	pKW599	pKW609
OPEN	VTHNLNR	DTSVLNR	RRHNLDR	pKW598	pKW608
OPEN	LTHNLRR	DRTTLKR	TQHNLDR	pKW600	—
OPEN	LTHNLRR	DPTVLKR	TTHNLKR	pKW601	pKW611
OPEN	LTHNLRR	ESTTLHR	RQHSLRR	pKW602[c]	—
OPEN	LSGNLRR	DRTTLHR	RTHSLAR	pKW603	pKW613[b]
GFP1	**GAA**	**GAT**	**GGT**		
Method	**Finger 3**	**Finger 2**	**Finger 1**	**ZFTF**	**ZFN**
OPEN[37]	QHPNLTR	VAHNLTR	TRQKLGV	—	GFP-ZFN1[d]
GFP2	**GAC**	**GAC**	**GGC**		
Method	**Finger 3**	**Finger 2[e]**	**Finger 1**	**ZFTF**	**ZFN**
OPEN[37]	EGGNLMR	DRSNLTR	APSKLDR	—	GFP-ZFN2[d]

GFP, green fluorescent protein; hTERT, human telomerase reverse transcriptase; ZFN, zinc finger nuclease; ZFTF, zinc finger transcription factor. [a]Amino acid sequences of individual zinc fingers are given in the –1 to +6 positional order of the α-helix. [b]Identified as highly active zinc finger nucleases in Cel1 assays, see **Figure 6**. [c]Identified as highly active zinc finger transcription factors. [d]Previously characterized zinc finger nucleases used as a positive control. [e]Modular zinc finger used.

Cel-I mutagenesis assay on the endogenous site. Our results present promising data towards developing new tools for not only studying the hTERT locus, but also creating new methods for treating telomerase-associated genetic disease.

Results

In our search for suitable target sites, we chose to limit our focus to the hTERT core promoter and exon-1 sequences for several reasons. First, these sequences are a very GC-rich region and as such are likely to have multiple high probability ZFP-binding sites.[14,15] Second, this part of the hTERT locus is thought to be where the majority of the transcriptional control occurs and contains a high density of both known and putative regulatory sequences. Finally, for ZFN applications, higher rates of gene conversion are possible if a DSB is delivered in close proximity to the desired site of change.[32,33] Thus, if gene editing to the core promoter or 5′-coding regions were to be accomplished, then it would be an advantage to engineer ZFNs to target sites within those sequences.

We identified five full ZFN target sites (10 target half-sites total) either within or in proximity to the hTERT core promoter and exon-1, which are listed in **Table 1**. All target half-sites in the chosen full ZFN target sites follow the 5′-GNNGNNGNN-3′ sequence motif, except for the hTERT5SR target half-site, which contains a CNN triplet. Furthermore, the hTERT5 and hTERT5C target sites overlap as a 3 bp frame shift of one another. For the remainder of this report, we will narrow our scope to the hTERT5 and hTERT6 target sites and data generated at all other sites can be found in the **Supplementary Tables S1–4**. The hTERT5R and hTERT6L target half-sites have been targeted previously but the full target sites we describe here have not.[20,34] Both the hTERT5 and hTERT6 target sites are located in exon-1 of the hTERT locus with the hTERT6 site containing the translational start codon within the hTERT6R target half-site (**Figure 1**).

Engineering ZFPs by modular assembly and OPEN methods

In order to create three-fingered ZFPs for the 10 target half-sites, we utilized three different methods. First, we used the B2H-based OPEN protocols to generate candidate DNA-binding domains for eight of the target half-sites, including those for hTERT5 and hTERT6 full sites (**Table 2** and **Supplementary Table S1**). Next, for the hTERT5SL and hTERT5SR half-sites, we installed a module finger (Addgene; http://www.addgene.org) in place of one of the ZFs within the three-fingered rZF libraries used in the OPEN selections due to the lack of coverage provided for some GNN[35] and CNN sequences.[36] We have successfully done this previously in making ZFPs to the GFP gene[37] (**Supplementary Table S1**). In addition to those ZFPs generated through OPEN protocols for hTERT6L and hTERT6R target half-sites, we constructed ZFPs made by modular assembly (**Table 2**). Thus, we were able to generate multiple candidate ZFPs for each target half-site. Although there is a high degree of conservation of the residues that mediate sequence recognition, there is also significant variation of the other residues. Since there is a component of context-dependence to DNA binding, we

screened variants for their activity. These results present a unique set of custom engineered, three-fingered ZFPs for the hTERT locus.

hTERT ZFTFs activate gene expression through hTERT promoter sequences

We converted many of the ZFPs for the hTERT5R, hTERT6L, and hTERT6R target half-sites into ZFTFs (23 total) by linking the VP16 transcriptional activation domain to the N-terminus of the candidate ZFP (**Table 2**).[38] We tested the activity of these ZFTFs using an extrachromosomal GFP reporter system. In this system, ~3 kb of the hTERT promoter and exon-1 were cloned upstream of a GFP reporter gene (pMC6). We hypothesized that the candidate ZFTF would bind to the cognate target site within this sequence and recruit the transcriptional machinery necessary to drive GFP expression (**Figure 2a**). We cotransfected the ZFTF-expressing plasmids and pMC6 into HEK293 cells (an hTERT$^+$ cell line) and measured the changes in %GFP$^+$ populations by flow cytometry to assess the efficacy of these ZFTFs (**Figure 2b**). We found that all of the ZFTFs acted as artificial transcription factors and increased the number of GFP$^+$ cells in these populations, in some instances, the fold stimulation could be as high as ~35-fold or greater (**Figure 2c**).

In our screen of all ZFTFs made, we found that stimulation of GFP expression could vary significantly between individual ZFTFs (**Figure 2c**). Overall, the ZFTFs made with ZFPs from the OPEN platform performed better than those made purely by modular assembly, with few exceptions.[17] These results support the idea that even though the major contact residues are conserved, that the non-contact residues are still important in determining the overall activity of the protein (**Table 2**).

Of the 23 ZFTFs screened, we chose the best performing ZFTFs from each target half-site set (KW602, KW620, and

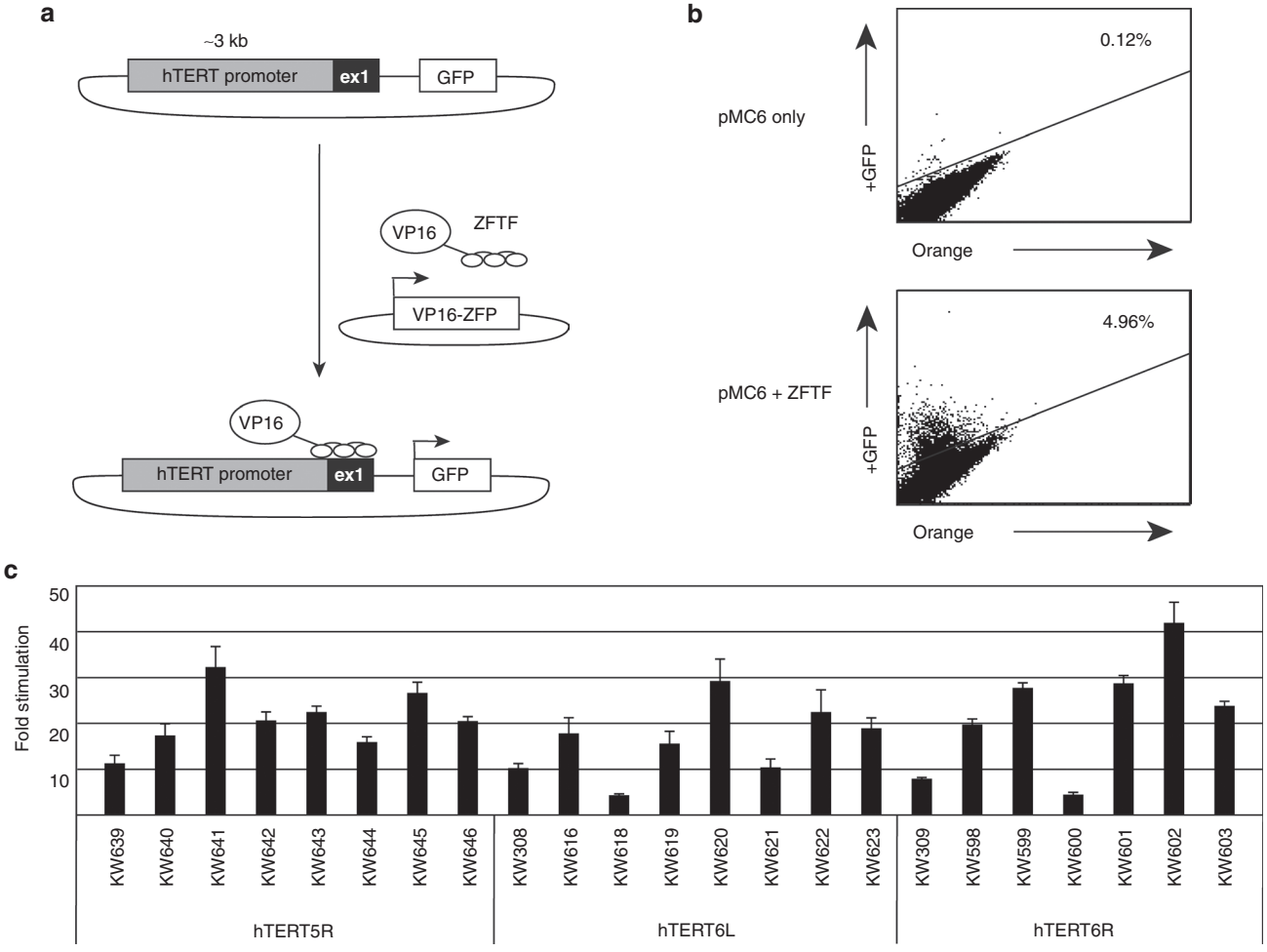

Figure 2 Screening human telomerase reverse transcriptase (hTERT) zinc finger transcription factors (ZFTFs) for transcriptional activation activity. (**a**) Schematic of the pMC6 transcriptional reporter, which contains 3 kb of the hTERT promoter and exon-1 sequence upstream of a green fluorescent protein (GFP) reporter gene. When cotransfected with a ZFTF-expressing plasmid, the ZFTF will bind to the pMC6 reporter plasmid and recruit the cellular machinery necessary to express GFP. (**b**) Flow cytometry plots demonstrating the increase in GFP$^+$ cells after cotransfection of 100 ng of the MC6 reporter plasmid with 700 ng of plasmid expressing the KW602 ZFTF in HEK293 cells. Here, the *x*-axis plots cellular autofluorescence as "orange" (abscissa) and the *y*-axis plots the fluorescent intensity of GFP expressed by pMC6 as "green". (**c**) All ZFTFs listed in **Table 2** were screened for transcriptional activation activity using pMC6. Our results identify which ZFPs efficiently recognize and bind the hTERT target half-sites as ZFTFs to stimulate increases in %GFP$^+$ over background (mean ± SD). ZFP, zinc finger protein.

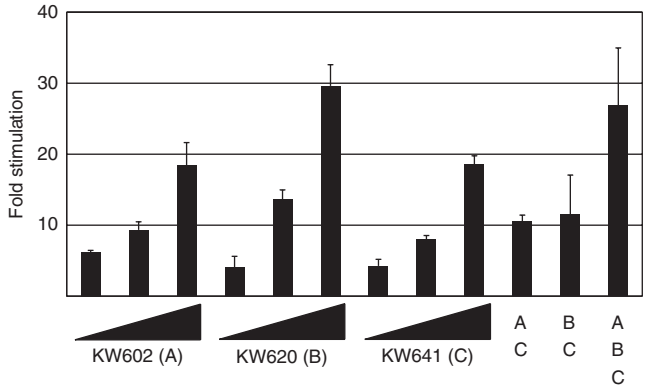

Figure 3 Human telomerase reverse transcriptase (hTERT) zinc finger transcription factors (ZFTFs) exhibit an additive response. The best performing ZFTF for each target half-site were transfected in increasing amounts of ZFTF-expressing plasmid (200, 400, and 600 ng) with 100 ng of pMC6 into HEK293 cells. The KW602, KW620, and KW641 ZFTFs were also cotransfected in pairs or all three combined (200 ng each, mean ± SD).

KW641) and tested them for a potential dose response. We cotransfected single ZFTFs in increasing amounts with the pMC6 reporter plasmid and found that as more ZFTF was transfected, more GFP expression was generated (**Figure 3**). We also tested whether combinations of ZFTFs could stimulate transcriptional activity and found that the individual ZFTFs did not interfere with each other. With the combinations, we found an additive response for different ZFTFs (**Figure 3**). This data reinforces previous findings demonstrating that ZFTFs can elicit a tunable response from a target gene and suggests that this may be possible for the hTERT locus as well.[22,39]

hTERT ZFNs can initiate repair of an extrachromosomal GFP reporter by SSA

To further test the ZFPs engineered to site specifically target the hTERT locus, we converted a total of 39 ZFPs into ZFNs by installing a wild-type (but not codon-optimized) FokI endonuclease domain (Fn) to the C-terminus of the ZFP (**Table 2** and **Supplementary Table S1**). Due to variations in activity seen in the ZFTFs and individual deviations from the consensus sequence in multiple ZFPs, we again designed a screening strategy to test pairing of ZFNs in a combinatorial manner for each target site. To determine the ability of a ZFN pair to recognize, bind, and cut the desired target sequence, we constructed four GFP-based g (SSA assay reporter plasmids where a full hTERT ZFN target site would be inserted between two repeated sequences within the GFP gene. The inserted ZFN target sites also include the full GFP1/2 site, which can be targeted by the GFP-ZFN1 and GFP-ZFN2 ZFN pair as a positive control and internal standard. In this assay, a GFP-based SSA reporter plasmid is cotransfected with ZFN-expressing plasmids together into HEK293 cells. After delivery of the ZFN-mediated DSB, the 5′ overhangs anneal together at the site of the repeats in the GFP gene. When repaired by the endogenous cellular SSA repair pathway, functionality is restored to the GFP reporter construct and GFP$^+$ cells are detected by flow cytometry (**Figure 4**). We report rates of GFP gene repair by hTERT ZFN activity as a relative percentage of

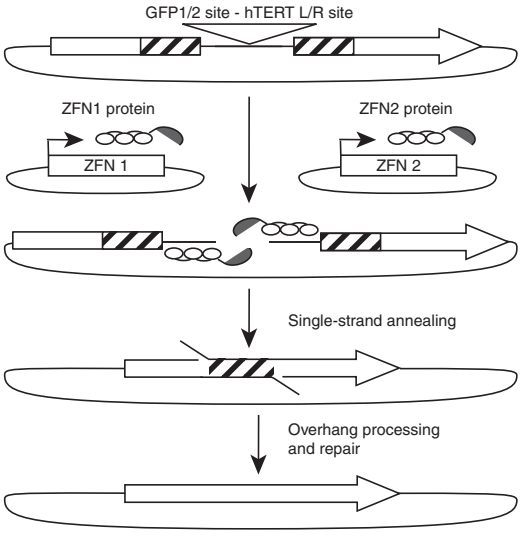

Figure 4 Diagram of the green fluorescent protein (GFP)-based single-strand annealing (SSA) assay. For the purpose of screening nuclease activities of various zinc finger nuclease (ZFN) pairs, we inserted the GFP1/2 ZFN full site and a human telomerase reverse trancriptase (hTERT) ZFN full site between two repeated sequences (hatch boxes) within a GFP reporter gene. Cotransfection of this reporter plasmid (20 ng) with ZFN-expressing plasmids (100 ng each) into HEK293 cells results in the delivery of a double-stranded break between the repeated GFP sequences which is then repaired by the endogenous SSA pathway (after the repeat sequences have annealed to each other) to produce a functional GFP reporter gene.

the nuclease activity measured for the GFP-ZFN1/2 internal standard and positive control (**Tables 3** and **4**; **Supplementary Tables S2–4**).[37]

After screening all five combinatorial sets of pairings, we found that the hTERT5 and hTERT6 ZFN sets were, broadly, the most active sets of ZFNs (**Tables 3** and **4**). For the ZFNs developed for the hTERT5 target site, the KW744/KW664 ZFN pairing exhibited equal nuclease activity to the GFP-ZFN1/2–positive control pair. Also, 8 out of 16 possible pairings were shown to have >50% activity relative to the control (**Table 3**). The hTERT6 ZFN set produced the most active nucleases out of those tested. In this combinatorial screen, the KW633/KW609 hTERT6 ZFN pair was more active than the GFP-ZFN1/2 standard by threefold. We also observed that all but six pairs demonstrated nuclease activity that was higher than the GFP-ZFN1/2 standard (**Table 4**). Therefore, using the SSA assay we identified a number of highly active ZFN pairs targeting the hTERT sequences.

Gene targeting by the hTERT6 ZFNs at a chromosomally integrated GFP reporter construct

We further studied the hTERT6 ZFNs using a chromosomally integrated GFP reporter system. Based on a previously described system, we designed another GFP-based reporter construct that was rendered non-functional through the insertion of an hTERT full ZFN target site and an I-SceI homing endonuclease recognition site as an internal standard.[24] This insertion does not disrupt the endogenous GFP1/2 target site that can be found in the GFP gene and the GFP-ZFN1/2 pair was used as a positive control in

Table 3 Combinatorial screen of hTERT5 ZFN pairs in the SSA assay

	GFP-ZFN2	KW663	KW664	KW665	KW666
GFP-ZFN1	100%[a]	—	—	—	—
KW741	—	57	61	63	64
KW744	—	75	104	78	67
KW745	—	37	32	13	24
KW746	—	33	34	25	30

GFP, green fluorescent protein; hTERT, human telomerase reverse transcriptase; SSA assay, single-strand annealing assay; ZFN, zinc finger nuclease.
[a]All hTERT ZFN pair activity is normalized as a mean percentage of the GFP-ZFN1/2 positive control pair (set to 100%).

Table 4 Combinatorial screen of hTERT6 ZFN pairs in the SSA assay

	GFP-ZFN2	KW608	KW609	KW610	KW611	KW613
GFP-ZFN1	100%[a]	—	—	—	—	—
KW632	—	106	194	59	166	122
KW633	—	281	298	35	255	208
KW634	—	228	203	12	183	142
KW635	—	239	171	46	231	195
KW637	—	281	238	15	238	223
KW638	—	236	280	22	280	226

GFP, green fluorescent protein; hTERT, human telomerase reverse transcriptase; SSA assay, single-strand annealing assay; ZFN, zinc finger nuclease.
[a]All hTERT ZFN pair activity is normalized as a mean percentage of the GFP-ZFN1/2 positive control pair (set to 100%).

this assay (**Figure 5**). Briefly, we generated a transgenic HEK293 cell line containing the stably integrated reporter construct and cotransfected the ZFN-expressing plasmids in pairs along with a donor plasmid. Function of the GFP gene is restored if the ZFNs cut their cognate target site and stimulated homologous recombination-mediated repair with the donor plasmid. Again, ZFN activity is normalized as a relative percentage of the activity measured for the internal standard, I-SceI (**Table 5**).

In repeating the hTERT6 ZFN combinatorial pairing screen, we found that all hTERT6 ZFN pairs stimulated less gene targeting than the GFP-ZFN1/2–positive control. The best performing pair, KW635/KW613, produced ~40% activity relative to I-SceI (**Table 5**). We again observed that, in general, the ZFNs made from ZFPs with purely module fingers showed low activity in this screen when compared with nucleases from ZFPs made by OPEN methodologies.[17] The KW610 ZFN, made from the same ZFP used to make the KW600 ZFTF, produced no evidence of nuclease activity in this gene-targeting assay even though we observed measurable activity in the SSA assay screens. Despite all other hTERT6 ZFN pairs showing higher activity in the SSA activity than the GFP-ZFN1/2 pair, in this chromosomal-based assay, the hTERT6 ZFNs did not have as much activity as the GFP-ZFN1/2 pair. A reason for this discrepancy is that a chromosomal gene-targeting assay is a more stringent screen than an extrachromosomal SSA assay. Using the chromosomal GFP-based reporter, we were able to narrow the candidate ZFNs to be tested for activity at the endogenous hTERT locus.

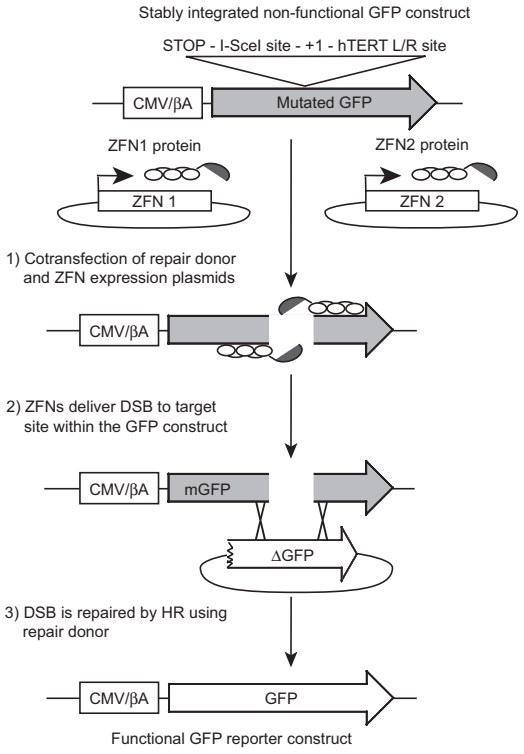

Figure 5 Schematic of the green fluorescent protein (GFP)-based gene-targeting reporter cell line. Based on a previously described strategy, we rendered a GFP reporter gene non-functional through the insertion of a stop codon, an I-SceI recognition site, a frame shift nucleotide, and the human telomerase reverse transcriptase 6 (hTERT6) zinc finger nuclease (ZFN) full site. Using this construct, we generated a stably integrated clonal HEK293 cell line in which we cotransfected pairs of ZFN-expressing plasmids and a repair donor plasmid. Nuclease activity of the ZFN pair is measured by the ZFN-mediated HR repair event between the cut GFP reporter and the donor plasmid producing a functional GFP reporter gene. The CAG promoter used in these experiments is a hybrid of the CMV intermediate-early enhancer and the chicken β-actin promoter. CMV, cytomegalovirus; DSB, double-stranded break; HR, homologous recombination.

ZFN-induced genomic modification at the hTERT locus

Future development of these ZFNs for gene targeting requires demonstrating efficacy for ZFN-mediated genomic modification at the endogenous hTERT locus. We chose the hTERT5 KW744/KW664 and hTERT6 KW635/KW613 ZFNs because they exhibited the best activity in our screening assays. To assay for genomic modification, we transfected the ZFN pairs into K562 cells and measured the rates of imperfect DSB repair by non-homologous end joining at the endogenous genomic locus using the Cel-I assay.[40] We found that the hTERT5 ZFNs gave ~19% allele modification activity and the hTERT6 ZFNs gave ~7% allele modification activity (**Figure 6**). For both sets of ZFNs, using a codon-optimized nuclease domain was critical to obtain maximal activity. While our screening assays suggested that the hTERT6 ZFNs would have greater activity than the hTERT5 ZFNs, we found that the reverse was true in K562 cells. The SSA assay may not be predictive as it is a transient transfection assay in with the reporter plasmid is extrachromosomal and present in multiple copies. Similarly, the assay for the ZFTFs

Table 5 Combinatorial screen of hTERT6 ZFN pairs in the gene-targeting assay

		KW99	KW608	KW609	KW610	KW611	KW613
I-SceI	100%[a]						
GFP-ZFN1/2	79 (19)						
KW98		0	5 (1)	6 (5)	0	2 (1)	2 (2)
KW632		0	28 (16)	18 (17)	0	13 (6)	11 (4)
KW633		0	8 (3)	15 (7)	0	34 (12)	27 (9)
KW634		1 (1)	16 (8)	4 (2)	0	19 (6)	10 (3)
KW635		0	28 (15)	16 (2)	0	21 (2)	42 (17)
KW637		0	19 (5)	18 (11)	0	22 (8)	30 (15)
KW638		0	36 (19)	12 (4)	0	35 (15)	27 (11)

GFP, green fluorescent protein; hTERT, human telomerase reverse transcriptase; ZFN, zinc finger nuclease.
[a]ZFN-mediated repair of the mutated GFP sequence by homologous recombination results in measurable changes in GFP expression in treated cells as read by flow cytometry. Rates of gene conversion for all hTERT ZFN pair activity was normalized as a relative percentage of the nuclease activity measured for the internal I-SceI standard, set to 100% (mean (SEM), $N = 3$). On average, there were 32,315 ISce-I generated repair events per million cells treated.

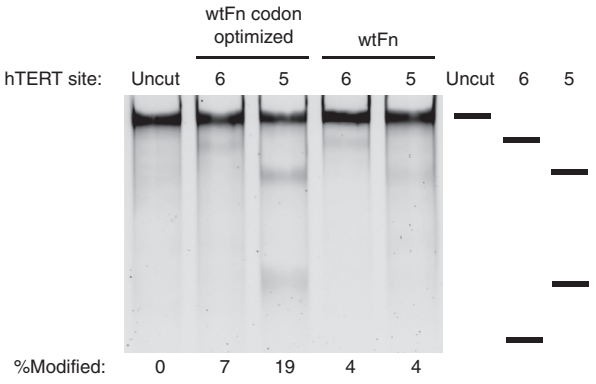

Figure 6 Zinc finger nuclease (ZFN)-induced modification of the genomic human telomerase reverse transcriptase (hTERT) locus in K562 cells. ZFN-treated cell populations were treated with either the hTERT5 (KW744/KW664) or hTERT6 ZFNs (KW635/KW613) and screened in bulk for mutations in the genomic that arose after repair of the ZFN-induced double-stranded break by NHEJ pathways. Mismatches in the mutated sequences are detectable by digestion with a form of the Cel1 nuclease (Surveyor). We included the use of human codon-optimized Fn domains in the ZFNs and expected digest products are given to the right of the figure. NHEJ pathway, non-homologous end joining pathway.

is also a transient extrachromosomal reporter assay and subject to the same limitations. In addition, further sources of discrepancy include: (i) that the hTERT6 ZFNs have more toxicity than the hTERT5 ZFNs (data not shown); (ii) that the chromatin differences of the target sites at the endogenous promoter in K562 cells is modulating the activity of the ZFNs; or (iii) that endogenous transcription or regulatory factor binding to the promoter is competing with ZFN binding.[7] In sum, we have demonstrated that both pairs of ZFNs have the potential to be used as tools in generating cell lines with specific hTERT gene modifications, but additional studies will be necessary to show this.

Discussion

Due to the role hTERT plays in telomere regulation, the hTERT locus is an important target for research and drug development.[41] The ability to direct hTERT transcription in a dose-dependent manner or edit the nucleotide content of the locus could yield useful insights into the regulation of this gene, cellular aging, and the oncogenic process. To this end, we have developed many ZFPs that recognize sequences within the hTERT promoter and exon-1 (**Table 1** and **Figure 1**). We built the ZFPs using modular assembly methods, the B2H-based OPEN platform, and a hybrid method that combines the two protocols.[14,15] This resulted in several candidate ZFPs for every target half-site.[42] Therefore, we used several different screens to identify the most appropriate ZFP that has the ability to recognize and direct activity to the cognate target site, whether as a ZFTF or a ZFN (**Table 2** and **Supplementary Table S1**).

As ZFTFs, we were able to demonstrate that many of our ZFPs linked to the VP16 effector domain could stimulate the transcription of an hTERT promoter exon-1–driven GFP reporter gene (**Figure 2**). We were also able to demonstrate that ZFTFs developed for sites in the hTERT locus could elicit transcriptional activation in coarsely tunable manner in which incremental increases in ZFTF produced an additive response from the GFP-based reporter (**Figure 3**). This is consistent with the findings of others using ZFTFs at other gene targets.[22,39] Our ZFTF screen also identifies some well-performing candidate ZFTFs (e.g., KW602, KW620, and KW641) that may be pursued for future research in endogenous hTERT transcriptional regulation. In comparison to the ZFTFs made by modular assembly described in Sohn *et al.* (2010), which were demonstrated to be effective repressors of hTERT transcription, we found the KW308 and KW309 modular assembly ZFTFs to be less effective at activating transcription than the OPEN-generated ZFTFs.[13,17,20] The differences in efficacy for modular assembly ZFPs as ZFTFs may be due to the differences in the module fingers used to construct the ZFPs, the effector domains, and design of the reporter assays.

To test the candidate ZFPs as nucleases, we screened sets of ZFN pairs in a combinatorial fashion for each hTERT target site identified using a GFP-based SSA assay (all sites) and a chromosomally integrated GFP gene-targeting assay (hTERT6 only) (**Figure 4**). ZFNs to the hTERT5 and hTERT6 site were the most active sets of nucleases in the SSA assays (**Tables 3** and **4**; **Supplementary Tables S2–4**). We then selected two ZFN pairs to demonstrate mutation of the endogenous genomic sequence and were able to achieve modification frequencies up to 18.7% (**Figure 6**). As a result, we have not only successfully custom-engineered ZFPs to sequences found in the hTERT promoter and exon-1 region, but have also shown that, as ZFNs, these artificial proteins can direct activity to the cognate target site within the genomic locus.

In summary, the data generated by our screening strategies provide the necessary results to move forward with using these ZFTFs and ZFNs as potential tools to modulate hTERT expression and edit the genomic locus for both research and clinical applications.

Materials and methods

Generating ZF DNA-binding domains to target sites at the hTERT locus. Five full ZFN target sites were identified in the hTERT core promoter and exon-1 and are listed in **Table 1**. We used the publically available OPEN platform and protein backbone sequence to construct three-fingered ZFP arrays for each target half-site.[14,15] For the hTERT5SL and hTERT5SR target half-sites, we generated ZFPs by including a module finger at the Finger 2 and Finger 1 positions (respectively) during the creation of the three-fingered cassettes used in the stage B2H selections due to the lack of coverage provided for some GNN[35] and CNN sequences.[36] In addition to the OPEN-based ZFPs made for the hTERT6L and hTERT6R target half-sites, we constructed ZFPs by assembling module fingers at each ZF position (KW308 and KW309). For each target half-site, the OPEN protocols produced multiple candidate ZFPs, some of which were chosen for further screening (**Table 1** and **Supplementary Table S1**).

Cloning ZFTFs and ZFNs. To create ZFTFs, chosen candidate ZFPs made by the OPEN-based methods were cloned into the PRK5.AD-GFZ3 vector provided by Toni Cathomen to link the VP16 transcriptional activator domain to the N-terminus of the ZFP.[43] ZFNs were made by cloning many of the same ZFPs into the previously characterized GFP-ZFN2-B2H vector to link a wild-type Fn domain of native sequence or codon-optimized to the C-terminus of the ZFP.[37,44] All cloning was performed using standard molecular biology techniques.

Screening hTERT ZFTFs for transcriptional activation activity. Approximately 3 kb of the hTERT promoter and exon-1 was installed upstream of a GFP reporter. In this plasmid construct (pMC6), the endogenous hTERT translational start codon was mutated from ATG to GTG, but the start codon for GFP was preserved. All ZFTFs were screened for transcriptional activation activity by cotransfecting 700 ng of ZFTF-expressing plasmid with 100 ng of pMC6 using lipofectamine into 1×10^5 HEK293 cells. GFP transcription was assayed by measuring %GFP$^+$ cells by flow cytometry on day 2 (FACS Calibur; BD Biosciences, Franklin Lakes, NJ). pMC6 was cotransfected with a blank vector as a negative control. Then, one ZFTF from each target half-site group (three total) was cotransfected singularly in increasing amounts (200–600 ng) and in combination (200 ng each) with pMC6 to assay for any dose response. In **Figure 2b**, the *x*-axis plots cellular autofluorescence as "orange" (abscissa) and the *y*-axis plots the fluorescent intensity of GFP expressed by pMC6 as "green". Fold stimulation of transcriptional activation was calculated as (experimental/background) and error is reported as SD.

SSA assays for screening hTERT ZFN pairs. Based on a previously described SSA strategy, reporters were cloned by inserting a hTERT full ZFN target site between repeated sequences within GFP gene (four total).[45] Each of these SSA reporters contains a single hTERT target site except for the hTERT5 reporter, which also carries the hTERT5C site due to the overlapping sequence shared (**Table 1**). The reporter constructs also include the GFP1/2 full ZFN target site (5′-ACCATCTTC-ttcaag-GACGACGGC-3′) as a positive control and internal standard. These extrachromosomal SSA reporter plasmids were used to assay for on-target nuclease activity for sets of combinatorial pairs of ZFNs developed for each hTERT target site. Briefly, 100 ng of each ZFN-expressing plasmid and 20 ng of appropriate reporter plasmid were cotransfected into 1×10^5 HEK293 cells using calcium phosphate and the GFP1/2 ZFN pair was used as a positive control. The repair of the GFP gene (after cutting by a pair of ZFNs) was measured by %GFP$^+$ cells using flow cytometry on day 2. The nuclease activities of all hTERT ZFN pairs tested were normalized to the activity of the positive control and are reported as a percentage of GFP1/2 ZFN activity. Blank vector-treated cell populations were on average 0.1% GFP$^+$ and GFP1/2 ZFN-treated cell populations were 0.62, 0.4, 0.57, 0.52, and 0.33% GFP$^+$ on the hTERT5, hTERT5C, hTERT5S, hTERT6, and hTERT6B SSA reporter constructs, respectively.

Gene-targeting assay for hTERT6 ZFNs. The hTERT6 ZFNS were tested for the ability to stimulate homologous recombination at a chromosomal target by using a previously described GFP gene-targeting assay.[24] This assay uses a GFP reporter that has been mutated by the insertion of an hTERT full ZFN target site and an I-SceI recognition site as a positive control and internal standard. Within the GFP gene, the GFP1/2 full ZFN target site can be found as well (see above). This reporter construct was electroporated into HEK293 cells and a monoclonal cell line was derived. ZFN-expressing plasmids were cotransfected by calcium phosphate into the monoclonal cell line in pairs (100 ng each) for a combinatorial screen with 300 ng of repair donor plasmid. Nuclease-mediated gene-targeting events were measured as %GFP$^+$ cells by flow cytometry on day 3. The nuclease activities of all ZFN pairs tested were normalized to the I-SceI–positive control and are reported as a relative percentage of that activity.

Cel-I assay to measure ZFN-induced genomic modification. Imperfect repair of a ZFN-induced DSB can result in mismatches in the DNA helix that bulge, creating the substrate for the Cel-I nuclease. Screening bulk genomic DNA for these mismatches at the hTERT locus allowed us to quantify percent modification after the application of our ZFNs into K562 cells. In brief, 1×10^6 K563 cells were nucleofected according to the manufacturer's instructions (Lonza, Basel, Switzerland) and genomic DNA was isolated from cell populations that had been nucleofected with the hTERT5 ZFNs (KW744/KW664) or hTERT6 ZFNs (KW635/KW613) that possessed a wild-type Fn domain of native sequence or was codon-optimized. Then, the hTERT promoter exon-1 sequence that contains both the hTERT5 and hTERT6 sites was PCR-amplified using the AccuPrime PCR kit (Invitrogen, Carlsbad, CA) and a melting temperature of 60 °C. When the primers 5′-GCCCGAGTTTCAGGCAGCGCTGCGTCCTG-3′ and 5′-TGATGTGCCTGCGCTGCTCTCCGCATGTCG-3′ were used, a 373 bp product was formed, which we gel-purified from agarose. A total of 200 ng of the

purified PCR product was then treated with the Surveyor (Cel1) nuclease kit (Transgenomic, Omaha, NE). If the genomic hTERT locus had been modified by the hTERT5 or hTERT6 ZFNs, then digest products of 248 + 125 bp or 308 + 65 bp would appear, respectively. We loaded Surveyor digest products onto a 10% acrylamide gel and imaged our results using ethidium bromide/ultraviolet. Analysis was performed using ImageJ software and calculations described by Miller *et al.* (2007) after normalizing for nucleofection efficiency (~70%).[46]

Supplementary material

Table S1. Zinc finger DNA-binding domains generated for the hTERT5C, hTERT5S, and hTERT6B target sites.
Table S2. Combinatorial screen of hTERT5C ZFN pairs in the SSA assay.
Table S3. Combinatorial screen of hTERT5S ZFN pairs in the SSA assay.
Table S4. Combinatorial screen of hTERT6B ZFN pairs in the SSA assay.

Acknowledgments. We acknowledge Sierra Sciences (Reno, NV) for supplying the human telomerase reverse transcriptase promoter and exon-1 sequences used for cloning pMC6. We thank Toni Cathomen (Hannover Medical School) for providing the PRK5.AD-GFZ3 plasmid used to clone the zinc finger transcription factors. This work was supported by National Institutes of Health (R01 HL079295), The March of Dimes, a career development award from the Burroughs Welcome Fund, and funding through University of Texas Southwestern Medical Center by the State of Texas. The authors declared no conflict of interest.

1. Smogorzewska, A and de Lange, T (2004). Regulation of telomerase by telomeric proteins. *Annu Rev Biochem* **73**: 177–208.
2. Bodnar, AG, Ouellette, M, Frolkis, M, Holt, SE, Chiu, CP, Morin, GB *et al.* (1998). Extension of life-span by introduction of telomerase into normal human cells. *Science* **279**: 349–352.
3. Hanahan, D and Weinberg, RA (2011). Hallmarks of cancer: the next generation. *Cell* **144**: 646–674.
4. Artandi, SE and DePinho, RA (2010). Telomeres and telomerase in cancer. *Carcinogenesis* **31**: 9–18.
5. Harley, CB (2002). Telomerase is not an oncogene. *Oncogene* **21**: 494–502.
6. Morales, CP, Holt, SE, Ouellette, M, Kaur, KJ, Yan, Y, Wilson, KS *et al.* (1999). Absence of cancer-associated changes in human fibroblasts immortalized with telomerase. *Nat Genet* **21**: 115–118.
7. Horikawa, I and Barrett, JC (2003). Transcriptional regulation of the telomerase hTERT gene as a target for cellular and viral oncogenic mechanisms. *Carcinogenesis* **24**: 1167–1176.
8. Takakura, M, Kyo, S, Kanaya, T, Hirano, H, Takeda, J, Yutsudo, M *et al.* (1999). Cloning of human telomerase catalytic subunit (hTERT) gene promoter and identification of proximal core promoter sequences essential for transcriptional activation in immortalized and cancer cells. *Cancer Res* **59**: 551–557.
9. Wick, M, Zubov, D and Hagen, G (1999). Genomic organization and promoter characterization of the gene encoding the human telomerase reverse transcriptase (hTERT). *Gene* **232**: 97–106.
10. Armanios, M, Chen, JL, Chang, YP, Brodsky, RA, Hawkins, A, Griffin, CA *et al.* (2005). Haploinsufficiency of telomerase reverse transcriptase leads to anticipation in autosomal dominant dyskeratosis congenita. *Proc Natl Acad Sci USA* **102**: 15960–15964.
11. Armanios, MY, Chen, JJ, Cogan, JD, Alder, JK, Ingersoll, RG, Markin, C *et al.* (2007). Telomerase mutations in families with idiopathic pulmonary fibrosis. *N Engl J Med* **356**: 1317–1326.
12. Tsakiri, KD, Cronkhite, JT, Kuan, PJ, Xing, C, Raghu, G, Weissler, JC *et al.* (2007). Adult-onset pulmonary fibrosis caused by mutations in telomerase. *Proc Natl Acad Sci USA* **104**: 7552–7557.
13. Kim, JS, Lee, HJ and Carroll, D (2010). Genome editing with modularly assembled zinc-finger nucleases. *Nat Methods* **7**: 91; author reply 91–91; author reply 92.
14. Maeder, ML, Thibodeau-Beganny, S, Osiak, A, Wright, DA, Anthony, RM, Eichtinger, M *et al.* (2008). Rapid "open-source" engineering of customized zinc-finger nucleases for highly efficient gene modification. *Mol Cell* **31**: 294–301.
15. Maeder, ML, Thibodeau-Beganny, S, Sander, JD, Voytas, DF and Joung, JK (2009). Oligomerized pool engineering (OPEN): an 'open-source' protocol for making customized zinc-finger arrays. *Nat Protoc* **4**: 1471–1501.
16. Sander, JD, Dahlborg, EJ, Goodwin, MJ, Cade, L, Zhang, F, Cifuentes, D *et al.* (2011). Selection-free zinc-finger-nuclease engineering by context-dependent assembly (CoDA). *Nat Methods* **8**: 67–69.
17. Ramirez, CL, Foley, JE, Wright, DA, Müller-Lerch, F, Rahman, SH, Cornu, TI *et al.* (2008). Unexpected failure rates for modular assembly of engineered zinc fingers. *Nat Methods* **5**: 374–375.
18. Sera, T (2009). Zinc-finger-based artificial transcription factors and their applications. *Adv Drug Deliv Rev* **61**: 513–526.
19. Jamieson, AC, Miller, JC and Pabo, CO (2003). Drug discovery with engineered zinc-finger proteins. *Nat Rev Drug Discov* **2**: 361–368.
20. Sohn, JH, Yeh, BI, Choi, JW, Yoon, J, Namkung, J, Park, KK *et al.* (2010). Repression of human telomerase reverse transcriptase using artificial zinc finger transcription factors. *Mol Cancer Res* **8**: 246–253.
21. Beerli, RR, Dreier, B and Barbas, CF 3rd (2000). Positive and negative regulation of endogenous genes by designed transcription factors. *Proc Natl Acad Sci USA* **97**: 1495–1500.
22. Snowden, AW, Zhang, L, Urnov, F, Dent, C, Jouvenot, Y, Zhong, X *et al.* (2003). Repression of vascular endothelial growth factor A in glioblastoma cells using engineered zinc finger transcription factors. *Cancer Res* **63**: 8968–8976.
23. Mattei, E, Corbi, N, Di Certo, MG, Strimpakos, G, Severini, C, Onori, A *et al.* (2007). Utrophin up-regulation by an artificial transcription factor in transgenic mice. *PLoS ONE* **2**: e774.
24. Porteus, MH and Baltimore, D (2003). Chimeric nucleases stimulate gene targeting in human cells. *Science* **300**: 763.
25. Durai, S, Mani, M, Kandavelou, K, Wu, J, Porteus, MH and Chandrasegaran, S (2005). Zinc finger nucleases: custom-designed molecular scissors for genome engineering of plant and mammalian cells. *Nucleic Acids Res* **33**: 5978–5990.
26. Wah, DA, Bitinaite, J, Schildkraut, I and Aggarwal, AK (1998). Structure of FokI has implications for DNA cleavage. *Proc Natl Acad Sci USA* **95**: 10564–10569.
27. Bibikova, M, Beumer, K, Trautman, JK and Carroll, D (2003). Enhancing gene targeting with designed zinc finger nucleases. *Science* **300**: 764.
28. Santiago, Y, Chan, E, Liu, PQ, Orlando, S, Zhang, L, Urnov, FD *et al.* (2008). Targeted gene knockout in mammalian cells by using engineered zinc-finger nucleases. *Proc Natl Acad Sci USA* **105**: 5809–5814.
29. Porteus, MH, Connelly, JP and Pruett, SM (2006). A look to future directions in gene therapy research for monogenic diseases. *PLoS Genet* **2**: e133.
30. Cathomen, T and Joung, JK (2008). Zinc-finger nucleases: the next generation emerges. *Mol Ther* **16**: 1200–1207.
31. Perez, EE, Wang, J, Miller, JC, Jouvenot, Y, Kim, KA, Liu, O *et al.* (2008). Establishment of HIV-1 resistance in CD4+ T cells by genome editing using zinc-finger nucleases. *Nat Biotechnol* **26**: 808–816.
32. Elliott, B, Richardson, C, Winderbaum, J, Nickoloff, JA and Jasin, M (1998). Gene conversion tracts from double-strand break repair in mammalian cells. *Mol Cell Biol* **18**: 93–101.
33. Stark, JM, Pierce, AJ, Oh, J, Pastink, A and Jasin, M (2004). Genetic steps of mammalian homologous repair with distinct mutagenic consequences. *Mol Cell Biol* **24**: 9305–9316.
34. Townsend, JA, Wright, DA, Winfrey, RJ, Fu, F, Maeder, ML, Joung, JK *et al.* (2009). High-frequency modification of plant genes using engineered zinc-finger nucleases. *Nature* **459**: 442–445.
35. Liu, Q, Xia, Z, Zhong, X and Case, CC (2002). Validated zinc finger protein designs for all 16 GNN DNA triplet targets. *J Biol Chem* **277**: 3850–3856.
36. Dreier, B, Fuller, RP, Segal, DJ, Lund, CV, Blancafort, P, Huber, A *et al.* (2005). Development of zinc finger domains for recognition of the 5'-CNN-3' family DNA sequences and their use in the construction of artificial transcription factors. *J Biol Chem* **280**: 35588–35597.
37. Pruett-Miller, SM, Connelly, JP, Maeder, ML, Joung, JK and Porteus, MH (2008). Comparison of zinc finger nucleases for use in gene targeting in mammalian cells. *Mol Ther* **16**: 707–717.
38. Triezenberg, SJ, Kingsbury, RC and McKnight, SL (1988). Functional dissection of VP16, the trans-activator of herpes simplex virus immediate early gene expression. *Genes Dev* **2**: 718–729.
39. Magnenat, L, Schwimmer, LJ and Barbas, CF 3rd (2008). Drug-inducible and simultaneous regulation of endogenous genes by single-chain nuclear receptor-based zinc-finger transcription factor gene switches. *Gene Ther* **15**: 1223–1232.
40. Guschin, DY, Waite, AJ, Katibah, GE, Miller, JC, Holmes, MC and Rebar, EJ (2010). A rapid and general assay for monitoring endogenous gene modification. *Methods Mol Biol* **649**: 247–256.
41. Philippi, C, Loretz, B, Schaefer, UF and Lehr, CM (2010). Telomerase as an emerging target to fight cancer–opportunities and challenges for nanomedicine. *J Control Release* **146**: 228–240.

42. Fu, F, Sander, JD, Maeder, M, Thibodeau-Beganny, S, Joung, JK, Dobbs, D *et al.* (2009). Zinc Finger Database (ZiFDB): a repository for information on C2H2 zinc fingers and engineered zinc-finger arrays. *Nucleic Acids Res* **37**(Database issue): D279–D283.
43. Alwin, S, Gere, MB, Guhl, E, Effertz, K, Barbas, CF 3rd, Segal, DJ *et al.* (2005). Custom zinc-finger nucleases for use in human cells. *Mol Ther* **12**: 610–617.
44. Urnov, FD, Miller, JC, Lee, YL, Beausejour, CM, Rock, JM, Augustus, S *et al.* (2005). Highly efficient endogenous human gene correction using designed zinc-finger nucleases. *Nature* **435**: 646–651.
45. Szczepek, M, Brondani, V, Büchel, J, Serrano, L, Segal, DJ and Cathomen, T (2007). Structure-based redesign of the dimerization interface reduces the toxicity of zinc-finger nucleases. *Nat Biotechnol* **25**: 786–793.
46. Miller, JC, Holmes, MC, Wang, J, Guschin, DY, Lee, YL, Rupniewski, I *et al.* (2007). An improved zinc-finger nuclease architecture for highly specific genome editing. *Nat Biotechnol* **25**: 778–785.

Supplementary Information accompanies this paper on the Molecular Therapy–Nucleic Acids website (http://www.nature.com/mtna)